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Morphogenesis in the nematode-trapping fungus *Arthrobotrys oligospora* – an extensive plasticity of infection structures

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Most nematode-trapping fungi are dependent on specific hyphal structures on or in which nematodes can be trapped mechanically or by adhesion. These structures are a prerequisite for the ability of the fungus to invade a host and are thus crucial for survival as well as virulence. The diversity of trapping structures is large and highly dependent on the environment of the fungus. Within one single species, *Arthrobotrys oligospora*, not only adhesive nets are formed but also so-called conidial traps, hyphal coils around hyphae of other fungi, and appressoria in the rhizosphere of agricultural crops. In this article these structures and the conditions for their development are described. Since the trapping structures influence the survival and the virulence of their producer, it is important that we know more about the molecular background of their development and function. The application of genomics to understand the function and the development of infection structures, therefore, has substantially increased the potential of *A. oligospora* to become a model system for fungal morphogenesis.

Keywords: adhesive networks, appressoria, *Arthrobotrys oligospora*, conidial traps, hyphal coils, morphogenesis, nematode-trapping, phenotypic plasticity

Morphogenesis plays a decisive role for infection and pathogenicity in many fungal systems. For example, yeast-mycelial differentiation in dimorphic fungi is important in both human and plant pathogens, germ tube differentiation into appressoria occurs in the rice blast fungus *Magnaporthe grisea* and in the insect pathogen *Metarhizium anisopliae*, and hyphal coiling is one of the infection mechanisms in mycoparasitic *Trichoderma* spp.

Nematophagous fungi use spores or mycelial structures called traps to capture vermiform nematodes, or hyphal tips to attack nematode eggs and cysts. Among the nematode-trapping fungi, differentiated structures such as adhesive nets, branches and knobs as well as mechanical traps called constricting or non-constricting rings are well known and typical of particular species. In addition, toxic hyphal stalks are developed by *Pleurotus ostreatus* and bottleneck-shaped phialides are used by *Hirsutella* spp., whereas *Haptoglossa* spp. differentiate into so-called gun cells. Several reviews have described this morphogenesis and evaluated its consequences for the fungal infection of the nematode, as well as its value in disease control (for review, see e.g. Barron, 1977, 1981; Dowe, 1987; Kerry & Jaffee, 1997; Dijksterhuis *et al*., 1994; Jansson & Lopez-Llorca, 2001; Nordbring-Hertz *et al*., 2002).

Thus there is a high diversity of trapping structures in nematophagous fungi, also reflected in their wide taxonomic distribution. While the typical nematode-trapping structures are well known, it is not widely appreciated that a single species – *Arthrobotrys oligospora* – can develop several different mycelial structures involved in infection and parasitism. These will be examined in the present review. Attention will be drawn not only to adhesive nets which are typical of the species, but also to other hyphal structures such as conidial traps and hyphal coils as well as the recently discovered appressoria.

Diversity of trapping structures in *Arthrobotrys*

The adhesive network trap. *Arthrobotrys oligospora* typically forms adhesive network traps (Fig 1), and nematodes easily induce the formation of these traps

from saprotrophic mycelium. The fungus thus enters the parasitic phase and captures nematodes on the surface of these structures (Fig 2). Nematodes are not the only factor inducing trap formation in *A. oligospora*. For instance, a low nutrient status of the environment favours this morphogenesis. In the laboratory, in combination with a low nutrient medium, induction of trap formation can be brought about by adding small peptides or their constituting amino acids, both to solid substrates (Nordbring-Hertz, 1973) and to liquid cultures (Friman *et al*., 1985).

The typical adhesive network trap consists of one to several loops attached to each other in a three-dimensional way as a result of several anastomoses. The first loop of the net develops from an initial on the parent hypha which is readily detected because of its bright appearance in the light microscope. An initial branch is formed and develops to a three-cell structure. It curves around to meet a branch formed on the parent hypha some 20-25 µm from the initial (Nordbring-Hertz *et al*., 1989).

Trap cells differ from hyphal cells in several ways. Apart from their unique ability to capture nematodes, irrespective of their age and stage of development they contain so-called dense bodies not present in hyphal cells (Fig 3). These dense bodies are cytosolic organelles which are peroxisomal in nature since they contain catalase and D-amino acid oxidase activity. They are only present in nematode-trapping fungi, but not in the so-called endoparasitic nematophagous fungi that infect their host with adhesive or non-adhesive spores. They do not seem to be involved in the adhesion of nematodes, but after penetration of the nematode cuticle they are translocated into the developing trophic hypha, suggesting that they have a role in supplying energy and/or building blocks to the invading hyphae (Veenhuis *et al*., 1985b, 1989). The extracellular fibrillar polymers of trap cell walls contain mainly proteins and carbohydrates (Tunlid, Johansson & Nordbring-Hertz, 1991). The adhesion of nematodes to the surface of the traps is considered as just one step in the infection (Tunlid *et al*., 1992), during which the fibrils become more dense and oriented in one direction (Veenhuis *et al*., 1985a).

The capture of nematodes by *Arthrobotrys* spp does not require a fully developed loop because nematodes can be trapped even on the first-formed branch. In an isolate of *A. superba*, nematodes were trapped on a basal cell, which later developed into either a fully developed trap or into a conidiophore, depending on environmental conditions (Jansson & Nordbring-Hertz, 1981). This study indicates that cells destined to become traps have the ability to trap nematodes long before the development of a full trap and that growth conditions and environmental factors strongly influence the direction of morphogenesis in this system. In Table 1 some morphological adaptations of *Arthrobotrys* spp. are summarized including one unusual *Arthrobotrys* species (Scholler & Rubner, 1999) that forms adhesive knobs. In the following some of

Table 1 Summary of hyphal structures in *Arthrobotrys* spp

Organism	Structure	Capture of nematodes	Dense bodies	Reference
A. oligospora ATCC 24927	Adhesive net	yes	yes	Nordbring-Hertz *et al.* (2002)
A. superba QM 1688, NCC	Adhesive branch and net	yes	yes	Jansson & Nordbring-Hertz (1981)
A. hertzianaq CBS 395.93	Adhesive knob	yes	n.d.	Scholler & Rubner (1999)
A. oligospora mutants MLC1, MLC2	Adhesive net, deformed	yes	n.d.	Lindeblad (2003)
A. oligospora ATCC 24927	Conidial trap	yes	yes	Dackman & Nordbring-Hertz (1992)
A. oligospora CT mutant, CBS 869.97	Conidial trap	yes	yes	Nordbring-Hertz *et al.* (1995)
A. oligospora ATCC 24927	Hyphal coils	no	no	Persson *et al.* (1985)
A. oligospora ATCC 24927	Appressoria	no	n.d.	Bordallo *et al.* (2002)

n.d.: not determined

these morphological adaptations will be described.

The conidial trap. Germination of conidia of *A. oligospora* usually takes place with one germ tube developing into a hypha, which in turn undergoes branching to form a mycelium. On this (preferably young) mycelium adhesive network traps may be formed in specific abiotic environments or as a result of the presence of nematodes. Alternatively, traps may be formed directly upon germination without an intermediate hyphal phase to form so-called conidial traps (CTs). These structures were found in natural environments such as cow dung (Dackman & Nordbring-Hertz, 1992) and rhizosphere soil (Persmark & Nordbring-Hertz, 1997). In these environments CTs were detected not only in *A. oligospora* but also in several other nematode-trapping fungi (Barron, 1977; Dowe, 1987; Persmark & Nordbring-Hertz, 1997). The production of conidial traps may indicate an increased potential of these fungi as antagonists to nematodes.

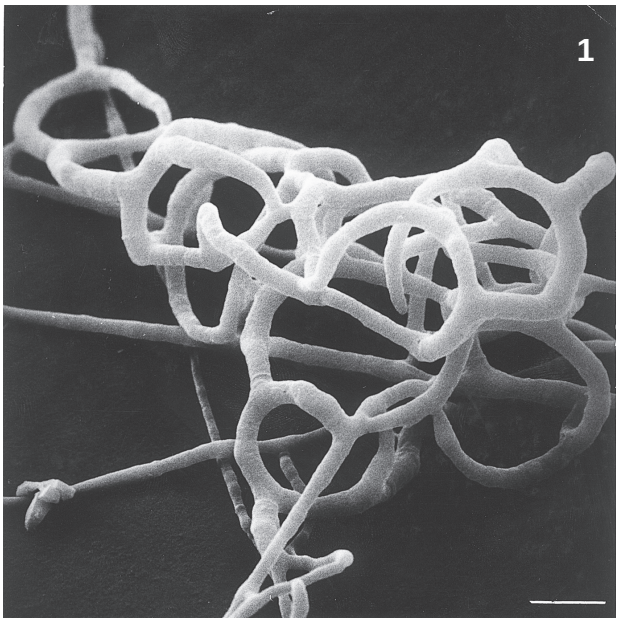
The conidial trap of *A. oligospora* (ATCC 24927) (Fig 4) was first detected during a study where the efficiency of *A. oligospora* as a biological control agent against animal-parasitic nematodes in cow faeces was evaluated. It should be noted that conidial traps of *A. oligospora* have never been detected in pure culture without the presence of natural substrates such as dung or soil. On the other hand, when conidia of *A.oligospora* were incubated in the vicinity of cow faeces on agar plates, about 90% germinated into conidial traps (Dackman & Nordbring-Hertz, 1992). Conidial traps are fully functional in trapping nematodes. They adhere to a passing nematode and may be carried away and spread by the nematode in a way similar to adhesive conidia of endoparasitic nematophagous fungi. In that sense they constitute an intermediate form between endoparasitic and nematode-trapping fungi. However, the adhesive trap nature was perfectly clear since the conidial trap contains numerous electron-dense bodies characteristic of normal hyphal network traps (Fig 5; Nordbring-Hertz *et al*., 1995).

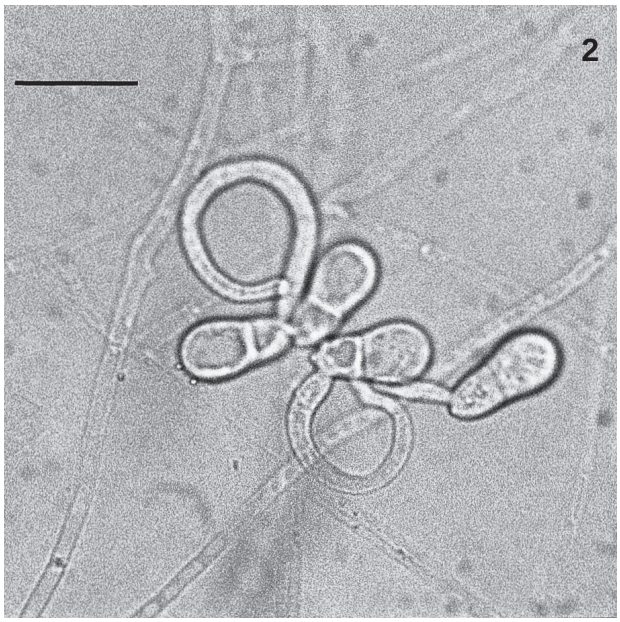
In order to obtain a more general view of CT formation in natural substrates we investigated nine fungi inoculated in the presence of soil or soil extracts. Conidial traps were observed in all but one species but the ability varied between species, with *A. dactyloides* and *Monacrosporium gephyropagum* being more efficient than *A. superba* and *A. oligospora* (Fig 6; Persmark & Nordbring-Hertz, 1997).

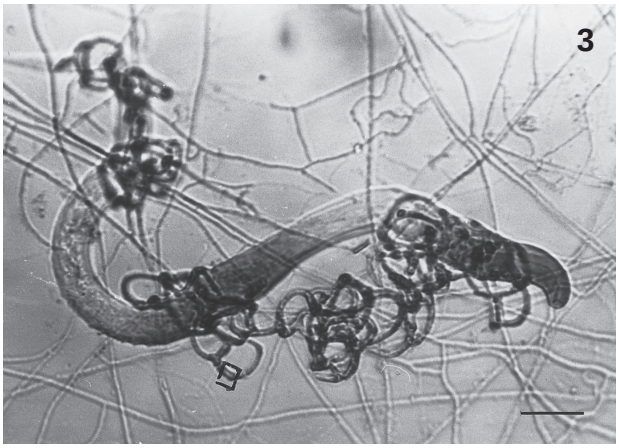
Conidial traps have been considered as survival structures just like the conventional adhesive network (Dackman & Nordbring-Hertz, 1992). This view is based on survival studies in the laboratory where adhesive net traps survived over long periods of time compared to normal hyphae (Veenhuis *et al*., 1985b). One reason was that traps contain large numbers of dense bodies that stay intact until the death of the cells. Furthermore, the occurrence of conidial traps in non-sterile soil and soil extracts strengthens the view of CTs as a survival structure. The mechanism of their formation was studied in soil extracts. The results showed that a low-nutrient level was necessary for CT formation. This result indicates competition for nutrients by microorganisms as being one of the reasons for conidia germinating directly into traps, thus overcoming fungistatic conditions in soil. Furthermore, rhizosphere soil was more efficient than root-free soil (Persmark & Nordbring-Hertz, 1997). It remains to be shown whether conidial traps develop in the vicinity of living roots.

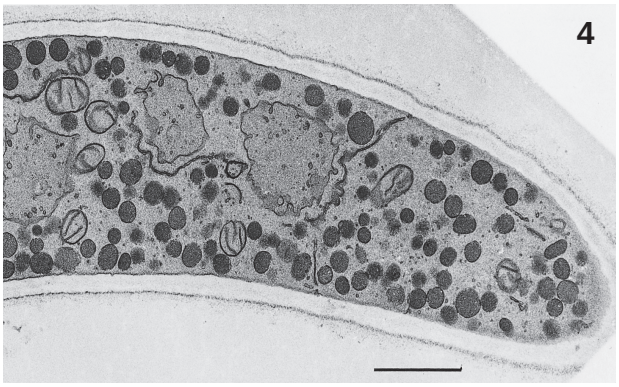
A mutant, *A. oligospora* CT (CBS 869.97), derived from *A. oligospora* (ATCC 24927) by a procedure analogous to microcycle conidiation (Nordbring-Hertz *et al*., 1995), was more efficient than the parent strain in formation of CTs in soil and in all treatments of soil extracts. We never succeeded to induce conidia of the mutant strain to germinate directly into CTs in pure culture, suggesting that some component(s) in natural substrates are necessary for this development (Persmark & Nordbring-Hertz, 1997). In a low temperature scanning electron microscopy investigation, conidia of the mutant strain were inoculated directly onto native soil particles (Jansson *et al*., 2000). The conidia germinated into CTs which were capable of capturing nematodes, thus confirming the results of the previous investigation. The most remarkable feature of this mutant, however, is that it forms CTs on conidia while still on standing conidiophores (Nordbring-Hertz *et al*., 1995). The mutant phenotype was very stable and constantly four growth phases on several trap-inducing media were observed. During the first 24h (phase 1), germination with 1-2 germ tubes took place, followed by normal trap formation on the young mycelium (3 d, phase 2). After about a week conidiophores and conidia developed (phase 3). Conidial traps on conidia still attached to upright conidiophores were produced after three weeks (phase 4). Thus the CT mutant not only formed CTs on upright conidiophores but also showed an increased normal trap formation on the mycelium compared to the parent strain.

When ungerminated conidia (growth phase 3) were spread onto a trap-inducing medium containing congo red, a compound which stains cell walls and interferes with cell wall synthesis, some germinated as conidial









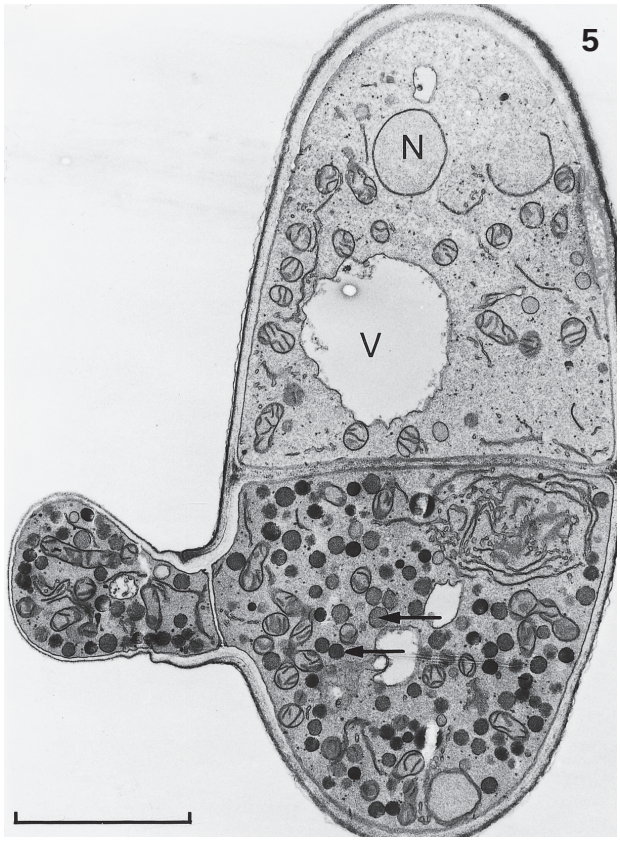


Fig 1 Scanning electron micrograph of typical peptide-induced adhesive trap of *Arthrobotrys oligospora*. Bar: 10 µm. From Lysek & Nordbring-Hertz (1983). *Forum Mikrobiologie* **6**: 201-208.
Fig 2 Light micrograph of a nematode captured in peptide-induced adhesive trap of A. oligospora. Bar: 20 µm. From Nordbring-Hertz, Veenhuis & Harder (1984). *Applied and Environmental Microbiology* **47**: 195-197. With permission by the American Society for Microbiology.
Fig 3 Light micrograph of conidial traps. Bar 20 µm.
Fig 4 Transmission electron micrograph of a trap cell of *A. oligospora*, containing numerous typical dense bodies. Bar: 1 µm. From Nordbring-Hertz (1984). In *The Ecology and Physiology of the Fungal Mycelium* (edited by D.H. Jennings and A.D.M. Rayner), pp. 419-432. Cambridge University Press.
Fig 5 TEM micrograph of germinating conidium of CT mutant. Note dense bodies both in CT and in the mother conidial cell (arrows). N: nucleus, V: vacuole. Bar: 5 µm. From Nordbring-Hertz *et al*. (1995) *Mycological Research* **99**: 1395-1398.

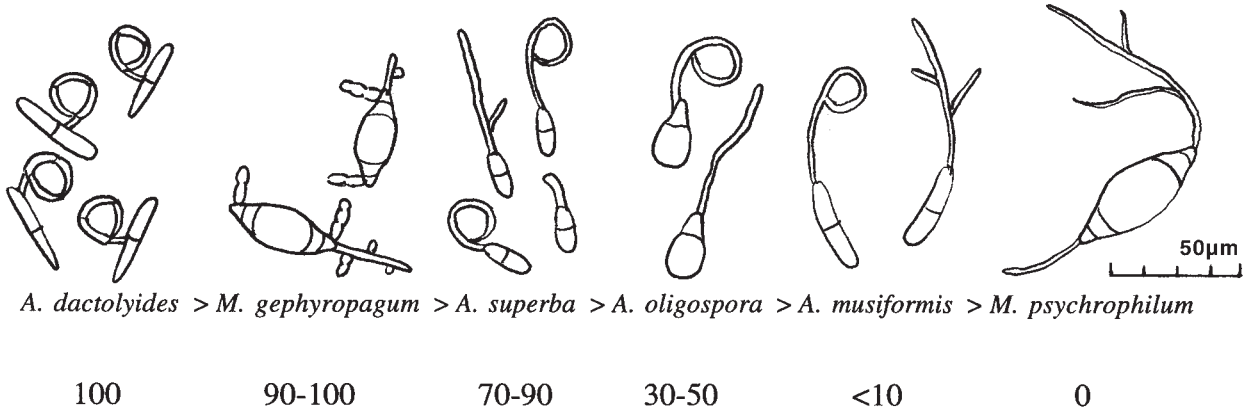


Fig 6. Conidial traps formed by different nematode-trapping fungi in an agar plate assay with soil. The numbers indicate percentages of conidia that developed CTs. Bar: 50 µm. *A. oligospora* in this experiment was the ATCC 24927 strain. The CT mutant, not included in this experiment, consistently formed conidial traps more easily than the parent strain, but still not to the same extent as *A. dactyloides*. From Persmark & Nordbring-Hertz (1997). *FEMS Microbiology Ecology* **22**: 313-323.

traps and others as normal hyphae, but only CTs and ungerminated conidia were stained (Fig 7). Both congo red and lactophenol blue, staining cytoplasm, stained the trap and the germinating conidial cell more heavily than the second conidial cell, or the hyphae. This is in concordance with the presence of dense bodies in metabolically highly active cells, such as the trap cell and the mother conidial cell (Figs 3 and 7).

Hyphal coils and appressoria. The above examples of morphogenesis are tightly connected with the trapping of nematodes and are induced by environmental signals connected with this function. However, nematode-trapping fungi are also capable of

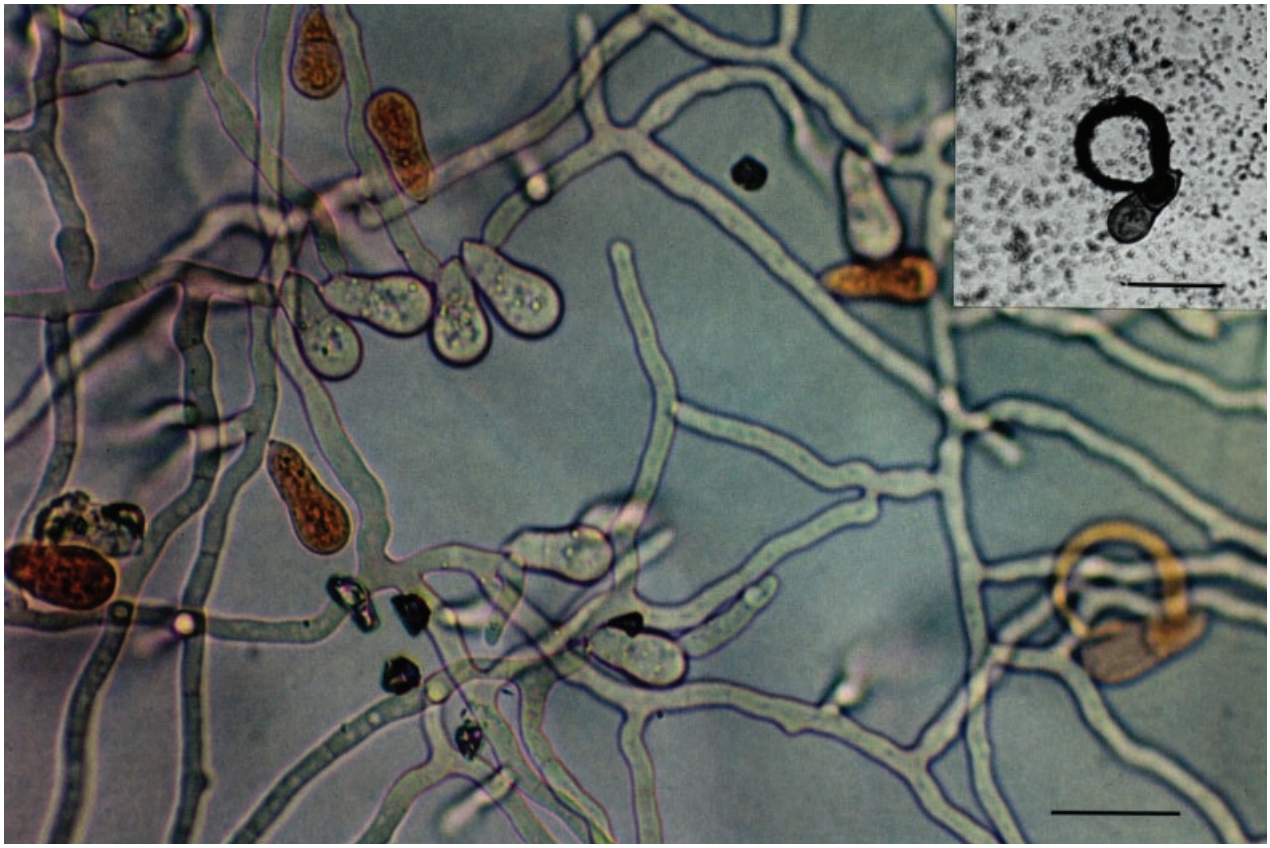


Fig 7 Conidial traps of the CT mutant of *A.oligospora*. Mutant conidia that had not yet formed CT (growth phase 3) were spread on a trap-inducing medium containing congo red (20 µg ml^{-1}). Germination took place as hyphae or as CTs. Note heavy staining of CT and of ungerminated conidia. Insert: same stage of CT development, stained with lactophenol blue. Bar: 20 µm.

morphogenetic responses towards other fungi which result in hyphal coils around their hyphae, and to plant roots, resulting in the formation of appressoria.

The phenomenon of hyphal coiling of one fungus around the hyphae of another, mycoparasitism, is well known as one of the main mechanisms involved in the antagonism of *Trichoderma* spp. as a biocontrol agent of soil borne plant pathogenic fungi (Chet, 1987). Hyphal coils of *A. oligospora* (Fig 8) were formed in response to

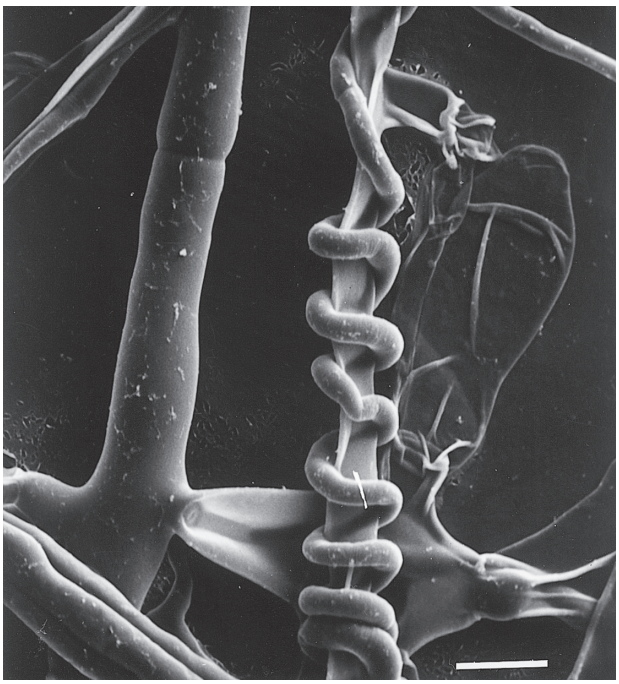


Fig 8 *A.oligospora* as a mycoparasite coiling around a hypha of *Rhizoctonia solani*. An unaffected *Rhizoctonia* hypha is shown to the left of the image. Bar: 10 µm. From Persson (1991) *Mycoparasitism by the nematode-trapping fungus Arthrobotrys oligospora*. Thesis, Lund University. With permission by Dr. Yvonne Persson.

6 out of 13 fungi tested (Persson *et al*., 1985). The overall morphology of the hyphal coil is very similar to adhesive network traps in that hyphal diameter and cell wall thickness differ from those of vegetative hyphae. Both traps and coils contain an abundance of cytoplasmic organelles that developed from the endoplasmic reticulum. Dense bodies typical of traps, however, are not present in coils. Also the biological function of the two structures is quite different. The attack of coils on host hyphae leads to cell wall proliferations of the host (*Rhizoctonia solani*) and disintegration of the host cytoplasm without penetration of intact cells. The interaction is interpreted in terms of competition for nutrients (Persson *et al*., 1985). In a later study the mycoparasitic nature of *A. oligospora* was further established. In double labeling experiments with ^{32}P-labelled *R. solani* and ^{33}P-labelled *A. oligospora*, the latter fungus, although a non-penetrating mycoparasite, derived a considerable proportion of nutrients from the host hyphae (Olsson & Persson, 1994).

From a biological control point of view the presence of nematophagous fungi in the rhizosphere of agricultural plants is important. Persmark & Jansson (1997) showed that out of 15 nematophagous species, *A. oligospora* was by far the most common, especially in the pea rhizosphere. In a recent study, Bordallo *et al*. (2002) compared the behaviour of a nematode-trapping fungus, *A. oligospora*, with an egg parasitic fungus, *Pochonia chlamydosporia* (*Verticillium chlamydosporium*), in the rhizosphere of axenic barley and tomato. Only *A. oligospora* responded by growing chemotactically towards the root surface. Both fungi grew inter- and intracellularly in barley and tomato roots, and formed appressoria during penetration of plant cell walls (Fig 9). The fungi colonized epidermis

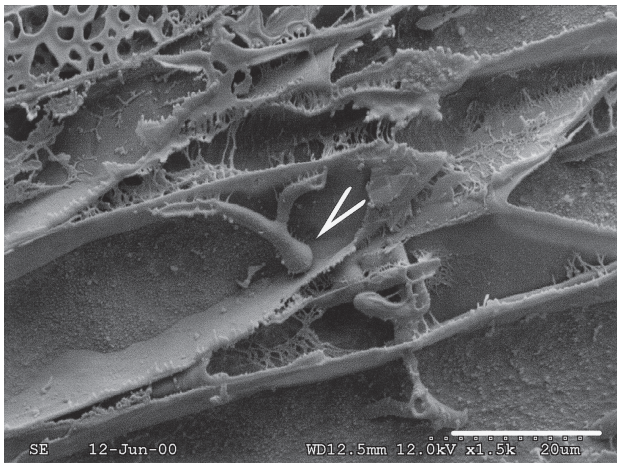


Fig 9 Early colonization of barley roots by *A. oligospora*. Cryoscanning electron micrograph of epidermis colonization. Arrow indicates appressorium. From Bordallo *et al*. (2002) *New Phytologist* **154**: 491-499. With permission by Dr. H.-B. Jansson, University of Alicante, Spain, and Blackwell Publishing, Oxford, UK.

and cortex but never penetrated the vascular tissues. They also induced plant defense reactions, e.g. papillae, but did not harm the development of the plants (Bordallo *et al*., 2002). The colonization of plant roots has been suggested to be endophytic (Jansson & Lopez-Llorca, 2004) and may render the plants more resistant to plant parasitic nematodes and/or other pathogens. Further research in this direction is under way (H.-B. Jansson, pers comm.).

Appressoria in *A. oligospora* are a further example of the ability of this species to respond morphogenetically to environmental signals. Furthermore, the colonization of the rhizosphere and the formation of appressoria by nematophagous fungi may have profound implications for their suitability as biocontrol

agents of plant parasitic nematodes (Bordallo *et al*., 2002).

Molecular approaches to the study of morphogenesis

Molecular tools to understand key steps in the transition of a nematode-trapping fungus from saprotrophic to parasitic growth are now available. A transformation system for *A. oligospora* to examine the function of virulence factors by constructing overexpressing strains and knockout mutants has been developed (Tunlid *et al*., 1999). A successful attempt to improve pathogenesis by genetic engineering of an extracellular serine protease, subtilisin, with nematotoxic activity was performed with *A. oligospora* (Åhman *et al*., 2002). This subtilisin (PII) is an important pathogenicity factor in *A. oligospora*. It immobilizes free-living nematodes in bioassays and hydrolyzes proteins of the nematode cuticle. Like other extracellular proteases it is also proposed to be involved in the differentiation of infection structures. High levels of PII were expressed when nematodes were colonized by the fungus after adhesion. Disruption of the PII gene by homologous recombination did not influence pathogenicity substantially, but mutants containing additional copies of the PII gene developed a higher number of infection structures. This resulted in an increased speed of capturing and killing nematodes.

Similarly, another virulence factor, the *A. oligospora* lectin AOL, was found to be expressed primarily after the capture of a nematode. AOL is a cytoplasmic lectin which functions as a storage protein. Late in the colonization process an increased trap formation was noticed, and translocation of the lectin through mycelial strands, another morphogenetic response, was recorded (Rosén *et al*., 1997). Thus both the serine protease and the lectin were expressed late in the infection process, resulting in an increase of traps outside the infected nematode, which in turn led to a decrease of nematode numbers.

Since the virulence of the nematode-trapping fungi clearly appears to be connected with morphogenesis, a major recent approach has been to isolate mutants that do not form traps, or mutants with other clear morphological defects. In order to generate and characterize trap mutants, insertional mutagenesis and restriction enzyme mediated integration (REMI) was used. Out of 4000 transformants (Lindeblad, 2003), two (MLC1 and MLC2) showed morphological changes in trap formation and were therefore further characterized. The loops in both mutants were unable to fuse with the parent hypha, while still being capable of trapping nematodes. An interesting question is whether these traps gradually lose their typical characteristics including dense bodies and extracellular adhesive while the traps become more similar to normal hyphae. Further light and transmission electron microscopy studies might answer this question. The mutants did not show any differences in growth rate, conidiation, or capability of anastomosis between undifferentiated hyphae compared to the wild type. Attempts to isolate genes responsible for the defect in trap formation have so far been unsuccessful.

Another approach was used to identify genes in the closely related knob-forming nematode-trapping fungus, *Monacrosporium haptotylum (Dactylaria candida)*, namely large-scale sequencing of expressed sequence tags (ESTs) and using EST data to construct cDNA arrays. One advantage of using this knob-forming fungus is that the knobs can be detached from the hyphae, while still being able to infect nematodes. Therefore, the transcriptome expressed during development of traps and infection of the nematode *Caenorhabditis elegans* could be analyzed. Significant differences between hyphae, knobs, and infected nematodes were observed with regard to distribution of ESTs into functional groups (Ahrén, 2002).

Molecular approaches are also being used to study the phylogeny of the nematode-trapping fungi. A phylogenetic tree was constructed using 18S rDNA sequences (Ahrén *et al*., 1998). According to this tree the nematode-trapping ascomycetes belong to a monophyletic clade. The phylogenetic pattern was concordant with the type of infection structures rather than the morphology of the conidia. Hagedorn and Scholler (1999) confirmed the monophyletic origin of the taxa forming various types of adhesive trapping devices. As a taxonomic consequence a new generic concept based on the morphological features of the trapping devices was proposed (Scholler *et al*., 1999).

Conclusions and future perspectives

Among the nematode-trapping fungi, *A. oligospora* is the best-studied species as regards its properties as a saprotroph and for exploring its transition to a parasitic lifestyle. It is one of the soil fungi that has been studied in depth, both in its natural environment and in the laboratory. This may explain why such a high developmental plasticity of trapping structures has been detected. The development of molecular tools for the understanding of the transition of a saprotroph to a parasite has tremendously increased the value of *A. oligospora* as a model organism. Methods developed in other fungi that depend on a morphological change for

infection of a host will be useful tools in this system. It is a challenge for future studies to understand how the development of infection structures is linked to the evolution of these and other parasitic fungi.

As we have seen above, there is a very delicate balance between hyphal growth and trap development, and thus between saprotrophic and parasitic lifestyles. In addition, many different structures may be formed by single species under the influence of external signals. It is remarkable that many of the structures have only been detected – or at least were first detected – in natural substrates. This is both promising and difficult. It is promising because an increased function as biocontrol agents is desirable in environments such as the rhizosphere of agricultural crops, animal pastures, faecal pellets etc. It is difficult because all factors involved in the morphogenesis are not completely defined to allow laboratory studies of high quality. For instance, knockout mutants may show an impaired response to environmental signals. For that reason, in every situation the development of relevant bioassays must receive careful attention.

Molecular methods to link morphological diversity in the natural environment to laboratory studies of differentiation and cellular physiology will be necessary. Therefore, the enhanced production of a serine protease, subtilisin, by a nematode-trapping fungus (Åhman *et al*., 2002) is a promising approach in this direction. The number of infection structures was increased, leading to increased trapping and killing of nematodes. In the future, a crucial point will be to find genes specifically involved in the morphogenesis of these fungi.

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