Trapping of root-knot nematodes
by the adhesive hyphae-forming fungus
Arthrobotrys oligospora

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Eefje den Belder

Trapping of root-knot nematodes by the adhesive hyphae-forming fungus *Arthrobotrys oligospora*

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STELLINGEN

1. Dat nematofage schimmels draden vormen waarmee plant-parasitaire nematoden worden gevangen, is een onderbelicht fenomeen in het onderzoek en een onderschatte mogelijkheid voor biologische bestrijding.
   Dit proefschrift

2. Klevende schimmeldraden van *Arthrobotrys oligospora* (CBS 289.82) zijn effectiever in het vangen van wortelknobbelaaltjes dan complexe vangstructuren van deze schimmel.
   Dit proefschrift

3. Voor de detectie en selectie van nematofage schimmels is het gebruik van plant-parasitaire nematoden te verkiezen boven het gebruik van niet plant-parasitaire modelnematoden.
   Dit proefschrift

4. De stelling dat in het algemeen bij nematoden-vangende schimmels het saprofytisch en predatoir vermogen elkaar uitsluiten, is onjuist.
   Cooke, R.C. (1963). Ecological characteristics of nematode-trapping Hyphomycetes. Ann. Appl. Biol. 52, 431-437.

5. Nematoden-vangende schimmels schakelen nematoden reeds uit door hechting aan vangstructuren. Wanneer op hechting geen infectie volgt dan vindt hierdoor tòch aantalsregulatie plaats, hetgeen niet het geval is bij endoparasitaire schimmels.

6. Het gebrek aan succes bij biologische bestrijding van nematoden hangt samen met de tot nu toe gevolgde trial-and-error methode.

7. De kans op succes bij biologisch bestrijding van nematoden wordt vergroot door ecologisch onderzoek aan nematofage schimmels en de ontwikkeling van criteria voor kandidaat-antagonisten.

8. Live as if you will die tomorrow, farm as if you will live forever.
   Angelsaksische zegswijze

9. De vraag naar de zin van ontwikkelingssamenwerking is verwant aan de vraag naar de zin van het leven. Het antwoord trouwens ook.

10. Met nestbescherming op agrarisch land zonder gebruiksbeperkingen kunnen meer weidevogels beschermd worden dan in natuurreservaten.

11. Mentaal afstand nemen levert een onderschatte bijdrage aan creatief onderzoek.

12. Wie gedetailleerde onderzoeksvoorstellen eist, wil bedrogen worden.

Stellingen behorend bij het proefschrift:
Trapping of root-knot nematodes by the adhesive hyphae-forming fungus *Arthrobotrys oligospora* door Eefje den Belder.

Wageningen, 19 april 1994

Contents

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Abstract

The present study addresses the ecology of a particular isolate of *Arthrobotrys oligospora* (CBS 289.82) in relation to its efficacy in controlling the root-knot nematode, *Meloidogyne hapla*.

This isolate was selected because it differs from most nematode-trapping fungi in that it captures nematodes with adhesive hyphae without having to form complex trapping devices. This characteristic may make it a very useful biological control agent. An attempt was made to gain insight into the factors controlling its capture ability.

In vitro experiments demonstrated that the *A. oligospora* (CBS 289.82) isolate was very effective in capturing *M. hapla* and *M. incognita*, compared to the ability of other fungi with other trapping devices. Mobile juveniles were all caught by the hyphae within one hour and in some cases attachment occurred within the very first contact (chapter 2).

Electron microscopic observations revealed that attachment of juveniles of *Meloidogyne* spp. to hyphae is mediated by a layer of extracellular material, about 0.1 µm thick, on the hyphae (chapter 3). Such a layer was never observed in hyphae of fungal cultures to which nematodes were not added, suggesting that its presence depends on an interaction of the fungus with the nematode.

The attachment of *Meloidogyne* second-stage juveniles was not affected by temperatures between 5 and 30°C. However, at 15°C ring structure development and growth of trophic hyphae were strongly hampered, which suggest that under prevailing soil temperatures in temperate regions, ring structure development and growth of trophic hyphae may proceed slowly whereas trapping would continue to occur (chapter 4).

Furthermore, the nutritional conditions during growth of the fungus did not correlate with the rapidity of nematode-hypha attachment. The results also provide evidence that the trapping ability of the isolate tested continued for over more than 70 days (chapter 4).

Arthrobotrys oligospora (CBS 289.82) covered dead, ruptured nematodes with a dense mycelium, whereas dead but otherwise intact nematodes were penetrated

through the buccal cavity by a corkscrew-like structure and were subsequently colonized by trophic hyphae. Colonization of living second-stage juveniles by trophic hyphae following attachment and penetration was faster than colonization of dead second-stage juveniles. The addition of dead juveniles to a fungal colony prior to the addition of living juveniles did not affect attachment or the development of trophic hyphae through the live juveniles. However, one day after the addition of live juveniles, the proportion of live nematodes with ring structures was higher than when living and dead juveniles were added at the same time. The development of trophic hyphae in dead second-stage juveniles was delayed in the presence of live second-stage juveniles. The results refute the commonly held assumption that poor possibilities for saprophytic growth are a prerequisite for the formation of trapping devices and the predacious mode of feeding in the fungus (chapter 5).

An important quality of fungi as potential biological control agents is their ability to form mycelium and capture structures in the soil at the place where their activity is desirable.

The establishment and capture activity of this isolate in a simple microcosm system at 20°C, was compared to that of other fungi from the *Dactylaria*-complex. Direct microscopic observations in microcosms confirmed the attachment of mobile juveniles of *M. hapla* to hyphae of *A. oligospora* (chapter 6). Application of about 30 mm hyphal fragments of *A. oligospora* (CBS 289.82) per gram soil resulted in 100-170 m of hyphae per gram oven-dry soil at 20°C within 12 days, a reduction of 90% in the number of living nematodes of *M. hapla* within one day after addition and the extermination of the nematodes within 10 days. In non-sterilized soil, the hyphae reached a total length of 10 m per gram oven-dry soil. This amount of hyphal mass was sufficient to reduce the number of nematodes by 70% as compared to the control within 10 days after nematodes were added to the soil. At 13°C, similar results were obtained. Even at low densities, this isolate is effective. Notwithstanding their ability to form the most extensive mycelial mats of all fungi tested and despite the fairly large amounts of *M. hapla* added, nematode capture in both adhesive ring-forming fungi *A. conoides* (CBS 265.83) and *A. oligospora* (ATCC 24927) was zero or low. This supports other observations that the adhesive ring-forming fungi are inefficient (chapter 6).

In chapter 7 the results on the ability of *A. oligospora* (CBS 289.82) to capture root-knot nematodes presented in the previous chapters are evaluated. The

mechanism of nematode trapping is also discussed from the perspective of screening nematode-trapping fungi and using the adhesive hyphae-forming fungi as biological control agents.

1

Introduction

In present agricultural practice, plant-parasitic nematodes are being increasingly controlled by the use of resistant cultivars and crop rotation, but still chemical control is widely practised. Most nematicides have undesired side effects with respect to the environment and affect the quality of drinking-water (Peoples *et al.*, 1980; Wixted *et al.*, 1987). The growing awareness of environmental pollution caused by such nematicides has stimulated the research on alternative control methods, such as the use of antagonists like Tardigrada, bacteria, and fungi, in addition to breeding resistant or tolerant varieties.
Although a paucity of quantitative data on the impact of antagonists on nematode populations remains, evidence is accumulating that under specific conditions antagonists are able to maintain nematode populations at levels that cause little crop damage (Stirling, 1991).
The limited understanding of the ecology of antagonists may be one of the principal reasons that they often fail in practice as biological control agents (Kerry, 1990; de Leij, 1992).
For biological control purposes the ideal antagonist should have a quick, functional and numerical response to the host and a high survival rate in soil. Potentially useful antagonists can only be identified on the basis of a thorough knowledge of their interaction with the target nematode species under realistic conditions. Basic laboratory and microcosm studies are necessary to understand the key factors that influence the relationship between antagonist and nematode, and for assessing the establishment of the antagonist and its survival in the soil (Stirling, 1991).

The present study addresses the ecology of a particular isolate of *Arthrobotrys oligospora* Fres. var. *oligospora* in relation to its efficacy in controlling the root-knot nematode, *Meloidogyne hapla*. After screening several fungi, this isolate was selected because of its ability to capture nematodes with undifferen-

tiated adhesive hyphae, i.e. without having to form complex trapping devices as other nematode-trapping fungi do. In the research presented here, an attempt has been made to gain insight into the factors controlling the capture ability of this fungus. At the organism level, the attachment of hypha to the nematode has been studied. At the population level, the effects of several biotic and abiotic factors on the capture and infection have been investigated *in vitro*. The microcosm experiments described here have focused on the establishment of the fungus and its control of nematodes in soil.

Root-knot nematodes

Nematodes of the genus *Meloidogyne*, the root-knot nematodes, are distributed worldwide. They have been found to affect many cultivated and wild plants. World-wide, over 90% of reported occurrences pertain to the species *Meloidogyne incognita* (Kofoid and White) Chitwood, *Meloidogyne arenaria* (Neal) Chitwood, *Meloidogyne javanica* (Treub) Chitwood and *Meloidogyne hapla* Chitwood. The serious economic damage caused by these species has been well documentated by Webster (1972). Worldwide crop losses are estimated to reach about 13%, with the tropics and subtropics being the most seriously affected regions (Sasser, 1979).

The target organism in the present study is the Northern root-knot nematode, *M. hapla*. This species is confined to temperate climates and to higher altitude zones in the tropics and subtropics. *M. hapla* is a highly polyphagous species attacking over 550 plant species and varieties including potato and sugar beet (Goodey *et al.*, 1965). Crop rotation can effectively suppress nematode populations, but the difficulty with *M. hapla* is that it has a very wide host range. In rotation with the potato, corn and wheat would be excellent crops to suppress *M. hapla* because they are not suitable hosts for *M. hapla*. Unfortunately both crops are suitable hosts for *M. chitwoodi*.

Like most nematodes, *M. hapla* has four juvenile stages (Figure 1.1). The first-stage juvenile moults within the egg. The infective second-stage juvenile (vermiform) emerges from the egg, moves freely in the soil and penetrates the host root behind the root cap. Giant cells are induced near the place where the nematode feeds from the vascular system, forming the characteristic galls or knots (Figure 1.2).

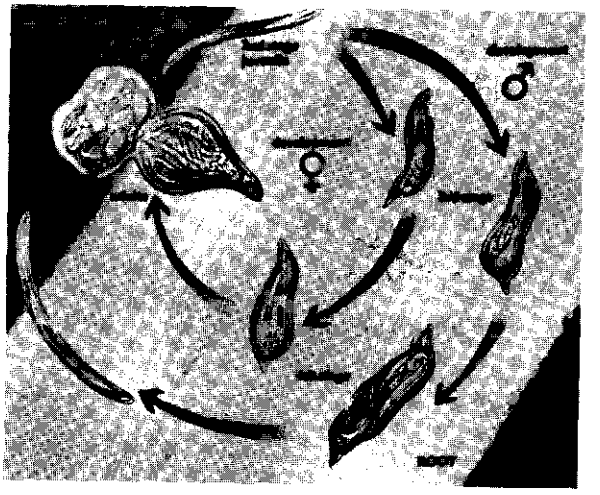

FIGURE 1.1 Life-cycle of a root-knot nematode.

FIGURE 1.2 Characteristic galls caused by root-knot nematodes.

The nematode undergoes two more moults before the adult stage. The females stay embedded in the root tissue while the mobile males leave the root and move in the soil. Generally reproduction of most *Meloidogyne* species is parthenogenetic, but with heavy infestations and on less suitable hosts, the proportion of males increases. The egg masses are deposited outside the female's body in a gelatinous protective matrix. The life cycle may be completed in less than a month depending on the temperature. Under favourable environmental conditions, new generations will be produced continuously (Guiran & Ritter, 1979).

Nematophagous fungi

Many species of nematophagous fungi occur in the soil. Stirling (1991) divided nematophagous fungi infecting mobile stages of nematodes into two categories: those which infect nematodes with small conidia (endoparasites) and those which capture nematodes with modified hyphal traps, the so-called nematode-trapping or predatory fungi.

The endoparasites do not produce an extensive mycelium, but rather sporulate on the nematode and survive in the soil as conidia. Nematodes are infected after adhering to their surface or after being ingested. In contrast, the nematode-trapping or predatory fungi produce extensive hyphal systems in the soil, trapping nematodes by a variety of structures. The hyphae themselves may be adhesive (mostly in Zygomycetes), or complex structures may develop into three-dimensional adhesive nets, branches, or knobs, or into constricting and non-constricting rings (mostly in Hyphomycetes, Gray, 1988). Irrespective of adhesion to conidia or capture by a trap the subsequent events in the infection process are sometimes immobilization of the nematode, penetration, colonization, possibly toxification, and digestion of the nematode contents (Jansson & Nordbring-Hertz, 1988). Most nematophagous fungi have retained nutritional requirements similar to that of other saprophytic fungi and show nutritional plasticity by switching from predation of nematodes to saprophytism (Gray, 1988).

Arthrobotrys oligospora

Like many other nematophagous fungi, *Arthrobotrys* species are fairly ubiquitous (Gray, 1988). Studying the presence of nematophagous fungi at one location in the Netherlands, van den Boogert (pers. comm.) has shown that *A. oligospora* was the predominant fungus. It has also been isolated from soils in recently reclaimed polder areas (Emden *et al.*, 1969). *Arthrobotrys oligospora* has been isolated from roots of different plants i.e. dwarf bean and barley (Parkinson *et al.*, 1963), tomato (Peterson & Katznelson, 1965), pine apple (Lindford & Yap, 1939) and citrus (Gaspard & Mankau, 1986).

The fungus produces erect conidiophores up to 800 µm in length yielding obovoidal to pyriform conidia. Chlamydospores are present. Generally nematodes are trapped by adhesive networks which are only formed in the presence of nematodes (van Oorschot, 1985).

In the last two decades, many papers have been published on the identification and general biology of nematode-trapping fungi (see for references Stirling, 1991). Most *in vitro* studies on the fungus-nematode infection process have been undertaken with a model system using *Panagrellus redivivus*, a bacteriophagous nematode, and an isolate of the nematode-trapping fungus, *Arthrobotrys oligospora* (Jansson & Nordbring-Hertz, 1980; Tunlid *et al.*, 1992) or *Drechmeria coniospora* (Dijksterhuis, 1993).

A limited number of plant-parasitic nematodes has been studied in relation to capture by *Arthrobotrys oligospora* (Cayrol & Brun, 1975; Jansson & Nordbring-Hertz, 1980). Most work on *Arthrobotrys* spp. and *Meloidogyne* spp. refers to empirical field studies assessing the fungal capacity to effectively control the nematodes. The results vary enormously. Reduction of damage was found in *M. incognita* in corn with *A. conoides* (Al-Hazmi *et al.*, 1982) and in tomato and kiwi with *A. irregularis* (Cayrol & Frankowski, 1980; Cayrol, 1983). *Dactylaria thaumasia* and *A. arthrobotryoides* did not reduce *M. incognita* in tomato or okra (Mankau, 1961). Seedcoating with *A. oligospora* did not control *M. hapla* on tomato and alfalfa either (Townshend *et al.*, 1989).

Interaction between the root-knot nematodes and the fungus

The control of mobile stages of *Meloidogyne* by nematode-capturing fungi primarily depends on the chance of encounters with the infective stage of the fungus. Since the nematode is much more mobile than the fungus, this chance mainly depends on the soil volume that has been colonized by fungal mycelium, the density of traps on the mycelium and the mobility of the nematode. Hence, the ability to produce vegetative hyphae (the saprophytic mode) and the ability to produce traps (the predacious mode) are both essential attributes for the success of nematode-trapping fungi. The saprophytic mode comprises the development of vegetative hyphae in the presence of other micro-organisms (saprophytic competence) as well as in the rhizosphere (rhizosphere competence). The predacious mode includes the development of effective trapping structures on the mycelium. Interestingly, the factors that govern the predacious mode in nematode-trapping fungi are poorly understood (Cooke, 1977).

Many nematophagous species are only able to produce traps in the presence of nematodes (the so-called non-spontaneous trap formers, NSTFs); other fungi can produce traps spontaneously without nematodes, the spontaneous trap formers, STFs (Gray, 1987). In both cases, biotic (age of the fungal colony) and abiotic factors (temperature, nutrient level) influence the transition from a vegetative growth to a trapping mode, the number of traps produced, their structure and their longevity (Nordbring-Hertz, 1987; Grønvold, 1989). Many authors consider saprophytism and predation to be mutually exclusive in fungi that capture nematodes either with adhesive networks, adhesive branches, adhesive knobs, non-constricting or constricting rings (Cooke, 1963a, b, c; Jansson & Nordbring-Hertz, 1979; Jansson, 1982). Spontaneous trap formation seems related to slower vegetative growth, a lower saprophytic ability and a higher ability to attract nematodes in comparison with NSTFs (Cooke 1963a, b, c, 1964; Jansson, 1982).

The present study

Among a range of nematode-trapping fungi screened for their ability to capture root-knot nematodes, was the fungus *Arthrobotrys oligospora* Fres. var. *oligospora* (CBS 289.82).

This isolate differs from most nematophagous fungi in that it captures nematodes with adhesive hyphae, i.e. without the need to form complex trapping devices. This characteristic may make it potentially more useful as a biological control agent.

This study deals with a number of basic questions that need to be answered in view of this possibility. Fundamental studies done concern the attachment of nematodes to fungal mycelium, infection of the nematode and factors that influence these phenomena in relation to the target nematode, *Meloidogyne hapla*.

In chapter 2, the ability to capture root-knot nematodes by direct attachment to unmodified adhesive hyphae is compared to capture by two STFs and four NSTFs including two other isolates of *A. oligospora*.

Chapters 2 and 3 describe light-microscopic studies on nematode-adhesive hypha attachment, focusing on the binding mechanism and the amount of capture sites along the mycelium. An ultrastructural study (transmission electron microscopy) on the nematode-hypha attachment was performed to elucidate more details of the binding mechanism such as the presence of extracellular material on the hyphae of *A. oligospora*.

In chapter 4, nematode capture is studied in *in vitro* experiments with respect to the effect of temperature on the attachment of nematodes to adhesive hyphae of *A. oligospora*, the effect of nutrients and light on the formation of adhesive hyphae and adhesive rings, and the effect of ageing of hyphae on nematode attachment and subsequent ring structure formation and infection.

Chapter 5 describes the capture of live nematodes by the fungus in the presence of an alternative dead substrate.

In chapter 6 results are given on the establishment and the capture ability of *A. oligospora* (CBS 289.82) and three other nematophagous fungi (selected because they vary in trapping strategy), after introduction of mycelial fragments to soil.

For assessing the ability of this adhesive hyphae-forming isolate as a biological control agent, a series of microcosm experiments was conducted in which the

nematophagous fungus was quantified by measuring hyphal lengths. The level of nematode mortality was estimated by counting the number of healthy nematodes and nematode capture was observed directly in the soil.
In chapter 7, nematode-trapping by the adhesive hyphae-forming fungus *A. oligospora* (CBS 289.92) is discussed from the perspective of controlling of root-knot nematodes.

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2

Capture of plant-parasitic nematodes by an adhesive hyphae-forming isolate of *Arthrobotrys oligospora* and some other nematode-trapping fungi*

Abstract

An isolate of *Arthrobotrys oligospora* (CBS 289.82) which forms adhesive hyphae and an isolate of the spontaneous knob-former *Monacrosporium cionopagum* (CBS 228.52) were shown to be the very effective in capturing *Meloidogyne hapla* and *M. incognita* in *in vitro* tests. *Arthrobotrys conoides* (CBS 265.83), *A. dactyloides* (CBS 109.37) and *A. scaphoides* (CBS 226.52) were intermediate in capturing ability. Capture by *A. oligospora* (ATCC 24927), *A. oligospora* (CBS 115.81) and *Duddingtonia flagrans* (CBS 565.50), which did not form traps spontaneously, did not occur irrespective of the age of the mycelium.
However, *A. oligospora* (CBS 289.82) revealed the ability to efficiently attach to root-knot nematodes; attachment to other species e.g. *Globodera pallida* and *G. rostochiensis* was very limited. Internal infection with trophic hyphae only occurred after development of ring structures around the nematode body. Capture efficacy and possible mechanisms are discussed.

Key words: root-knot nematodes, nematophagous fungi, adhesive hyphae, capture ability

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Introduction

Most *in vitro* studies on the successive events of the fungus-nematode infection process have been undertaken on a model system using *Panagrellus redivivus,* a bacteriophagous nematode, and an isolate of the nematode-trapping fungus, *Arthrobotrys oligospora* (Jansson & Nordbring-Hertz, 1980; Tunlid *et al.*, 1992). Some authors have studied the capture of animal-parasitic nematodes (Grønvold, 1989; Murray & Wharton, 1990), mycophagous (Monoson, 1968; Heintz, 1978) or entomophagous nematodes (Poinar & Jansson, 1986). Data on the capture and infection of plant-parasitic nematodes and the physiological and biochemical processes of the fungus-nematode interaction are more limited (Jansson & Nordbring-Hertz, 1980; Tunlid *et al.*, 1992).

Several nematode-capturing fungi seem to be highly specific in the attraction, capture and infection of particular nematode species (Jansson & Nordbring-Hertz, 1980; Esser *et al.*, 1991). Thus results obtained with other than plant-parasitic nematodes may have limited value in predicting capture ability of the latter.

In this paper the hypothesis was tested that an isolate of *A. oligospora,* found to capture nematodes by direct attachment to morphologically unmodified hyphae, captures root-knot nematodes as efficiently as fungi, which spontaneously form traps and that the capture of root-knot nematodes was greater than by fungi which form traps only in the presence of nematodes.

Variability in capturing efficacy needs to be assessed in order to find an effective agent to suppress economically important plant-parasitic nematodes. Trapping ability was firstly compared on agar plates (present study) before soil experiments were conducted. In this study the adhesive hyphae-forming isolate was compared with: *Monacrosporium cionopagum* (CBS 228.52) which spontaneously forms adhesive knobs; *Arthrobotrys dactyloides* (CBS 109.37) which spontaneously forms constricting rings; and four ring-forming fungi which do not form traps spontaneously, *A. conoides* (CBS 265.83), *A. oligospora* (CBS 289.82, CBS 115.81 and ATCC 24927), *A. scaphoides* (CBS 226.52) and *Duddingtonia flagrans* (CBS 565.50).

Also the hypothesis was tested that this adhesive hyphae-forming isolate of *A. oligospora* (CBS 289.82) which readily traps *Meloidogyne hapla* can also capture *Globodera pallida, G. rostochiensis* and *Pratylenchus penetrans.*

Materials and methods

Fungal species and culture methods Stock cultures of *Arthrobotrys conoides* (CBS 265.83), *A. dactyloides* (CBS 109.37, ex nematodes), *A. oligospora* (CBS 289.82, ex *Meloidogyne* sp.), *A. oligospora* CBS 115.81 (=ATCC 24927, ex garden soil, Sweden), *A. oligospora* (ATCC 24927, ex garden soil, Sweden), *A. scaphoides* (CBS 226.52, ex nematodes), *Duddingtonia flagrans* (CBS 565.50) and *Monacrosporium cionopagum* (CBS 228.52) were maintained on corn meal agar (Oxoid, CMA 1:1, 1.5% agar) in Petri-dishes (diameter 88 mm) at 25 ± 1°C, with monthly routine transfers to fresh medium.

Nematodes A population of root-knot nematodes, *M. incognita* originally isolated from tomato plants in Dutch glasshouses, was obtained from the Department of Nematology, Agricultural University Wageningen. *Meloidogyne hapla*, originally isolated from rose plants, was obtained from the DLO-Centre for Plant Breeding and Reproduction Research, Wageningen. Since 1988, both root-knot nematode species have been maintained continuously on tomato plants (*Lycopersicon esculentum* Mill. cv. Moneymaker) in riversand at 22-25°C and 20°C respectively, in a glasshouse.

Newly hatched second-stage juveniles were obtained by incubating egg masses on a 50 µm sieve in water for 2 days at 20°C. The juveniles of *Meloidogyne* spp. were surface sterilized in a mixture of 0.02% (w/v) ethoxy-ethylmercury chloride (Aretan) and 0.1% (w/v) streptomycin sulphate for about 2 h in a 10 ml conical centrifuge tube and subsequently washed three times in sterile water (s'Jacob & van Bezooyen, 1984). Because of possible changes in the nematode surface or activity as a result of the sterilization procedure, which might influence the trapping ability of the fungi tested, in one test non-sterilized and water-washed juveniles of two different populations of *M. hapla* were included (isolated from rose and *Bergenia* sp.).

To ensure purity of cultures, the *Meloidogyne* species were routinely characterized on the basis of enzyme phenotypes of females (Esbenshade & Triantaphyllou, 1985). Difference between species were based on their characteristic esterase, malate dehydrogenase and superoxide dismutase phenotypes.

A population (juveniles and adults) of the root-lesion nematode, *Pratylenchus penetrans* (Cobb), was obtained from the Research Station for Floriculture,

Aalsmeer; second-stage juveniles of the potato-cyst nematodes, *Globodera rostochiensis* (Wollenweber), and *G. pallida* (Stone) pathotypes Ro1 and Pa3, were obtained from a stock culture in our institute.
These three nematode species were surface sterilized in streptomycin-Aretan mixture for 30 minutes in a Büchner funnel and subsequently washed once in 0.1% streptomycin sulphate and three times in sterile water. Contamination by micro-organisms was regularly checked after inoculating nematodes on bouillon broth agar or water agar.

Trapping ability of an adhesive hyphae-forming isolate of *A. oligospora* compared with other fungi from the *Dactylaria*-complex Individual 4-mm plugs cut from the periphery of a growing stock colony of respectively *A. conoides, A. dactyloides, A. oligospora* (CBS 289.82, CBS 115.81, ATCC 24927), *A. scaphoides, D. flagrans* and *M. cionopagum* were placed upside down in the centre of small Petri-dishes (44 mm) on CMA 1:10 (thickness 1-2 mm) and removed several days after inoculation.
The Petri-dishes (Lux) had a coverglass bottom, thus facilitating microscopic observations with an inverted microscope (Zeiss Axiovert 10). Twenty eight day-old fungal colonies were used. In those cases where fungal colonies could not cover the whole agar surface of the Petri-dishes during the incubation period, tissue-culture plates with a smaller diameter than the Petri-dishes (24 wells of 15 mm diameter) were used. They were kept at 25 ± 1°C during the incubation time.
Subsequently, experiments were performed at 25 ± 1°C, 90-100% R.H., pH= 5.5 and without light (Waalwijk *et al.*, 1990). In each test, a drop adjusted to contain about 50 second-stage juveniles of *M. hapla* or *M. incognita*, was added to the fungal cultures. Juveniles moved actively from the inoculation point. At 25°C the mobility of juveniles was about 7.3 mm/h on CMA 1:10, 1.5% agar, so frequent nematode-hypha encounters occurred (den Belder & Jansen, 1994, chapter 4). At regular time intervals, the number of captured nematodes was counted and expressed as a percentage of the number of introduced nematodes. Mobile and immobile captured individuals were counted separately.
Assays usually consisted of three replicate plates and three repetitions in time. Differences in percent capture and immobilization were analyzed for days 1, 2, 6 and 9 by performing one-way analysis of variance (ANOVA) on the arcsin-

transformed values. Differences were further analyzed by comparing the means for the different fungi using Student's t-test.

Because *D. flagrans* and two isolates of *A. oligospora* did not show any ability to capture root-knot nematodes, experiments were repeated with younger fungal colonies (7, 14 and 21 days old). For *A. conoides*, *A. dactyloides* and *A. scaphoides* the tests were repeated with 10 and 17 day-old fungal colonies. Because no differences were found between the colonies with different growth time, results are only given for one set of data.

Capture of four different plant-parasitic nematodes by the adhesive hyphae-forming isolate of *A. oligospora* The ability of *A. oligospora* (CBS 289.82) to capture four different plant-parasitic nematode species, *G. rostochiensis* (Ro1) and *G. pallida* (Pa3), *M. hapla* and *P. penetrans*, was also tested using 28 day-old fungal cultures in small Petri-dishes. In each test, a drop adjusted to contain about 50 nematodes was added to the fungal cultures. Total numbers of captured nematodes and the number of nematodes associated with the ring structures and trophic hyphae were counted.

Observations were made 6 h, 1, 3, 6, 10 and 16 days after the addition of nematodes. Assays usually consisted of 3 replicate plates and the experiment was repeated twice. Differences were analyzed by comparing the means using a generalized linear model (GLM) for binary data (McCullagh & Nelder, 1989) followed by Student's t-test (RPAIR procedure of Genstat 5, Payne *et al.*, 1987).

Capture mechanism in *A. oligospora* After it was demonstrated that nematodes could attach to morphologically unmodified hyphae of *A. oligospora* (CBS 289.82), 30 active second-stage juveniles of *M. hapla* were observed individually on the mycelial mat of a 28 day-old culture from the moment of addition until attachment to hyphae (maximum observation time 45 minutes). The number of nematode-hypha encounters was counted.

To check if nematodes were also attached to newly formed hyphae, 4-mm plugs cut from a stock colony were placed upside down in 24 well-plates on CMA 1:10 and removed 24, 48, 72, 96 h after inoculation.

The area which had been in contact with the mycelium from the plug was removed with a cork borer and subsequently filled with fresh agar to guarantee that nematodes could attach only to newly formed young hyphae. Attachment to the fungus was observed 6 h after addition of about 50 nematodes.

Scanning electron microscopy To ascertain whether ring structures developed at the initial point of attachment, the infection processes were studied on 20 juveniles of *M. hapla*. Pieces of agar (4 mm diameter, and 1-2 mm deep), containing recently attached nematodes were mounted on copper stubs with a thin film of Tissue Tek (Hexland Ltd., England). The samples were immediately frozen by immersion (plunge cooling) in nitrogen slush in the EMSCOPE SP2000 Cryogenic-Preparation System. The stubs were transferred under vacuum to the preparation chamber where surface ice was etched by conductive heating (den Belder *et al.*, 1993). The specimens were then sputtered with gold and transferred to a scanning electron microscope (Jeol JSM 35C) equipped with a cryo-stage. The specimens were photographed at 15 kV accelerating voltage using a Kodak 35-mm Pan X film.
The term ring structures has been used instead of trap structures because in *A. oligospora* (CBS 289.82) three-dimensional structures develop after the nematode has adhered to the hypha.

Saprophytic growth rate An experiment on saprophytic growth rate of several nematode-capturing fungi at different temperatures was performed in Petri-dishes (diameter 88 mm) with those species which had trapped juveniles of *Meloidogyne* spp. in the earlier tests. Plugs of 14 day-old cultures of *A. conoides, A. dactyloides, A. oligospora* (CBS 289.82), *A. scaphoides* and *M. cionopagum* were inoculated on CMA 1:10.
Five Petri-dishes with each species were kept at 5, 10, 15, 20, 25, 30 and 35°C. The average mycelial growth was recorded as the average distance from the centre of the inoculated agar piece to the outer rim of the growing hyphae (at days 3 and 7) and expressed in mm per day.
Saprophytic growth rate was also measured for three isolates of *A. oligospora:* CBS 289.82, CBS 115.81, ATCC 24927 (twelve replicates) at 25°C. Hyphal density (mm^2 mycelium per 100 mm^2 area) was measured in two areas of each of 10 Petri-dishes for each isolate of *A. oligospora* using image analysis (GOP-302, Context Vision). Density was recorded by selecting an optimum threshold value for the contrast line (hypha)-background (agar). The cultures were 28 days old and grown at 25°C (den Belder & Jansen, 1994, chapter 4).

Results

Capture mechanism in *A. oligospora* *Arthrobotrys oligospora* (CBS 289.82) captured nematodes by direct attachment to morphologically unmodified hyphae and to coiled hyphae, at places not markedly differentiated (Figure 2.1A and B).

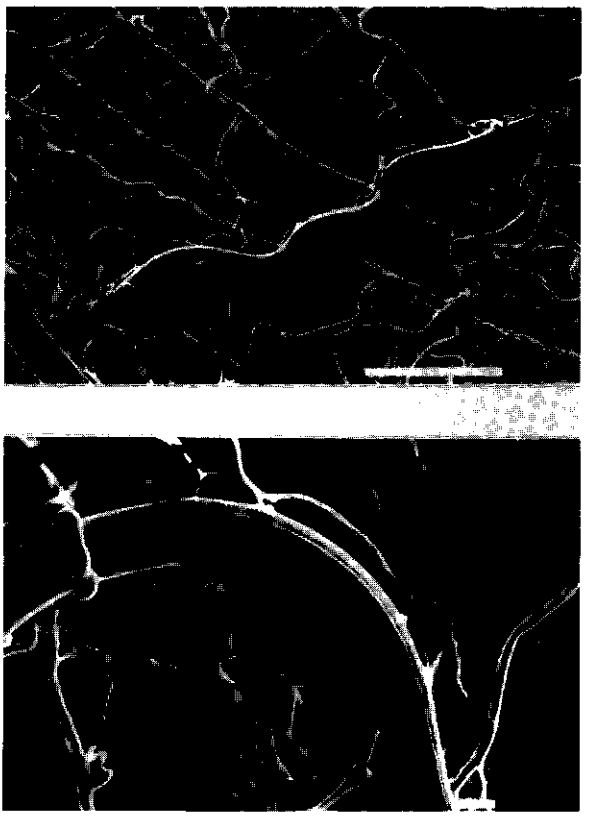


FIGURE 2.1 A-B Cryo-SEM micrographs of second-stage juveniles of *Meloidogyne hapla* captured by means of adhesive hyphae (AH) of *Arthrobotrys oligospora* (CBS 289.82). Multicellular ring structures (RS) develop after attachment of the nematodes to the hypha. They did not necessarily develop at the places of initial attachment.
A: bar = 100 µm; B: bar = 10 µm.

Because this has not previously been described for this species, its identity was confirmed twice by het Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, through identification of conidia developed on nematode-infested cultures. *Arthrobotrys oligospora* (CBS 289.82) attached to almost 45% of the motile juveniles of *M. hapla* during one of the first contacts with a hypha (Figure 2.2). Average nematode-hypha encounters before attachment was about 100; two juveniles remained unattached after 320 encounters with a hypha within the observation period of 45 minutes (Figure 2.2).
Nematodes were also attached to newly formed hyphae (24 h old) and these hyphae were able to produce ring structures around the nematodes.
Following attachment of the nematodes to the hyphae, 50% of the ring structures did not develop at the initial attachment site (Figure 2.1A and B). Development of an infection bulb and of trophic hyphae was dependent on the formation of the ring structure and did not occur if nematodes were only attached to hyphae.

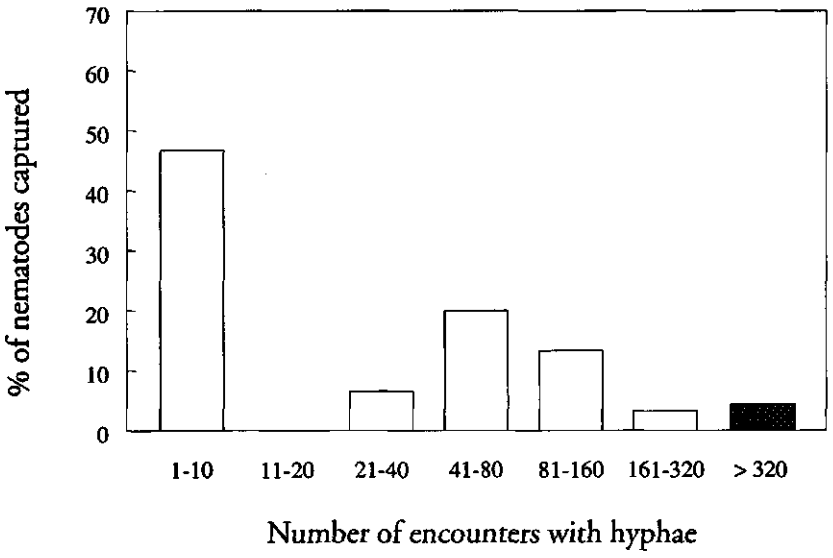


FIGURE 2.2 Capture of second-stage juveniles of *Meloidogyne hapla* by *Arthrobotrys oligospora* (CBS 289.82). Juveniles of *M. hapla* (N=30) were followed individually while moving on the mycelial mat. Number of nematode-hypha encounters was enumerated between start of the experiment and attachment. Open bar: % of nematodes captured by adhesive hyphae; filled bar: % of non captured nematodes.

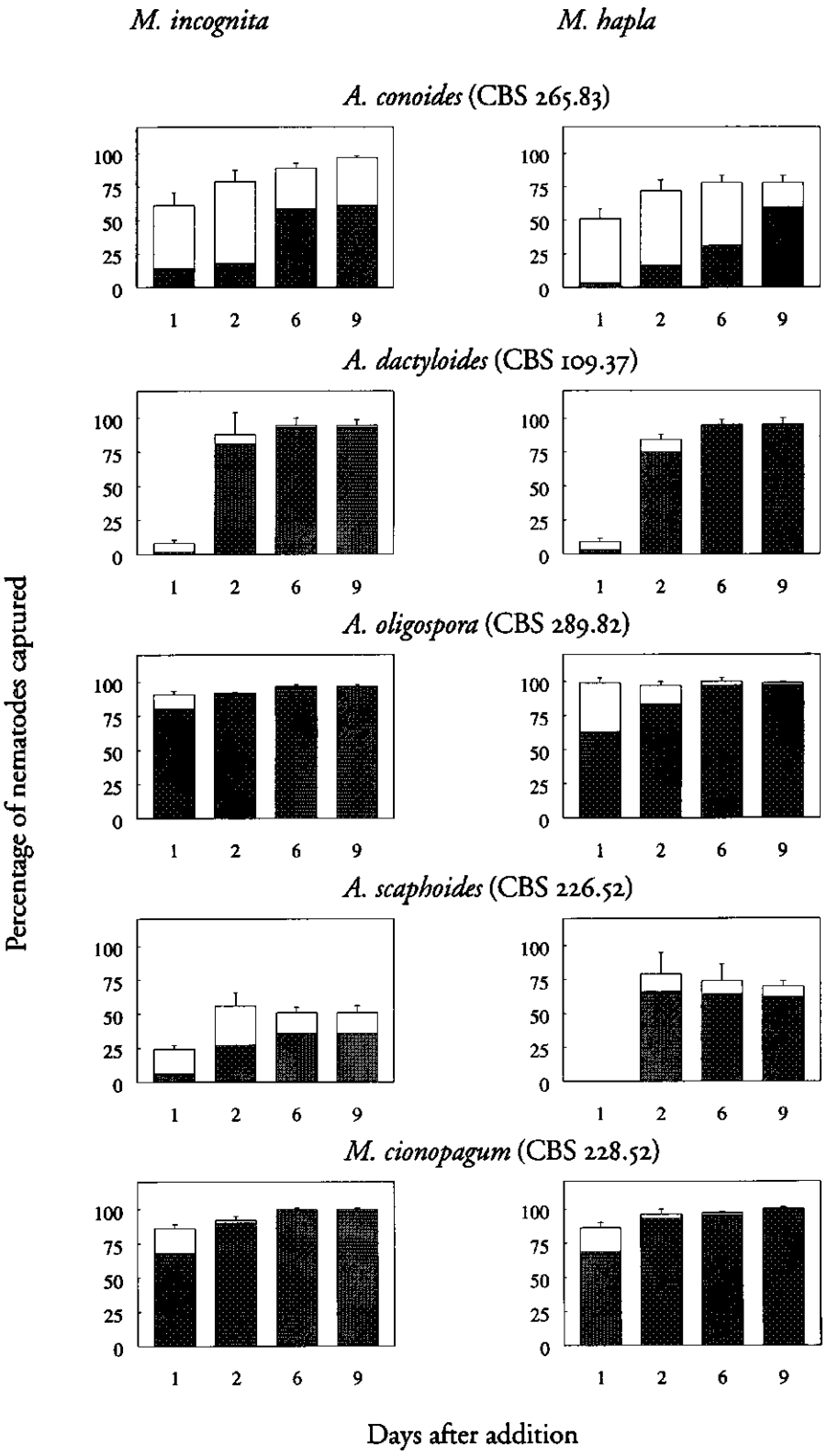
Trapping ability of the adhesive hyphae-forming isolate of *A. oligospora* compared with other fungi from the *Dactylaria*-complex After one day, significant differences could be observed between the five effective fungi both in percentage of nematodes captured ($P<0.001$) and percentage of immobilized nematodes ($P<0.001$, Figure 2.3). *Arthrobotrys oligospora* (CBS 289.82) and *M. cionopagum*, which formed their traps spontaneously, captured more *M. hapla* and *M. incognita* (96 and 86%, respectively) than *A. conoides* (51 and 61%, respectively).

Significantly fewer juveniles of these nematode species were captured by *A. dactyloides* and *A. scaphoides*: 0-25% ($P<0.001$), irrespective of the age of the mycelium and presence of traps. In *A. dactyloides* constricting rings developed spontaneously but abundant ring formation occurred after addition of the nematodes. In *A. scaphoides* adhesive rings were formed only after addition of the nematodes.

Two days after the start of the experiment *A. scaphoides* had caught significantly fewer juveniles of *M. incognita* than the other four fungi ($P<0.01$). The proportion of nematodes captured by the other four fungal species reached a maximum (70-100%) between days 2 and 9. *Arthrobotrys conoides* and *A. scaphoides* were less effective in immobilizing nematodes than the other fungi ($P<0.001$).

Although by day 9, *A. scaphoides* had captured only 51% of the *M. incognita* juveniles, most of them were immobilized, as with the more effective *Meloidogyne*-capturing fungi. In contrast with the other less effective fungus, *A. conoides*, 18% of *M. hapla* juveniles and 37% of *M. incognita* juveniles were still active after 9 days even when ring structures were present around the nematode's body.

FIGURE 2.3 Ability of five species of the *Dactylaria*-complex to capture *Meloidogyne hapla* and *Meloidogyne incognita* on CMA (1:10) at 25°C. Capture by *Arthrobotrys oligospora* (CBS 115.81), *Arthrobotrys oligospora* (ATCC 24927) and *Duddingtonia flagrans* (CBS 565.50) was very limited or did not occur. Entire bars represent % capture and the black lower part the % immobilized nematodes. Bars indicate standard deviation for capture.


M. incognita
M. hapla
A. conoides (CBS 265.83)
A. dactyloides (CBS 109.37)
A. oligospora (CBS 289.82)
A. scaphoides (CBS 226.52)
M. cionopagum (CBS 228.52)
Percentage of nematodes captured
0
25
50
75
100
1
2
6
9
Days after addition

Both *Meloidogyne* spp. were captured with approximately the same efficacy by *A. conoides*, *A. dactyloides*, *A. oligospora* (CBS 289.82), *A. scaphoides* and *M. cionopagum* (Figure 2.3). *Duddingtonia flagrans* failed to capture *M. hapla* and *M. incognita* juveniles, irrespective of the age of the mycelium as no adhesive networks developed.

Induction of capture structures in *A. oligospora* (CBS 115.81) and *A. oligospora* (ATCC 24927) and subsequent capture of *M. hapla* and *M. incognita* was infrequent, which could not be due to the age of the mycelium; when the bacteria-feeding nematode *Panagrellus redivivus* was added to 28 day-old fungal colonies within 24 h ring structures have been developed and almost all nematodes were captured (data unpublished).

The surface sterilisation treatments did not affect trapping in the effective isolate of *A. oligospora* (CBS 289.82); 100% of the nematodes were captured in non-sterilized and in water-washed juveniles as well as in the streptomycin-Aretan treated juveniles.

Capture of four different plant-parasitic nematodes by the adhesive hyphae-forming *A. oligospora* Significant differences were observed in the ability of *A. oligospora* (CBS 289.82) to capture *G. pallida*, *G. rostochiensis*, *M. hapla* and *P. penetrans*, as well as in the formation of ring structures and the development of trophic hyphae in the interior of the various species (Figure 2.4).

Of the four plant-parasitic nematode species tested, all *M. hapla* juveniles were captured within 6 h and those of *P. penetrans* (plus the adults) within 24 h. However, significantly fewer juveniles of *G. pallida* and *G. rostochiensis* were captured, irrespective of their activity in the agar plates. The formation of ring structures around all the nematode species began within 24 h. After one day, 30% of *M. hapla* juveniles had fungal ring structures around the body compared to only 10% in the other nematodes species. After 16 days more than 90% of the captured *M. hapla* juveniles showed one or more ring structures around the body. The remaining 10% of the nematodes remained motionless alongside the hyphae by which they had been captured. However, only 60% of *P. penetrans* individuals were surrounded by ring structures; 30% of those attached continued to wriggle at the place of attachment. With the two *Globodera* species, development of ring structures was delayed; after 16 days only 27% of the nematodes were surrounded by a ring, while trophic hyphae

inside the nematode body had not developed. At day 16, more than 80% of *M. hapla* juveniles were filled with trophic hyphae, only 40% of *P. penetrans* (Figure 2.4). Development of trophic hyphae occurred only when ring structures were present. *Globodera* species were not normally colonised by the fungus under the test conditions.

Saprophytic growth rate and hyphal density of several nematode-capturing fungi *Arthrobotrys conoides, A. oligospora* (CBS 289.82) and *M. cionopagum* grew slowly at 5°C but no growth was observed in the two other species (Table 2.1). There was no growth of any species at 35°C. Radial growth rate optima were at 15-20°C or at 20-25°C. *Arthrobotrys scaphoides*, the least effective of the capturing fungi, was the fastest growing species.

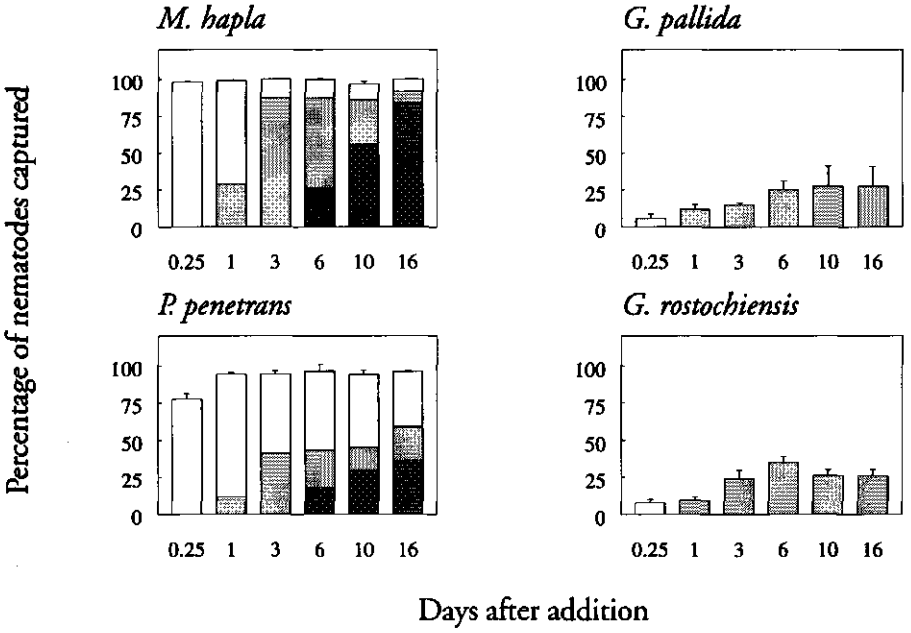


FIGURE 2.4 Ability of *Arthrobotrys oligospora* (CBS 289.82) to capture juveniles of *Meloidogyne hapla, Pratylenchus penetrans, Globodera pallida* (Pa3) and *Globodera rostochiensis* (Ro1). Open bar: % of nematodes captured by adhesive hyphae; dotted bar (light): % of nematodes captured by adhesive hyphae and surrounded by ring structures; dotted bar (dark): % of nematodes captured by adhesive hyphae, surrounded by ring structures and filled with trophic hyphae.

Growth rates of different *A. oligospora* isolates were significantly different: at 25°C; *A. oligospora* (CBS 289.82), the most effective isolate in capturing *Meloidogyne* spp., grew half as fast as the two other isolates on agar plates and hyphal density was also significantly lower (Table 2.2).

TABLE 2.1 *Saprophytic growth rates of nematode-capturing Hyphomycetes (mm/day) on CMA 1:10.*

	A. conoides (CBS 265.83)	*A. dactyloides* (CBS 109.37)	*A. oligospora* (CBS 289.82)	*A. scaphoides* (CBS 226.52)	*M. cionopagum* (CBS 228.52)
Temp. (°C)					
5	0.1 ± 0.1[1]	0	0.1 ± 0.1	0	0.1 ± 0.3
10	0.4 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
15	0.8 ± 0.1	2.3 ± 0.1	0.9 ± 0.4	2.0 ± 0.1	1.7 ± 0.6
20	1.7 ± 0.3	2.3 ± 0.2	2.3 ± 0.1	2.2 ± 0.3	2.1 ± 0.2
25	2.0 ± 0.3	1.7 ± 0.3	2.9 ± 0.1	5.0 ± 0.3	1.5 ± 0.2
30	2.0 ± 0.1	1.5 ± 0.2	2.5 ± 0.2	0.3 ± 0.2	0.3 ± 0.1
35	0	0	0	0	0

[1] Mean ± s.e.

TABLE 2.2 *Saprophytic growth rates (mm per day) and hyphal densities (mm^2 mycelium per 100 mm^2 area) of three isolates of* Arthrobotrys oligospora *on CMA 1:10 at 25°C.*

	Arthrobotrys oligospora		
	CBS 289.82	CBS 115.81	ATCC 24927
Saprophytic growth	2.9 ± 0.2[1] a[2]	4.4 ± 0.3 b	4.3 ± 0.3 b
Hyphal density[3]	21.8 ± 0.4[1] a[2]	23.5 ± 0.6 b	25.4 ± 0.6 c

[1] Mean ± s.e.

[2] Different letters indicate significant differences between isolates ($P<0.001$)

[3] Hyphal density was measured using image analysis

Discussion

In predatory fungi of the *Dactylaria*-complex, specialized capture structures generally are considered a prerequisite for attachment of nematodes (Eren & Pramer, 1978). A trapping strategy in which unmodified hyphae are adhesive, was until now considered to be restricted to the lower non-septate Zygomycetes (Gray, 1988).

The present study clearly demonstrates that *A. oligospora* (CBS 289.82) attaches to nematodes through hyphae without prior formation of complex networks. The possible existence of fungi with septate hyphae (Hyphomycetes) capturing nematodes directly by means of adhesive hyphae was first suggested by Barron (1977). Since then few studies have been published on the capture of nematodes by vegetative hyphae (Barron, 1979; Gray, 1985).

More than 40% of the *Meloidogyne* juveniles attached to hyphae of *A. oligospora* (CBS 289.82) during the first ten contacts with an individual hypha or coiling. Casual contacts between nematode and the networks of *A. oligospora* (ATCC 24927) did not result in immediate capture (Nansen *et al.*, 1988). Possible explanations for this phenomena are 1) the absence/presence of an adhesive on certain cells of the vegetative hyphae, 2) the presence/absence of the secretion of adhesive shortly after contact with the prey (as observed in plant-pathogenic fungi: Hamer *et al.*, 1988; Gubler & Hardham, 1988), 3) the reorganization/activation of polymers at the surface of the fungal hyphae by nematodes as suggested by Tunlid *et al.* (1991) or a combination of the above alternatives. Microscopic observations of hyphae of *A. oligospora* (CBS 289.82) revealed the formation of a characteristic layer between the nematode and the hypha at the site of attachment of this isolate and juveniles of *M. hapla* (den Belder *et al.*, 1994a, chapter 3).

Our observation that nematode-fungus attachment might not be restricted to complex capture structures but may be accomplished by vegetative hyphae that are not visibly differentiated suggests a less clear distinction between saprophytic phase and the predacious phase in comparison to fungi which form adhesive networks, knobs or constricting rings.

Second-stage juveniles attach to the young hyphae, as well as mature hyphae of *A. oligospora* (CBS 289.82) and attachment sites are not necessarily identical

to sites of multicellular ring formation. In many instances, rings around the nematodes developed at some distance from the attachment site.
In assays, *G. pallida, G. rostochiensis, M. hapla* and *P. penetrans* differed greatly in the rate of attachment to the hyphae of *A. oligospora* (CBS 289.82); all nematode species tested were actively moving and frequent nematode-hypha encounters occurred. Differences in induction of trapping sites thus the subsequent capture might be partly explained by differences in locomotive behaviour of the nematodes (Tunlid *et al.*, 1992). Nematode behaviour probably determines frequency and duration of contact with the hyphae and this factor may be decisive for direct attachment, as well as induction of the mechanism of attachment through possible secretion of an adhesive.
However, fungus-nematode contact is not enough to attach nematodes to the fungus in all instances: trap-forming fungi such as *A. dasguptae*, which forms adhesive knobs spontaneously (Boag *et al.*, 1988) and endoparasitic fungi such as *Meria coniospora* (Jansson *et al.*, 1985) show different patterns of adhesion to the cuticles of different nematode species.
Mere adhesion of nematodes to the hyphae of *A. oligospora* (CBS 289.82) is not sufficient for infection of the nematode. Formation of ring structures is a prerequisite for the development of an infection bulb and trophic hyphae. However, the presence of ring structures does not imply subsequent formation of a bulb and trophic hyphae as shown in the attached nematodes of *P. penetrans, G. pallida* and *G. rostochiensis.* Failure to infect the nematodes, demonstrated in some of the experiments discussed above, might be related to the properties of the cuticle, such as its composition and thickness, or to the internal turgor pressure of the nematode (Jansson *et al.*, 1985).

The present study demonstrated that capture of *M. hapla* and *M. incognita* by the adhesive hyphae-forming isolate of *A. oligospora* (CBS 289.82) and *M. cionopagum* (CBS 228.52) was much more effective than that of the other species of the *Dactylaria*-complex tested on agar plates. Our experiments showed evidence of intraspecific variability in the induction of traps of *M. hapla* and *M. incognita* by *A. oligospora:* in 4 and 28 day-old fungal colonies of *A. oligospora* (CBS 115.81) and *A. oligospora* (ATCC 24927) no capture devices were induced (not even after more than a week) by these nematodes, whereas fungal colonies of the same age produced many network traps if nematodes of *P. redivivus* were added.

This same phenomenon has been described for isolates of *A. conoides*, *A. dactyloides* and *A. oligospora*, although no quantitative results were given (von Leuprecht, 1986).

The question arose whether adhesive hyphae of *A. oligospora* (CBS 289.82) would capture nematodes under more natural conditions and at circumstances different from those that induce complex capture structures. Several authors reported that the nematode capturing fungi they studied, including other isolates of *A. oligospora*, did not respond to nematodes at low temperatures (Grønvold, 1989) while our experiments show that *A. oligospora* (CBS 289.82) still forms hyphae at low temperatures. In experiments in agar plates, capture of juveniles of *M. hapla* by *A. oligospora* (CBS 289.82) has been tested while varying several abiotic factors (den Belder & Jansen, 1994, chapter 4). Nevertheless, dependency on special conditions for trap induction on agar may be of less importance in soil (Jaffee *et al.*, 1992).

In experiments in sterilized soil the capture ability of the adhesive hyphae-forming isolate of *A. oligospora* has been compared with several with the above mentioned fungi including an isolate of *A. oligospora* (ATCC 24927), which did not produce capture structures on agar plates when root-knot nematodes were added (den Belder *et al.*, 1994b, chapter 6).

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3

Adhesive hyphae of *Arthrobotrys oligospora*: an ultrastructural study*

Abstract

Electron microscopic studies of the adhesive hyphae-forming isolate of *Arthrobotrys oligospora* (CBS 289.82) and second-stage juveniles of *Meloidogyne hapla* strongly suggest that attachment of nematodes to hyphae was mediated by a layer of extracellular material on the hyphae with a thickness of about 0.1 µm. After attachment this layer was irregularly distributed over the fungal cell surface, in some cases covering it entirely, in others covering only the side of nematode attachment. Serial sectioning of hyphae, adjacent to the site of attachment revealed that the layer could also be present at some distance from the exact site of capture. Such a layer was never observed in thin sections of hyphae of fungal cultures to which no nematodes had been added suggesting that its presence depends on contact with the nematode. At higher magnification the extracellular layer seemed amorphous and were fibrils absent.

Key-words: *Arthrobotrys oligospora*, *Meloidogyne*, adhesive hyphae, TEM, ultrastructure, video-enhanced contrast light microscopy

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Introduction

Strong adhesion to the host surface seems to be a prerequisite for penetration of live nematodes by nematophagous fungi (Nordbring-Hertz & Stålhammar-Carlemalm, 1978).

In Zygomycetes (non-septate fungi), species of the genera *Cystopage* and *Stylopage* catch nematodes by adhesion (Drechsler, 1934). Apparently all parts of the mycelium are capable of capturing nematodes (Gray, 1984). With conventional light microscopy a yellow adhesive substance could readily be observed. Depending on the fungal species it seemed to be either secreted over the entire surface of the hypha or to be only produced at the place of contact with the nematode (Drechsler, 1934; Sachchidananda & Swarup, 1966).

Most of the nematophagous fungi of the *Dactylaria*-complex capture nematodes with specialized capture devices, which spontaneously develop on the vegetative hyphae or in response to nematodes or certain environmental conditions (Nordbring-Hertz, 1977; Gray, 1985; Tunlid *et al.*, 1992). Transmission electron microscopy of captured nematodes revealed a layer bridging fungus and nematode (Nordbring-Hertz & Stålhammar-Carlemalm, 1978; Dowsett & Reid, 1979). Some authors reported the layer to be present before nematodes were attached (Saikawa, 1982), others observed that the stimulus of a nematode was needed (Veenhuis *et al.*, 1985). The layer was composed of a number of radiating fibrils (Saikawa, 1982; Tunlid *et al.*, 1992). So far, only few Hyphomycetes are known to capture nematodes by hyphae: *Arthrobotrys botryospora* (Barron, 1979) and *Monacrosporium psychrophilum* (Gray, 1985) and an isolate of *A. oligospora* (den Belder & Jansen, 1994a, chapter 2). In *A. superba*, especially in very old cultures, nematodes are trapped on hyphae prior to network formation (Fritsch & Lysek, 1989).

Light microscopical studies revealed that within one hour after addition of nematodes, all second-stage juveniles of *Meloidogyne hapla* attach to hyphae of *A. oligospora* (CBS 289.82) irrespective of test temperature (between 5 to 30°C) and irrespective of differences in nematode mobility; varying nutritional conditions did not influence nematode-hypha attachment either (den Belder & Jansen, 1994b, chapter 4).

In the present study we present results from video-enhanced contrast microscopy (Wyss & Zunke, 1986) and ultrastructural observations on the initial stages of nematode-hypha attachment with emphasis on:

- whether a hypha do attach at any point along its length which comes in contact with second-stage juveniles of *M. hapla*
- whether the attachment may be mediated by an extracellular layer
- whether nematodes are required to stimulate its production
- whether its structure is fibrillar.

Materials and methods

Organisms and growth conditions *Arthrobotrys oligospora* (CBS 289.82) was grown on corn meal agar (Oxoid, CMA 1:1, 1.5%) in Petri-dishes (diameter 88 mm) at 25 ± 1°C with monthly routine transfers to fresh medium. Individual 4-mm plugs cut from the periphery of the actively growing stock colony were placed upside down in small Petri- dishes (Lux, diameter 44 mm) on CMA 1:10 (den Belder & Jansen, 1994a, chapter 2). The Petri-dishes used in the experiments described below and subsequently used for microscopical observations have a hole in the bottom lid covered by a coverglass glued to the lid, thus facilitating microscopic observations with an inverted microscope.

Meloidogyne hapla Chitwood, was reared on tomato plants (*Lycopersicon esculentum* Mill. cv. Moneymaker). Second-stage juveniles were harvested and surface sterilized (axenized) as described by den Belder & Jansen (1994a, chapter 2).

Fungus-nematode interaction Studies of the interactions between *A. oligospora* (CBS 289.82) and *M. hapla* were performed in the small Petri-dishes mentioned above. Juveniles were added to a part of the fungal colony (28 days old) at room temperature. In the controls no nematodes were added.

Video-enhanced contrast light microscopy The process of attachment was followed light microscopically with an enhanced contrast video system (Wyss & Zunke, 1986). All observations were performed using the observation chamber described by Wyss (1992). The video-system has been made up with the following elements: Carl Zeiss inverted interference contrast microscope Axiovert 10 with 100x /1.3 N.A. and 40x /0.75 N.A. planneofluar oil immersion

objectives and achromatic, aplanatic oil condenser 1.40 N.A.. There was a connection to a monochrome camera Zeiss/Grundig 76, converted to 960 lines/50 Hz vertical frequency, equipped with a PASECON tube (Heimann XQ 1467), contrast enhancement 1-30x, with remote camera control for brightness, contrast and shading correction. The processes could be followed on a Zeiss/Sony 14 monitor (converted to 960 lines/50 Hz vertical frequency) and were recorded with a Panasonic time lapse S-VHS video recorder model AG-6720A. The temperature at the microscope stage was kept constant at 23 ± 1°C. Nematodes were followed individually.

Transmission electron microscopy To prevent removal of hyphae or attached nematodes during subsequent steps of fixation and embedding, samples were covered with 2% water agar prior to fixation. By pouring liquid agar on the samples, the few non-attached nematodes were removed.
Areas, 4 mm in diameter and 1-2 mm deep, from fungal colonies without or with nematodes, were cut with a sterile agar borer and immersed in 6% (v/v) glutaraldehyde in 0.2 M sodium cacodylate buffer (pH=7.2) for at least 1 h at room temperature, washed in the same buffer (3 times) and subsequently fixed in 1% (w/v) osmium tetroxide in the same buffer, for 1 h at room temperature in the dark and washed in distilled water (3 times). After fixation the samples were dehydrated in a graded ethanol series and embedded in Epon LX 112 or Spurr. Ultrathin serial sections, cut with a diamond knife, were mounted on copper grids (100 mesh, diameter 3 mm) and examined in a Philips EM 400 after post-staining with 2% (w/v) uranylacetate in water and Reynolds lead citrate (0.4 % in 0.1 M sodium hydroxide) for 7 minutes each. The specimens were photographed using a Kodak 35-mm Pan X film.

Results

Video-enhanced contrast light microscopy Second-stage juveniles of *M. hapla* added to the fungal colony firmly adhered to the hyphae. Observations revealed that the hyphae do not attach at any particular point along its length which comes in contact with second-stage juveniles of *M. hapla*. Time between addition of the nematode and attachment varied greatly: in some cases attachment occurred within seconds after addition, at the first contact with the hypha (Figure 3.1). In other cases, the whole nematode moved over a hypha at one and the same place and finally the posterior part of the tail became attached. Sometimes the nematode was moving for more than 30 minutes over the hyphae before attachment occurred.

Also the area over which nematodes became attached varied enormously: the nematode could be caught at the head or tail only, but attachment over a length of more than 50 µm has been observed. In the latter case several fungal cells might be involved.

Both apical cells and those in the middle of a long hypha were seen to attach to nematodes. All fungal cells had in common that attachment to a nematode coincided with intense traffic of cellular components near the point of contact with the nematode. In some cases a hypha holding a nematode cross-linked to another hypha.

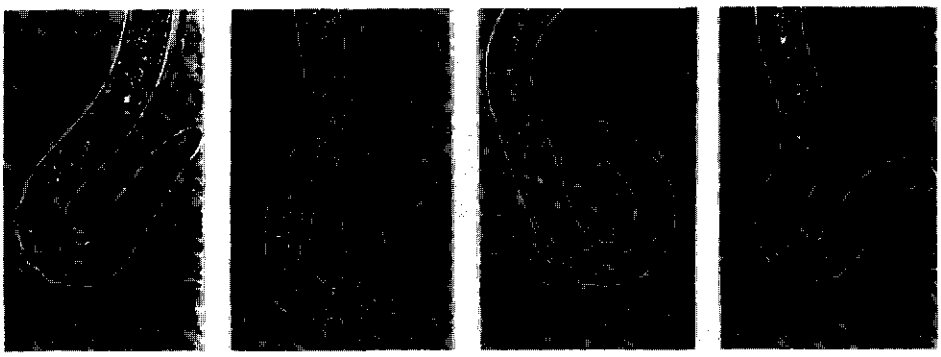


FIGURE 3.1 Captured second-stage juvenile of *Meloidogyne hapla* moving heavily to get free from an adhesive hypha of *Arthrobotrys oligospora* (CBS 289.82). A: fixed point; arrow, place where the hypha sticks to the nematode.

Transmission electron microscopy Ultrathin sections prepared from hyphae of *A. oligospora* (CBS 289.82) of 28 day-old fungal colonies showed organelles such as the nucleus, the endoplasmic reticulum, mitochondria, vacuoles and lipid droplets. No evidence was present for a layer of extracellular material all over the hyphae (Figure 3.2A and B). In some cases multivesicular bodies and microtubuli were visible (not shown). Occasionally, *A. oligospora* (CBS 289.82) produced intrahyphal hyphae (Figure 3.2C).

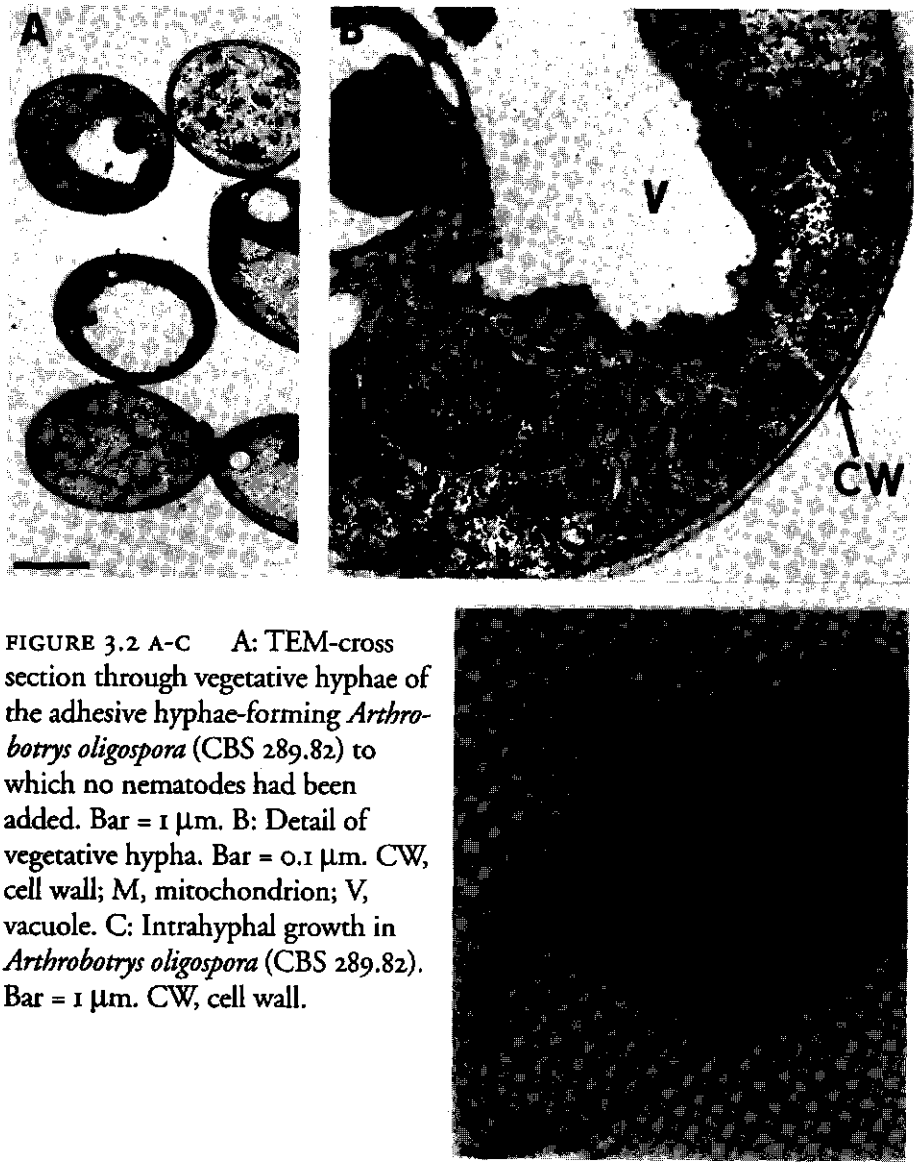


FIGURE 3.2 A-C A: TEM-cross section through vegetative hyphae of the adhesive hyphae-forming *Arthrobotrys oligospora* (CBS 289.82) to which no nematodes had been added. Bar = 1 µm. B: Detail of vegetative hypha. Bar = 0.1 µm. CW, cell wall; M, mitochondrion; V, vacuole. C: Intrahyphal growth in *Arthrobotrys oligospora* (CBS 289.82). Bar = 1 µm. CW, cell wall.

Ultrathin sections of nematodes fixed 1 h after their addition to the fungal colony, showed the presence of a layer of extracellular material between the hypha of *A. oligospora* (CBS 289.82) and the juvenile. The thickness was about 0.1 µm (Figure 3.3A and B). Nematodes could be seen attached at different places along the entire body length, thought attachment often occurred along the lateral fields.

The layer of extracellular material was distributed irregularly on the surface of the hypha. In some cases the entire cell seemed to be covered, in other cases the layer could only be seen at the site of attachment.

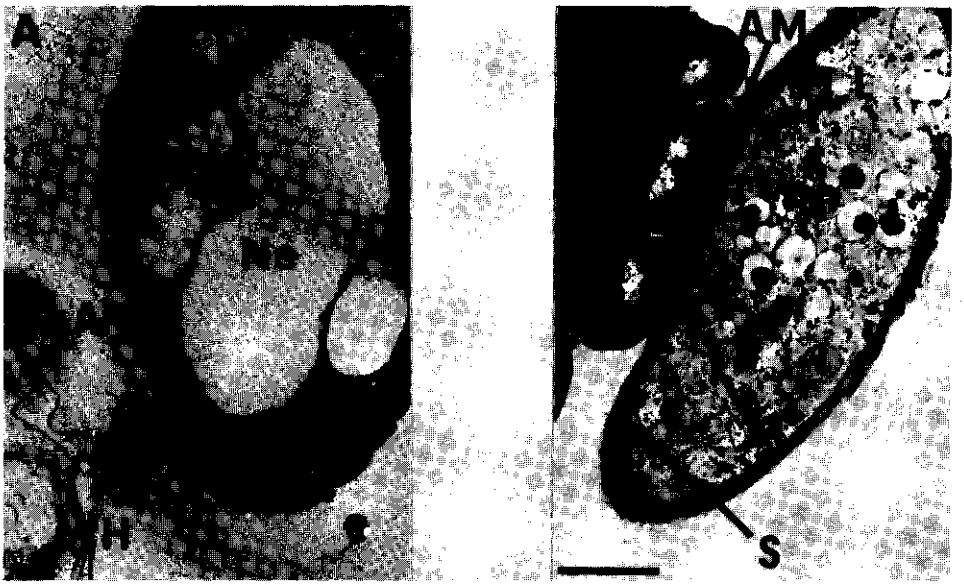
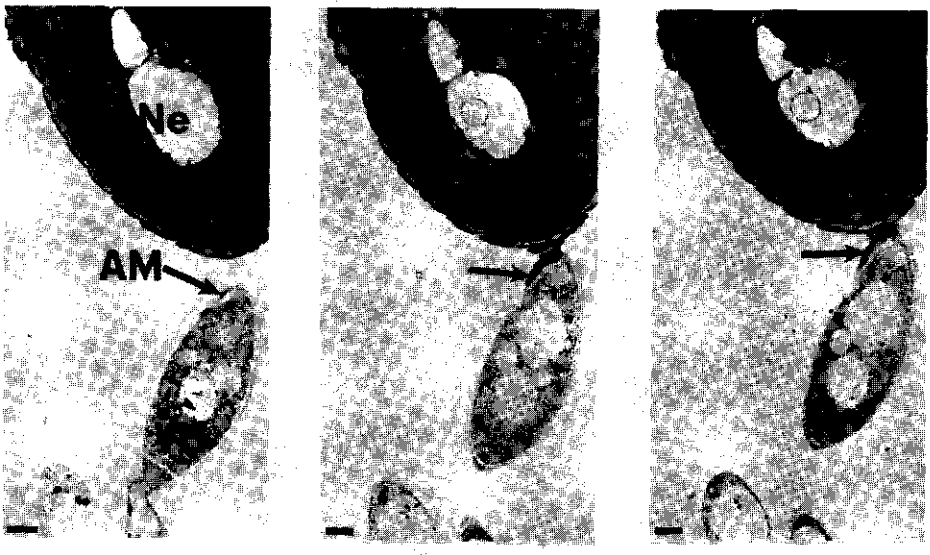


FIGURE 3.3 A-B A: TEM-cross section through a captured second-stage juvenile of *Meloidogyne hapla* attached to a hypha of *Arthrobotrys oligospora* (CBS 289.82) one hour after addition of the nematode. Bar = 1 µm. AM, adhesive material; Ne, nematode; VH, vegetative hypha. B: Idem. Bar = 1 µm. AM, adhesive material; L, lipid; M, mitochondrion; Ne, nematode; S, septum.

Serial sectioning of nematodes attached to hyphae revealed that the extracellular material could be present at some distance from the point of attachment (Figure 3.4 A-C). In fungal colonies to which no nematodes were added such a layer of extracellular material was never observed.
One hour after addition of nematodes no obvious structure was observed within the layer. Also at higher magnifications the layer remained amorphous without evidence of any fibrils (Figure 3.4 D).



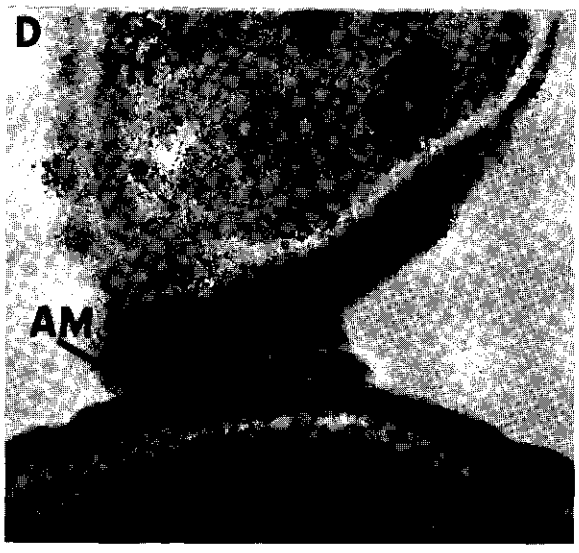


FIGURE 3.4 A-D Micrographs from a series of sections demonstrating that adhesive material can be present on a vegetative hypha on short distance from the exact site of capture of the nematode. Bar = 1 µm. AM, adhesive material; Ne, nematode; VH, vegetative hypha. Arrow, adhesive material. D: Detail. Bar= 1 µm. AM, adhesive material; Ne, nematode; VH, vegetative hypha. Arrow, adhesive material.

Evidence for the presence of numerous electron-dense bodies in the cytoplasm in the hyphae as described by Veenhuis *et al.* (1989) was not obtained. However, in hyphal cells attached to nematodes numerous mitochondria and large vacuoles could be observed frequently. Apart from a layer of extracellular material, size and form of hyphal cells attached to nematodes appeared fairly similar morphologically to hyphae to which no nematodes had been added.

Discussion

Attachment of juveniles to presumably adhesive layers on specialized capture structures has been observed in many nematophagous fungi and consequently adhesion mechanisms in nematophagous fungi have been extensively discussed (Tunlid *et al.*, 1992). Nevertheless the stickiness of non-differentiated hyphae is a largely unexplored area.

Light microscopical observations did not reveal the presence of lumps of adhesive substances as can be seen easily in the Zygomycetes and can have a diameter several times the diameter of the hypha (Drechsler, 1934; Wood, 1983). There is no evidence that all nematophagous fungi employ the same mechanism to bind to nematodes or that several binding mechanisms are not involved concurrently. Until present three types of adhesive mechanisms have been distinguished:

1 extracellular polymers present on trap cells of nematode-trapping fungi, even prior to interaction with nematodes. In some cases fibrils present in this layer are orientated into one direction after contact with the nematode (*A. oligospora* ATCC 24927, Nordbring-Hertz & Stålhammar-Carlemalm, 1978; Dowsett & Reid, 1979; Tunlid *et al.*, 1991a). The origin of this adhesive is still unclear (Tunlid *et al.*, 1992)
2 in other cases a rigid radiated structure in the adhesive layer was already shown prior to the presence of nematodes and does not show a reorganization following attachment to the nematode (in conidia of *Drechmeria coniospora*: Saikawa, 1982; Dijksterhuis *et al.*, 1990)
3 an extracellular layer formed only at the site of contact and not observed before attachment to the nematode (in conidia of *Harposporium subuliforme:* Saikawa & Morikawa, 1985; zoospores of *Catenaria anguillulae:* Tunlid *et al.*, 1991b).

Our studies suggest that attachment of hyphae of *A. oligospora* (CBS 289.82) to second-stage juveniles of *M. hapla* is mediated by a layer of extracellular material. Its thickness (about 0.1 µm) is comparable to similar layers found on other nematophagous fungi (Tunlid *et al.*, 1991a) and less than the lumps of adhesive substances present on hyphae of Zygomycetes (Drechsler, 1934; Wood, 1983).

A layer of this size was never recognized in thin sections of hyphae to which no nematodes had been added, suggesting that its presence depends on contact with the nematode host as in some of the above-mentioned interactions.

Extracellular polymers, exclusively confined to trap cells of the adhesive network-forming fungus *A. oligospora* (ATCC 24927, Nordbring-Hertz & Stålhammar-Carlemalm, 1978; Veenhuis *et al.*, 1985), were recently isolated from vegetative hyphae in *A. oligospora* (ATCC 24927, Tunlid *et al.*, 1991a). These polymers were more loosely packed than the polymers in the layer bridging trap and nematode and they seemed to be distributed unevenly over the surface. It cannot been excluded that at several places of the hyphae of *A. oligospora* (CBS 289.82) very small amounts of such polymers can be found.

Until present, no evidence has been obtained for a fibrillar zone as observed on conidia *D. coniospora* (Dijksterhuis *et al.*, 1990) or on trap cells of *A. oligospora* (ATCC 24927, Nordbring-Hertz & Stålhammar-Carlemalm, 1978).

So far, one hour after attachment there is no evidence for the presence of numerous so-called dense bodies as observed in trap cells in the ring structure forming isolate of *A. oligospora* (ATCC 24927) (Veenhuis *et al.*, 1985, 1989; Dijksterhuis, 1993). Decrease of the number of mitochondria observed in individual mature trap cells (Veenhuis *et al.*, 1984) was not found. On the contrary, cells attached to the nematode cuticle contained many mitochondria.

Even at 5°C all nematodes added to mycelium became attached to it (den Belder & Jansen, 1994b, chapter 4) and time required for attachment may be short: a few seconds after the first contact with a hypha firm binding may occur. This might imply that simple binding with preformed substances, rather than a complex metabolic process is the basis for attachment. This is not exceptional. In encysting *Phytophthora* zoospores only 30-60 seconds are required to deliver the adhesive compound that helps attachment to host surfaces (Gubler & Hardman, 1988). The material is localized in small vesicles in the cell periphery (Bartnicki-Garcia & Sing, 1987). In *Magnaporthe grisea* mucilage is excreted

from a periplasmic deposit at the apex of the spore within several seconds after moisturing because the material is prepacked and no metabolism is required (Hamer *et al.*, 1988).

Any adhesive reaction observed in a simple adhesion assay performed *in vitro* should not automatically be assumed to represent adhesion in the natural environment (Kennedy, 1990). *In situ* observations and experiments in soil have revealed that also under more realistic circumstances the sticky hyphae do catch *M. hapla* (In press, den Belder *et al.*, 1994).

It is clear from our study that a layer of extracellular material with a thickness of about 0.1 µm formed only is present after the nematodes touched the hyphae of *A. oligospora* (CBS 289.82). Elucidation of the underlying mechanisms requires biochemical characterization of the substances involved.

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4

The influence of temperature, nutrition, light and the growth time of the mycelium on capture and infection of *Meloidogyne hapla* by *Arthrobotrys oligospora*[*]

Abstract

An isolate of *Arthrobotrys oligospora* (CBS 289.82) captured the second-stage juveniles of a population of *Meloidogyne hapla* by attachment to its hyphae. The effects of temperature, substrate, light and ageing of fungal mycelium on the ability to capture nematodes was studied in the laboratory. Firm binding of the second-stage juveniles of *M. hapla* to hyphae of *A. oligospora* (CBS 289.82) occurred within one hour irrespective of temperature (between 5 to 30°C) and irrespective of differences in nematode mobility. Subsequent development of ring structures (comparable to adhesive networks) around the nematode, however, was significantly slower at the lower temperatures (5 to 10°C) than at the higher temperatures (15 to 30°C). The colonization of the nematode by trophic hyphae was clearly affected by temperature. At temperatures below 15°C development of trophic hyphae was significantly reduced in comparison to higher temperatures. Furthermore, the nutritional conditions tested did not correlate to the efficacy in nematode-hypha attachment, however the subsequent development of adhesive networks was delayed on water agar.

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* Belder, E. den & Jansen, E. (1994). The influence of temperature, nutrition, light and the growth time of the mycelium on capture and infection of *Meloidogyne hapla* by *Arthrobotrys oligospora*. Fundam. Appl. Nematol. 17, 57-66.

The present results provide evidence that the trapping ability in the isolate tested continues over the whole test period of more than 70 days. Light did not influence the ability of the fungus to capture nematodes.
Our results suggest that the formation of an adhesive hypha is less temperature and nutrient dependent than the development of the morphologically more complex adhesive networks. This observation suggests that the range of conditions under which this isolate of *A. oligospora* captures nematodes is wider than in isolates where adhesive networks are the only capture devices, which makes this isolate interesting for evaluation as a possible control agent.

Key words : *Arthrobotrys oligospora, Meloidogyne,* adhesive hyphae, temperature, nutrient level, light, ageing, capture, trophic hyphae

Introduction

Micro-organisms need to be active in soil conditions which prevail in the field and to survive unfavourable conditions, in order to be effective and widely applicable as biological control agents against soil-inhabiting nematodes (Stirling, 1991).
In former studies it was clearly demonstrated that *A. oligospora* (CBS 289.82) can attach to second-stage juveniles of *Meloidogyne hapla* and *M. incognita* by morphologically unmodified hyphae without the formation of adhesive networks. The chronological stages of the infection process are attachment nematode-hyphae, development of an adhesive network (generally composed by one ring structure if juveniles of *Meloidogyne* spp. are added), penetration and the formation of an infection bulb and subsequently the development of trophic hyphae throughout the nematode body.
Little is known about the influence of abiotic and biotic conditions such as temperature, light, substrate and the age of the mycelium, on the formation of the adhesive hyphae in nematophagous fungi, on subsequent capture and infection of nematodes.
Grønvold (1989) found a significant effect of temperature on the adhesive network development in *Arthrobotrys oligospora* (ATCC 24927): mycelium did not respond to juveniles or responded only slowly with the development of adhesive networks at temperatures below 15°C. Also *Dactylella* spp. captured

larger proportions of nematodes between 20 and 24°C than at lower temperatures (Feder, 1963). The question arises if adhesive hyphae are active in a broader temperature range than adhesive networks.

Generally nutrients available in the environment of the nematophagous fungus have effects on its metabolism and its morphogenesis (Blackburn & Hayes, 1966; Hayes & Blackburn, 1966). *In vitro* studies on the nutrition of nematophagous fungi, including *A. oligospora*, showed that mycelial growth and formation of nematode-induced adhesive networks are differently influenced by the composition of the media and extensive mycelial growth is not always correlated with high predacity. Few adhesive networks are developed in *A. oligospora* (ATCC 24927) on water agar (Nordbring-Hertz, 1977), whereas vegetative hyphae develop normally (Soprunov, 1966) and the question raise if the predacity of adhesive hyphae-forming fungi is affected by composition of the media.

Morphological responses to light have been described for many fungi (Leach, 1971). Grønvold (1989) reported that light suppresses development of adhesive networks in *A. oligospora* (ATCC 24927), whereas Olthof & Estey (1965) did not observe any influence of light on the vegetative growth of another isolate of this fungus.

Loss of virulence of old cultures of nematode-capturing fungi was observed by Couch (1937) and Feder (1963) but details on the age of the fungal colony were not given. Such loss of virulence by nematode-capturing fungi is of special significance because it may limit their usefulness for nematode control. More recent work by Heintz (1978) showed that ageing of mycelium of *A. dactyloides* and *A. cladodes* resulted in a reduction of the ability to capture nematodes. Loss of adhesiveness of adhesive networks of *Dactylella megalospora* was found within seven days (Esser *et al.*, 1991), while in adhesive networks of *A. oligospora* (ATCC 24927) this occurred within seven weeks, depending on temperature (Grønvold, 1989).

In the present paper the hypothesis has been tested that:

1 attachment of *Meloidogyne* sp. to adhesive hyphae of *Arthrobotrys oligospora* (CBS 289.82) is less temperature- dependent than the development of the adhesive networks themselves
2 adhesive hyphae can be developed on a nutrient lower culture medium than adhesive networks

3 light suppresses the formation of adhesive hyphae less than that of adhesive networks and
4 ageing of the fungal mycelium results in a reduction of attachment of nematodes to adhesive hyphae and subsequent ring structure formation and infection of nematodes.

Materials and methods

Organisms *Arthrobotrys oligospora* (CBS 289.82) was cultured on corn meal agar (Oxoid, CMA 1:1, 1.5%) in Petri-dishes (diameter 88 mm) at 25 ± 1°C, and transferred monthly to fresh medium. In experiments described below, generally the fungus was transferred to small Petri-dishes (Lux, diameter 44 mm) on CMA 1:10 and removed a few days after incubation (den Belder & Jansen, 1994, chapter 2).
Meloidogyne hapla Chitwood originally isolated from rose plants, was obtained from the DLO-Centre for Plant Breeding and Reproduction Research, Wageningen. Since 1988, this species was maintained continuously on tomato plants (*Lycopersicon esculentum* Mill. cv. Moneymaker) in riversand at a temperature of 20°C in a greenhouse. Newly hatched second-stage juveniles were obtained by incubating egg masses on a 50 µm sieve in water for 2 days at 20°C. The outer surface of 2-day old juveniles of *Meloidogyne* spp. was sterilized in a mixture of 0.02% (w/v) ethoxy-ethylmercury chloride (Aretan) and 0.1% (w/v) streptomycin sulphate for about 2 h in a 10 ml conical centrifuge tube and subsequently washed three times in sterile water (s'Jacob & van Bezooyen, 1984). The juveniles had been acclimatized to the various test temperatures for 2 days.
Capture ability of the fungus and subsequent infection of nematodes were assessed after addition of a drop containing about 50 axenic second-stage juveniles of *M. hapla* to each fungal colony.
All experiments were performed with *A. oligospora* (CBS 289.82) unless indicated otherwise.

Temperature experiments The conditions during incubation and testing in the various temperature experiments are summarized (Table 4.1). Twenty-

eight or 42 day-old cultures of *A. oligospora* grown at 25°C (Experiments 1 and 2 respectively) were placed at each of the following constant temperatures, 48 h prior to the addition of nematodes: 5, 10, 15, 20, 25, 30°C (and also 35°C in Experiment 2) and tested at those temperatures. In Experiment 3, 78 day-old fungal cultures, grown for 42 days at 25°C and subsequently 36 days at 5, 10 or 15°C, were tested while in Experiment 4 and 5 the cultures were grown and tested at 5, 10 and 15°C

TABLE 4.1 *Experimental conditions of Temperature Experiments 1 to 5.*

	time of culturing fungus before experiment (days)	temperature during culturing before experiment (°C)	test temperature (°C)
Exp. 1	28	25	5, 10, 15, 20, 25, 30
Exp. 2	42	25	5, 10, 15, 20, 25, 30, 35
Exp. 3	42	25	
	+36	5, 10, 15	5, 10, 15
Exp. 4	42	5, 10, 15	5, 10, 15
Exp. 5	28	15	5, 10, 15

In Experiments 1, 2 and 3 observations on numbers of juveniles captured by hyphae and on those surrounded by adhesive networks and filled with trophic hyphae started at day 1 and were repeated several times during one week. As in interactions with *Meloidogyne* spp. juveniles, this network consisted of only one adhesive ring, the term 'ring structures' has been used. Nevertheless this ring structure is the most simple form of an adhesive network as found in other species of the *Dactylaria*-complex.

Numbers were expressed as a percentage of the total number of nematodes counted immediately after the start of the experiment (at the start of the experiments the number of dead nematodes was nihil). In Experiment 4 observations on capture started 6 h after addition of the juveniles. In Experiment 5 observations commenced 1 h following addition of the juveniles.

At the end of each experiment the mycelial mat was examined for any morphological changes.

The proportion of nematodes captured by hyphae, surrounded by ring structures and filled with trophic hyphae were analyzed with a generalized linear model (GLM) for binary data (McCullagh & Nelder, 1989), leading to an analysis of deviance for quantal data and subjected to a Student's t-test for pairwise comparison of treatments on a logit-scale. This was only possible if the mean proportions were not equal to 0 or 1. For these means significance was obtained by considering confidence intervals. Analysis were carried out with Genstat (Payne *et al.*, 1987).

In addition to the variables mentioned above, in Experiment 1 and 2 the colonization of each nematode was also determined at regular intervals (day 1, 2, 5, 8, 12, 15, 21, 27, 36, 44, 63) and expressed as a percentage of body length filled with trophic hyphae. Five classes were distinguished: 0 (no visible trophic hyphae), 1-25, 25- 50, 50-75 and 75-100% body length filled with hyphae. The products of the total percentage counts in each of the groups and the midpoints of these groups was summed to give a percentage estimate of the body length filled with trophic hyphae. The technique is based on the assumption that the presence of trophic hyphae is indicative of colonization. T_{50} and T_{95} (days after addition, 50% and 95% of the nematode body length was filled with trophic hyphae) were calculated under the assumption of logistic growth increase of trophic hyphae filled body length. T_{50} and T_{95} were estimated for each Petri-dish (containing about 50 nematodes) and subsequently analyzed by analysis of variance followed by Student's t-test for pairwise comparison of treatments. Because both experiments showed the same trends results are given for only one experiment.

Effect of temperature on activity of second-stage juveniles of *M. hapla*

Nematode activity was measured in order to analyze whether any temperature effects on the capture of nematodes was due to the ability of the fungus to capture juveniles of *M. hapla* or to the activity of the juveniles.

Fifty axenic second-stage juveniles of *M. hapla* (from the same batch as in Experiment 2) were inoculated in the centre of a Petri-dish with CMA 1:10, 1.5% agar. Two hours after introduction, nematode mobility was assessed by counting the number of juveniles in four concentric zones of 5 mm from the inoculation point. Tests were performed at 5, 10, 15, 20, 25, 30 and 35°C.

For each temperature an average mobility was calculated using the following formula:

$$M = \sum_{i=0}^{i=3} f \,.\, Di$$

f = fraction of nematodes present in area i
Di = average distance (mm) from centre for area i

i=0	$0 < r \leq 5$ mm	Di= 2.5 mm
i=1	$5 < r \leq 10$ mm	Di= 7.5 mm
i=2	$10 < r \leq 15$ mm	Di=12.5 mm
i=3	$15 < r \leq$ rim Petri-dish	Di=17.5 mm

The nematode mobility for different temperatures was analyzed by using one-way ANOVA, followed by a Student's t-test for comparison of means.

Nutrient experiment The fungus was grown on different substrates: water agar 1.5%, corn meal agar (CMA 1:10) and a low nutrient mineral salts medium with or without 200 µgr thiamin/litre and 5 µgr biotin/litre (LNM+ and LNM- respectively, Nordbring-Hertz, 1973). Seven day and 28 day-old fungal cultures of *A. oligospora* grown at 25°C, were inoculated with 50 axenic second-stage juveniles of *M. hapla* at 25°C. Observations on number of attached and infected nematodes were made 1 and 6 h and 1, 2, 6 and 16 days after addition of nematodes. The average number of ring structures around the attached nematodes was counted one day after the start of the experiment. Each treatment consisted of three replicates and the experiment was repeated three times.
The number of captured nematodes, nematodes surrounded with ring structures and nematodes with trophic hyphae were analyzed as described for the temperature experiments.
In order to analyze if differences in capture of nematodes were due to a higher frequency of nematodes encountering hyphae and or ring structures, mycelium growth, hyphal density and number of spontaneous ring structures (freely formed and not surrounding nematodes) were determined. Average mycelial growth rate (mm/day) was recorded as the average distance from the inoculated

agar piece to the outer rim of the growing hyphae (at day 3 and 5 in 10 replicates). The hyphal density, expressed as the area of mycelium mm^2 per 100 mm^2 was measured in two areas (each 50 mm^2) in each of 10 Petri-dishes, using the image analyzer GOP-302 (Context Vision). The advantage of the used hardware was, that beside conventional grey level thresholding, structures could be detected by texture analysis. The actual detection of the hyphae was done with a line detection operation, which generated an output image in which the grey level intensity represented the amount of estimated line energy. By thresholding a certain grey level range in the output image, a selection of the most dominant lines could be made. So by variation of the lower threshold level a selection could be made of the hyphae on the surface or the hyphae growing through the agar. Because hyphae grown in the agar were a minor part of the total amount and did not influence the total density significantly, data are only given for those hyphae growing on the agar. The areas in which the hyphae density was measured were selected by a computer controlled scanning stage on equal distance (1 cm) left and right from the initial inoculation point with the plug. For each nutrient level in 28 day-old fungal colonies the hyphal density was estimated.

Ring structure formation was observed one day before and one day after addition of nematodes, by counting the number of ring structures in 20 fields (at 200x magnification).

The effects of the growth time of the fungal mycelium on the capture and infection of nematodes were conducted as parts of the experiments on the temperature and nutrient level by using fungal cultures varying in growth time.

Light experiment Twenty-eight day-old cultures of *A. oligospora* were inoculated with 50 axenic second-stage juveniles of *M. hapla* and incubated at 25°C. Three Petri-dishes were placed in the dark and three Petri-dishes were incubated under constant artificial light (Philips Pls lamp 11 Watt, wavelength 310-765 nm, 8.6 $J/cm^2/h$). Numbers of nematodes captured by hyphae, surrounded by ring structures and filled with trophic hyphae were counted at regular intervals (day 1, 2, 4, 7, 9, 11, 14, 23). The number of ring structures surrounding juveniles was counted 3 and 13 days after the start of the experiment. On each Petri-dish ten randomly selected fields of sight were examined (at 200x magnification). Only completely closed ring structures were enumerated.

In one experiment two isolates of *A. oligospora* (ATCC 24927 and CBS 115.81) have been included under the same experimental conditions.

Results

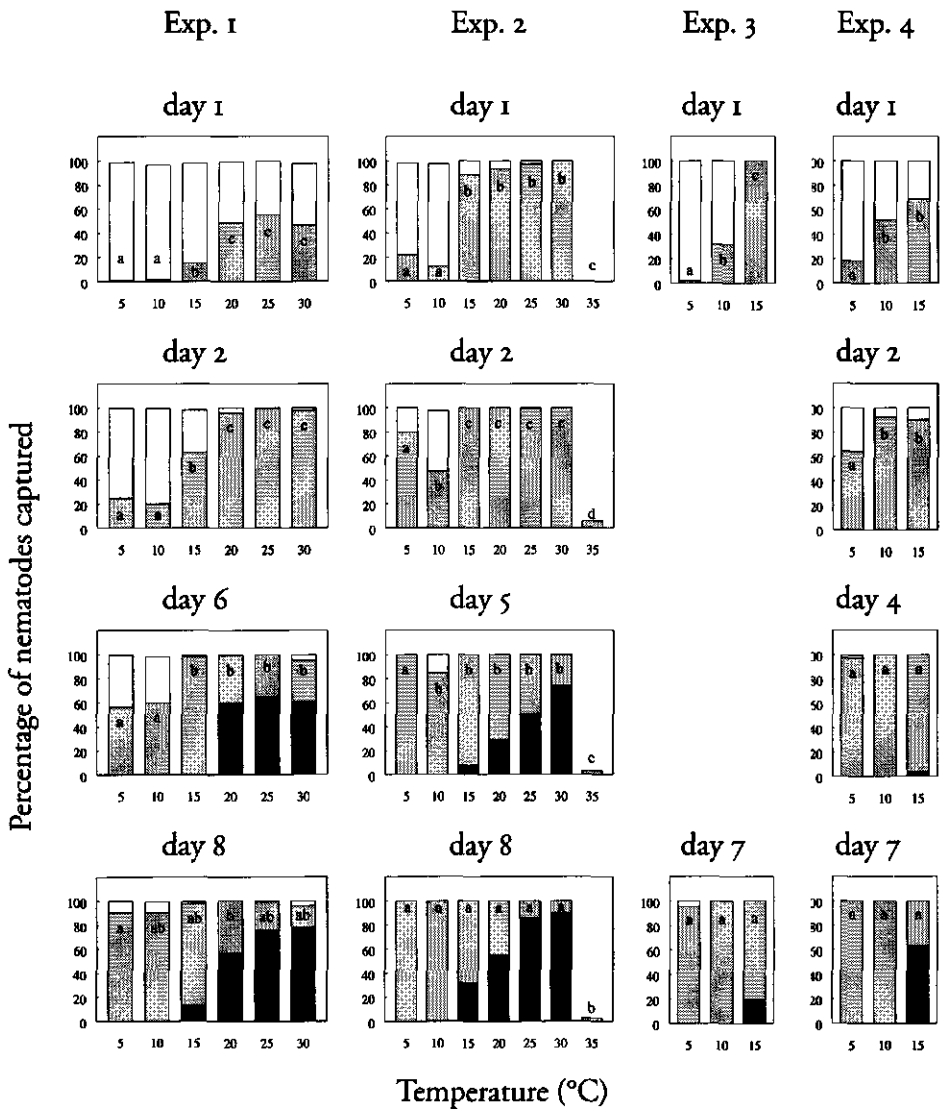
Temperature

Effect of temperature on nematode-hypha attachment At the first observation 24 h after addition of the nematodes, all second-stage juveniles of *M. hapla* were captured by the hyphae of 28 day-old cultures of *A. oligospora* at all temperatures below 35°C (Experiment 1 and 2, Figure 4.1). Nematodes became attached at any part of the body. At 35°C, hyphae failed to attach to the nematodes. Mycelium of *A. oligospora* grown under suboptimal temperatures for vegetative growth (Experiment 3 and 4, Figure 4.1), did not show any decline in nematode attachment compared to hyphae grown at the optimum temperature for vegetative growth (Experiments 1 and 2, Figure 4.1). In experiment 4, all nematodes were captured at the first observation after six hours, irrespective of the temperatures tested. At day 1 the development of the ring structures was started in all temperatures tested but was significantly lower at 5°C (Figure 4.1). Immediate observations one hour after the start of Experiment 5 showed that all nematodes were captured at the temperatures tested.

Effect of temperature on ring structure development Subsequent development of ring structures around the nematodes differed significantly between tested temperatures during the first six days after capture (Experiment 1, Figure 4.1). At 5 and 10°C only 1 and 2% of the nematodes became surrounded, at 15°C the number of nematodes surrounded by ring structures was 15% and at 20, 25 and 30°C this number was significantly higher. At day 2 these differences were still significant although less pronounced. At 5 and 10°C the number of surrounded nematodes reached only 20%, at 15°C this number reached about 60% and at the three highest temperatures tested, the proportion of nematodes surrounded by ring structures reached almost 100%. At day 8 at all temperatures the ring structure development was 90% or higher. In the second temperature experiment the development of ring structures around nematodes progressed more rapidly (Figure 4.1). After one day, almost all nematodes were surrounded by

ring structures at 15°C, whereas at 5 and 10°C these numbers were less than 40%. At days 7 and 8 all nematodes were surrounded at all temperatures ≤ 30°C even when the mycelium was grown at low temperatures (Experiment 3 and 4, Figure 4.1).

FIGURE 4.1 Ability of *Arthrobotrys oligospora* (CBS 289.82) to capture and infect second-stage juveniles of *Meloidogyne hapla* at different temperatures. Exp. 1 carried out with 28 day-old cultures at 25°C followed by two days at the test temperatures. Exp. 2 as Exp. 1 but with 42 day-old cultures. Exp. 3 carried out with a culture grown for 42 days at 25°C and followed by 36 days at the test temperatures. Exp. 4 carried out with a cultures grown for 42 days at the experimental temperatures. Open bar: % of nematodes captured by adhesive hyphae; dotted bar: % of nematodes captured by adhesive hyphae and surrounded by ring structures; filled bar: % of nematodes captured by adhesive hyphae, surrounded by ring structures and at least filled with trophic hyphae. Different letters indicate significant differences between means of % of juveniles surrounded by ring structures within each experiment and each day ($P<0.05$).


Exp. 1
Exp. 2
Exp. 3
Exp. 4
day 1
day 1
day 1
day 1
day 2
day 2
day 2
day 6
day 5
day 4
day 8
day 8
day 7
day 7
Percentage of nematodes captured
Temperature (°C)

Effect of temperature on trophic hyphae development Development of trophic hyphae in the nematode body (Figure 4.2) was significantly influenced by temperature (Figure 4.3).

FIGURE 4.2 Trophic hyphae (TH) of *Arthrobotrys oligospora* (CBS 289.82) in a typical wave form in a second-stage juvenile of *Meloidogyne hapla.*

This resulted in significant differences in the number of days after which 50% or 95% of the nematode body length was filled with hyphae (Figure 4.3, Table 4.2). In mycelium of *A. oligospora*, grown or kept under suboptimal conditions for vegetative growth (Experiment 3 and 4), development of trophic hyphae was not altered in comparison to the earlier experiments (Figure 4.1).

Forty-two day-old cultures of *A. oligospora* did not show a decline in capture and infection of nematodes in comparison to 28 day-old cultures (Experiment 2 and 1 respectively, Figure 4.1).

Capture ability of *A. oligospora* and subsequent infection by trophic hyphae did not show any decline after keeping mycelium at low temperatures during a prolonged period of time (Experiment 3, 78 days: 42 days at 25°C and subsequently 36 days at 5, 10 or 15°C; Experiment 4, 42 days at 5, 10 or 15°C). In this respect there was no significant difference with Experiment 1. In none of the experiments morphologically aberrant hyphae were found in the time course of the experiments.

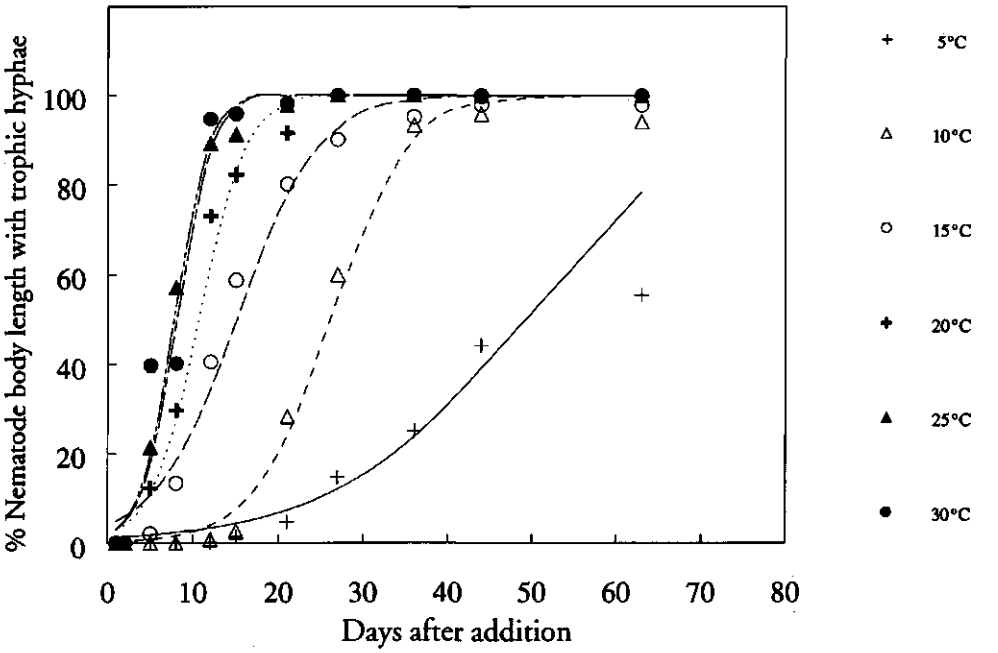


FIGURE 4.3 Development of trophic hyphae of *Arthrobotrys oligospora* (CBS 289.82) inside *Meloidogyne hapla* at different temperatures. Trophic hyphae development was quantified through estimation of the percentage of the nematode body length filled with trophic hyphae. Observed values are in markers, calculated values (Fieller-procedure in Genstat 5) in lines.

TABLE 4.2 *Development of trophic hyphae of* Arthrobotrys oligospora *(CBS 289.82) in second-stage juveniles of* Meloidogyne hapla *at different temperatures, expressed in days after addition when 50% and 95% of the nematode body length was filled with trophic hyphae (T_{50} and T_{95}).*

Temperature (°C)	T_{50}		T_{95}	
5	47.6 ± 3.8[1]	a[2]	77.2 ± 8.9	a
10	24.8 ± 1.0	b	35.4 ± 4.7	b
15	15.1 ± 1.8	c	28.3 ± 2.8	bc
20	10.6 ± 0.8	cd	18.5 ± 0.3	cd
25	7.9 ± 0.3	d	13.4 ± 1.1	d
30	7.8 ± 0.9	d	13.5 ± 0.7	d
35	∞	n.t.	∞	n.t.

1 Mean ± s.e.

2 T_{50} and T_{95} were estimated for each Petri-dish and subsequently tested by analysis of variance followed by Student's t-test for pairwise comparison of treatments. Different letters indicate significant differences between means in the column ($P<0.05$)
n.t.: Not tested

Nematode mobility in response to temperature All tested juveniles of *M. hapla* dispersed over the agar. The nematode activity is shown in Figure 4.4. Mobility differed significantly between the temperatures tested: at 25°C the highest mobility was reached, 7.3 mm/h, while at 5°C a mobility of 2.2 mm/h was observed.

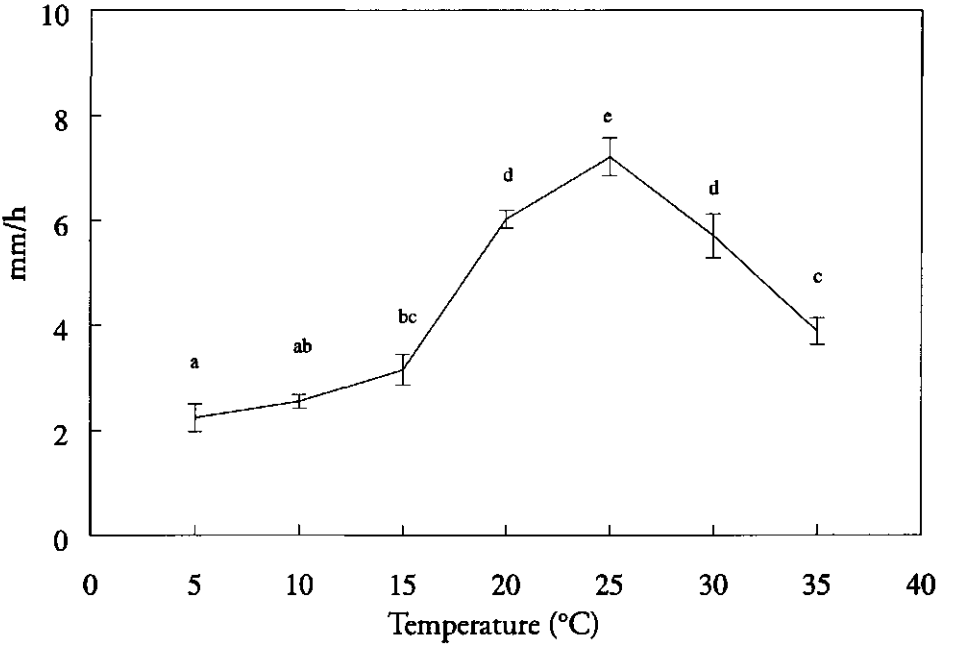


FIGURE 4.4 Mobility of second-stage juveniles of *Meloidogyne hapla* on CMA (1:10, 1.5% agar) at different temperatures. Different letters indicate significant differences between means (analysis of variance followed by a Student's t-test, $P<0.05$).

Effect of growth substrates on capture ability The number of nematodes surrounded by ring structures and number of nematodes with trophic hyphae was affected by the different substrates (Figure 4.5). Within one hour following addition of nematodes, all second-stage juveniles of *M. hapla* were captured by the hyphae of *A. oligospora*, irrespective of differences in hyphal density on the different media (Table 4.3). One day after the start of the experiment a higher percentage of nematodes was surrounded by ring structures on LNM+ (76%) as compared to LNM- and CMA 1:10 (61 and 63%, respectively), while a

significantly fewer juveniles were surrounded by ring structures (39%) on WA. After two days this percentage increased to 84% on WA and to about 97% on the other substrates. The number of ring structures around the nematodes was significantly larger on LNM+ and LNM-, than either on water agar or corn meal agar (Table 4.3).

At day 6 and 14 the development of trophic hyphae in the interior of the nematode body reached about 55% and 85% in LNM-, CMA and LNM+. On WA these percentages were significantly lower: in only 25% and 50 % of the nematodes trophic hyphae developed.

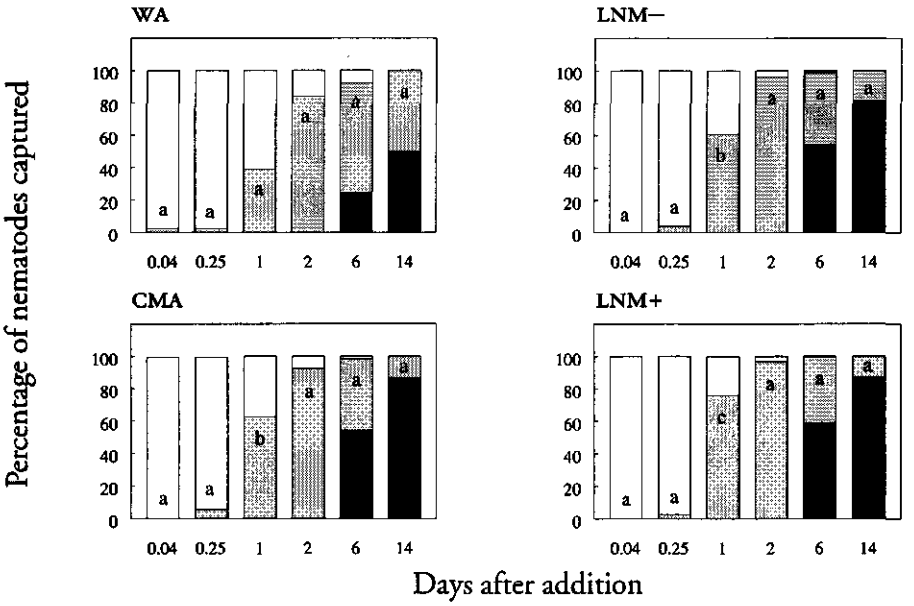


FIGURE 4.5 Ability of *Arthrobotrys oligospora* (CBS 289.82) to capture and infect second-stage juveniles of *Meloidogyne hapla* on different substrates; WA: water agar 1.5%, CMA: corn meal agar 1:10, LNM+ and LNM-: low nutrient mineral salts media respectively with and without 200 µgr/litre thiamin and 5 µgr biotin/litre.

Open bar: % of nematodes captured by adhesive hyphae; dotted bar: % of nematodes captured by adhesive hyphae and surrounded by ring structures; filled bar: % of nematodes captured by adhesive hyphae, surrounded by ring structures and filled at least partly with trophic hyphae. Different letters indicate significant differences between means of % of juveniles surrounded by ring structures within each day ($P<0.05$).

TABLE 4.3 *Growth rate, mycelial density and formation of ring structures in* Arthrobotrys oligospora *(CBS 289.82) on different substrates before and after addition of second-stage juveniles of* Meloidogyne hapla.

Medium		Vegetative hyphae		Number of ring structures		
	pH	Growth[1]	Density[2]	Before addition of *M. hapla*[3]	After addition of *M. hapla*[3]	Number of ring structures around *M. hapla*[4]
1.5% water agar	5.5	2.7 ± 0.1 a[5]	23.2 ± 1.5[5] a	5	4	2.8 ± 1.3[5] a
LNM–[6]	6.5	3.1 ± 0.1 b	19.9 ± 2.8 b	3	2	4.0 ± 1.8 b
LNM+[6]	6.5	3.4 ± 0.1 c	20.7 ± 2.6 bc	2	2	4.6 ± 1.6 b
CMA 1:10	5.5	2.9 ± 0.1 ab	21.5 ± 1.5 c	1	1	2.5 ± 1.4 a

[1] Radial growth: mm/day

[2] Mycelial density: mm^2 mycelium per 100 mm^2 in 28 day-old fungal colonies

[3] Number of ring structures in 20 fields of sight (at 200x magnification)

[4] Number of ring structures around one juvenile one day after addition of the nematodes

[5] Mean ± s.e. Different letters indicate significant differences (Student's t-test, $P<0.05$)

[6] Low nutrient mineral salts medium with(+) and without(-) thiamin and biotin

Effect of light on capture ability As the results of all treatments in each of three experiments on the influence of light (8.6 J/cm^2/h, 310-765 nm) on attachment of juveniles to hyphae, development of ring structures and trophic hyphae were alike, detailed results are not given. Light did not influence the number of nematodes captured by *A.oligospora* by adhesive hyphae, nor did have any significant effect on the number of ring structures induced or the development of trophic hyphae in nematodes. At day 1 all nematodes already attached to the hyphae, while over 60% of nematodes had been surrounded by ring structures. The number of ring structures surrounding juveniles (at 3 and 13 days after start of the experiments), was not influenced significantly by light (at day 3: 3.3 and 3.6 and at day 13, 3.6 and 3.7 respectively). The first trophic hyphae were observed at day 4, irrespective light or dark regime and their growth was similar in both treatments.

Discussion

Part of research on nematode-capturing fungi has focused on the transition from saprophytic to predacious behaviour and factors that induce trap formation. For many species trap structure development appears conditioned by environmental factors.

The observation that nematode-fungus attachment is not only realized by complex capture structures but can also be accomplished by not visibly differentiated vegetative hyphae (den Belder & Jansen, 1994, chapter 2) raised the question whether adhesive hyphae are active in a temperature range or under nutrient conditions different from those that induce formation of adhesive networks.

The present work demonstrated that while temperatures between 5 and 30°C clearly affected ring structure development, attachment of nematodes to hyphae of *A. oligospora* (CBS 289.82) was not affected. All active second-stage juveniles of *M. hapla* were attached to hyphae within a very short time after addition to fungal colonies, irrespective of the temperature even though the nematode mobility at 5°C is one third of that at 25°C. At the first observation, one hour after the addition of the nematodes, all juveniles observed were already attached to the hyphae at 5, 10 as well at 15°C. This confirms earlier observations at 25°C in which 28 out of 30 juveniles became attached to hyphae within

45 minutes (den Belder & Jansen, 1994, chapter 2). At temperatures occurring in the field in temperate regions this fungal isolate and the juveniles of this nematode species are both sufficiently active to ensure capture if contact occurs. The temperature independent capture of nematodes by hyphae appears quite exceptional in comparison with fungi that capture nematodes through more complex structures only. Several authors reported that the nematode capturing fungi they studied, including other isolates of *A. oligospora*, did not respond to nematodes at low temperatures (Soprunov, 1966; Cayrol & Brun, 1975). Studies on the induction of adhesive networks in *A. oligospora* (ATCC 24927) showed a total failure at 5, 30 and 35°C (Grønvold, 1989). Our isolate of *A. oligospora* also developed adhesive networks over a wider temperature range than isolates that have adhesive networks as the only capture device such as in isolate *A. oligospora* ATCC 24927 (Grønvold, 1989) or *A. superba* (Cayrol & Brun, 1975). Nevertheless at temperatures below 15°C the development was significantly slower than at 15 to 30°C.

Our results show that colonization of the nematode body by trophic hyphae was clearly affected by temperature. At temperatures below 15°C development of trophic hyphae was very slow as compared to higher temperatures. Hence, under common soil temperatures of temperate regions colonization of this nematode species would be a very slow process.

The present investigation demonstrated that both hyphae of *A. oligospora* (CBS 289.82), developing on water agar and those growing on low nutrient salt media or corn meal agar, are able to capture all *M. hapla* juveniles present within one hour. Under nutritional conditions ranging from simple to more complex, the rate of nematode-hypha attachment did not appear to be influenced. A critical point or range in nutrients determining the development of capture devices, as suggested by Olthof & Estey (1966), was not observed for the attachment to hyphae grown on the media tested.

The delay in ring structure development on the water agar in comparison with other media, was similar to results obtained with several other isolates of *A. oligospora* (ATCC 24927) (Nordbring-Hertz, 1968; Jansson & Nordbring-Hertz, 1980).

This observation is not in agreement with the hypothesis that capture structure development in nematophagous fungi would be increased when few energy sources are available (Cooke, 1962a, b). Apparently the fungus requires at least

some nutrients from the medium to form adhesive networks or initiate formation of trophic hyphae.

Light, whether continuous or alternating with darkness, had little effect on mycelial growth of *A. oligospora*, *A. conoides* and *A. brochopaga* (Olthof & Estey, 1965). Our results showed that light did not affect nematode-hypha attachment or ring structure development in *A. oligospora* (CBS 289.82).
It has already been shown (den Belder & Jansen, 1994, chapter 2) that very young mycelium, 24 h old, was able to attach to nematodes and to develop ring structures around them.
The present results showed that the trapping ability of hyphae of isolate CBS 289.82 was comparable to younger colonies, even when the hyphae were kept for 36 days at 5°C and few hyphae were formed. Loss of adhesiveness as found for *D. megalospora* after 7 days (Esser *et al.*, 1991) or a reduction of adhesiveness after several weeks as found in *A. oligospora* (Grønvold, 1989) was never found. In colonies in which nematodes mainly encountered aged hyphae (mycelium, developed during 42 days at 25°C and subsequently kept for 36 days at 5°C) any change in attachment efficacy or development of trophic hyphae was found in comparison to mycelium grown for 28 days at 25°C.
Development of conidiophores or complex three-dimensional adhesive networks developing on the ring structure surrounding the nematodes as observed by Poinar & Jansson (1986), Nordbring-Hertz *et al.* (1987) and Jaffee *et al.* (1992) after the contents of the nematode were absorbed never occurred in the tested isolate.

Under adverse temperatures that do not favour adhesive network development or vegetative growth, or under poor nutritional conditions for adhesive network development, our isolate tested shows the capacity to capture nematodes with adhesive hyphae. This clearly illustrates lower demands on temperature and nutrition than needed for ring structure formation. This implies that the range of circumstances for this fungus to capture nematodes and to be active as a control agent, may be much broader for fungi in which adhesive networks are the only capturing devices.

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5

Saprophytic and predacious abilities in *Arthrobotrys oligospora* in relation to dead and living root-knot nematodes*

Abstract

An adhesive hyphae-forming isolate of *Arthrobotrys oligospora* clearly responded to the condition of its food source, i.e. living, inactivated or dead second-stage juveniles of the root-knot nematode *Meloidogyne hapla*. Second-stage juveniles immobilized by heating and only able to move the anterior region or the stylet, were surrounded by ring structures similar to fully mobile juveniles. However, ring structures were principally developed around the moving head. The fungus penetrated dead, but intact juveniles (obtained after treatment with gamma-irradiation or sodium azide), through its buccal cavity with a corkscrew-like structure.

Dead juveniles with a broken cuticle were totally overgrown by the fungus with thin vegetative hyphae. Evidently, *A. oligospora* (CBS 289.82) switched between nutritional modes while exploiting different food sources. The saprophytic and predacious ability appeared not to be mutually exclusive.

Addition of dead juveniles to a fungal colony prior to live juveniles did not affect attachment or the development of trophic hyphae through the latter. But one day after addition of the living juveniles, the proportion of live juveniles with ring structures raised in comparison with all juveniles added at the same time. The development of trophic hyphae in dead juveniles was delayed in the presence of live juveniles. The results refute the commonly held assumption

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that poor conditions for saprophytic growth are a prerequisite for the predacious mode.

Key-words: *Arthrobotrys oligospora*, *Meloidogyne*, adhesive hyphae, morphogenesis, saprophytic, predacious, nutritional modes

Introduction

Many fungi show a degree of flexibility in nutritional modes throughout their life-cycle (Lewis, 1972; Luttrell, 1974; Cooke & Whipps, 1980; Cooke & Whipps, 1987). Nematode-capturing fungi are also able to switch from one nutritional mode to another. Knowledge about the nutrition of facultative nematophagous fungi, however, is fragmentary (Blackburn & Hayes, 1966; Hayes & Blackburn, 1966; James & Nowakowski, 1968; Nordbring-Hertz, 1968). Nevertheless, nematophagous fungi may capture bacteria, Tardigrada, amoeba and other living soil organisms as well as digest dead nematodes and bacteria (Nordbring-Hertz & Stålhammar-Carlemalm, 1978; Fermor & Wood, 1981).

Both the ability to grow saprophytically and their predacious habit are prerequisites for those nematophagous fungi that capture nematodes with structures developed along the vegetative hyphae. Hence the effect of fungi on nematodes does not depend solely on predation, but also on the ability of the fungus to grow and compete saprophytically (Pramer, 1964). However, the significance of nutritional versatility of nematophagous fungi in relation to their use in biological control is difficult to assess.

Arthrobotrys oligospora has been found in a broad range of niches (Gray, 1983; Dackman *et al.*, 1987; Fritsch & Lysek, 1989).

Comparison of isolates of *A. oligospora* with respect to radial growth *in vitro*, formation of ring structures, attraction of nematodes and the capture of *Meloidogyne hapla* on agar medium or in sterilized soil, showed intraspecific variability (Jansson & Nordbring-Hertz, 1979; den Belder & Jansen, 1994a, chapter 2). Hence, individual isolates may have different saprophytic and predacious properties.

The ability of a fungus to form traps may reflect its evolutionary adaptation to nutritional stress imposed by competition with other soil micro-organisms for

energy sources (Cooke, 1963a, b). He also suggested that the evolution of predacious efficacy or the ability to reduce soil populations of nematodes has been generally accompanied by a loss of characteristics associated with an efficient saprophytic existence in the soil such as rapid vegetative growth and good competitive saprophytic ability.

The present study was initiated to obtain a better understanding of the relative predacious and saprophytic abilities of *A. oligospora* (CBS 289.82). This isolate may be considered as an effective predator because it captures nematodes by its morphologically undifferentiated vegetative hyphae, at low temperatures and under poor nutritional conditions (den Belder & Jansen, 1994b, chapter 4).

On the basis of comments and conclusions by various authors (Cooke, 1963b; Jansson & Nordbring-Hertz, 1979; Jansson, 1982; Jaffee & Zehr, 1985) it was anticipated that the ability of this isolate to use non-living material to be limited due to an inverse relation between saprophytic and predacious abilities.

Factors affecting or regulating switches from the saprophytic to the predacious feeding mode are largely unknown (Jaffee & Zehr, 1985; Quinn, 1987). Also in other well-studied nematophagous fungi such as the egg parasites *Paecilomyces lilacinus* and *Verticillium chlamydosporium* or in entomophagous fungi little is known of the switches which are responsible for changes in the trophic state (Jatala, 1986; de Leij, 1992). Adhesive network-forming fungi such as *A. oligospora* are considered as facultative predators not capturing prey under nutrient rich conditions (Cooke, 1963a, b). Consequently it was postulated that attachment of live *Meloidogyne* sp. to *Arthrobotrys oligospora* and subsequent formation of ring structures around, and trophic hyphae through, the nematode body, should be suppressed in the presence of added dead juveniles.

Materials and methods

Organisms *Arthrobotrys oligospora* (CBS 289.82) was cultured on corn meal agar (Oxoid, CMA 1:1, 1.5%) in Petri-dishes (diameter 88 mm) at 25 ± 1°C with monthly transfers to fresh medium. Individual 4-mm plugs cut from the periphery of the actively growing stock colony were placed upside down in

small Petri-dishes (Lux, diameter 44 mm) on CMA 1:10. The Petri-dishes used in the experiments had an oval hole in the bottom lid (length 35 mm, width 18 mm) covered by a coverglass glued to the lid, thus facilitating microscopic observations with an inverted microscope. Fungus cultures (28 day-old) on CMA 1:10, were inoculated with freshly extracted juveniles of *M. hapla*.

Meloidogyne hapla was reared on tomato plants (*Lycopersicon esculentum* Mill. cv. Moneymaker). Second-stage juveniles were harvested and surface sterilized as described by den Belder & Jansen (1994a, chapter 2).

Response of the mycelium of *A. oligospora* to vitality of *M. hapla* (Experiment 1) The response of the fungus was studied after addition of different substrates: juveniles of *M. hapla* living or either partly immobilized, either dead with either an intact or damaged cuticle. The different qualities of juveniles were obtained by applying them a variety of physical stresses. The following treatments were included:

1 Axenic, healthy 2 day-old juveniles, actively moving (control)
2 Axenic juveniles suspended in sterile water and incubated for 24 h at 35°C, slightly moving the anterior region of the body or the stylet but lacking clear rhythmic muscular movements along the body
3 SON treatment: axenic juveniles suspended in sterile water and sonicated in 60-second bursts with a Branson Sonic Power sonicator until all juveniles appeared to be broken when examined through an inverted light microscope (40 bursts, 60 Watt)
4 SDS– treatment: axenic juveniles heated in 5 ml sodium dodecyl sulphate-containing 0.1 M tris-HCl buffer at 100°C in a water bath for 2 minutes (STP buffer, Reddigari *et al.*, 1986); they appeared to be broken when examined through a light microscope
5 SDS+ treatment: following the SDS– treatment, juveniles were washed in sterile water 3 times in a centrifuge (5000 rpm, 10 minutes); appearing broken when subsequently observed through a light microscope
6 SON/SDS– treatment: axenic juveniles were suspended in buffer (0.05 M tris-HCl containing 1 mM phenylmethylsulfonyl fluoride, pH=7.0) and sonicated in 60-second bursts with a Branson Sonic Power sonicator until juveniles appeared to be broken when examined with a light microscope. The nematode suspension was centrifuged for 10 minutes at 2000 rpm and

washed in the tris-buffer before being treated with the SDS containing buffer at 100°C in a water bath for 2 minutes

7 SON/SDS+ treatment: After the SON/SDS– treatment the juveniles were washed 3 times in sterile water in a centrifuge (5000 rpm, 10 minutes) to remove residual detergent

8 100°C heating: axenic juveniles were heated at 100°C in a water bath for 2 minutes; the juveniles appeared broken when observed with a light microscope

9 NaN_3 treatment: axenic juveniles were killed by suspension in sodium azide (0.1 M NaN_3) for 24 h and were washed subsequently in sterile water in a centrifuge (5000 rpm, 10 minutes). Sodium azide inhibits the respiration process (Schlegel, 1986). The juveniles appeared intact when observed through the light microscope.

Experiment 2 Also the response of the fungus was studied, after addition of axenic irradiated juveniles of *M. hapla.* They have been exposed to gamma-irradiation from a cobalt-60 source (at the Pilot Plant for Food Irradiation, Wageningen) in a small amount of sterile water in a Greiner tube. The doses, ranging from 0.50 to 100 kGy, were realized by varying distance from the source and exposure time.

This range was chosen using results on irradiation of other species: about 1 kGy was needed to immobilize *Ditylenchus dipsaci* (Green & Webster, 1965), 7 and 13 kGy was needed to kill juveniles of *Trichinella spiralis* and *Panagrellus* sp. respectively (Myers & Dropkin, 1959; Myers, 1960) and about 10 kGy was needed to break the juveniles of *Ditylenchus dipsaci* (Green & Webster, 1965). In preliminary studies no differences were found in the reaction of 28 day-old fungal colonies to juveniles irradiated with doses from 0.50, 1.00, 1.50 and 1.75 kGy, in comparison to untreated juveniles. In a second series, juveniles exposed to 2, 4, 20 and 100 kGy were added to 28 day-old fungal colonies.

In Experiment 1 and 2 about 100 juveniles were added to 28 day-old fungal colonies (volume 80-100 µl). Each treatment was repeated three times and as a control axenic juveniles were added to the fungal colonies.

Fungal activity was observed at regular time intervals with a light microscope (Zeiss Axiovert 10) and a distinction was made between: juveniles captured by

adhesive hyphae and surrounded by ring structures, juveniles infected via the body orifices and juveniles surrounded by a loose mesh.

Response of the mycelium of *A. oligospora* to simultaneous addition of live/dead nematodes In order to analyze the effects of the presence of dead nematodes on the capture and infection of live nematodes, gamma-ray killed juveniles were added together with or 14 days before the live juveniles.
Aliquots counting 50 or 500 gamma-ray killed juveniles (10 kGy) were pipetted over a 1 cm^2 area left and right from the centre of the Petri-dish. In each test, a drop adjusted to contain about 50 living nematodes was added to the fungal cultures. Assays usually consisted of three replicate plates and the experiment was repeated twice.
In all experiments, the response of the fungus was observed at regular time intervals with a light microscope. A distinction was made between: juveniles surrounded by ring structures, juveniles infected via the body orifices and juveniles surrounded by a loose mesh (and in the live nematodes attachment to hyphae was included).
Development of trophic hyphae was quantified by counting the number of juveniles containing trophic hyphae.
Also the relative length of every nematode body containing trophic hyphae was determined at intervals (days 1, 2, 3, 6, 10, 14, 17, 22, 29). Five classes were distinguished: 0, 1-25, 25-50, 50-75, 75-100% body length filled with hyphae. T_{50} and T_{95} (days after addition when 50 and 95% of the nematode body length was filled with trophic hyphae) were estimated and subsequently analyzed by analysis of variance followed by Student's t-test for pairwise comparison of treatments (den Belder & Jansen, 1994b, chapter 2).

Light and fluorescence microscopy Observations were made using a Zeiss Axiovert 10 light microscope (objective, planneofluar 20x long distance) and differential interference contrast (DIC). Images were documented using Kodak Ektachrome 160. In several cases a fluorescent mycology stain was used (Fungi-Fluor, Polysciences, Inc., Warrington) to visualize trophic hyphae and observations were made using the same microscope with fluorescence illumination (filter block 2Fl, 365-440 nm).

Low-temperature scanning electron microscopy For detailed observations of fungal structures, cryo-SEM was employed. Specimens were mounted on custom designed copper stubs and immediately frozen by immersion in a nitrogen slush (60 K) in the EMSCOPE SP2000 Cryogenic-Preparation System, etched by conductive heating and subsequently sputtered with gold for 2 minutes (den Belder *et al.*, 1993). The frozen specimens were transferred to the scanning electron microscope (Jeol JSM 35C) which had been modified with a cryo-stage to maintain specimens at a temperature of 110 K.

Results

Response of the mycelium of *A. oligospora* to vitality of *M. hapla* Significant differences were observed in Experiment 1 in the reaction of the fungus to axenic juveniles and juveniles killed by sonication, heating or a respiration inhibitor (Table 5.1). Juveniles immobilized after 24 h at 35°C except for slight head and stylet movements still induced ring structures. None of the dead juveniles stimulated the development of ring structures.

In treatments where SDS was not removed from the juveniles (SDS– and SON/SDS–) the fungus did not respond to the presence of juveniles (Figure 5.1 A). In those treatments where juveniles were broken due to sonication or heating (SON, SDS+, SON/SDS+ and 100°C heating), the juveniles were surrounded by many vegetative hyphae resulting in a mesh totally overgrowing the juveniles (Figure 5.1B, 5.2A).

In treatments where the juveniles were dead but not broken (NaN_3) the fungus responded by forming a corkscrew-like structure penetrating the buccal cavity (or in some cases the anus) of the nematode (Figure 5.1C). The hypha which formed this spiral structure tended to be of the same diameter as the vegetative hyphae (Figure 5.2D). Subsequently trophic hyphae developed throughout the nematode body (Figure 5.2C). In some cases vegetative hyphae grew closely alongside the nematode body but never as many as in the SON, SDS+ or the SON/SDS+ treatments.

TABLE 5.1 *Effects of physico-chemical treatments of second-stage juveniles of* Meloidogyne hapla *on the response of the mycelium of* Arthrobotrys oligospora *(CBS 289.82).*

Treatment of nematodes		Days after addition	No reaction of the fungus[1]	Vegetative hyphae[1]	Corkscrew-like structures[1]	Ring structures[1]
None	Active juveniles	1				90
		7				100
35°C heating	Partly paralysed juveniles with intact cuticle	1				90
		7				100
SON[2]	Broken juveniles	1		25		
		7		100		
SDS–[3]	Broken juveniles with SDS-residues	1	100			
		7	100			
		21	100			
SDS+	Broken juveniles	1		40		
		7		100		
SON/SDS–	Broken juveniles with SDS-residues	1	100			
		7	100			
		21	100			
SON/SDS+	Broken juveniles	1		40		
		7		100		
100°C heating	Broken juveniles	1		25		
		7		100		
NaN_3	Dead juveniles with intact cuticle	1			80	
		7			100	

[1] % of juveniles

[2] SON = sonication

[3] SDS = sodium dodecyl sulphate

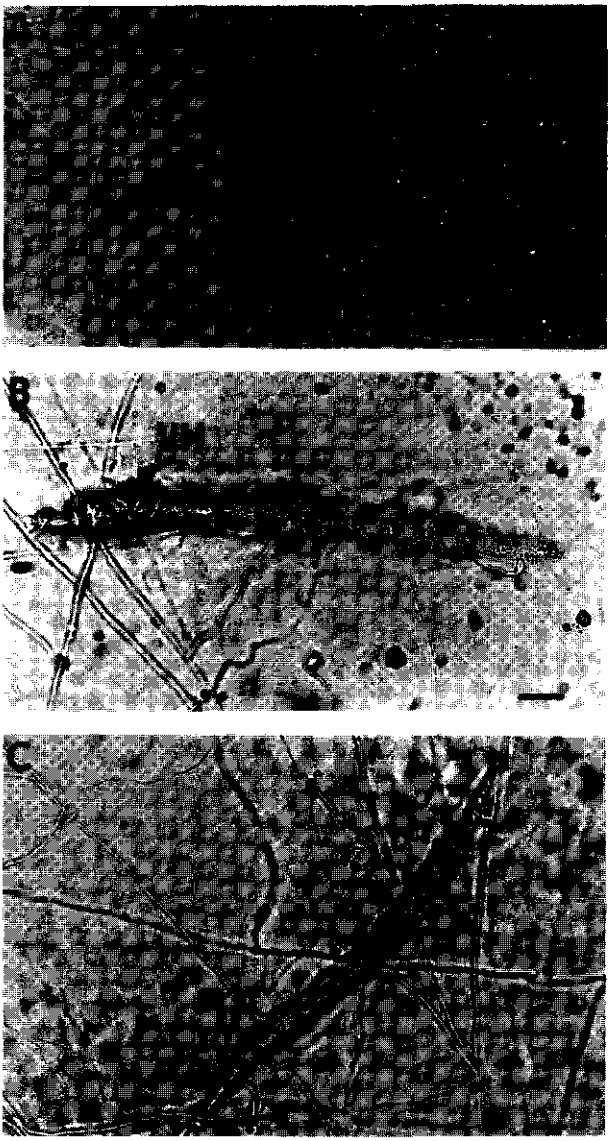

FIGURE 5.1 A-C *Arthrobotrys oligospora* (CBS 289.82) on differently treated second-stage juveniles of *Meloidogyne hapla.* A: after SDS– or SON/SDS– treatment of juveniles the fungus did not respond to the presence of juveniles; B: after sonication of juveniles, the fungus formed a meshwork of vegetative hyphae (VH); C: after NaN_3 treatment of juveniles (in which the nematodes were dead but not broken) the fungus responded by forming a corkscrew-like structure (CS) approaching the buccal cavity (or in some cases the anus). Subsequently trophic hyphae (TH) were formed. Bar = 30 µm.

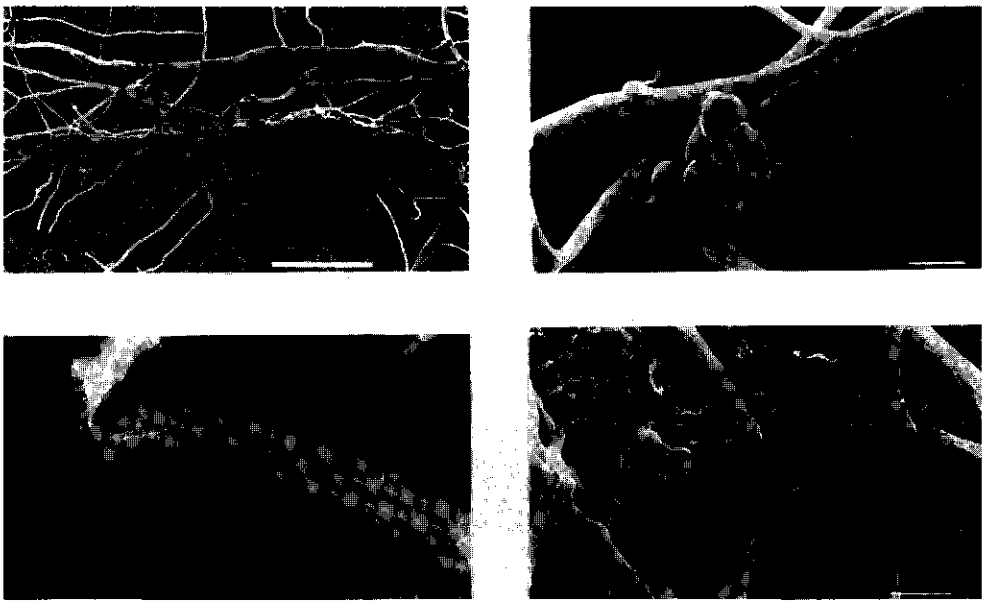

FIGURE 5.2 A-D A: *Arthrobotrys oligospora* (CBS 289.82) on broken, sonicated second-stage juveniles of *Meloidogyne hapla*, six days after addition of second-stage juveniles to the fungal colony, VH= vegetative hyphae; B: simultaneous development of a ring structure (RS) and a corkscrew-like structure (CS) in a gamma-ray irradiated second-stage juvenile of Meloidogyne hapla (dose 2 kGy); C: trophic hyphae (TH) of *Arthrobotrys oligospora* in a second-stage larva of *Meloidogyne hapla* killed by gamma-irradiation; D: corkscrew-like structure (CS) of *Arthrobotrys oligospora* approaching the buccal cavity of a second-stage juvenile of *Meloidogyne hapla*. Bar equivalents: A = 100 µm; B,D = 10 µm; C = 5 µm.

Experiment 2 Irradiated juveniles (0.5 to 1.75 kGy) or untreated juveniles did not differ as inducers of capture and ring structure development in *A. oligospora* (CBS 289.82) when added immediately after the irradiation of the juveniles. However, increased radiation doses applied to juveniles (2 kGy and higher) resulted in significant differences in the reaction of the fungus. The fungus reacted positively by forming a corkscrew-like structure, approaching the buccal cavity of the nematode similarly as in the case of juveniles killed with NaN_3 (Figure 5.2B). At 2 kGy, also ring structures were induced around all moving juveniles (60%) (Table 5.2). Irrespective of the formation of these ring structures, also 70% of all juveniles were approached by corkscrew-like structures. Both types of responses occurred finally on 30% of the juveniles (Figure 5.2B). At 4, 20 and 100 kGy ring structures were not formed at all, neither at

the start of the experiment nor after digestion of the nematode body. The juveniles were invaded only after the formation of the corkscrew-like structures near the buccal cavity and in some cases the anus (Table 5.2).

Development of trophic hyphae through the nematode body occurred both following either development of ring structures or development of corkscrew-like structures.

Sixteen days following addition trophic hyphae were developed in all juveniles radiated with 2 to 20 kGy gamma-irradiation. However in 20% of the nematodes irradiated with 100 kGy no development of trophic hyphae occurred.

TABLE 5.2 *Effects of gamma-irradiation of second-stage juveniles of* Meloidogyne hapla *on the response of the mycelium of* Arthrobotrys oligospora *(CBS 289.82).*

Dose of gamma-irradiation (kGy)	Days after addition	Nematodes surrounded by ring structures (%)	Nematodes approached by a corkscrew-like structure (%)
0	1	60	0
	10	100	0
1.75	1	60	0
	10	100	0
2	1	60	30
	10	60	70
4	1	0	60
	10	0	100
20	1	0	60
	10	0	100
100	1	0	70
	10	0	95

Response of the mycelium of *A. oligospora* to simultaneous addition of live/dead nematodes At the first observation, one day after addition of living nematodes, all juveniles of *M. hapla* were captured by the hyphae of the 28 day-old fungal cultures irrespective of addition of 50 or 500 irradiated juveniles or the timing of the addition of the live juveniles (Table 5.3).

The presence of live nematodes did not affect the proportion of dead nematodes invaded through a corkscrew-like structure. The proportion of the irradiated juveniles with a corkscrew-like structure in front of the buccal cavity increased from 60% after one day to 90% two days after addition of nematodes.

Subsequent development of ring structures around the juveniles differed significantly between treatments: addition of dead juveniles prior to live juveniles, stimulated the ring structure development around the latter. One day after addition of the live nematodes (no dead nematodes were added) about 15% more juveniles were surrounded by ring structures than when dead nematodes were added two weeks earlier ($P<0.0001$).

Development of trophic hyphae in those juveniles that were added alive was not significantly influenced by the presence of dead juveniles neither when 50 nor when 500 juveniles were added (Table 5.4). However, development of trophic hyphae through irradiated nematodes was significantly slower if live juveniles were added at the same time. This resulted in significantly more days needed to reach 50% or 95% nematode body length filled with trophic hyphae.

TABLE 5.3 *Response of* Arthrobotrys oligospora *(CBS 289.82) on living second-stage juveniles of* Meloidogyne hapla *when gamma-ray killed juveniles were added before or at the same time to 28 day-old fungal colonies.*

Addition of nematodes		Results				
		% living nematodes captured by hyphae	% living nematodes surrounded by ring structures		% dead nematodes with corkscrew-like structure	
At day 1	At day 14	One day after addition	One day after addition	Two days after addition	One day after addition	Two days after addition
0 dead + 50 living	+ 0 living	100	73.1 ± 11.2[1] ab[2]	98.0 ± 1.4 a[1]	–	–
50 dead + 50 living	+ 0 living	100	72.2 ± 13.4 ab	98.0 ± 1.2 a	60.1 ± 12.3 a	94.1 ± 1.2 a
50 dead + 0 living	+ 50 living	100	88 ± 2.3 b	99.1 ± 0.6 a	60.7 ± 3.8 a	90.6 ± 2.3 a
500 dead + 50 living	+ 0 living	100	64.2 ± 2.5 a	100 ± 0.0 a	58.7 ± 2.7 a	88.0 ± 2.0 a
500 dead + 0 living	+ 50 living	100	92.3 ± 0.8 b	100 ± 0.0 a	59.3 ± 3.8 a	87.6 ± 5.2 a
0 dead + 0 living	+ 50 living	100	75.2 ± 1.9 a	100 ± 0.0 a	–	–

[1] Mean ± s.e.

[2] Different letters indicate significant differences between the means in the column (Student's t-test, $P<0.05$)

TABLE 5.4 *Development of trophic hyphae of* Arthrobotrys oligospora *in second-stage juveniles expressed in days after addition when 50% and 95% of the nematode body was filled with trophic hyphae (T_{50} and T_{95}).*

Addition of nematodes		Results			
		T_{50}		T_{95}	
At day 1	At day 14	Living nematodes	Dead nematodes	Living nematodes	Dead nematodes
0 dead + 50 living	+ 0 living	10.7[1] ± 0.9 a[2]	–	20.4 ± 1.6 a	–
50 dead + 50 living	+ 0 living	11.3 ± 0.3 a	15.4 ± 1.3 a	23.0 ± 0.6 a	30.6 ± 6.9 a
50 dead + 0 living	+ 50 living	11.6 ± 0.7 a	9.1 ± 0.6 b	19.9 ± 1.8 a	19.7 ± 1.4 b
500 dead + 50 living	+ 0 living	12.7 ± 0.6 a	13.7 ± 0.3 a	25.2 ± 2.0 a	26.5 ± 0.6 a
500 dead + 0 living	+ 50 living	12.5 ± 0.1 a	9.1 ± 0.2 b	23.4 ± 0.3 a	17.8 ± 1.7 b
0 dead + 0 living	+ 50 living	11.0 ± 0.4 a	–	20.0 ± 0.4 a	–

[1] Mean ± s.e.

[2] Different letter indicate significant differences between the means in the column (Student's-t-test, $P<0.05$)

Discussion

It is clear from this study that the condition of the nematode, in this case living, inactivated or dead juveniles of *M. hapla* is of decisive importance for the response of the mycelium of *A. oligospora* (CBS 289.82).
Live juveniles, only able to move the head or the stylet, are principally surrounded by ring structures around the head. Similarly in juveniles attached to adhesive hyphae, ring structures developed in 50% at the initial attachment site but at the sites the nematodes were vigorously moving (den Belder & Jansen, 1994a, chapter 2). Signals triggering ring structure formation include direct contact of the living nematode with the hyphae (Nordbring-Hertz, 1987). A positive correlation between nematode motility and the ability to induce traps in *A. oligospora* (ATCC 24927) was found by Jansson & Nordbring-Hertz, 1980). Initiation of ring formation could be the consequence of changes in membrane potential following nematode movement (Nordbring-Hertz, 1977). Besides physical stimuli, the importance of specific chemical cues in the formation of fungal structures have been illustrated (Charnley, 1989). The lack of a response to nematodes might have been due to the fact the nematode was unrecognizable in the presence of sodium dodecyl sulphate. On the other hand the fungus might have avoided contact with this aggressive detergent. It confirms that morphogenesis in fungi seems to be easily affected by inhibitory concentrations of various chemicals and volatiles (Nordbring-Hertz, 1987).

Dead nematodes with an intact cuticle, were invaded by the fungus after development of a corkscrew-like structure. In contrast, the fungus reacted to nematodes with a broken cuticle by totally overgrowing them with vegetative hyphae along the nematode cadaver which was colonized. Growth of vegetative hyphae into intact nematodes as described by Nordbring-Hertz (1968), was never observed.
Recognition of the status of the prey elicits a defined functional and morphological response. When juveniles were irradiated with a non-lethal dose of gamma-irradiation, in 30% of the nematodes a ring structure and a corkscrew-like structure developed. This phenomenon might be explained by the fact that during the course of the experiment the nematode died. Stimuli initially given by the living juvenile might have been absent finally. Whether the fungus responds to chemical or to physical stimuli that might very well differ according

to viability of the nematode in a way comparable to that of spores of plant-pathogenic fungi to plants (Wynn, 1976; Hoch *et al.*, 1987; Bourett & Howard, 1990), warrants further research.

The lack of response to nematodes treated with very high doses gamma-irradiation (100 kGy) clearly illustrates the role of chemical stimuli.

Nematodes filled with trophic hyphae of *A. oligospora* (ATCC 24927) as observed in *Panagrellus redivivus* 24 h after attachment to ring structures (Nordbring-Hertz *et al.*, 1986) or complete consumption of juveniles of *Neoaplectana* or *Heterorhabditis* after three days, conidia emerging from the cadavers were never observed in *M. hapla* (Poinar & Jansson, 1986). Our results showed that the fungus needed ten days to occupy 50% of the nematode body length. When living and dead juveniles were added together to the fungal colony attachment to the live juveniles was not diminished, presenting evidence that the presence of food in the form of dead juveniles did not affect attachment. This confirms former results where nutritional conditions ranging from simple to more complex, did not influence nematode attachment (den Belder & Jansen, 1994b, chapter 4).

An increased proportion of nematodes with ring structures one day after addition of the live nematodes indicates that the fungus apparently uses the nutrient supply from the dead juveniles in the development of ring structures or is triggered/stimulated by the dead nematodes, to produce such structures. This refutes the hypothesis that capture structure development would be increased at low nutrient concentrations (Cooke, 1963a, b). On the contrary, the fungus appeared to be more capable of forming ring structures. This agrees with the finding that the development of ring structures occurred sooner on corn meal agar than on water agar (den Belder & Jansen, 1994b, chapter 4) and also with the observation that an increase in nutrient level of the medium resulted in an increase in coiling frequency of *A. oligospora* (ATCC 24927) around *Rhizoctonia solani* (Persson & Bååth, 1992).

A delay in ring structure development on water agar in comparison with other media (den Belder & Jansen, 1994b, chapter 4) might be compensated by the presence of dead nematodes.

The presence of living juveniles did not affect the proportion of dead juveniles infected through a corkscrew-like structure by *A. oligospora* (CBS 289.82). However, trophic hyphae grew more slowly through dead juveniles when live ones were present than when dead juveniles were added alone. Our results do

not only show that this fungus is able to feed on live and dead nematodes simultaneously, but also that development of trophic hyphae through living juveniles occurred sooner than through dead juveniles. Living nematodes not only form an alternative nutrient source (Blackburn & Hayes, 1966) but they are digested more rapidly by the fungus.

This isolate of *A. oligospora* shows, through the adaptation of vegetative hyphae, a plasticity to exploit different nutrient sources: the same mycelium can adapt in such a way that living nematodes are attached and subsequently surrounded by ring structures or dead material is penetrated through the formation of a corkscrew-like structure or covered by mycelium so that the nutrients will be used. This isolate of *A. oligospora* does produce different vegetative structures in response to changing conditions encountered by a dynamic mycelium growing under heterogeneous conditions. Thus different parts of the colony may fulfil different functions. There is no evidence in this isolate for a limited saprophytic ability.

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6

Establishment and nematode capture ability of an adhesive hyphae-forming isolate of the fungus *Arthrobotrys oligospora* in soil*

Abstract

In situ observations of an isolate of *Arthrobotrys oligospora* (CBS 289.82) in sterilized and untreated soil revealed attachment of second-stage juveniles of the root-knot nematode *Meloidogyne hapla* to hyphae, development of rings around the nematode body and nematode colonization by trophic hyphae. Abundant growth of rings from infected nematodes was never observed. Establishment and nematode capture by this isolate was compared to those of several other fungi from the *Dactylaria*-complex in a simple soil microcosm system. Application of hyphal fragments of *A. oligospora* (CBS 289.82), about 30 mm/g oven-dry soil, resulted in hyphal lengths of 100-170 m/g oven-dry soil, reduction of 90% of living juveniles of *M. hapla* within one day and extermination of the nematodes after ten days. *Arthrobotrys oligospora* (CBS 289.82) performed far better than any of the other fungi.

Establishment in non-sterilized soil was less well, but still total hyphal length of about 10 m/g oven-dry soil resulted in 70% reduction of living nematodes at day 10. At 13°C results were similar to those at 20°C.

The results support the conclusion that adhesive hyphae-forming fungi may be useful for the control of nematodes in soil.

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Key-words: *Arthrobotrys*, *Meloidogyne*, soil, *in situ* observations, capture ability, hyphal length, temperature

Introduction

In vitro studies revealed that the formation of adhesive hyphae in the adhesive hyphae-forming isolate of *Arthrobotrys oligospora* (CBS 289.82) is less temperature and nutrient dependent than the development of adhesive ring structures (den Belder & Jansen, 1994a, chapter 4). This observation suggests that the range of conditions under which this isolate of *A. oligospora* captures nematodes is wider than in isolates where adhesive networks are the only capture devices. Nevertheless in both cases the nematophagous fungus must be able to form a mycelial mat. Studies on the survival or growth of nematophagous fungi introduced in non-sterilized soil generally showed a limited mycelial extension when compared with that under sterile soil conditions (Cooke & Satchuthanathavale, 1968). A number of fungi, tested against numerous nematode species in different environments, showed great inconsistency with respect to reduction of nematode populations in sterilized and non-sterilized soil (Stirling, 1991). For a variety of reasons these fungi did not give adequate control. Factors mentioned are related to arrival at, establishment in and exploitation of the resource by the fungus. A reduced capture could be expected at lower temperatures due to a reduced establishment of the mycelial network and subsequently a lower frequency of nematodes encountering hyphae (den Belder & Jansen, 1994a, chapter 4).

In the present study the establishment and the capture ability of the adhesive hyphae-forming isolate of *A. oligospora* (CBS 289.82) and three other nematophagous fungi, selected because they vary in trapping strategy, were quantified, after introduction of mycelial fragments to soil. The nematophagous fungi included were: the adhesive knob former (spontaneously) *Monacrosporium cionopagum* (CBS 228.52), the adhesive network formers (non-spontaneously) *A. conoides* (CBS 265.83) and *A. oligospora* (ATCC 24927). These fungi have been tested previously on agar plates showing the highest capture by the adhesive hyphae-forming isolate *A. oligospora* (CBS 289.82) and a decrease in capture ability in the above mentioned order (den Belder & Jansen, 1994b, chapter 2).

The objective of the present paper is to quantify the attachment of *Meloidogyne* spp. to adhesive hyphae of *Arthrobotrys oligospora* (CBS 289.82) in soil. The capture ability of this isolate is compared to that of three other fungal species which employ other capture strategies. The hypothesis is tested that because of a better developed mycelial mat, the capture of *A. oligospora* (CBS 289.82) at 20°C is better than at 13°C. Experiments in non-sterilized soil will show if the capture of nematodes is reduced due to depressed mycelial extension of *A. oligospora* (CBS 289.82) in comparison with the sterilized soil.

Materials and methods

For assessing the ability of this adhesive hyphae-forming isolate as a biological control agent, a series of experiments was conducted in which the nematophagous fungus was quantified by measuring hyphal lengths, the level of nematode mortality was estimated by counting the number of healthy nematodes and nematode capture was observed directly in soil.

Fungal species and culture methods Stock cultures of *Arthrobotrys conoides* (CBS 265.83), *Arthrobotrys oligospora* (CBS 289.82, ex *Meloidogyne* sp.), *Arthrobotrys oligospora* ATCC 24927 (ex garden soil, Sweden) and *Monacrosporium cionopagum* (CBS 228.52) were maintained on oat meal agar (Oxoid, 1:1, 1.5% agar) in Petri-dishes (diameter 88 mm) at 25 ± 1°C and were subcultured monthly.

Nematodes Second-stage juveniles of *Meloidogyne hapla*, from a population originally isolated from rose and obtained from the DLO-Centre for Plant Breeding and Reproduction Research, Wageningen, were used in all tests. Since 1988 this population was maintained continuously on tomato plants (*Lycopersicon esculentum* Mill. cv. Moneymaker) in riversand at a temperature of 20°C in a glasshouse. Newly hatched second-stage juveniles were obtained by incubating egg masses on a 50 µm sieve on water for 2 days at 20°C. The outer surface of 2 day-old juveniles of *M. hapla* was sterilized in a mixture of 0.02% (w/v) ethoxy-ethylmercury chloride (Aretan) and 0.1% (w/v) streptomycin sulphate for about 2 h in a 10 ml conical centrifuge tube and subsequently washed three times in sterile water (s'Jacob & van Bezooyen, 1984).

Soil In capture experiments a loamy sand from Lelystad was used (organic matter 0.5%, pH-KCl=6.6). Prior to use, water was added to approximately 13% water content (w/w).
For *in situ* observations, a sandy soil (organic matter 3.7%, pH-KCl=7.3) and a coarse sand (<0.05% organic matter) were used in addition.

In situ observations of nematode capture in soil (Experiment 1) To verify capture of nematodes in soil through attachment to hyphae, adhesive networks or knobs several methods were used:

1 Gelatine-embedding method (Anderson, 1978), in which soil contained in glass cylinders (diameter 1 cm, 3 ml soil) was covered with 22 µm nylon filter and impregnated with a 20% (w/v) gelatine solution under low pressure, using a water jet pump held in a water bath at 37°C.
2 Gelatine-embedding method, in which individual wells of tissue-culture plates (12 wells of 15 mm diameter) were filled with 3 ml soil and were covered with gelatine. The impregnation took place in a vacuum desiccator in an incubator at 37°C. In both gelatine-embedding methods, the gelatine was cooled at 4°C and cut into blocks. In some cases the samples were stained (before or after impregnation) with cotton-blue or Fungi-Fluor (Polysciences, Inc.).
3 Buried slide technique (Barron, 1971): glass slides (3 cm length) were covered with a thin film of water agar and buried individually in 500 ml glass pots containing 200 g autoclaved soil (once for 1 h at 120°C). The soil was inoculated with five 4-mm plugs cut from the periphery of a growing stock colony of the fungi tested. Two ml of a nematode suspension were added 8 days after incubation of the fungus at 25°C. After another 3 days the object glasses were removed and observed immediately under a binocular microscope.
4 Soil samples (about 1 cm^3) taken from fungus-infested soil obtained as described under method 3, were placed on a small Petri-dish with a sealed-in coverglass bottom and dissected carefully with two preparation needles (Forster, 1979). Observations were made using an inverted microscope (Zeiss Axiovert, Achroplan, 20x), 7 h, 24 h and 10 days after addition of the nematodes. Two ml of water were added to promote disintegration of soil aggregates.

Fungal growth and capture ability in sterilized soil In Experiment 2A and 2B plastic containers (75 ml) filled with 65 g Lelystad soil were placed individually in plastic boxes (200 ml) and sterilized through exposure to gamma- irradiation (25kGy) from a cobalt-60 irradiation source. To prepare fungal inocula, one 4-mm plug was cut from a stock colony on oat meal agar, macerated in 1 ml liquid oat meal medium (Difco, 0.14% dry weight) using a Potter glass homogenizer. Two ml of the homogenate (thus 2 plugs) containing hyphal fragments measuring less than 1 mm, were randomly syringed as small drops through the soil. The containers were then incubated for 10 days at 20°C. For controls, oat meal agar disks without fungi, were prepared in the same way.

Fungal growth in soil Total hyphal length in soil was measured as an estimate of the fungal biomass, at regular intervals after inoculation of the fungus at day 7, 10, 13 and 25 (day 12, 16, 25 and 30 in the low temperature experiment). Four soil samples (10 g fresh weight) were taken randomly from a container using a cork borer and added to 100 ml demi-water containing 0.05% detergent (Bosamite AL-5). The suspension was homogenized in a homogenizer at half-speed for 30 seconds (Bardgett, 1991). Usually 4 replicates per treatment were counted and 4 subsamples per replicate. Hyphal length was estimated using a microscope slide with a 3x3 squared grid (10 µl), an ocular micrometer and 400x magnification. The total hyphal lengths of 2 randomly selected grid fields were averaged for each subsample. Total hyphal length expressed as m/g fresh soil was converted to m/g oven-dry soil on the basis of soil moisture content. Live or dead hyphae were not differentiated.

Capture ability Ten days after the inoculation of the fungus, 8000 axenic second-stage juveniles of *M. hapla* in 0.6 ml were added to the container.
The number of living juveniles remaining in the soils was estimated after extraction of soil by a mystifier extraction technique (Seinhorst, 1950), after 1 and 6 h and 1, 2, 3, 4, 7 and 10 days incubation (6 replicates per incubation time).
In preliminary tests three nematode extraction techniques were compared: a mystifier extraction technique (Seinhorst, 1950), the Oostenbrink funnel technique (Oostenbrink, 1954; 1960) and a method in which juveniles had to migrate through a cotton-wool filter on a nylon gauze (Stemerding, 1963).

These tests revealed that the mystifier extraction technique of Seinhorst yielded the highest recovery of nematodes.
The content of each container was placed carefully upside down on a sieve with two filters (Ederol 100, quality nr. 261) in a mist chamber (17-18°C, water velocity 0.75 ml/cm^2/h). Juveniles were collected in a shallow tray, placed below the sieves. Unless otherwise stated, the nematodes were counted after a 48 h extraction period. This experiment was repeated twice (experiment 2A and B).

Fungal growth and capture ability in non-sterilized soil In Experiment 3, total hyphal length in soil was measured as an estimate of the fungal biomass, at day 7, 10 and 20. Capture ability in non-sterilized Lelystad soil was compared with that in sterilized soil for both *A. oligospora* isolates (CBS 289.82 and ATCC 24927) at 20°C following the same procedures as described above. Approximately 9000 juveniles in 0.5 ml were added to each container.

Fungal growth and capture ability at 13 and 20°C In Experiment 4, total hyphal length in soil was measured at regular intervals after inoculation of the fungus at day 12, 16, 25 and 30. Capture ability of *A. oligospora* (CBS 289.82) at 13°C was compared to that at 20°C in sterilized as well as in non-sterilized Lelystad soil. The same procedure was followed as in the former experiments, except for the following modifications: the fungus was incubated for 15 days instead of 10 days and the number of living juveniles was estimated 1, 2, 4, 10 and 15 days after addition. Approximately 8000 juveniles in 0.6 ml were added to each container. Extraction of the number of living nematodes from non-sterilized soil also revealed the presence of some other nematodes.

Statistical analysis of the capture ability Each experiment was conducted in a randomized complete block design with 6 replications. Differences between numbers of living juveniles obtained after extraction were tested by analysis of variance after log-transformation. Means of treatments were subjected to a Student's t-test for pairwise comparison on a log-scale (α=0.05).

Presence of captured nematodes in the mycelium mat In order to verify capture of nematodes by *A. oligospora* (CBS 289.82 or ATCC 24927) at the end of the experiment, the content of a container (65 g fungus-inoculated soil) was washed very gently through a sieve (300 µm) with about 1.5 l water. The

recovered mycelium was observed under the light microscope to verify attachment, infection and development of ring structures or complex networks from the nematode body.

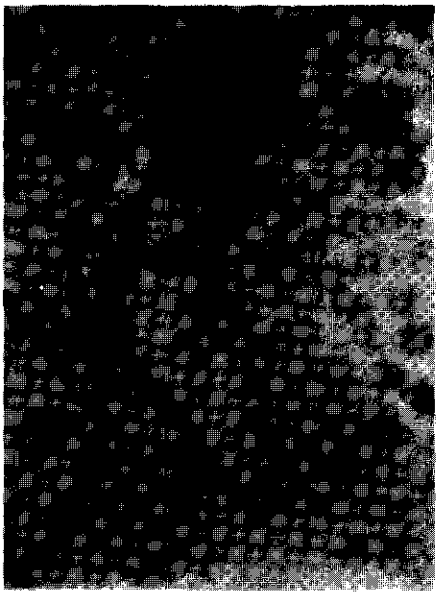
Identification of fungal species To identify fungi other than the fungi applied, samples of the control soils were spread over water agar containing tetracycline chloride (100 µg/l) which was used to isolate slow growing fungi, Dhingra & Sinclair, 1985). Fungal colonies (including those applied) were isolated and identified by het Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

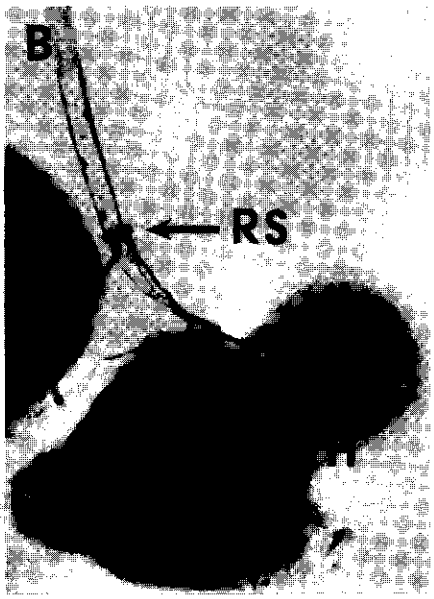
Results

In situ observations of nematode capture in soil (Experiment 1)

Following embedding of soil with either of both gelatine-embedding methods, mycelium could hardly be recognized. Staining with cotton-blue or Fungi-Fluor (Polysciences, Inc., Warrington), before or after gelatine impregnation did not improve contrast against the background of gelatine. The buried slides did allow observation of juveniles of *M. hapla* attached to hyphae of *A. oligospora* (CBS 289.82). Dissected soil samples, however, allowed for the best observation. Hyphae growing between and on soil particles, showed nematodes attached to them. Juveniles, attached to hyphae of *A. oligospora* (CBS 289.82), struggling to get free were frequently visible at the first observation 7 h following their addition (Figure 6.1A). At 24 h, juveniles surrounded by a ring could also be observed (Figure 6.1B). In samples taken after 11 days and in mycelium obtained from sterilized and non-sterilized soil by wet sieving (21 days after addition of the nematodes), captured and infected second-stage juveniles of *M. hapla* could be observed (Figure 6.1C). Ring development around the nematodes was only rarely observed.

In soil with *M. cionopagum*, adhesive knobs, captured and infected juveniles were evident. In contrast, samples with *A. oligospora* (ATCC 24927) did not show adhesive networks or captured nematodes, neither in the buried slides nor in the mycelial mats obtained after wet sieving of the soil 21 days after addition of the nematodes.





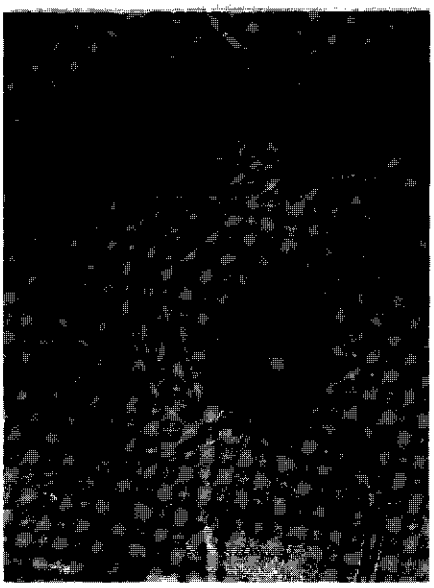


FIGURE 6.1 A-C A: Second-stage juvenile of *Meloidogyne hapla* captured by means of adhesive hyphae of *Arthrobotrys oligospora* (CBS 289.82) in sterilized soil, 7 h after addition of the nematode. VH, vegetative hypha. B: Second-stage juvenile of *Meloidogyne hapla* surrounded by a ring structure of *Arthrobotrys oligospora* (CBS 289.82) in sterilized soil, 24 h after addition of the nematode. RS, ring structure; TH, trophic hypha; VH, vegetative hypha. C: Mycelium of *Arthrobotrys oligospora* (CBS 289.82) from non-sterilized soil, infecting a second-stage juvenile of *Meloidogyne hapla* with trophic hyphae.VH, vegetative hypha.

Fungal growth in soil (Experiments 2-4) Results on growth of the fungal isolates in sterilized soil (Experiment 2B) are presented in Table 6.1.
In the non-inoculated sterilized soil hyphae were nevertheless observed, but far and significantly less than in the inoculated soil. Highest values were obtained for the sticky hyphae-forming *A. oligospora* (CBS 289.82).

Results on growth of *A. oligospora* (CBS 289.82) and *A. oligospora* (ATCC 24927) in non-sterilized soil (Experiment 3) are presented in Table 6.2. In both control soils, total hyphal lengths were significantly below those in soils inoculated with one of the isolates of *A. oligospora*. Hyphal length measured was for both isolates significantly higher during the entire observation period in the sterilized soil than in the non-sterilized soil.
The temperature experiment (Experiment 4) revealed that hyphal growth was not significantly different in soil incubated at 13 or 20°C (Table 6.3). As in the experiments described above, hyphal length in sterilized soil was significantly above that in the non-sterilized soil: the average hyphal length of *A. oligospora* (CBS 289.82) for the entire period in sterilized soil was about 110 m/g oven-dry soil at 13°C and 125m/g oven-dry soil at 20°C and 10 m/g oven-dry soil in non-sterilized soil.

TABLE 6.1 *Hyphal lengths (m/g oven-dry soil) in sterilized loamy sand soil after inoculation with mycelium of:* Arthrobotrys oligospora *(CBS 289.82)*[1], Arthrobotrys oligospora *(ATCC 24927)*, Monacrosporium cionopagum *(CBS 228.52) and* Arthrobotrys conoides *(CBS 265.83) at 20°C (Experiment 2B).*

Days after inoculation[2]	Control	*A. oligospora* (CBS 289.82)	*A. oligospora* (ATCC 24927)	*M. cionopagum* (CBS 228.52)	*A. conoides* (CBS 265.83)
7	18.5 ± 9.6[3]	200.1 ± 69.5	133.7 ± 3.1	60.9 ± 17.6	146.1 ± 3.5
10	12.8 ± 7.9	224.8 ± 16.0	134.6 ± 35.4	76.5 ± 2.5	226.1 ± 69.0
13	9.7 ± 7.2	n.o.	76.1 ± 1.7	64.6 ± 8.8	111.4 ± 10.3
25	8.0 ± 1.2	104.9 ± 18.0	60.2 ± 26.7	65.9 ± 11.5	67.3 ± 5.7
Whole period[4]	11.9 ± 3.2 a[5]	173.7 ± 25.6 d	128.0 ± 21.6 cd	66.6 ± 4.9 b	94.6 ± 14.0 bc

[1] About 30 mm/g oven-dry soil

[2] Nematodes were added at day 10

[3] Mean ± s.e.

[4] Average total hyphal length for the four sampling dates together

[5] Different letters indicate significant differences between the means (Students' t-test, $P<0.05$)

n.o. Not observed

TABLE 6.2 *Hyphal lengths (m/g oven-dry soil) in sterilized and non-sterilized loamy sand soil after inoculation with mycelium of* Arthrobotrys oligospora *(CBS 289.82)*[1] *and* Arthrobotrys oligospora *(ATCC 24927) at 20°C (Experiment 3).*

	Sterilized soil			Non-sterilized soil		
Days after inoculation[2]	Control	*A. oligospora* (CBS 289.82)	*A. oligospora* (ATCC 24927)	Control	*A. oligospora* (CBS 289.82)	*A. oligospora* (ATCC 24927)
7	5.3 ± 0.9[3]	118.5 ± 3.0	96.1 ± 30.8	7.1 ± 1.1	12.7 ± 3.1	16.7 ± 3.9
10	4.7 ± 0.7	81.2 ± 15.8	96.8 ± 15.3	5.0 ± 0.7	13.8 ± 1.9	11.7 ± 0.4
20	6.4 ± 0.4	40.1 ± 7.9	48.7 ± 7.4	9.4 ± 1.8	11.0 ± 1.1	9.9 ± 0.5
Whole period[4]	5.5 ± 0.5 a[5]	76.4 ± 11.5 b	80.5 ± 12.6 b	7.2 ± 0.9 a	12.5 ± 1.4 b	12.8 ± 1.2 b

[1] About 30 mm/g oven-dry soil

[2] Nematodes were added at day 10

[3] Mean ± s.e.

[4] Average total hyphal length for the three sampling dates together

[5] Different letters indicate significant differences between means per soil (Students' t-test, $P<0.05$)

TABLE 6.3 *Hyphal lengths (m/g oven-dry soil) in sterilized and non-sterilized loamy sand soil after inoculation with mycelium of* Arthrobotrys oligospora *(CBS 289.82)*[1] *and incubation at 13 or 20°C (Experiment 4).*

	13°C				20°C			
	Sterilized soil		Non-sterilized soil		Sterilized soil		Non-sterilized soil	
Days after inoculation[2]	Control	*A. oligospora* (CBS 289.82)	Control	*A. oligospora* (CBS 289.82)	Control	*A. oligospora* (CBS 289.82)	Control	*A. oligospora* (CBS 289.82)
12	3.4 ± 0.5[3]	96.8 ± 7.7	7.0 ± 0.8	8.2 ± 2.3	5.6 ± 1.3	119.5 ± 8.7	8.9 ± 1.5	11.9 ± 0.8
16	3.1 ± 0.5	93.2 ± 1.1	7.9 ± 1.0	12.1 ± 2.3	3.2 ± 0.5	128.4 ± 21.7	6.3 ± 0.7	9.9 ± 1.6
25	2.9 ± 0.4	122.7 ± 3.1	3.1 ± 0.5	8.4 ± 0.3	3.5 ± 0.6	91.4 ± 19.7	5.6 ± 0.4	9.3 ± 1.1
30	1.9 ± 0.4	123.4 ± 6.2	3.8 ± 0.1	7.3 ± 1.3	4.1 ± 0.5	159.6 ± 13.3	8.2 ± 2.5	6.9 ± 0.8
Whole period[4]	2.8 ± 0.3 a[5]	107.7 ± 2.5 c	5.5 ± 0.3 a	9.7 ± 0.9 b	4.1 ± 0.4 a	124.8 ± 8.3 b	7.3 ± 0.8 a	9.5 ± 0.6 a

[1] About 30 mm/g oven-dry soil

[2] Nematodes were added at day 15

[3] Mean ± s.e.

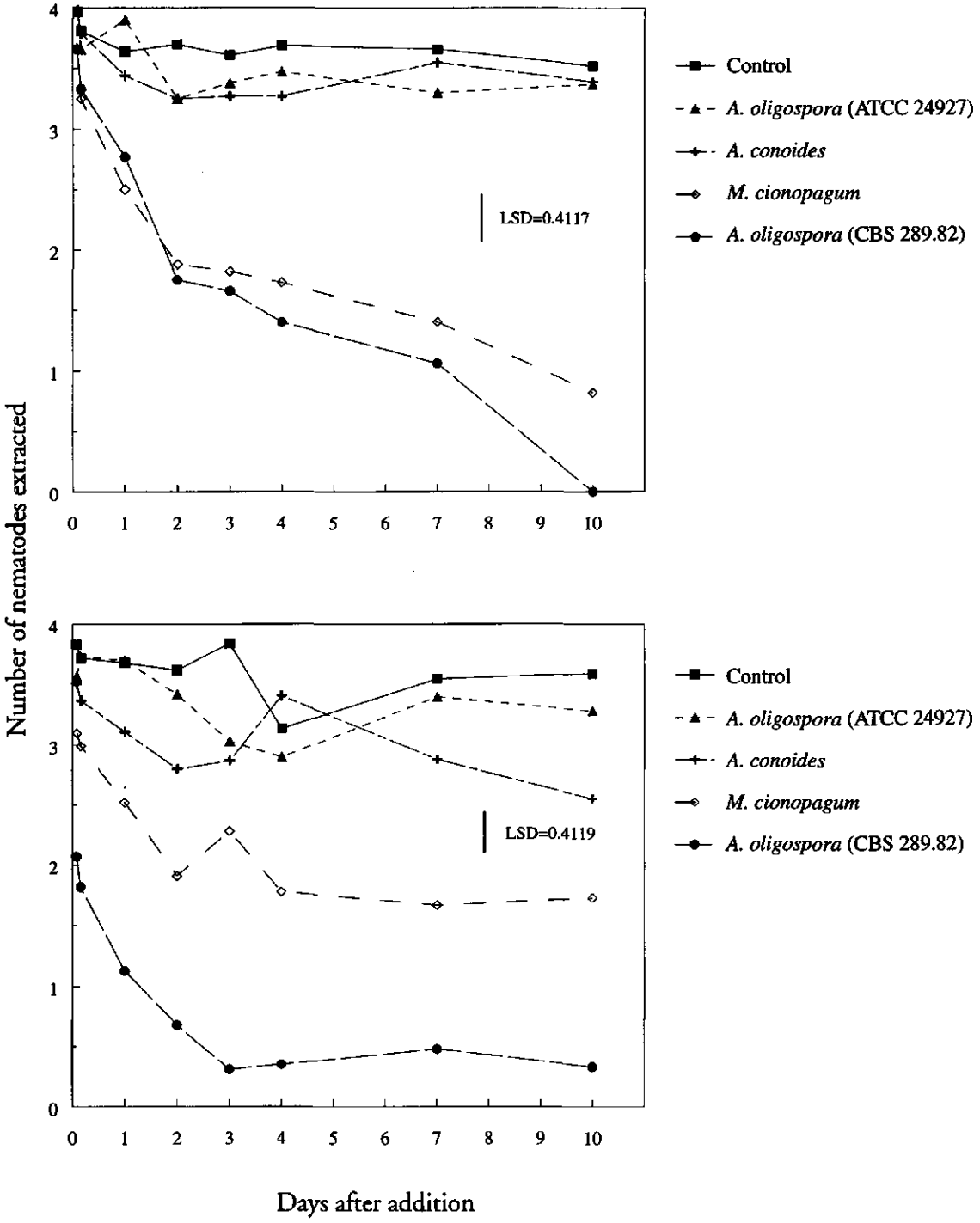
[4] Average total hyphal length for the four sampling data together

[5] Different letters indicate significant differences between the means per temperature (Students' t-test, $P<0.05$)

Capture ability in sterilized soil Experiments 2A and B showed the same tendencies. By far the lowest number of living juveniles was extracted from soil inoculated with *Arthrobotrys oligospora* (CBS 289.82), yielding less than 100 juveniles after a 48 h incubation period, respectively, and virtually none after 10 days (Figure 6.2).

Monacrosporium cionopagum also showed good capture ability, but the number of living juveniles extracted after a 10-day incubation period still was between 10 and 100 juveniles (Figure 6.2). *Arthrobotrys oligospora* (ATCC 24927) and *A. conoides* hardly retained any free-moving juveniles in soil in comparison with the control. In all these treatments, the number of juveniles added, decreased to about 50% of the original number during a 10-day incubation period. Soil inoculated with *A. conoides* resulted in significantly lower numbers of living juveniles at day 10 in comparison to the control in the second experiment, but in the first experiment the number in the soil inoculated with *A. conoides* equalled the number in the control (Figure 6.2).

FIGURE 6.2 Number of living second-stage juveniles of *Meloidogyne hapla* extracted with a mystifier technique after nematode incubation in the presence of a nematophagous fungus added to sterilized soil (Experiment 2A + B). Fungi were incubated for 10 days before addition of the nematodes. Each point in the figure represents 6 replicates. Bar represents $LSD_{\alpha=0.05}$.


Number of nematodes extracted
Days after addition
LSD=0.4117
LSD=0.4119
Control
A. oligospora (ATCC 24927)
A. conoides
M. cionopagum
A. oligospora (CBS 289.82)
Control
A. oligospora (ATCC 24927)
A. conoides
M. cionopagum
A. oligospora (CBS 289.82)

Capture ability in non-sterilized soil In Experiment 3, in sterilized soil inoculated with *A. oligospora* (CBS 289.82), the number of living juveniles was reduced to the same degree as described above: 96 h after inoculation with nematodes only 25 juveniles could be extracted from the soil (Figure 6.3). In the non-sterilized soil inoculated with *A. oligospora* (CBS 289.82) the number of living nematodes that could be extracted was much higher than in the sterilized soil, but significantly lower than in the non-sterilized control soil (at day 10 about 1000 and 5000 juveniles, respectively).

As shown in earlier experiments, the presence of *A. oligospora* (ATCC 24927) did not result in significantly lower numbers of living juveniles when compared to non-inoculated soil, whether sterilized or non-sterilized.

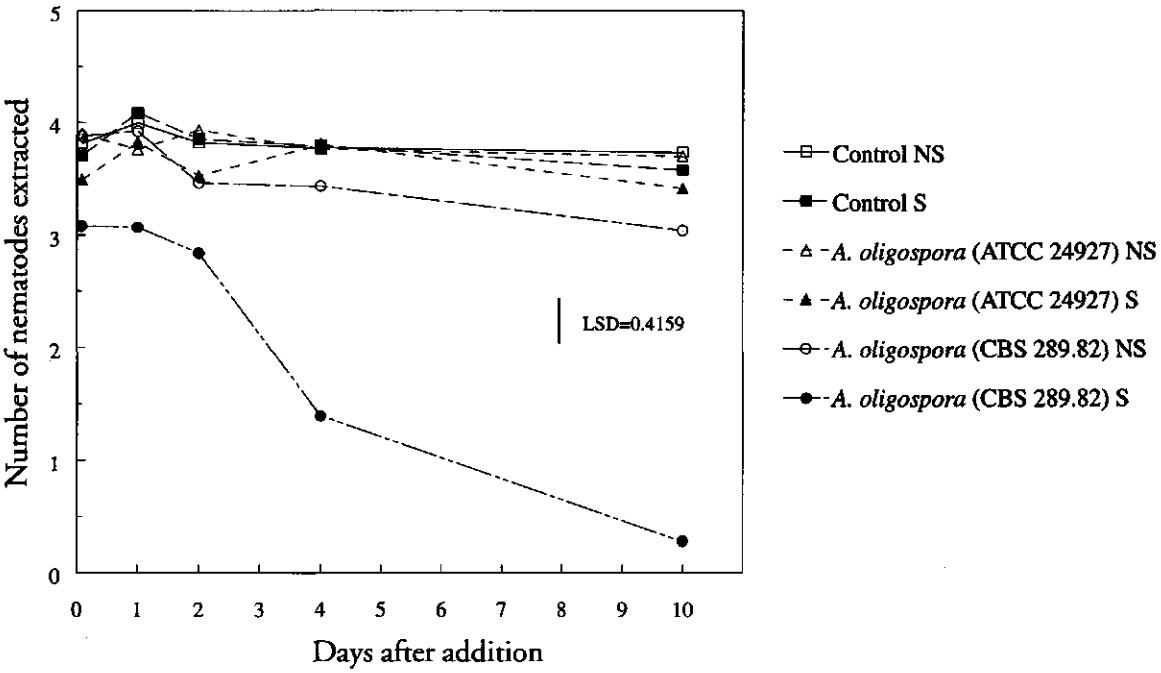
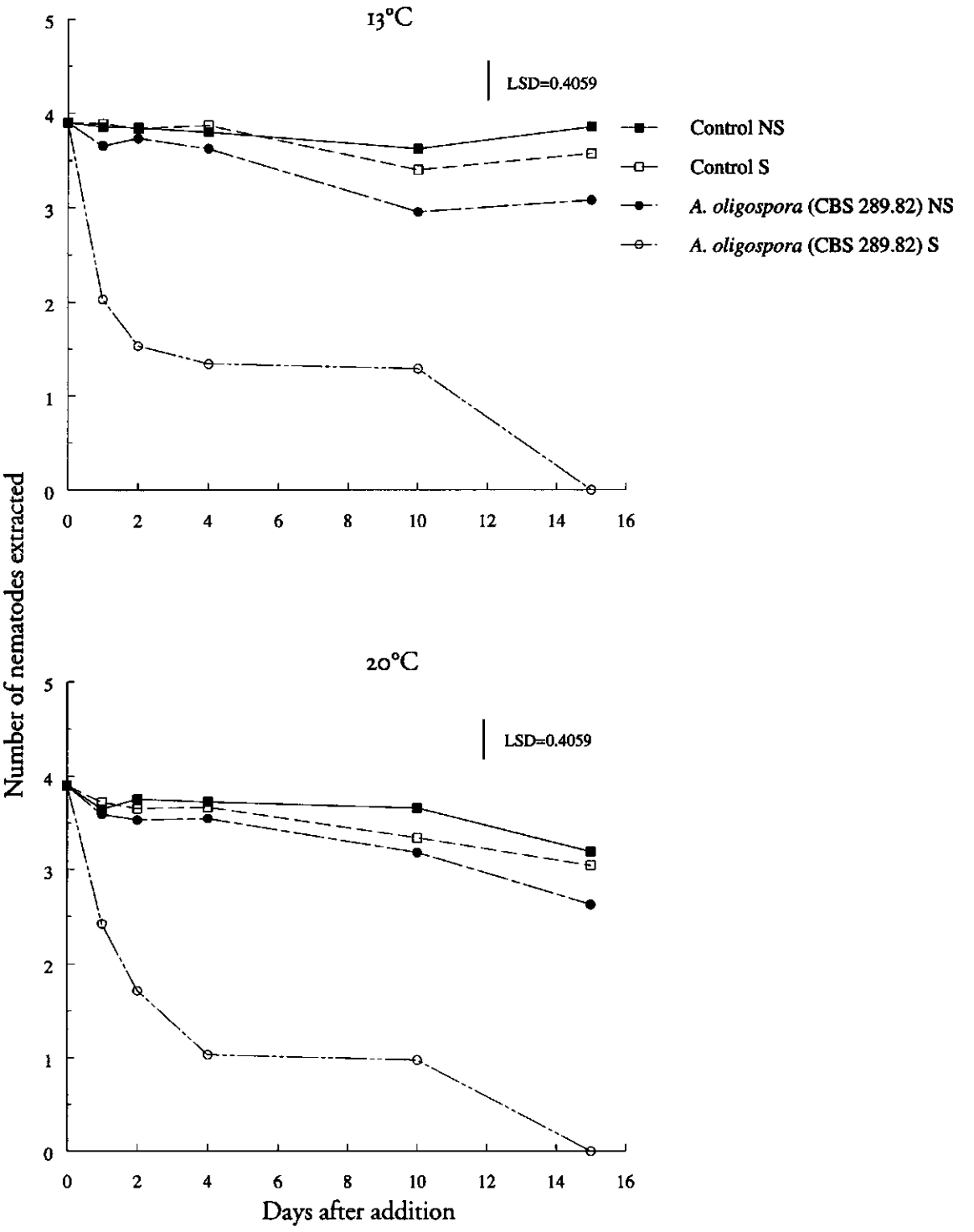


FIGURE 6.3 Number of living second-stage juveniles of *Meloidogyne hapla* extracted with a mystifier technique after nematode incubation in the presence of an isolate of *Arthrobotrys oligospora* (CBS 289.82 or ATCC 24927) in sterilized (S) or non-sterilized soil (NS, Experiment 3). Fungi were incubated for 10 days before addition of the nematodes. Each point in the figure represents 6 replicates. Bar represents $LSD_{\alpha=0.05}$.

Capture ability at 13 and 20°C In Experiment 4, in sterilized soil inoculated with *A. oligospora* (CBS 289.82) and incubated at 13°C, the number of living nematodes that could be extracted was comparable to the number recovered from soil incubated at 20°C (Figure 6.4). Within 96 h after inoculation only 10 and 20 nematodes (at 13 and 20°C, respectively), were extracted from the fungus-infested soil, while in the controls about 7000 and 5000 nematodes were extracted. After 15 days, at both temperatures no live nematodes were extracted from the fungus-inoculated sterilized soil. Also in non-sterilized soil the number of living nematodes in *A. oligospora* (CBS 289.82) inoculated soil decreased significantly, both at 13 as well as at 20°C, when compared to the control (Figure 6.4). After 10 days the number of living nematodes recovered from the soil were 900 and 1500 at 13 and 20°C, respectively, while in the controls about 5000 living nematodes were extracted. Only at the last observation at day 15, the number of living nematodes recovered from the non-sterilized soil incubated at 13°C was higher than at 20°C.

FIGURE 6.4 Number of living second-stage juveniles of *Meloidogyne hapla* extracted with a mystifier technique after incubation in the presence the adhesive hyphae-forming isolate of *Arthrobotrys oligospora* (CBS 289.82) in sterilized (S) and non-sterilized soil (NS), at 13 and 20°C (Experiment 4). Fungi were incubated for 15 days before addition of the nematodes. Each point in the figure represents 6 replicates. Bar represents $LSD_{\alpha=0.05}$.


13°C
LSD=0.4059
Control NS
Control S
A. oligospora (CBS 289.82) NS
A. oligospora (CBS 289.82) S
20°C
LSD=0.4059
Number of nematodes extracted
Days after addition
0
1
2
3
4
5
0
2
4
6
8
10
12
14
16

Isolation of other micro-organisms From the sterilized soil (Experiments 2A and B) fungi of the genera *Monilia*, *Phialophora* and *Penicillium* were isolated. No conidia of endoparasites could be isolated from nematodes extracted with the Oostenbrink funnel technique and pipetted on different media. In the non-sterilized soil experiment the following fungi were isolated: *Penicillium* spp., *Trichoderma harzianum*, *Trichoderma inhamatum* and one time an unidentified Zygomycete.

Discussion

In situ observations of nematode capture in soil Direct observations of nematode capture in soil are very scarce (Ko, 1971; Kliejunas & Ko, 1975). A good impression of capture of nematodes by nematode-trapping fungi could be obtained in samples from soil, placed on a coverglass with a small amount of water and observed with an inverted microscope. Observations of these samples revealed the attachment of second-stage juveniles of *Meloidogyne hapla* to hyphae of *A. oligospora* (CBS 289.82), development of several rings around the nematode body and development of trophic hyphae through the body, illustrating that capture by adhesive hyphae of *A. oligospora* (CBS 289.82) and subsequent infection does occur in soil, as observed previously on agar plates (den Belder & Jansen, 1994b, chapter 2). In soil, development of complex networks or abundant sticky knobs from infected nematodes was never observed, neither in *A. oligospora* (CBS 289.82) nor in *M. cionopagum* (CBS 228.52). This observation is consistent with those in Petri-dishes on corn meal agar where nematodes have been observed for more than 60 days (den Belder & Jansen, 1994a, chapter 4). However, these observations do not support the work of Jaffee *et al.* (1992) who suggested that nematode-trapping fungi typically produce traps when growing from infected nematodes in soil extracts. Evidence that hyphae are readily covered by micro-organisms, causing a drastic reduction of trapping, as suggested by Barron (1979) was never obtained.

Establishment of fungi and capture ability in sterilized soil The present studies show that application of mycelium of the *Dactylaria*-complex (about 30 mm/g oven-dry soil) to sterilized loamy sand soil (organic matter 0.5%,

pH-KCl=6.6), resulted in the establishment of all fungi tested, with hyphal lengths ranging from 70-170 m/g oven-dry soil after 25 days of incubation at 20°C. These data show that they are in good agreement with those from field soils: Bååth & Söderström (1980) found values between 90 and 280 m/g wet soil and Elmholt & Kjøller (1987) found 40 to 400 m/g oven-dry soil. Contrary to observations by Tribe (1980) rapid lysis of vegetative hyphae in sterilized soil has not been observed in all experiments. In some experiments, a decline was evident, nevertheless in other hyphal lengths have been increased.

Nematode capture by *A. conoides* (CBS 265.83) did not show a clear pattern. Notwithstanding its ability to form one of the most extensive mycelial mats of all fungi tested, a very limited or zero reduction of living juveniles recovered from soil 10 days after addition of the nematodes was obtained. This observation agrees with those of Mankau (1961) and Duthoit & Godfrey (1963) who also reported this network forming fungus as rather ineffective. Trap formations has not been quantified in the different soil types so it is difficult to say if soil characteristics could explain the lack of morphological change in these strains. Nevertheless bad trapping of *Meloidogyne* by *A. oligospora* (ATCC 24927) in soil is comparable to results in Petri-dishes (den Belder and Jansen, 1994b, chapter 2). In these *in vitro* experiments *A. oligospora* (ATCC 24927) reacts to *Panagrellus redivivus* with the production of high number of ring structures while in the presence of *M. hapla* no reaction was shown. This could indicate that the lack of reaction to *M. hapla* is due to specific interaction nematode-species/fungus. Differences in trap induction has also been found in the adhesive hyphae-forming isolate of *A. oligospora* (CBS 289.82) in relation to several plant-parasitic nematodes (den Belder & Jansen, 1994b, chapter 2).

Arthrobotrys oligospora (ATCC 24927), also a good colonizer of sterilized soil, reached a density of about 100 m/g oven-dry soil. Nevertheless the number of living juveniles did not differ significantly from that of the control, even using a fairly large nematode inoculum of about 9000 nematodes per container, corresponding to approximately 150 nematodes/g soil, i.e. about three times the nematode number found in natural soil (Jaffee *et al.*, 1992). Comparable to results on agar plates hyphae of *A. oligospora* (ATCC 24927) did not produce traps (den Belder and Jansen, 1994b, chapter 2).

Predacity does not simply depend on the amount of mycelium. This is illustrated by *Monacrosporium cionopagum*, which formed the lowest hyphal length among the fungi tested, whereas 2 days after addition only 10-20% of the nematodes could be recovered from the soil. Clearly spontaneous trap production can result in fast capture.

In the sticky hyphae-forming *A. oligospora* (CBS 289.82) capture of *M. hapla* was most efficient. Application of about 30 mm mycelium of *A. oligospora* (CBS 289.82) resulted in 70-170 m/g oven-dry soil, reduction of 90% in the number of living juveniles of *M. hapla* within 24 h and extermination of the nematodes after 10 days.

Mycelium of *A. oligospora* (CBS 289.82), obtained through wet sieving at the end of the experiments, contained many juveniles attached to hyphae, surrounded by ring structures and filled with trophic hyphae. This observation supports of the suggestion that the reduction of juveniles was due to capture by the fungus.

Establishment of fungi and capture ability in non-sterilized soil Fungal occurrence as measured by hyphal length was considerably reduced in non-sterilized soil, when compared to that in sterilized soil clearly showing the soil fungistasis (Mankau, 1962; Gray, 1987). The total hyphal length of *A. oligospora* (CBS 289.82) and *A. oligospora* (ATCC 24927) reached at most 15% that in sterilized soil: 10-13 m/g oven-dry soil. Poor mycelial development in non-sterilized soils was also observed by other authors in several other nematode-capturing fungi. Mankau (1962) reported limited hyphal extension on agar, hyphae even lysed if, prior to the tests, the fungus had been exposed to soil. Cooke & Satchuthananthavale (1968) found a lower mycelium growth rate (9-60%) on non-sterilized than on sterilized soil.

Two *Trichoderma* spp. known for their suppressiveness of several plant-parasitic fungi (Chet, 1987; Harman & Taylor, 1990) were isolated from the soil. So far there is no evidence for mycoparasitism of the tested nematophagous fungi or vice versa, nematode-trapping fungi as mycopathogens for *Trichoderma* (Tzean & Estey, 1978).

Notwithstanding the relatively poor establishment of *A. oligospora* (CBS 289.82), with a total hyphal length of about 10 m/g oven-dry soil (about 0.01% v/v), the number of living nematodes was significantly reduced.

The mycelia of *A. oligospora* (CBS 289.82), obtained by wet sieving of the soil in the containers, showed a high density of caught and infected juveniles. This clearly demonstrates the capacity of this isolate to establish in non-sterilized soil when inoculated as small mycelial fragments and its ability to capture nematodes under such conditions. The other isolate tested, *A. oligospora* (ATCC 24927), did not produce traps in the loamy sand used in our experiments. This observation is consistent with results obtained on agar plates (CMA 1:10) where *M. incognita* and *M. hapla* did not induce capture structures either (den Belder & Jansen, 1994b, chapter 2).

Establishment of fungi and capture at 13 and 20°C Based on *in vitro* results reduced hyphal growth of *A. oligospora* (CBS 289.82) could be expected at 13°C in comparison to 20°C (den Belder & Jansen, 1994b, chapter 4). However, this was not observed. Probably other factors are limiting growth through the soil than temperature.
Both at 13 and at 20°C, only 10-20 living nematodes were extracted from the fungus inoculated soil after 96 h, whereas in the controls between 5000 and 7000 juveniles were recovered. Several authors reported that the nematode-capturing fungi they studied, including network-forming isolates of *A. oligospora*, did not respond to nematodes at low temperatures (Soprunov, 1966; Cayrol & Brun, 1975). Our data show that at soil temperatures typical for temperate regions, proliferation of vegetative hyphae and capture may result in a significant reduction of the nematode population.
In non-sterilized soil inoculated with *A.oligospora* (CBS 289.82) the number of living nematodes decreased significantly slower than in sterilized soil both at 13 and at 20°C. This suggests that in these experiments fungistasis was more important as a limiting factor than temperature.

The distinction made by Jansson & Nordbring-Hertz (1980) and Jansson (1982) on the basis of the work of Cooke (1963a, b) of the fungi infecting vermiform nematodes into several groups can be extended with the group of adhesive hyphae formers.

In the ecological continuum of nutritional dependencies, largely predacious species occur at one end and saprophytic species at the other (Cooke, 1963a; Jansson, 1982; Gray, 1987). Based on the knowledge we obtained on predacious

abilities in soil and *in vitro* studies (den Belder & Jansen, 1994a, b), *A. oligospora* (CBS 289.82) can be placed near the group of the predacious species because, as soon as growing hyphae are produced in soil it may capture root-knot nematodes, while firm binding occur irrespective of temperature. Also the other network formers *A. botryospora* (Barron, 1979), *A. superba* (Fritsch & Lysek, 1989) and *Monacrosporium psychrophilum* (Gray, 1985) producing adhesive hyphae, might be placed in this group.

The above results obtained on *A. oligospora* (CBS 289.82) support the conclusion that such species are able to capture nematodes in soil. Consequently trapping of nematodes by fungi may be of greater importance than hitherto considered.

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7

General discussion

Introduction Nematode-trapping fungi are receiving more and more attention as potential agents for biological control of nematodes. In the literature on this subject, two types of research prevail. On the one hand, there are *in vitro* studies on the mechanisms of nematode capture and infection at the organism level such as attraction, attachment, binding mechanisms, penetration, and toxification. On the other hand, there are studies at a very high level of integration under natural conditions, using plant growth and injury levels as derived parameters to measure the efficacy of trapping. The lack of knowledge at the intermediate level, the ecology of nematode trapping fungi, precludes satisfactory explanations for the inconsistent effects of these fungi in the complex soil environment. De Leij (1992) stressed the importance of studies on the ecology of antagonists and their nematode target as a prerequisite for development of effective biological control methods.
In this study, trapping by the adhesive-hyphae forming fungus *Arthrobotrys oligospora* in relation to the target nematode, *Meloidogyne hapla*, has been investigated by *in vitro* studies and microcosm experiments. Factors most likely to affect trapping activity have been emphasized. Experiments were meant to contribute to a better understanding of the potential of this type of fungi as biological control agents.

Mechanisms of nematode trapping and infection The possible existence of fungi with septate hyphae (Hyphomycetes) are able to capture directly nematodes by means of adhesive vegetative hyphae was first suggested by Barron (1977). Since then four studies have reported the capture of nematodes by such hyphae. Barron (1979) decribed the phenomenon for *Arthrobotrys botryospora*; Gray (1985) for *Monacrosporium psychrophilum*; Fritsch & Lysek (1989) for *Arthrobotrys superba* and in the present study, den Belder & Jansen

for *A. oligospora*. Unfortunately, the species of nematodes were not mentioned by the first three authors.

The isolate *Arthrobotrys oligospora* (CBS 289.82) proved to be very effective in capturing *M. hapla* and *M. incognita* by morphologically undifferentiated vegetative hyphae (chapter 2).

In *in vitro* experiments, mobile juveniles of *M. hapla* were all caught by the hyphae within one hour. In some cases attachment occurred at the very first contact and more than 40 % of the juveniles became attached during the first ten encounters (chapter 2).

Microscopic observations revealed the presence of small amounts of extracellular material 0.1 µm thick bridging hyphae and nematode. Lumps of adhesive substances, such as those found in the Zygomycetes (Drechsler, 1934; Wood, 1983) which reach a diameter of 35 µm, were never observed. A possible explanation for such binding might be that the movement of *Meloidogyne* spp. juveniles induced excretion and/or accumulation of the extracellular material and its reorganization to a strong adhesive (Tunlid *et al.*, 1992). In the case of certain nematode-hyphae interactions in which the nematode moves over the hypha with its entire length along, it might explain why the posterior part of the body becomes attached. Examples for such thigmotropic responses in fungi-plant attachment abound (Hoch & Staples, 1987).

The chemical composition of nematodes (Nordbring-Hertz & Mattiasson, 1979) and fungal surfaces are also likely to determine attachment (Tunlid *et al.*, 1992). Treatment of hyphae of *A. oligospora* (CBS 289.82) with Pronase E, known to modify the structure of extracellular proteins, inhibits the subsequent attachment of nematodes (den Belder, unpublished). This agress with results on the adhesive ring forming isolate *A. oligospora* ATCC 24927 (Tunlid *et al.*, 1991). These and similar observations strongly suggest involvement of extracelluar proteins, possibly glycoproteins, in the surface-binding properties of the hyphae.

Lectin-carbohydrate binding could be postulated, as this would properly explain the very short time required for firm attachment and the virtual temperature independence of the process. However, the simultaneous involvement of more binding mechanisms, including absorbins, at the same time cannot be excluded (Tunlid *et al.*, 1992).

Differences in physical and chemical qualities of the cuticle of different plant-parasitic nematode species (Tunlid *et al.*, 1992) might explain the significant

differences in the ability of *A. oligospora* (CBS 289.82) to capture the nematode species tested, such as *Globodera pallida, G. rostochiensis, Meloidogyne hapla* and *Pratylenchus penetrans.*

A large proportion of second-stage juveniles of *M. hapla* were consistently trapped by *A. oligospora* (CBS 289.82), both *in vitro* and *in situ* in soil. These experiments suggest a very high density of capture sites along the hyphae, indicating the potential to capture high numbers of nematodes by *A. oligospora* (CBS 289.82). nematodes.Independent of age of the fungus *Meloidogyne* second-stage juveniles attached equally well to recently formed and mature hyphae (chapter 4). This is quite different from other species of the *Dactylaria*-complex described so far, which form complex trapping structures (Feder, 1963; Esser *et al.*, 1991; Grønvold, 1989) and also different from other adhesive hyphae-forming Hyphomycetes such as *Monacrosporium psychrophilum* (Gray, 1985). A comparable high density of capture sites was found in a nematophagous Zygomycete *Cystopage intercalaris* (Gray, 1984). Eventually, capture continued until all hyphal surfaces were completely occupied, leaving no space for any more nematodes.

Following the attachment of *Meloidogyne* to the hyphae ring structures are formed, 50% of which did not develop at the initial attachment site. Development of an infection bulb and trophic hyphae was dependent on the formation of the ring structure and did not occur if nematodes were only attached to hyphae.
A ring structure may prevent loss of contact between the fungal wall and the nematode, counteracting the mechanical forces exerted during penetration (Veenhuis *et al.*, 1985). The requirement of an intermediate structure, 'appresorium' prior to penetration has been demonstrated in spores of several endoparasitic fungi (Dijksterhuis, 1993).
Failure to infect particular nematode species such as *Pratylenchus penetrans* and *Globodera* spp. might be related to the properties of the cuticle, such as its composition and thickness, or to the internal pressure the nematode. Although infection failure has been studied in detail in *Drechmeria coniospora,* the precise nature of the factors which regulate penetration are still obscure (Jansson *et al.*, 1985, 1987; Dijksterhuis, 1993). Their observations show that nematode attachment is not necessarily followed by penetration and colonization.

The consequences of an incomplete infection differs for nematode-trapping fungi and endoparasites: attachment to endoparasitic conidia does not result in a reduction of nematodes, while attachment to nematode-trapping fungi prevents plant-parasitic nematodes from reaching the host plant. This implies that the incapability of nematode-trapping fungi to penetrate and colonize the nematode has less consequences for its role as a biological control agent than for endoparasites.

Growth requirements of the fungus in relation to the efficacy of trapping nematodes Several authors have reported that nematode-capturing fungi, including the adhesive ring-forming isolates of *A. oligospora*, did not respond or resonded only slowly to nematodes at temperatures below 10°C in developing ring structures (Soprunov, 1966; Grønvold, 1989).
The attachment of *Meloidogyne* second-stage juveniles to *A. oligospora* isolate CBS 289.82 was not affected by temperatures between 5 and 30°C. However, ring structure development and growth of trophic hyphae were strongly influenced by temperature (chapter 4). Both processes were strongly hampered below 15°C in comparison to higher temperatures (chapter 4). This implies that under the prevailing soil temperatures in temperate regions, ring structure development and growth of trophic hyphae may proceed slowly, whereas trapping would continue to occur. At soil temperatures typical for temperate regions, our isolate *A. oligospora* (CBS 289.82), showed that proliferation of vegetative hyphae and capture may result in a significant reduction of the nematode population (chapter 6).

A similar tendency was found with respect to nutrition. A poor substrate like water agar slowed down the development of ring structures, and hence internal colonization by trophic hyphae, but did not influence attachment. Also, the presence of food in the form of dead *Meloidogyne* juveniles did not influence attachment efficacy to live nematodes (chapter 4). Combining these observations, it appears that nematode attachment to hyphae is largely independent of environmental conditions. This implies that the range of circumstances in which this fungus can be effective as a biological control agent may be very broad. In contrast, fungi with complex capture devices are severely influenced by environmental factors (Blackburn & Hayes, 1966; Nordbring-Hertz, 1977; Rosenzweig, 1984; Nordbring-Hertz, 1988; Grønvold, 1989; Dijksterhuis, 1993).

Our isolate showed that it also had the capacity to consume dead nematodes. Dead and ruptured nematodes were covered by a dense mycelium, whereas dead intact nematodes were penetrated through their buccal cavities by corkscrew-like hyphal structures and were subsequently colonized by trophic hyphae.

Our observations show that the colonization of living second-stage juveniles by trophic hyphae following attachment and penetration was faster than colonization of killed second-stage juveniles of *M. hapla*. This may be explained in part by activation of the live nematode's autolytic enzymes. (Veenhuis *et al.*, 1985).

Under optimal conditions *A. oligospora* (CBS 289.82) required 10 days to occupy 50% of the nematode body length. Nematodes filled with trophic hyphae 24 h after attachment to ring structures, as in *Panagrellus redivivus* with *A. oligospora* (ATCC 24927, Nordbring-Hertz *et al.*, 1986), or the complete consumption aafter 3 days as in juveniles of *Neoaplectana* or *Heterorhabditis* (Poinar & Jansson, 1986), were never observed in *M. hapla* with *A. oligospora* (chapter 4). Hence, it appears that the speed of infection of the nematode and the nutrient acquisition by the fungus vary with nematode species.

An important quality of fungi as potential biological control agents is their ability to form mycelium and trapping structures in the soil at the place where their activity is desirable. Although quite some work has been done on the occurrence and distribution of *A. oligospora* (Gray, 1987), few quantitative studies exist on population dynamics and survival in soil (Stirling, 1991). Generally, populations of antagonistic soil-inhabiting fungi are difficult to quantify because of their filamentous nature (Dackman *et al.*, 1987; Stirling, 1991). In this study, observations made in an *in vitro* system were confirmed by direct microscopic observations in microcosms with sterilized or non-sterilized soil. The confirmation included the attachment of mobile juveniles of *M. hapla* to hyphae of *A. oligospora* (CBS 289.82), development of rings around the nematode body and development of trophic hyphae through the body (chapter 6).

In this study the establishment of fungi in soil, following the introduction of hyphal fragments, was also demonstrated. The fungus introduced into sterilized soil increased its hyphal length 3500 to 7000x in 12 days. When *M. hapla*

second-stage juveniles were subsequently introduced into the microcosm, 90% was captured within one day and 100% within 10 days (chapter 6).
Fungal occurrence as measured by hyphal length of *A. oligospora* (CBS 289.82) was considerably reduced in non-sterilized soil when compared to that in sterilized soil. Reduced growth in non-sterilized soil also has been reported by various authors (Mankau, 1962; Cooke & Satchuthananthavale, 1968) and is most likely a result of fungistasis.
However, within 10 days, 70% of the second-stage juveniles were trapped. This indicates that this isolate is highly effective, even at a low density.
The re-isolation of mycelium and infected nematodes from the soil by wet sieving at the end of the experiments, clearly showed that the reduction of second-stage juveniles was due to capture by the fungus (chapter 6).

Notwithstanding their ability to form the most extended mycelial mats of all fungi tested and despite the fairly large amounts of *M. hapla* added, *A. conoides* (CBS 265.83) and *A. oligospora* (ATCC 24927) were not able to develop sufficient capture devices in soil. In sterilized soil, nematode capture by both adhesive ring forming fungi tested, *A. conoides* and *A. oligospora* ATCC 24927, was zero or very low (chapter 6). This supports observations by Cooke (1962) and Gray (1987) that adhesive ring forming fungi are inefficient.

Implications for the screening of nematode-trapping fungi This study has shown that differences between isolates of *A. oligospora* are large with regard to mycelium growth and formation of trapping structures and their efficacy. Until recently, most studies on nematophagous fungi were performed with single isolates (Stirling, 1991), overlooking within-species variability. The variation in virulence found between isolates of *A. oligospora* in relation to root-knot nematodes strongly suggests that the screening of nematophagous fungi as potential biocontrol agents should involve different isolates of one species.
Obviously, screening for rapid development of complex trapping structures only is not appropiate, as this would lead to the neglect of adhesive hyphae-forming fungi. It is also recommended to screen fungi using the target nematode species. In many studies, model nematodes are used to detect nematophagous fungi (Jansson, 1982) and although this may be attractive from the point of view of convenience of rearing of the nematode, it may lead to selection of fungal species and isolates that are little effective as biocontrol

agents. Deacon (1991) has rightly stated in this respect that 'we get what we select'.

One way of looking at the role of nematophagous fungi as biological control agents is to study nematode suppression in relation to naturally occurring population levels of these fungi. It may well be, however, that the existence of adhesive hyphae formers has remained unnoticed because they do not have traps or produce traps in a later phase, they are rather inconspicuous and only readily noticed when nematodes have been caught in the hyphae.

There is a need to make surveys for nematophagous fungi, preferably in soils where the pest nematode is indigenous and in soils where it causes less problems than would be expected, the so called suppressive soils (Kerry, 1988). Variability in nematode distribution is usually attributed to soil physical factors or cropping history, but there are probably situations where nematodes are suppressed by biological means.

This study supports arguments for a different approach in screening nematophagous fungi. Factors which are very difficult to manipulate e.g. temperature, host specificity, competitive saprophytic ability and rhizosphere competence have to be included in an early stage of the screening. Experiments should be conducted at realistic temperatures.

If we want to exploit the antagonistic potential of the adhesive hyphae-forming fungi in agricultural ecosystems, we have to use methods with which adhesive hyphaeformers can be detected.

In ordinary soil fungal analyses, nematophagous fungi are rarely isolated. So to detect nematophagous fungi, specialized techniques for their recovery have to be used (Barron, 1982; Gray, 1984). Baiting fungi with living nematodes is an adequate technique for the detection of nematophagous fungi (Dackman *et al.*, 1987).

Within this group of nematophagous fungi, the poorly sporulating or slow-growing fungi tend to be even more overlooked, particularly when using the dilution plate technique. The soil washing technique described by Gams & Domsch (1969) and Gams (1992) seems to be a good alternative.

Implications for biological control When discussing the biology and ecology of nematophagous fungi it is essential to consider two factors that may greatly limit their potential as biological control agents. First, there is the

complex soil microbial community that can seriously hamper their establishment. Second, it would generally be uneconomical to introduce nematophagous fungi into the soil in amounts that are sufficiently high to obtain direct control (Stirling, 1991). Consequently, it is essential to select nematophagous fungi with a strong competitive saprophytic ability. The microcosm studies showed that the CBS 289.82 isolate of *A. oligospora* rapidly colonized non-sterilized soil, starting from a small amount of initial inoculum, and trapped high numbers of *M. hapla* second-stage juveniles. The combination of its saprophytic ability, its moderate temperature requirements for vegetative growth, and temperature indifference for nematode trapping makes this isolate a promising candidate for biological control. Further research is needed to establish its saprophytic competence and effectiveness in the natural soil environment, particularly in the rhizosphere (Ahmad & Baker, 1987). The fate of the introduced antagonist should be carefully monitored. This may be achieved most readily by a stepwise increase in the complexity of the test system. Examples of such an approach with *Heterodera schachtii – Hirsutella rhossiliensis* (Jaffee *et al.*, 1992a, b) and *Verticillium chlamydosporium – Meloidogyne* sp. (Kerry, 1988; de Leij, 1992) have yielded valuable information on the dynamics of host plant/plant-parasitic nematode/ nematophagous fungi systems. Such studies should lead to insight in fungal application rates necessary for control, and adequate inoculation techniques. The choice of the latter can have a major effect on the success of nematophagous fungi as biological control agents (Harman, 1991). In this respect, seed treatment seems particularly interesting because it would give the fungus a competitive advantage as initial colonizer of the rhizosphere and would require smaller amounts of initial inoculum in comparison to soil inoculation (Harman, 1991).

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Summary

In this thesis the ecology of an isolate of a particular fungus in the *Dactylaria*-complex, *Arthrobotrys oligospora* (CBS 289.82) which captures juveniles of root-knot nematodes *Meloidogyne* spp. directly with hyphae instead of through complex structures, is studied in relation to its potential as biological control agent.

In vitro tests (chapter 2) demonstrated that the isolate of *A. oligospora* forming adhesive hyphae was very effective in capturing *M. hapla* and *M. incognita* in comparison with fungi with other capture devices.
Following adhesion, penetration of the nematodes and internal colonization with trophic hyphae of *A. oligospora* (CBS 289.82) did not occur until development of ring structures around the nematode body had taken place.
Electron microscopic studies strongly suggested that attachment of nematodes to hyphae is mediated by a layer of extracellular material on the hyphae with a thickness of about 0.1 µm. After attachment this layer was distributed irregularly over the fungal surface, in some cases covering it entirely, in others covering only the side where nematode attachment took place. Such a layer was never observed in hyphae of fungal cultures to which no nematodes were added, suggesting that its presence depends on interaction of the fungus with the nematode. At higher magnification the extracellular layer appeared amorphous, and without fibrils (chapter 3).

Development of capture structures and attachment in most nematode-trapping fungi is conditioned by temperature, substrate, light and ageing of fungal mycelium. How these factors affect the development of capture structures in *A. oligospora* (CBS 289.82) was investigated. Firm binding of the second-stage juveniles of *M. hapla* to hyphae of *A. oligospora* (CBS 289.82) occurred within one hour, independent of temperature (between 5 to 30°C) and independent of differences in nematode mobility. Furthermore, the nutritional conditions during growth of the fungus did not correlate with the rapidity of nematode-hypha attachment.

The results provide evidence that the trapping ability in the isolate tested continued over more than 70 days. Light did not appear to influence the ability of the fungus to capture nematodes. Subsequent development of ring structures around the nematode, however, was significantly slower at the lower temperatures (5 and 10°C) than at the higher temperatures (between 15 and 30°C). At temperatures of 15°C and below development of trophic hyphae was significantly reduced in comparison to higher temperatures tested. Also on a poor medium, water agar, this process was slower than on richer media. This is contrary to the assumption that a poorly nourished fungus would switch to nematode capture.

These results suggest that the formation of adhesive hyphae is less temperature and nutrient dependent than the development of the morphologically more complex capture devices. This suggests that the range of conditions under which this isolate of *A. oligospora* captures nematodes is wider than that of isolates where adhesive ring structures (or three-dimensional networks) are the only capture devices (chapter 4).

This adhesive hyphae-forming isolate of *A. oligospora* strongly responded to the condition of its prey. Second-stage juvenile nematodes immobilized by heating and only capable of moving the anterior region or the stylet, were surrounded by ring structures similar to mobile juveniles. However in this case, allmost all ring structures were mainly developed around the moving head. The fungus penetrated dead, but otherwise intact second-stage juveniles (treated with gamma-irradiation or sodium azide), through their buccal cavity with hyphae with a corkscrew-like structure.

Dead second-stage juveniles with a ruptured cuticle were totally overgrown by the fungus with vegetative hyphae. Apparently, this isolate of *A. oligospora* switched between modes of nutrition while exploiting different food sources. So far, the saprophytic and predacious ability appear not to be mutually exclusive.

Addition of dead juveniles to a fungal colony prior to live juveniles did not affect attachment or the development of trophic hyphae through the live juveniles. However, one day after the addition of the live juveniles, the proportion of live nematodes with ring structures was higher than where living and dead juveniles were added at the same time. The development of trophic hyphae in dead second-stage juveniles was delayed in the presence of live

second-stage juveniles. The results refute the commonly held assumption that poor possibilities for saprophytic growth are a prerequisite for the formation of capture devices and the predacious mode of feeding in the fungus (chapter 5).

In order to identify some of the factors affecting capture ability of the adhesive hyphae-forming isolate of *A. oligospora* (CBS 289.82) in soil, establishment and capture of this isolate was compared to that of other fungi from the *Dactylaria*-complex in a simple microcosm system at 20°C.
Application of about 30 mm hyphal fragments of *A. oligospora* (CBS 289.82) per gram of sterilized soil resulted in 100-170 m per gram oven-dry soil at 20°C, a reduction of 90% in the number of live juveniles of *M. hapla* within one day and an extermination of the nematodes within 10 days. In non-sterilized soil, the hyphae reached a total length of about 10 m per gram oven-dry soil. Such hyphal mass was sufficient to reduce the number of live nematodes by 70% as compared to the control within 10 days after adding the nematodes to the soil. At 13°C similar results were obtained.
These results indicate that this adhesive hyphae-forming isolate has a potential to control nematodes under realistic conditions (chapter 6).

In chapter 7 the results on the ability of *A. oligospora* (CBS 289.82) to capture root-knot nematodes presented in the previous chapters are evaluated and the mechanism of nematode trapping by hyphae is discussed from the perspective of screening nematode-trapping fungi and using the adhesive hyphae-forming fungi as biological control agents.

Because of the variation found in capture ability between isolates of *A. oligospora* in relation to root-knot nematodes different isolates of species of nematophagous fungi have to be considered in the screening as potential biological control agents.
It is recommended to use the target nematode species in detection and screening of nematode-trapping fungi since the use of a model nematode in screening can easily lead to under-and overestimation of particular fungal species. Screening for fast development of complex trapping devices only is not enough as this would lead to the neglect of adhesive hyphae-forming species.

The present thesis gives evidence that it is of utmost importance to conduct experiments at temperatures comparable to those in the field. Most of the published work has been performed at higher temperatures.

The combination of its saprophytic ability and its temperature independence for nematode trapping makes the adhesive hyphae-forming isolate of *A. oligospora*, used in the present study, a promising agent for biological control of nematodes.

Further research is needed to establish its saprophytic competence and effectiveness in the natural soil environment particularly in the rhizosphere of several crops.

Samenvatting

De ontdekking van een schimmel binnen de groep van het *Dactylaria*-complex, die in staat in nematoden direct met behulp van schimmeldraden te vangen, in plaats van met complexe vangstructuren, riep vragen op over zijn capaciteit om economisch belangrijke nematodesoorten te bestrijden.
Dit proefschrift gaat over de vangcapaciteit van deze bijzondere schimmel, *Arthrobotrys oligospora* (CBS 289.82), ten aanzien van *Meloidogyne* soorten die een plaag vormen in tal van gewassen.

In vitro experimenten toonden aan dat het isolaat van *A. oligospora* dat kleefdraden vormt één van de meest efficiënte *M. hapla* en *M. incognita*-vangende schimmels is in vergelijking met schimmels die andere vangstructuren vormen. Na de hechting vond het binnendringen van de nematode en de inwendige kolonisatie door verteringshyfen niet plaats voordat een ringstructuur rond het lichaam van de nematode was gevormd (hoofdstuk 2).
Transmissie-elektronenmicroscopische studies suggereerden in sterke mate, dat een laag van extracellulair materiaal op de hyfen ter dikte van ongeveer 0,1 µm, een rol speelt in de hechting van de nematode aan de schimmel. Deze laag was onregelmatig verdeeld over het schimmeloppervlak; in sommige gevallen was de hyfe in het geheel overdekt, in andere gevallen zat het materiaal alleen aan die kant waar de nematode vastzat. Op schimmeldraden uit een kolonie zonder nematoden werd een dergelijke extracellulaire laag echter nooit aangetroffen, wat erop duidt dat de aanwezigheid ervan afhangt van de interactie van de schimmel met de nematode. De extracellulaire laag lijkt bij hogere vergroting amorf en zonder fibrillen (hoofdstuk 3).

Ontwikkeling van hechtplaatsen en de daaropvolgende hechting van de nematoden wordt in vele nematoden-vangende schimmels door o.a. temperatuur, het substraat waarop de schimmel groeit, licht en de leeftijd van de schimmel beïnvloed.

Onafhankelijk van de temperatuur, welke varieerde van 5 tot 30°C, en de mobiliteit van de nematoden, lagen binnen één uur alle juvenielen van *M. hapla* stevig vast aan de schimmeldraden van *A. oligospora* (CBS 289.82).
De snelheid van hechting tussen nematode en schimmeldraad werd evenmin beïnvloed door de voedingsomstandigheden waaronder de schimmel groeide. Daarnaast kwam in deze studie naar voren dat het vangvermogen van dit isolaat over meer dan 70 dagen constant bleef. Licht had evenmin effect op het vermogen van de schimmel om de nematoden te vangen.
De snelheid waarmee de schimmel vervolgens ringstructuren rond de nematoden ontwikkelde was beduidend lager bij temperaturen van 5 en 10°C dan bij 15°C en hoger. Ook op een voedingsarm medium (wateragar) verliep dit proces trager dan op rijkere media. Dit is tegengesteld aan de algemene aanname dat op een arm medium een schimmel overgaat op het vangen van nematoden.
Deze resultaten suggereerden dat het ontstaan van een kleefdraad minder temperatuurs- en voedingsafhankelijk is dan de ontwikkeling van morfologisch meer complexe vangstructuren. Dit impliceerde dat dit isolaat van *A. oligospora* onder een bredere reeks van omstandigheden nematoden vangt dan isolaten waarvan ringstructuren (of drie-dimensionale netwerken) de enige vangstructuur zijn (hoofdstuk 4). Het isolaat was daarom interessant voor een uitgebreidere evaluatie met het oog op de biologische bestrijding van *Meloidogyne* soorten.
Het kleefdraden-vormend isolaat van *A. oligospora* reageerde in sterke mate op de conditie van de prooi. Juvenielen van *M. hapla* in het tweede larvale stadium, door een warmtebehandeling geïmmobiliseerd en alleen in staat hun kop of stekel te bewegen, werden net zoals de normaal bewegende nematoden omgroeid door een ringstructuur. Deze ringen ontstonden bij de geïmmobiliseerde nematoden echter overwegend rond de kop van de nematode. Dode juvenielen met een intacte cuticula behandeld met gamma-straling of natriumazide, werden via de mondopening door middel van hyfen met een kurketrekkerachtige structuur gepenetreerd. Dode nematoden met een gescheurde cuticula werden volledig overgroeid door vegetatieve hyfen. Klaarblijkelijk kan dit isolaat naar verschillende voedingswijzen overschakelen om de verschillende voedselbronnen te benutten. Saprofytische en predatoire eigenschappen sluiten elkaar in dit isolaat niet uit.

De toevoeging van dode nematoden aan een schimmelkolonie voordat er levende nematoden werden aangeboden had geen invloed op het vangen van de levende nematoden of de kolonisatie van deze laatste groep door verteringshyfen. Eén dag na toevoeging van de levende nematoden echter, was het aantal levende nematoden met ringstructuren groter dan wanneer dode en levende nematoden op hetzelfde moment werden aangeboden. De ontwikkeling van verteringshyfen in de dode nematoden werd vertraagd indien ook levende nematoden aanwezig waren. De algemene veronderstelling dat ongunstige omstandigheden voor saprofytische groei een vereiste zijn voor de vorming van vangstructuren en voor de predatie van nematoden gaat voor dit isolaat niet op (hoofdstuk 5).

In een microkosmos-systeem werd bij 20°C de vestiging en het vangen van het kleefdraden-vormend isolaat van *A. oligospora* (CBS 289.82) vergeleken met dat van andere schimmels uit het *Dactylaria*-complex, teneinde een aantal factoren te bepalen die de vangcapaciteit beïnvloeden. Toediening van stukjes schimmeldraad aan gesteriliseerde grond (30 mm per gram grond) resulteerde uiteindelijk in een hoeveelheid van 100-170 m hyfe per gram gedroogde grond en een reductie van 90% van het aantal levende juvenielen van *M. hapla* binnen één dag. Na 10 dagen werd een reductie van 100% gevonden.
In niet-gesteriliseerde grond was de gemeten totale hyfenlengte van *A. oligospora* van ongeveer 10 m per gram gedroogde grond toereikend om het aantal levende nematoden met 70% te reduceren. In proeven bij 13°C werden vergelijkbare resultaten verkregen. Deze uitkomsten ondersteunen de verwachting dat dit kleefdraden-vormend isolaat van *A. oligospora* potentie heeft voor de biologische bestrijding van wortelknobbelaaltjes in de praktijk (hoofdstuk 6).
In hoofdstuk 7 worden de resultaten van de vangcapaciteit van *A. oligospora* (CBS 289.82) geëvalueerd en wordt het vangmechanisme bediscussieerd, in het perspectief van de selectie van nematoden-vangende schimmels en hun potentie in de biologische bestrijding.
Selectie op basis van een snelle ontwikkeling van complexe vangstructuren is onvolledig en leidt ertoe dat aan kleefdraden-vormende schimmels voorbij wordt gegaan.
De grote verschillen die zijn gevonden in vangcapaciteit tussen de isolaten van *A. oligospora* in relatie met wortelknobbelaaltjes illustreren dat met verschillen

tussen isolaten van één soort in de selectie van nematoden-vangende schimmels rekening moet worden gehouden.
Hoewel het zeer aantrekkelijk is met een niet plant-parasitaire modelnematode te werken omdat deze eenvoudig te kweken is, kan dit makkelijk leiden tot een onder- of overwaardering van bepaalde isolaten. Het is daarom aan te bevelen met de te bestrijden nematodesoort te werken bij de selectie van nematode-vangende schimmels.
Veel onderzoek naar het vangen van nematoden wordt op dit moment bij kamertemperatuur uitgevoerd. Het is echter van groot belang dit onderzoek bij realistische, meestal lagere bodemtemperaturen uit te voeren.
De combinatie van saprofytisch vermogen en temperatuursonafhankelijkheid van het vangen van nematoden maken *A. oligospora* (CBS 289.82)tot een veelbelovende kandidaat in de biologische bestrijding van *Meloidogyne* soorten. Voortzetting van het onderzoek zal nodig zijn om het competitief saprofytisch vermogen en de vangcapaciteit in een aantal grondsoorten en in de rhizosfeer van een aantal gewassen vast te stellen.

Curriculum vitae

Eefje den Belder werd op 21 oktober 1955 in Haarlem geboren. Na voltooiing van het V.W.O. B op het Coornhertlyceum in Haarlem begon zij in 1974 haar studie Planteziektenkunde aan de toenmalige Landbouwhogeschool Wageningen. Gedurende de praktijktijd in Nicaragua werd onderzoek gedaan naar natuurlijke aantalsregulatie van de legerrups *Spodoptera frugiperda* in traditionele teeltsystemen. Dit gebeurde in het kader van een onderzoeksproject van de Voedsel- en Landbouworganisatie van de Verenigde Naties (FAO).
Tijdens de doctoraalfase deed zij entomologisch onderzoek in het DLO-Instituut voor Planteziektenkundig Onderzoek (IPO-DLO) naar de invloeden van klaver als ondergroeigewas op het optreden van Lepidoptere plagen in spruitkool. Bij de vakgroep Virologie volgde een onderzoek naar de effecten van een kernpolyedervirus op de Floridamot. Een gedeelte van dit onderzoek werd uitgevoerd bij het Proefstation voor de Bloemisterij in Nederland, te Aalsmeer.
Direct na het afsluiten van haar studie in 1981 kwam zij in dienst bij de Vakgroep Fytopathologie van de Landbouwuniversiteit Wageningen. Zij werd projectuitvoerder van het Gewasbeschermingsproject 'Sanidad Vegetal UNAN-LUW' in Managua, Nicaragua. Vanaf 1985 was zij tevens coördinator van de Nederlandse universitaire samenwerkingsprojecten aldaar. Na terugkomst in Nederland in 1987 werkte ze enige maanden bij de vakgroep Entomologie van de Landbouwuniversiteit. Vervolgens trad zij in dienst bij het IPO-DLO en startte ze onderzoek naar de mogelijkheden van biologische bestrijding van wortelknobbelaaltjes met behulp van nematofage schimmels. Een deel van dit onderzoek werd gefinancierd door de Programmacommissie Landbouwbiotechnologie van het ministerie van Landbouw, Natuurbeheer en Visserij.
Op 1 januari 1994 heeft zij haar werkterrein verlegd naar de biologische bestrijding van insektenplagen in de sierteelt. Daarnaast blijft ze betrokken bij de biologische bestrijding van aaltjes. In het IPO-DLO maakt ze deel uit van de Wetenschappelijke Raad.