European Journal of Plant Pathology **102:** 471–478, 1996.

Adhesive hyphae of *Arthrobotrys oligospora*: an ultrastructural study

E. den Belder[1], E. Jansen[1] and J. Donkers[2]
[1]*Department of Biological and Integrated Control, DLO Research Institute for Plant Protection (IPO-DLO), P.O.Box 9060 6700GW, Wageningen, The Netherlands (Fax: 0317 410113);* [2]*Agrotechnological Research Institute (ATO-DLO)*

Accepted 1 February 1996

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

Abstract

Electron microscopic studies of the interaction of second-stage juveniles of *Meloidogyne hapla* and *Arthrobotrys oligospora* (CBS 289.82) strongly suggest that hyphae attachment to nematodes was mediated by a 0.1μm thick layer matrix between the fungus and prey after contact with the nematode cuticle. The amorphous electron opaque matrix was irregularly distributed over the fungal surface, in some cases covering only the side attached to the nematode. Serial sections of adhesive hyphae showed that the extracellular matrix spread from the exact site of capture. A matrix with this thickness was never observed in the absence of nematodes.

Introduction

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

The Zygomycetes *Cystopage* spp. and *Stylopage* spp. 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 can 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; Sachchidanandia and 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 and Stålhammar-Carlemalm, 1978; Dowsett and Reid, 1979). Some authors observed that the stimulus of a nematode was needed as in *Arthrobotrys oligospora* (Veenhuis et al., 1985).

Others reported a mechanism during which a layer was present before nematodes were attached as in *Drechmeria coniospora* an endoparasite (Saikawa, 1982; Dijksterhuis et al., 1990).

So far, only few Hyphomycetes are known to capture vermiform stages of nematodes by hyphae: *Arthrobotrys botryospora* (Barron, 1979) and *Monacrosporium psychrophilum* (Gray, 1985) and an isolate of *A. oligospora* (den Belder and Jansen, 1994a). In *A. superba*, especially in very old cultures, nematodes are trapped on hyphae prior to network formation (Fritsch and Lysek, 1989). Also Jansson and Nordbring-Hertz (1981) described the capture of nematodes by more or less differentiated hyphae.

Light microscopic 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 and 30 °C) and irrespective of differences in nematode mobility; varying nutritional conditions did not influence nematode-hypha attachment either (den Belder and Jansen, 1994b).

In this study we present results from video-enhanced contrast microscopy (Wyss and Zunke, 1986) and ultrastructural observations on nematode-hypha attachment. Nematode capture is studied in order to see if hyphae attach randomly at the nematode surface or if attachment is restricted to specific sites on the cuticle (Dijksterhuis et al., 1990).

Furthermore the outer surface of the hyphae is examined to determine if a layer is present between the hypha and the nematode at the site of attachment and if its structure is fibrillar similar to other fungi (Tunlid et al., 1992).

Materials and methods

Organisms and growth conditions. Arthrobotrys oligospora (CBS 289.82) was grown on corn meal agar (Oxoid, CMA 1:10, 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 et al., 1993). The Petri-dishes used in the experiments described below and subsequently used for microscopical observations have a hole in the bottom covered by a coverglass, 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 et al. (1993).

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 (14–28 days old) at room temperature. In the controls no nematodes were added.

Video-enhanced contrast light microscopy. The process of attachment was followed by light microscopy with an enhanced contrast video system (Wyss and Zunke, 1986). All observations were performed using the observation chamber described by Wyss (1992). The video-system was constructed 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 attachment 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. 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, mounted on copper grids (100 mesh, diameter 3 mm) and examined in a Philips EM 400 after post-staining with 2% (w/v) uranyl acetate in water and Reynolds lead citrate (0.4% in 0.1 N sodium hydroxide) for 7 min each. The specimens were photographed using 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. Juveniles do not attach at any particular point on the hyphae. Time required for attachment varied greatly: in some cases attachment occurred within seconds, at the first contact with the hypha (Figure 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 min 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

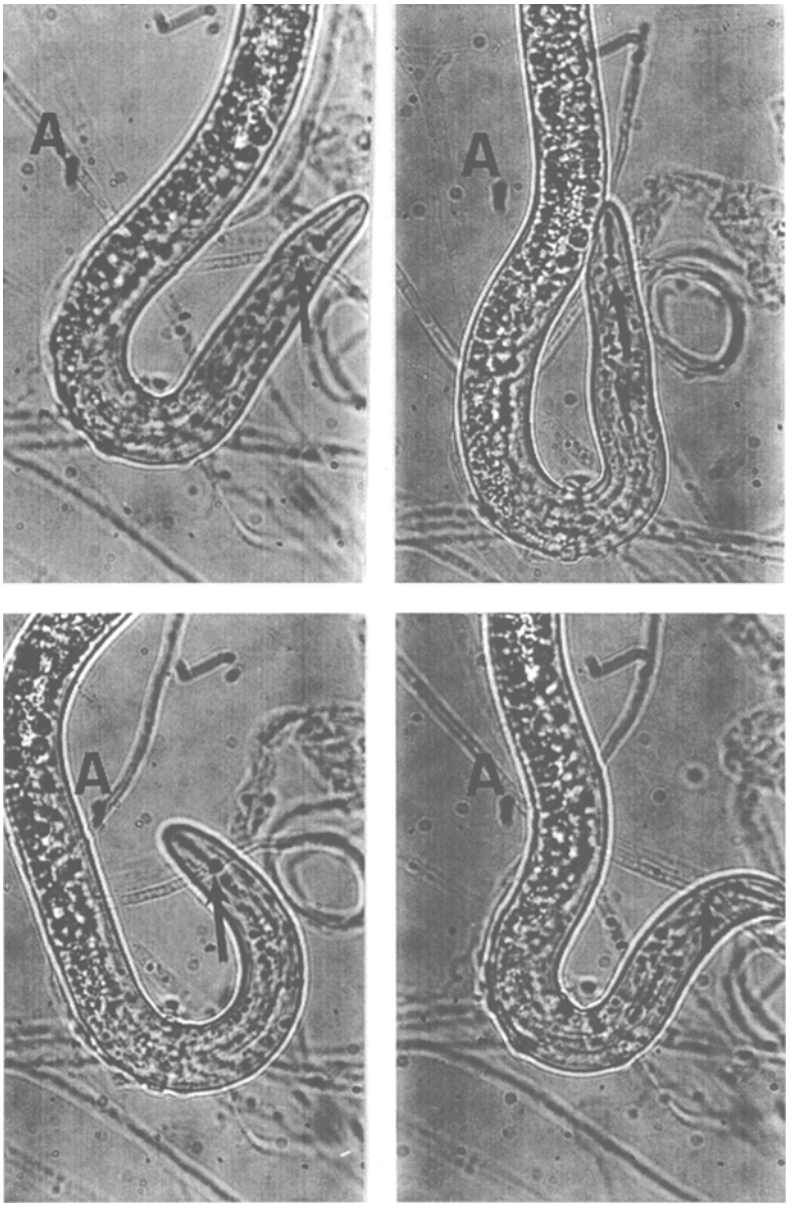

Figure 1. Captured second-stage juvenile of *Meloidogyne hapla* moving heavily to get free from an adhesive hypha of *Arthrobotrys oligospora* (CBS 289.82) A: fixed point; arrow, place where the hypha sticks to the nematode.

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.

Transmission electron microscopy. Ultrathin sections prepared from hyphae of *A. oligospora* (CBS 289.82) of 14 to 28 day-old fungal colonies did not provide evidence for a layer of extracellular material all over the hyphae (Figure 2A and B). In some cases multivesicular bodies and microtubules were visible (not shown). Occasionally, *A. oligospora* (CBS 289.82) produced intrahyphal hyphae (Figure 2C).

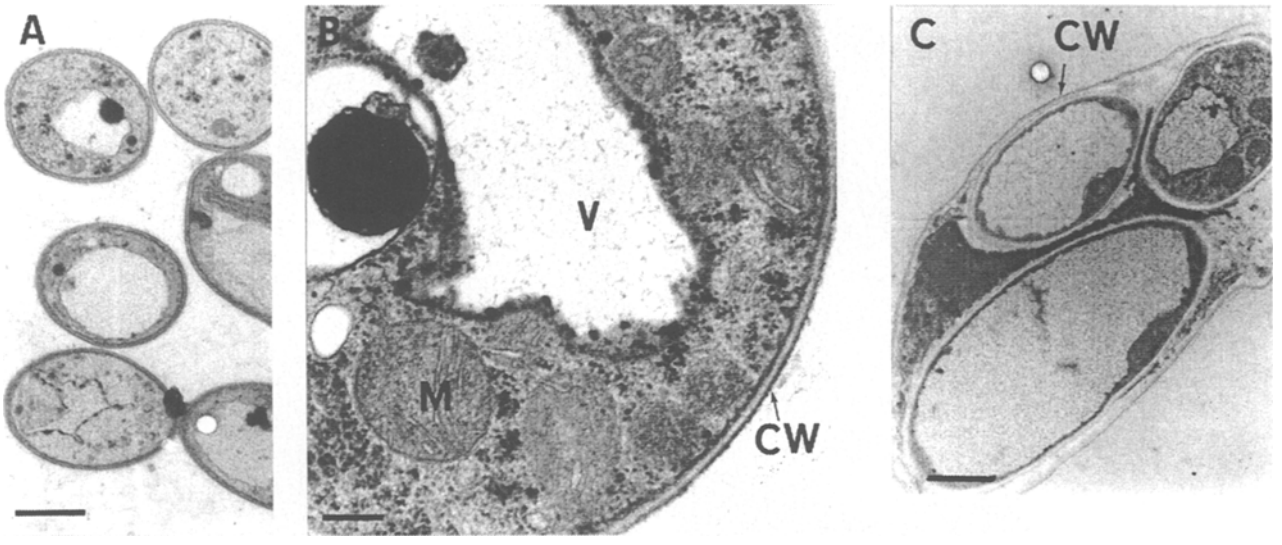

Figure 2. A: TEM-cross section through vegetative hyphae of the adhesive hyphae forming *Arthrobotrys oligospora* (CBS 289.82) to which no nematodes had been added. Bar = 1 μm. B: Detail of vegetative hypha. Bar = 0.1 μm. CW, cell wall; M, mitochondrion; V, vacuole. C: Intrahyphal growth in *Arthrobotrys 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 nematode. The thickness was about 0.1 μm (Figure 3A and B). Nematodes could be seen attached at different places along the entire body length, though attachment often occurred along the lateral fields.

The layer of extracellular material was distributed irregularly on the surface of the hypha (Figure 3B). In some cases the entire cell seemed to be covered, in other cases the layer could only be seen at the site of attachment.

Serial sectioning of nematodes attached to hyphae revealed that the extracellular material could be present at some distance from the point of attachment (Figure 4A-C). In fungal colonies to which no nematodes were added a layer of this thickness was never observed.

One hour after addition of nematodes no obvious structure within the layer was observed, it remained amorphous without evidence of any fibrils (Figure 4D).

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 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 of *A. oligospora* (CBS 289.82) did not reveal the presence of lumps of adhesive substances as can be seen easily in the Zygomycetes (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 four 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 and Stålhammar-Carlemalm, 1978; Dowsett and

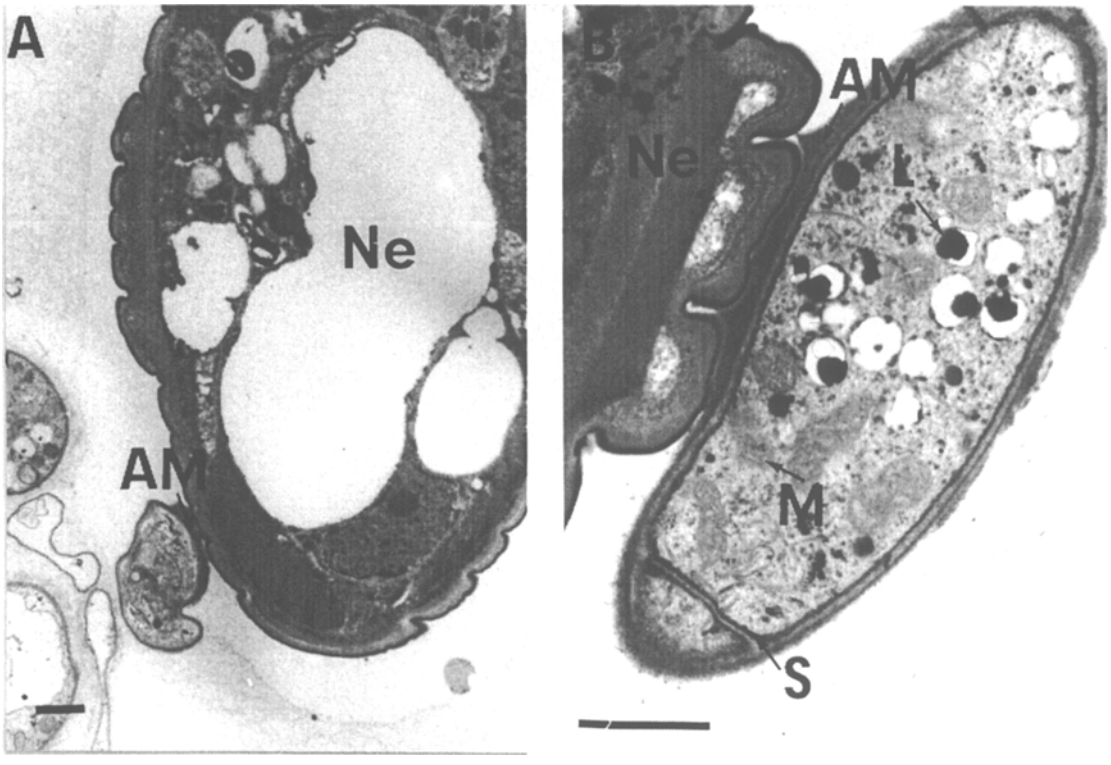

Figure 3. A: TEM-cross section through a captured second-stage juvenile of *Meloidogyne hapla* attached to a hypha of *Arthrobotrys oligospora* (CBS 289.82) one hour after addition of the nematode. Bar = 1 μm. AM, adhesive material; Ne, nematode; VH, vegetative hypha. B: Idem. Bar = 1 μm. AM, adhesive material; L, lipid; M, mitochondrion; Ne, nematode; S, septum.

Reid, 1979; Veenhuis et al., 1985; 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 is visible prior to the presence of nematodes and shows 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 and Morikawa, 1985; zoospores of *Catenaria anguillulae*: Tunlid et al., 1991b).

4) the presence of lumps of adhesive substances in the Zygomycetes (Wood, 1983).

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 not even closely associated hyphae (2A). This suggests that its presence depends on contact with the nematode host as in some of the above-mentioned interactions. Nevertheless it is important to stress that different fixation techniques can result in substantial dissolution or disruption of this layer during preparation of the samples for microscopical investigation (Veenhuis et al., 1985; Wharton and Murray, 1990).

Extracellular polymers, exclusively confined to trap cells of the adhesive network-forming fungus *A. oligospora* (ATCC 24927, Nordbring-Hertz and Stålhammar-Carlemalm, 1978; Veenhuis et al., 1985), were recently isolated from both traps and 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 nema-

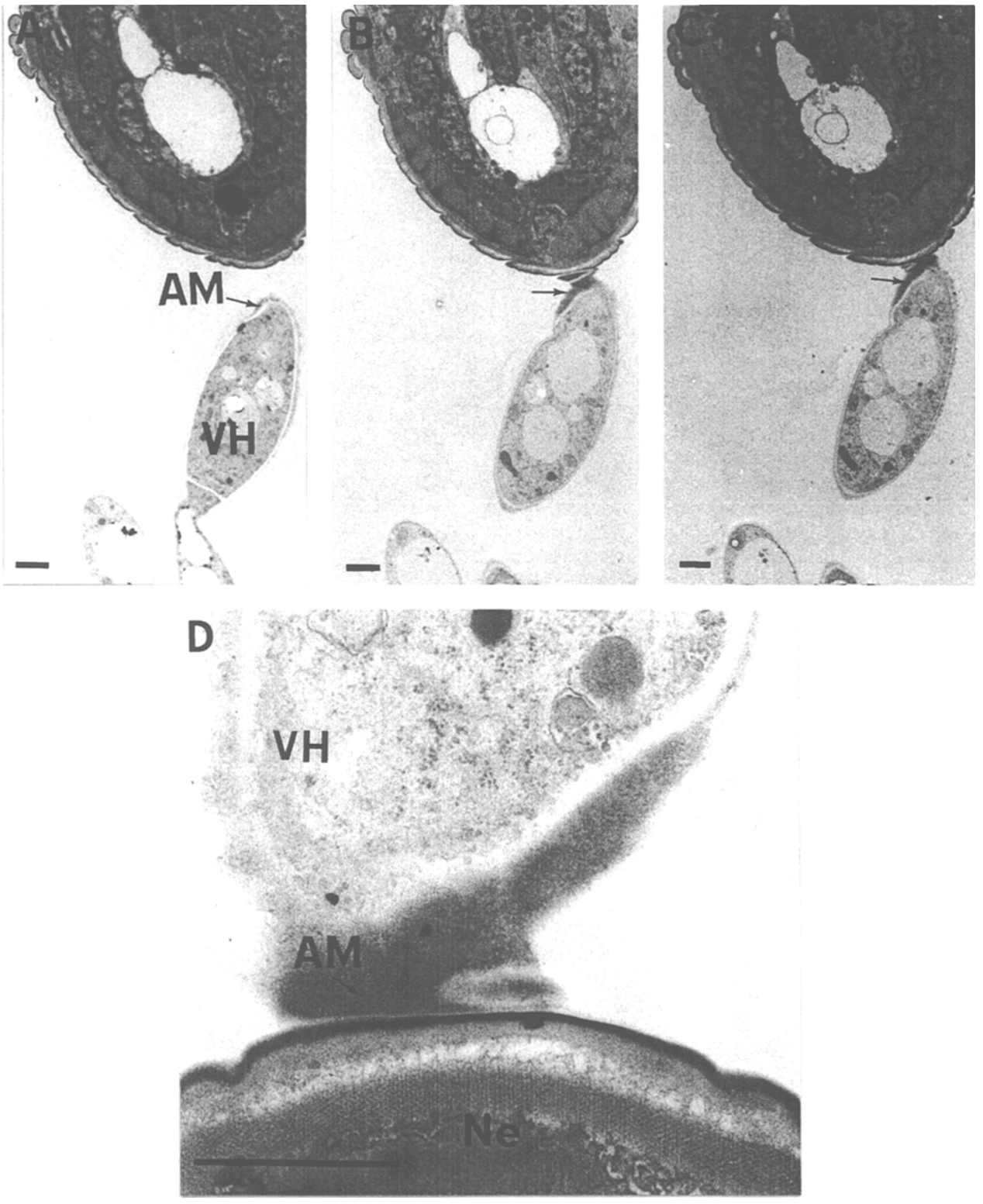

Figure 4. Microphotographs from a series of sections demonstrating that adhesive material can be present on a vegetative hypha on short distance from the exact site of capture of the nematode. Bar = 1 μm. AM, adhesive material; Ne, nematode; VH, vegetative hypha. Arrow, adhesive material. D: detail. Bar= 1 μm. AM, adhesive material; Ne, nematode; VH, vegetative hypha. Arrow, adhesive material.

tode. They seemed to be distributed unevenly over the surface.

The structure of the adhesive layer can be quite complex and attachment can be accompanied by morphological changes (Tunlid et al., 1991).

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 and Stålhammar-Carlemalm, 1978) or changes in the adhesive layer.

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). In some cases several dense bodies were present which is comparable with the initial stage in trap development in *A. oligospora* (ATCC 24927, Veenhuis et al., 1985). 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 (den Belder and Jansen, 1994b) and the time required for attachment can 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. In encysting *Phytophthora* zoospores only 30–60 seconds are required to deliver the adhesive compound that helps attachment to host surfaces (Gubler and Hardham, 1988). The material is localized in small vesicles in the cell periphery (Bartnicki-Garcia and 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).

That sometimes the nematode was moving more than 30 minutes over the hyphae before attachment occured, may be explained in several ways such as differences in surface properties (Jansson and Nordbring-Hertz, 1983; Dürschner-Pelz and Atkison, 1988).

Any binding 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 sterilized and untreated soil have revealed that also under more realistic circumstances the second-stage juveniles of *M. hapla* are attached to the hyphae (den Belder et al., 1994c). Also under adverse temperatures that do not favour adhesive ring structure development or pour nutritional conditions for adhesive ring structure development this isolate showed the ability to capture nematodes with adhesive hyphae (den Belder and Jansen, 1994b). This clearly illustrates that trapping of nematodes by undifferentiated hyphae without specific structures takes place under a broader range of conditions than for fungi in which adhesive rings are the only capture devices. Thus it is possible that adhesive hyphae forming fungi play a greater role in nematode trapping in soil than hitherto is considered.

Acknowledgements

We like to thank Prof. Dr. L. Brussaard and Dr. J.W.D.M. Henfling for valuable comments on the manuscript. Dr. U. Wyss, Institute for Phytopathology at Kiel, FRG, is gratefully acknowledged for providing the prototype of the observation chamber for the video-enhanced contrast microscopy.

References

Bartnicki-Garcia S and Sing VO (1987) Adhesion of zoospores of *Phytophthora* to solid surfaces. In: Fuller MS and Jaworski A (eds), Zoosporic Fungi in Teaching and Research (pp. 279–283)

Barron GL (1979) Nematophagous fungi: a new *Arthrobotrys* with nonseptate conidia Can J Bot 57: 1371–1373

Belder E den, Boekestein A, van Esch JWJ and Thiel F (1993) Low-temperature scanning electron microscopy of fungus-nematode interaction. Scanning 15: 37–42

Belder E den and Jansen E (1994a) Capture of plant-parasitic nematodes by an adhesive hyphae forming isolate of *Arthrobotrys oligospora* and some other nematode-trapping fungi. Nematologica 40: 423–437

Belder E den and Jansen E (1994b) 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

Belder E den, Edens JT and Jansen E (1994c) Establishment and nematode capture ability of the adhesive hyphae forming *Arthrobotrys oligospora* in soil. Soil Ecol 1: 171–183

Dowsett JA and Reid J (1979) Observations on the trapping of nematodes by *Dactylaria scaphoides*, using optical, transmission and scanning-electron microscopic techniques. Mycologia 71: 379–391

Drechsler C (1934) Organs of capture in some fungi preying on nematodes. Mycologia 26: 135–144

Dürschner-Pelz UU and Atkinson AJ (1988) Recognition of *Ditylenchus* and other nematodes by spores of the endoparasitic fungus *Verticillium balanoides*. J Invert Pathol 51: 97–106

Dijksterhuis J, Veenhuis M and Harder W (1990) Ultrastructural study of adhesion and initial stages of infection of nematodes by conidia of *Drechmeria coniospora*. Mycol Res 94: 1–8

Dijksterhuis J, Harder W, Wyss U and Veenhuis M (1991) Colonization and digestion of nematodes by the endoparasitic nematophagous fungus *Drechmeria coniospora*. Mycol Res 95: 873–878

Dijksterhuis J (1993) Nematode-fungal interactions: structure-function relationships. PhD thesis State University Groningen, The Netherlands

Fritsch A and Lysek G (1989) Nematode-capturing Hyphomycetes from soils over xerophytic calcareous rock in upper Bavaria. Botanica Acta 102: 270–275

Gray NF (1984) Ecology of nematophagous fungi: predatory and endoparasitic species new to Ireland. Ir Nat J 21: 337–341

Gray NF (1985) *Monacrosporium psychrophilum*, a nematode-destroying fungus new to Ireland. Irish J Agr Res 24: 129–132

Gubler F and Hardham AR (1988) Secretion of adhesive material during encystment of *Phytophthora cinnamomi* zoospores, characterized by immunogold labelling with monoclonal antibodies to components of peripheral vesicles. J Cell Sci 90: 225–235

Hamer JE, Howard RJ, Chumley FG and Valent B (1988) A mechanism for surface attachment in spores of a plant pathogenic fungus. Science 239: 288–290

Jansson HB and Nordbring-Hertz B (1983) The endoparasitc nematophagous fungus *Meria coniospora* infects nematodes specifically at the chemosensory organs. J Gen Microbiol 129: 1121–1126

Kennedy MJ (1990) Models for studying the role of fungal attachment in colonization and pathogenesis. Mycopathologia 109: 123–137

Nordbring-Hertz B (1977) Nematode-induced morphogenesis in the predacious fungus *Arthrobotrys oligospora*. Nematologica 23: 443–451

Nordbring-Hertz B and Stålhammar-Carlemalm M (1978) Capture of nematodes by *Arthrobotrys oligospora*, an electron microscope study. Can J Bot 56: 1297–1307

Nordbring-Hertz B and Odham G (1980) Determination of volatile nematode exudates and their effects on a nematode-trapping fungus. Microb Ecol 6: 241–251

Sachchidanandia J and Swarup G (1966) Nematophagous fungi in Delhi soils. Indian Phytopathol 19: 279–284

Saikawa M (1982) An electron microscope study of *Meria coniospora*, an endozoic nematophagous Hyphomycete. Can J Bot 60: 2019–2023

Saikawa M and Morikawa C (1985) An electron microscope study of initiation of infection by conidia of *Harposporium subuliforme*, an endozoic nematophagous fungus. Trans Mycol Soc Japan 26: 215–219

Tunlid A, Johansson T and Nordbring-Hertz B (1991a) Surface polymers of the nematode-trapping fungus *Arthrobotrys oligospora*. J Gen Microb 137: 1231–1240

Tunlid A, Nivens DE, Jansson HB and White DC (1991b) Infra-red monitoring of the adhesion of *Catenaria anguillulae* zoospores to solid surfaces. Exp Mycol 15: 206–214

Tunlid A, Jansson HB and Nordbring-Hertz B (1992) Fungal attachment to nematodes. Mycol Res 96: 401–412

Veenhuis M, Nordbring-Hertz B and Harder W (1984) Occurrence, characterization and development of two different types of microbodies in the nematophagous fungus *Arthrobotrys oligospora*. FEMS Microbiol Lett 24: 31–38

Veenhuis M, Nordbring-Hertz B and Harder W (1985) An electron microscopical analysis of capture and initial stages of penetration of nematodes by *Arthrobotrys oligospora*. Antonie van Leeuwenhoek J Microbiol Serol 51: 385–398

Veenhuis M, Wijk C van, Wyss U, Nordbring-Hertz B and Harder W (1989) Signi1 ficance of electron dense microbodies in trap cells of the nematophagous fungus *Arthrobotrys oligospora*. Antonie van Leeuwenhoek J Microbiol Serol 56: 251–261

Wharton DA and Murray DS (1990) Preparation of the nematode-btrapping fungus *Arthrobotrys oligospora* for scanning electron microscopy by freeze-substitution. J Microscopy 158: 81–85

Wood SN (1983) *Stylopage anomala* sp. nov. from dung. Trans Br Mycol Soc 80: 368–370

Wyss U and Zunke U (1986) The potential of high resolution video-enhanced contrast microscopy in nematological research. Rev Nématol 9: 91–94

Wyss U (1992) Observations on the feeding behaviour of *Heterodera schachtii* throughout development, including events during moulting. Fundam Appl Nematol 15: 75–89