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MODE OF ACTION AND INTERACTIONS OF NEMATOPHAGOUS FUNGI

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Abstract. Nematophagous fungi are potential candidates for biological control of plant-parasitic nematodes, and an important constituent in integrated pest management programs. In this chapter we describe various aspects on the biology of these fungi. Nematophagous species can be found in most fungal taxa, indicating that the nematophagous habit evolved independently in the different groups of nematophagous fungi. Regarding their mode of action we discuss recognition phenomena (e.g. chemotaxis and adhesion), signaling and differentiation, and penetration of the nematode cuticle/eggshell using mechanical, as well as enzymatic (protease and chitinase) means. The activities of nematophagous fungi in soil and rhizosphere is also discussed.

1. INTRODUCTION

The term "nematophagous fungi" is used to describe a diverse group of organisms with the ability to infect and parasitize nematodes for the benefit of nutrients. The first description of their nematophagous habit came in the late 1800's and has been followed by work of many scientists describing this fascinating group of fungi. Apart from infecting nematodes, nematophagous fungi also have the ability to colonize and parasitize other organisms, such as plants and even other fungi. Some of them are obligate parasites of nematodes, but the majority are facultative saprophytes.

Because of their capability to parasitize plant- and animal-parasitic nematodes they have a potential for development as biocontrol agents. In the current chapter we describe and discuss some of the research that has been performed on nematophagous fungi. We will focus on fundamental aspects such as their mode of action and interactions, especially regarding their behaviour in the rhizosphere and their endophytic behaviour within the scenario of a complex trophic web, with the soil and its biota as background. Our working hypothesis is that an adequate management of this ecosystem will lead to the establishment of long-term nematode suppression as it happens under natural conditions in a wide array of

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soils worldwide. The plant host defences are triggered unspecifically by biotic and abiotic factors. Therefore, better knowledge about the mode of action of nematophagous fungi, especially regarding the host plant, may lead to control of other root pathogens such as fungi and may in turn improve plant growth.

2. NEMATOPHAGOUS FUNGI

2.1. Biology

Depending on their mode of attacking nematodes, the nematophagous fungi are divided into four groups: (*i*) nematode-trapping (formerly sometimes called predacious or predatory fungi), (*ii*) endoparasitic, (*iii*) egg- and female-parasitic and (*iv*) toxin-producing fungi (Jansson & Lopez-Llorca, 2001). Some of the characteristics of these grops are resumed and shown in Fig. 1.

The nematode-trapping fungi, as the name implies, capture nematodes with the aid of hyphal trapping devices of various shapes and sizes, e.g. adhesive three-dimensional nets, adhesive knobs, non-adhesive constricting rings. A few "nematode-trappers" capture nematodes without visible traps in an adhesive substance formed on their hyphae, e.g. *Stylopage* spp.

Endoparasitic fungi use their spores (conidia or zoospores) to infect nematodes. The propagules adhere to the nematode cuticle, and the spore contents is then injected into the nematode, or the spores are swallowed by the host. Most of these fungi are obligate parasites of nematodes and live their entire vegetative stages inside infected nematodes.

The egg- and female-parasitic fungi infect nematode females and the eggs they contain, using appressoria or zoospores. Finally, the toxin-producing fungi immobilize the nematodes by a toxin, prior to hyphal penetration through the nematode cuticle. In all four nematophagous groups, nematode parasitism results in a complete prey or egg digestion, activity which supplies the fungus with nutrients and energy for continued growth.

2.2. Taxonomy and Phylogeny

Nematophagous fungi are found in most fungal taxa: Ascomycetes (and their hyphomycete anamorphs), Basidiomycetes, Zygomycetes, Chytridiomycetes and Oomycetes (Fig. 2). It therefore appears that the nematophagous habit evolved independently in the different fungal taxonomic groups. Barron (1992) suggested that the nematophagous habit evolved from lignolytic and cellulolytic fungi, as an adaptation to overcome competition for nutrients in soil.

Recently, the egg-parasitic fungi previously placed within the genus *Verticillium* were transferred to the new genus *Pochonia*, in parallel with entomopathogenic species of *Verticillium*, which were transferred to the genus *Lecanicillium* based both on morphological and molecular characters (Zare & Gams, 2001; Zare, Gams, &

Evans, 2001). The teleomorphs of the *Pochonia* species are located within *Cordyceps*. The best known species of egg parasites are *P. chlamydosporia* and *P. rubescens*, but species of other genera such as *Paecilomyces lilacinus* and *Lecanicillium lecanii,* are also known to parasitize nematode eggs.

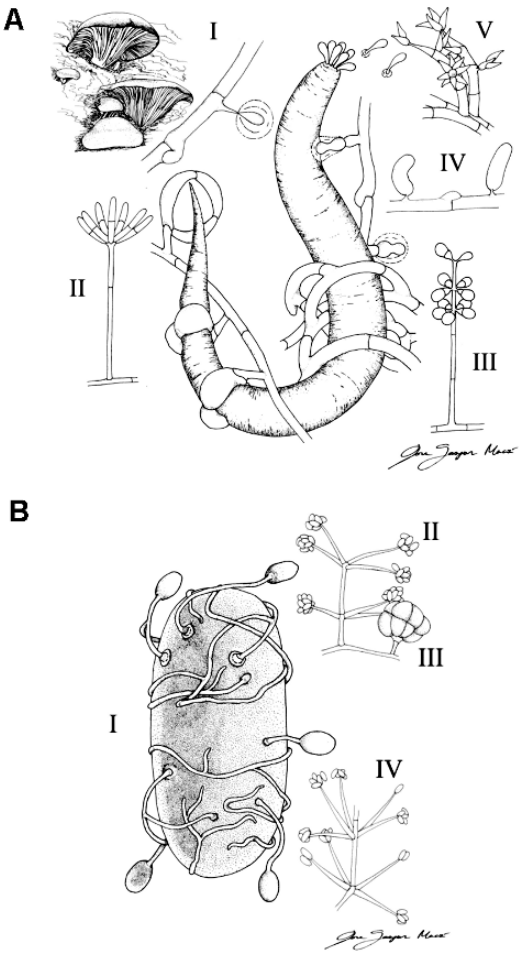

Figure 1. Biology of nematophagous fungi. Vermiform (motile) nematode (A) displaying infection structures: (I) toxin-producing fungus [Pleurotus *sp.*], *nematode-trapping fungi (II)* Drechslerella *sp. (III)* Arthrobotrys *sp., (IV)* Nematoctonus *sp. and (V) endoparasitic* Drechmeria *sp.. Nematode (sedentary) egg (B) (similar features can be found in egg masses, females and cysts) displaying infection structures: penetrating hyphae and appressoria of egg-parasitic fungi (I), conidia (II) and chlamydospores (III) of* Pochonia *sp., and conidia of* Lecanicillium *sp. (IV).*

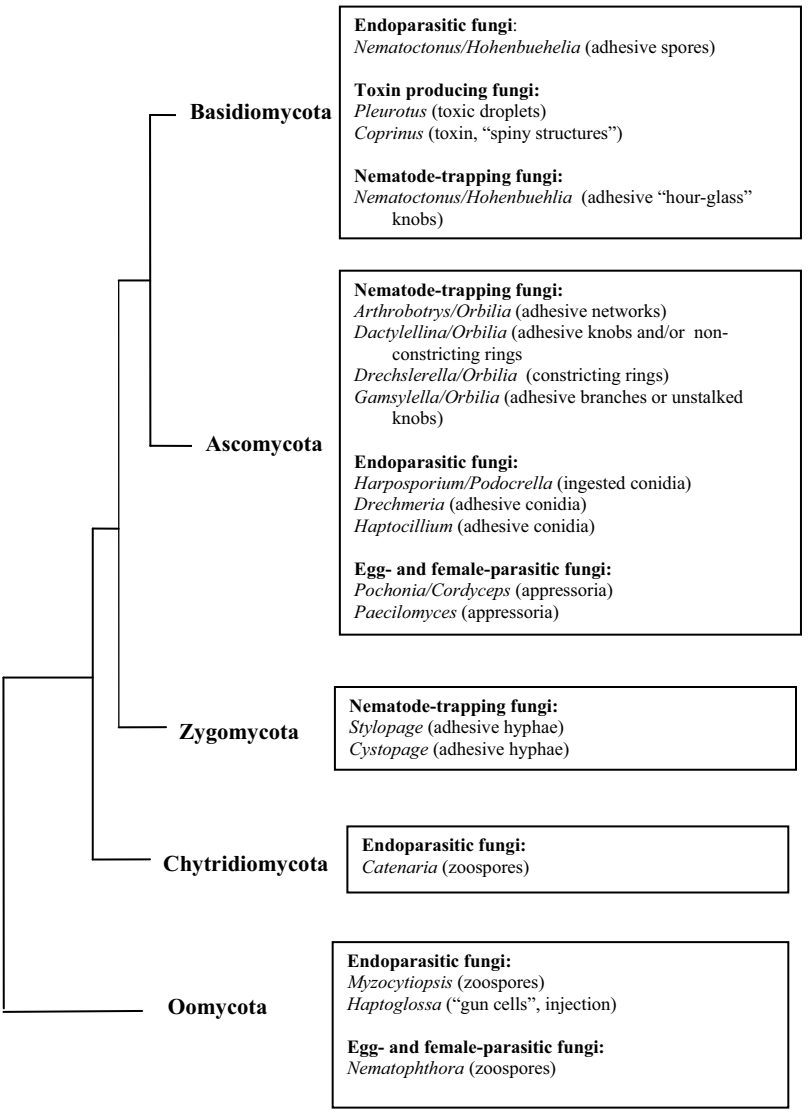

Figure 2. Taxonomic position of nematophagous fungi with examples of genera. The first genus names are anamorphs, and genus names after slashes indicate known teleomorphs. Infection structures are shown in parenthesis.

Most nematode-trapping species have a teleomorph within *Orbilia*, and their taxonomic positions have been arranged according to their type of trapping device (Ahrén, Ursing, & Tunlid, 1998). Scholler, Hagedorn, and Rubner (1999) suggested the following classification based on molecular data: *Arthrobotrys* (adhesive three-dimensional networks), *Dactylellina* (stalked adhesive knobs and/or non-constricting rings), *Drechslerella* (constricting rings) and *Gamsylella* (adhesive branches and unstalked knobs). This classification was questioned by Li et al. (2005) who suggested that the species in *Gamsylella* should be transferred to either *Arthrobotrys* or *Dactylellina* based on more and refined DNA sequencing. In this review we follow the taxonomy suggested by Scholler et al. (1999). Li et al. (2005) put forward a hypothesis of an evolutionary pathway of traps of the nematode-trapping Orbiliales. According to this hypothesis, two lines have evolved originating from adhesive knobs, in one line the adhesive was lost and evolved to form constricting rings, whereas the other evolutive line retained the adhesive and became three-dimensional networks.

Much less is known about the taxonomy/phylogeny of the endoparasitic fungi. Some of these are placed in the Chytridiomycetes, e.g. the zoosporic *Catenaria anguillulae*, others in *Haptocillium* (formerly *Verticillium*), *Harposporium* or *Drechmeria*. The teleomorph of *Harposporium* spp. has recently been transferred from *Atricordyceps* to *Podocrella* (Chaverri, Samuels, & Hodge, 2005). The basidiomycete genus *Hohenbuehelia* (anamorph: *Nematoctonus*) contains fungi that can be classified as both nematode-trapping and endoparasites (Thorn & Barron, 1986). The genus *Pleurotus* includes species, such as the oyster mushroom *P. ostreatus,* and constitutes the toxin-producing fungi. Recently, *Coprinus comatus* was shown to have similar capabilities (Luo, Mo, Huang, Li, & Zhang, 2004), suggesting that the nematophagous habit may be more widespread among Basidiomycetes than previously thought.

2.3. Fungal Parasites of Invertebrates

Entomopathogenic and nematophagous fungi are generally facultative parasites, usually implying a low host specificity and consequently a wide host range. They can also colonize a wide array of habitats and their main species can be found worldwide.

Entomopathogenic and nematophagous fungi bear multiple similarities. The most important species of both fungal groups have been described as soil inhabitants, where they spend most of the saprophytic growth phase. Soil is also the environment of nearly all plant-parasitic nematodes and of soil dwelling insects such as roots pests or other underground plant organs. For further details on these aspects see Lopez-Llorca and Jansson (2006).

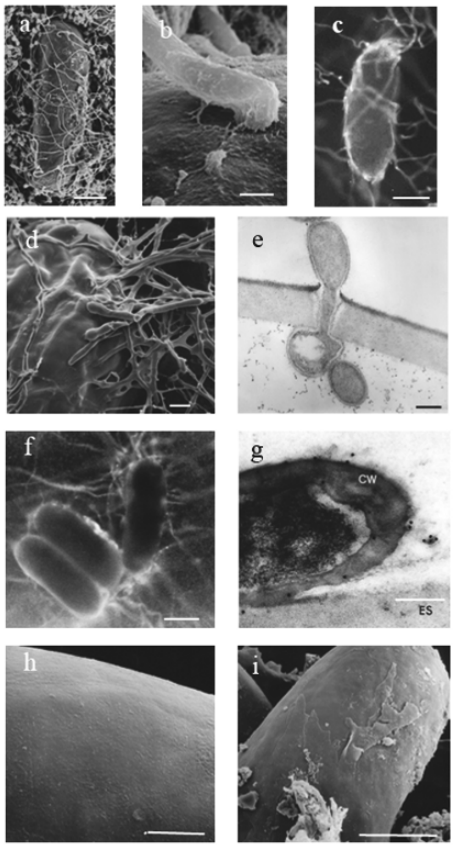

*Figure 3. Mode of action of fungal parasites of nematode eggs. (a) Field emission scanning electron microscopy (FESEM) of nematode (*Heterodera schachtii*) egg inoculated with conidia of* Pochonia rubescens *(Bar = 25 µm). (b) Detail of the fungus appressoria showing adhesive secretions on the eggshell (Bar = 2 µm). (c) Labelling of nematode-infected egg with Con A lectin fluorescently labelled (Bar = 25 µm). (d) Detail of advanced infection by* P. rubescens *showing fully developed appressoria on eggshell (Bar = 5µm). (e) Eggshell penetration by* P. rubescens *(Bar = 0.25 µm). (f) immunofluorescence detection of P32 protease produced by* P. rubescens *(Bar = 5 µm). (g) Immunogold detection of P32 (Bar = 1 µm) (Lopez-Llorca & Robertson, unpublished). (h) and (i) Effect of purified P32 on eggshell of* H. schachtii*. (h) control (Bar = 5 µm) and (i) P32-treated. (Bar = 10 µm). (FESEM, Lopez-Llorca & Claugher, unpubl.). (a) From Lopez-Llorca and Claugher, 1990, courtesy of Elsevier. (c) From Lopez-Llorca, Olivares-Bernabeu, Salinas, Jansson, and Kolattukudy, 2002b, courtesy of Elsevier. (d) and (e) adapted from Lopez-Llorca and Robertson, 1992b, courtesy of Springer. (f) From Lopez-Llorca and Robertson, 1992a, courtesy of Elsevier.*

3. MODE OF ACTION

The infection of nematodes and their eggs by various nematophagous fungi follows a similar, general pattern. This is illustrated here by infection of nematode eggs by *Pochonia rubescens* (Fig. 3) and also by the zoospores of *Catenaria anguillulae*, which infect vermiform nematodes (Fig. 4).

Penetration of nematode eggs by *P. rubescens* starts with contact of the hyphae with the egg (Fig. 3a) and subsequent formation of an appressorium (Figs. 3b, d). An extracellular material (ECM) or adhesive, is formed on the appressorium, and is revealed by labelling with the lectin Concanavalin A (Con A), indicating that it contains glucose/mannose residues (Fig. 3c). From the appressorium the fungus penetrates the nematode eggshell (Fig. 3e) by means of both mechanical and enzymatic components. The nematode eggshell contains mainly chitin and proteins (Bird & Bird, 1991) and therefore chitinases and proteases play an important role during eggshell penetration (Lopez-Llorca, 1990; Tikhonov, Lopez-Llorca, Salinas, & Jansson, 2002). The ECM contains the protease P32 that can be immunologically detected using both fluorescent stains (Fig. 3f) or colloidal gold (Fig. 3g). The proteolytic activity causes the degradation of eggshells (Fig. 3i).

The life cycle of *C. anguillulae* starts with uniflagellate zoospores which become attracted to natural orifices (mouth, anus, excretory pores, etc.) of nematodes (Figs. 4a, 4b). The flagellar movement is supported by the mitochondria at the base of the flagellum (Fig. 4c). Upon contact with the nematode cuticle the zoospores show an "amoeboid movement" before encystment takes place (Fig. 4d). During encystment a cell wall is formed covered by an adhesive, and the flagellum is withdrawn (Fig. 4e). The encysted zoospore forms an infection peg which penetrates the nematode cuticle (Fig. 4f). Within 24 hours the developing fungus invades and digests the nematode contents, and zoosporangia are formed (Fig. 4g) from which the zoospores are released (Fig. 4h) to infect new hosts. *Catenaria anguillulae* also has the ability to infect nematode eggs (Wyss et al., 1992).

3.1. Recognition: Chemotaxis and Adhesion

Nematodes infection starts with a recognition phase including attraction, host chemotaxis towards fungal hyphae or traps, or chemotaxis of zoospores towards the host's natural openings (Jansson & Nordbring-Hertz, 1979; Jansson & Thiman, 1992). The compounds involved in chemotactic events are not known (Jansson & Friman, 1999; Bordallo et al., 2002). The adhesive on the traps of *A. oligospora* switches from an amorphous to a fibrillar appearance after contact with a nematode, which is in contrast to the adhesive on conidia of *D. coniospora* which always appears fibrillar (Jansson & Nordbring-Hertz, 1988). The adhesive on the appressoria of *P. chlamydosporia* and *P. rubescens* can be labelled with the lectin Concanavalin A, suggesting a glycoprotein nature with mannose/glucose moieties (Lopez-Llorca et al., 2002b). Involvement of a Gal-NAc-specific lectin of *A. oligospora* (Nordbring-Hertz & Mattiasson, 1979) and a sialic acid-specific lectin of *D. coniospora* (Jansson & Nordbring-Hertz, 1984) in nematode recognition have been suggested. Infection events eventually lead to a signalling cascade necessary

for penetration and colonisation of the nematode prey (Tunlid, Jansson, & Nordbring-Hertz, 1992).

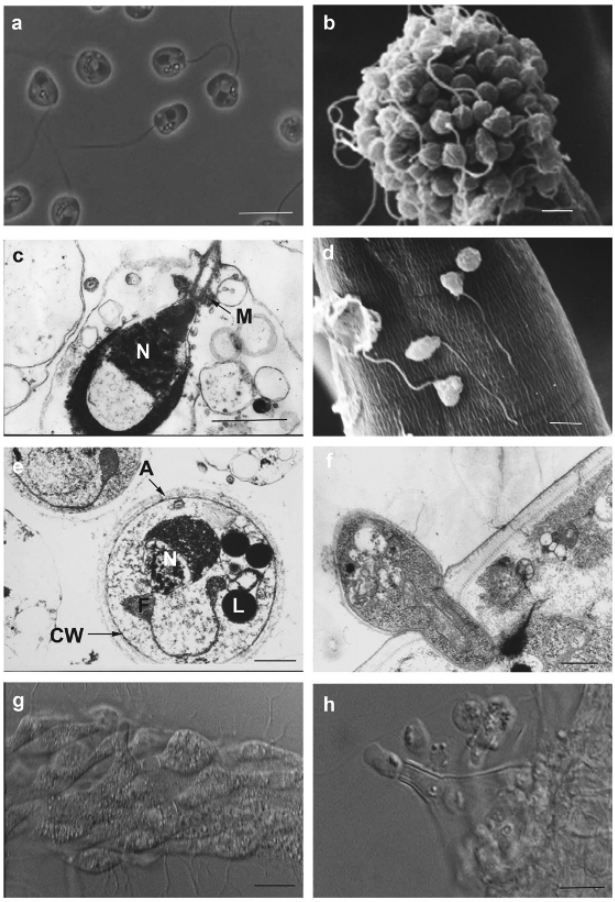

Figure 4. Infection of nematodes by the zoosporic fungus Catenaria anguillulae*. Monoflagellate zoospores (a). Zoospores (b) accumulated at the mouth of a nematode. Ultrastructure of a zoospore (c): N = nucleus and nuclear cap, M = mitochondrium at flagellar base. Zoospores show typical amoeboid movement prior to encystment (d). Encysted zoospore (e): A = adhesive, CW = cell wall, F = withdrawn flagellum, L = lipid droplet, N = nucleus and nuclear cap. Penetration of nematode cuticle (f) and development of zoosporangia (g) inside an infected nematode. The cycle is completed by the release of zoospores (h). Scale bars: a, b, d, h = 2 µm; c, e, f = 1 µm; g = 5 µm. (Figs. a, g, h) from Jansson et al., 1995, courtesy of IWF Wissen und Medien, Göttingen; (b) from Jansson & Thiman, 1992, courtesy of Mycological Society of America; (e) from Tunlid, Nivens, Jansson, and White, 1991b, courtesy of Experimental Mycology; (c, d and f) H–B. Jansson, unpublished.*

After contact, an extracellular material, or adhesive, is formed which keeps the fungus onto the nematode surface (Figs. 3b, 3c, 4e). Nematophagous fungi adhesives commonly contain proteins and/or carbohydrates (Tunlid, Johansson, & Nordbring-Hertz, 1991a; Tunlid et al., 1991b).

Carbohydrates present on the surface of nematodes are involved in the recognition step of lectin binding, but also appear to be involved in nematode chemotaxis (Zuckerman & Jansson, 1984; Jansson, 1987). The main nematode sensory organs, amphids and inner labial papillae, are located in the cephalic and labial region, around their mouth (Ward, Thomson, White, & Brenner, 1975).

A hypothesis of the involvement of carbohydrates in nematode chemoreception was put forward by Zuckerman (1983) and Zuckerman and Jansson (1984). The chemoreceptors, purportedly glycoproteins, could be blocked by lectins (Concanavalin A binding to mannose/glucose residues, and Limulin binding to sialic acid) resulting in loss of chemotactic behaviour of bacterial-feeding nematodes to bacterial exudates (Jeyaprakash, Jansson, Marban-Mendoza, & Zuckerman, 1985). Furthermore, treating nematodes with enzymes (mannosidase, sialidase) obliterating the terminal carbohydrates also decreased chemotactic behaviour (Jansson, Jeyaprakash, Damon, & Zuckerman, 1984), showing the role of carbohydrate moieties in nematode chemotaxis.

The endoparasitic nematophagous fungus *D. coniospora* infects nematodes with conidia which adhere to the host chemosensory organs (Jansson & Nordbring-Hertz, 1983). Conidial adhesion was suggested to involve a sialic acid-like carbohydrate since treatment of nematodes with the lectin Limulin, and treatment of spores with sialic acid, decreased adhesion (Jansson & Nordbring-Hertz, 1984). Furthermore, nematodes with newly adhered spores lost their ability to respond chemotactically to all attraction sources tested, i.e. conidia, hyphae and bacteria, indicating a connection between adhesion and chemotaxis through carbohydrates present on the nematode surface (Jansson & Nordbring-Hertz, 1983).

The conidia of *D. coniospora* adhere to the chemosensory organs of *Meloidogyne* spp., but do not penetrate and cannot infect the nematodes. Irrespective of the lack of infection, the fungus was capable of reducing root galling in tomato in a biocontrol experiment (Jansson, Jeyaprakash, & Zuckerman, 1985), again indicating the involvement of chemotactic interference.

Interfering with nematode chemotaxis, thereby inhibiting their host-finding behavior, may be a possible way of controlling plant-parasitic species. In a pot experiment using tomato as host plant and *Meloidogyne incognita* as parasitic nematode, addition of Concanavalin A and *Limax flavus* agglutinin (sialic acid specific lectin) resulted in decreased plant damage by the nematode compared to controls (Marban-Mendoza, Jeyaprakash, Jansson, & Zuckerman, 1987). Addition of lectins (or enzymes) on a field is not feasible, but the possibility to use, for instance, lectin-producing leguminous plants have been shown to reduce galling by root knot nematodes (Marban-Mendoza, Dicklow, & Zuckerman, 1992).

3.2. Signalling and Differentiation

Most pathogenic fungi differentiate appressoria (Fig. 3b) when sensing the host´s surface or even artificial surfaces. Appressoria development has been studied in detail in plant pathogenic fungi infecting leaves (Lee, D´Souza, & Kronstad, 2003; Basse & Steinberg, 2004). A hypothesis of signalling events during appressorium formation of the insect pathogen *Metarhizium anisopliae* was put forward by St. Leger (1993), partly based on knowledge acquired on plant pathogenic fungi.

Nematophagous fungi, especially egg parasites, differentiate appressoria on their hosts (Lopez-Llorca & Claugher, 1990). Very little is known about the signalling pathways leading to nematodes infection by nematophagous fungi. Recently, using expressed sequence tag (EST) techniques, it was shown that genes involved in the formation of infection structures and in fungal morphogenesis were expressed during trap formation of the nematophagous fungus *Dactylellina haptotyla* (syn. *Monacrosporium haptotylum*) (Ahrén et al., 2005). Similar results have also been presented for the entomopathogen *M. anisopliae* (Wang & St. Leger, 2005).

Fungi infecting vermiform nematodes differentiate several trapping organs as a response to environmental stimuli, chemical as well as tactile. The constricting ring traps function through the inflation of the three ring cells which form the trapping device. When a nematode starts touching the inner ring wall, an unknown mechanism triggers its inflation and closure, a process which takes about 0.1 seconds. The cells of the ring can also be manipulated to close in the laboratory by mild heat, pressure or Ca^{2+}. Chen, Hsu, Tsai, Ho, and Lin (2001) investigated signalling taking place in ring closure of the constricting ring trap of *D. dactyloides* using activators and inhibitors of G-proteins, and suggested a model in which the nematode exerts a pressure on the ring which activates G-proteins. This leads to an increase in cytoplasmic Ca^{2+}, activation of calmodulin and finally to opening of water channels resulting in trap inflation and nematode capture.

3.3. Penetration of Nematode Cuticles and Eggshells

After firm attachment to the host surface, nematophagous fungi penetrate the nematode cuticle (Fig. 4f) or eggshell (Fig. 3e). As in many other instances of fungal penetration of host surfaces, nematophagous fungi appear to use both enzymatic and physical means. The nematode cuticle mainly contains proteins (Bird & Bird, 1991) and therefore the action of proteolytic enzymes (Table 1) may be important for penetration. A serine protease, PII, from *A. oligospora*, has been characterized, cloned and sequenced (Åhman, Ek, Rask, & Tunlid, 1996). The expression of PII is increased by the presence of proteins, including nematode cuticles (Åhman et al., 1996). PII belongs to the subtilisin family and has a molecular mass of 32 kDa.

Table 1. Serine proteases and chitinases isolated and characterized from different nematophagous fungi.

Nematophagous species	*Enzyme*	*kDa*	*pI*	*Optimum pH*	*References*
			Proteases		
			Nematode-trapping fungi		
Arthrobotrys oligospora	PII	35	4.6	7–9	Tunlid, Rosén, Ek, and Rask (1995) Åhman et al. (1996)
A. oligospora	Aoz	38	4.9	6–8	Zhao, Mo, and Zhang (2004)
Arthrobotrys (Monacrosporium) microscaphoides	Mlx	39	6.8	9	M. Wang, Yang, and Zhang (2006)
Arthrobotrys (Dactylella) shizishanna	Ds1	35	-	10	R.B. Wang, Yang, Lin, Y. Zhang, and K.Q. Zhang (2006)
			Egg-parasitic fungi		
Pochonia rubescens	P32	32	6.2	8.5	Lopez-Llorca (1990) Olivares-Bernabéu (1999) Lopez-Llorca and Robertson (1992b)
Pochonia chlamydosporia	VCP1	33	10.2	-	Segers, Butt, Kerry, and Peberdy (1994); Segers, Butt, Keen, Kerry, and Peberdy (1995)
Paecilomyces lilacinus	PL	33.5	>10.2	10.3	Bonants et al. (1995)
Lecanicillium psalliotae	Ver112	32	-	10	Yang et al. (2005a, 2005b)
			Chitinases/chitosanases		
P. rubescens	CHI43	43	7.6	5.2–5.7	Tikhonov et al. (2002)
P. chlamydosporia	CHI43	43	7.9	5.2–5.7	Tikhonov et al. (2002)
P. lilacinus	-	23	8.3	6	Chen, Cheng, Huang, and Li (2005)

Another serine protease from *A. oligospora* (Aoz1), with a molecular mass of 38 kDa showing 97% homology with PII was recently described (Zhao et al., 2004). Other serine proteases have been isolated and characterized from the nematode-trapping fungi *Arthrobotrys* (syn. *Monacrosporium*) *microscaphoides* designated Mlx (M. Wang et al., 2006) and *Arthrobortys* (syn. *Dactylella*) *shizishanna* (Ds1) (R. B. Wang et al., 2006) both showing high homology with the *A. oligospora* serine proteases (M. Wang et al., 2006).

Nematode eggshells mostly contain protein and chitin (Clarke, Cox, & Shepherd, 1967) organized in a microfibrillar and amorphous structure (Wharton, 1980). Therefore, a search for extracellular enzymes degrading those polymers was carried out. A 32 kDa serine protease (P32) was first purified and characterized from the egg parasite *P. rubescens* (Lopez-Llorca, 1990). Involvement of the enzyme in pathogenesis was suggested by quick *in vitro* degradation (Fig. 3i) of *Globodera pallida* egg shell proteins (Lopez-Llorca, 1990), but most of all by its immunolocalization (Fig. 3f, 3g) in appressoria of the fungus infecting *Heterodera schachtii* eggs (Lopez-Llorca & Robertson, 1992b).

Although pathogenesis is a complex process involving many factors, inhibition of P32 with chemicals and polyclonal antibodies reduced egg infection and penetration (Lopez-Llorca et al., 2002b). The similar species *P. chlamydosporia* also produces an extracellular protease (VcP1) (Segers et al., 1994) which is immunologically related to P32 and similar enzymes from entomopathogenic fungi (Segers et al., 1995). VcP1-treated eggs were more easily infected than untreated eggs, suggesting a role of the enzyme in eggshell penetration by egg-parasitic fungi.

Recently a serine protease (Ver112) was isolated and characterized from *Lecanicillium psalliotae* showing similarities with the *Arthrobotrys* proteases (PII and Aoz1) of ca 40%, and ca 60% homology with serine proteases of egg-parasitic fungi (Yang et al., 2005a, 2005b).

Other proteases from nematophagous fungi have been partly characterized, e.g. a chymotrypsin-like protease from conidia of the endoparasite *D. coniospora* (Jansson & Friman, 1999), and a collagenase produced by the nematode-trapping *Arthrobotrys tortor* (Tosi, Annovazzi, Tosi, Iadarola, & Caretta, 2001). Non-nematophagous fungi such as the mycoparasites *Trichoderma harzianum* and *Clonostachys rosea* (syn. *Gliocladium roseum*) are also sources of serine proteases with nematicidal activity (Suarez, Rey, Castillo, Monte, & Llobell, 2004; Li, Yang, Huang, & Zhang, 2006).

Several chitinolytic enzymes of *Pochonia rubescens* and *P. chlamydosporia* have been detected. One of those accounting for most of the activity was a 43 kDa endochitinase (CHI43) (Tikhonov et al., 2002). When *G. pallida* eggs were treated with both P32 and CHI43 damage to eggshell was more extensive than with each enzyme alone, suggesting a cooperative effect of both enzymes to degrade egg shells (Tikhonov et al., 2002). Recently a chitosanase was isolated and characterized from the egg-parasitic fungus *P. lilacinus* (Chen et al., 2005).

3.4. Fungal Pathogen Genomics and Proteomics

In the era of genomics, fungal pathogens are suitable candidates for the analysis under this new paradigm in modern biology. In the dawn of fungal pathogen genomics under the Fungal Genome Initiative, important fungal pathogens have been or are being sequenced (Xu, Peng, Dickman, & Sharon, 2006). A direct bonus is the finding of unique fungal genes and characterization of genome structure and

function. Available gene predictions in genomes of fungal plant pathogens indicate 30% of no homologues. This situation, which could be similar in nematophagous fungi, indicates that new fungal genes or gene products (e.g. proteins of unknown function) can soon be discovered.

The re-evaluation of the study of fungal pathogenicity-related genes with a genomic approach is underway. One example is appressorium development. This awaits to be applied in nematophagous fungi. Signalling/reception are other fields which will follow.

Proteomic approaches complement genomics. There are expression, localization and interactions, which are unique to this global strategy. Our preliminary results indicate that plant-host fungal invertebrate pathogen "cross-talk" can be approached this way

The assembly of the Fungal Tree of Life project (Spatafora, 2005; Kuramae, Robert, Snel, Weiss, & Boekhout, 2006) which is at a very advanced stage, could represent a useful tool for deciding on how to proceed to establish genomic approaches. EST approaches to understand the pathogenicity of nematophagous fungi are already being used (Ahrén et al., 2005).

4. SOIL AND RHIZOSPHERE ENVIRONMENT

4.1. Activities in Soil

Nematophagous fungi are generally regarded as soil organisms (Dackman, Jansson, & Nordbring-Hertz, 1992), although there are reports on their frequent occurrence also in aquatic environments, especially in shallow, unpolluted water (Hao, Mo, Su, & Zhang, 2005). Most nematophagous fungi can live saprophytically in soil, but in presence of a host they change from a saprophytic to a parasitic stage. The exact mechanism behind this is not known. Nematophagous fungi inhabit soil pores where infection structures are formed and nematodes are captured (Fig. 5). The zoosporic fungi are obviously dependent of soil water films for their function.

When nematophagous species have to be applied to manage plant parasitic nematodes they have to be delivered to soil. Several approaches for introducing them have been used (see Stirling, 1991), but very little efforts have been paid to follow the fate of nematophagous fungi in the soil/rhizosphere environment, after their release.

Nematophagous fungi grow in almost all types of soil, but are generally regarded as being more frequent in soils with high organic matter (Duddington, 1962). Generally, they have few nutritional and vitamin requirements for growth, and hence are ubiquitous. Additions of glucose (Cooke, 1962) and chopped organic matter, e.g. grass (Duddington, 1962) increased activity of nematode-trapping species. This effect was probably due to an increase in the numbers of microbivorous nematodes. *Arthrobotrys* spp. have a teleomorph in *Orbilia*, which are weak wood decomposers (Pfister, 1997), and the wood decomposing *Pleurotus* spp. suggests that decomposition of wood may be an important supply of carbon and energy for the fungi. Capturing nematodes may hence support the fungi with

nitrogen (Barron, 1992). In Petri dishes and sterilized microcosms there is a heavy reduction of nematodes due to nematophagous fungi (Jansson, 1982b), and a density dependence relationship exists between nematodes and endoparasites (Jaffee, Gaspard, & Ferris, 1989).

In field soil, there is no clear correlation between nematophagous fungi and nematodes (Persmark, Banck, & Jansson, 1996a) and nematode-trapping fungi are known to be sensitive to soil mycostasis (Cooke & Satchuthananthavale, 1968), as well as to feeding by soil enchytraeids (Jaffee, 1999).

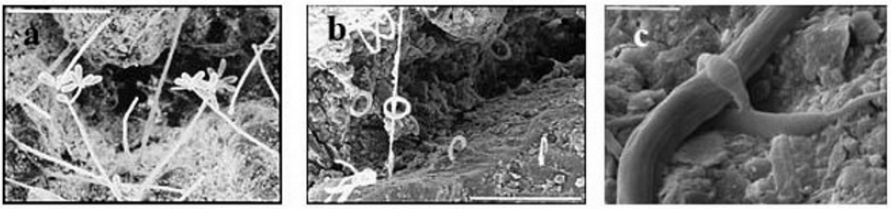

Figure 5. Low temperature scanning electron micrographs (LTSEM) of nematophagous fungi in soil. (a) Conidiophores with conidia of the nematode-trapping fungus Arthrobotrys superba *(bar = 100 µm). (b) Constricting ring traps of* Drechslerella dactyloides *(bar = 50 µm). (c) Nematode captured in constricting ring of* D. dactyloides *(bar = 50 µm). From Jansson, Persson, and Odselius (2000), courtesy of Mycological Society of America.*

Introduction of nematophagous fungi, and most microbial biocontrol agents, to soil has been problematic due to both biotic and abiotic factors. Biocontrol experiments using the egg-parasite *P. chlamydosporia* showed low control efficiency against root-knot nematodes, and furthermore, the fungus was detected at very low rates, mainly in the rhizosphere of the test plants (Verdejo-Lucas, Sorribas, Ornat, & Galeano, 2003). One of the reasons for this may be that the soil was not receptive to the fungus.

We have used an *in vitro* assay to be able to easily study soil receptivity for nematophagous fungi (Monfort, Lopez-Llorca, Jansson, & Salinas, 2006). Using a soil-membrane technique 0, 25, 50, 75 and 100% sterilized soil was inoculated with several isolates of the nematophagous fungi *P. chlamydosporia* and *P. lilacinus*. After 4 weeks, colony radius was measured (expressed as relative growth) as well as hyphal density on the membrane placed on top of the soils.

When comparing two sandy soils (Spanish and Australian) with similar physico-chemical properties, large differences between the receptivity to the fungi were found, both regarding isolates as well as between soils. For instance, an Australian isolate of *P. chlamydosporia* was most inhibited in the Spanish soil, but the least inhibited in the Australian soil. The result suggests that a soil can be more receptive to indigenous isolates than to non-indigenous ones.

4.2. Nematophagous Fungi as Root Endophytes

Since nearly all plant-parasitic nematodes attack plant roots, the rhizosphere biology of nematophagous fungi is important from the point of view of a biological control strategy. Nematode-trapping fungi (Peterson & Katznelson, 1965; Gaspard & Mankau, 1986; Persmark & Jansson, 1997) and egg-parasitic fungi (Bourne, Kerry, & De Leij, 1996; Kerry, 2000) have been found to be more frequent in the rhizosphere than in the bulk soil.

External root colonisation varies between plant species. The pea rhizosphere harboured by far the highest frequency and diversity of nematode-trapping fungi compared to other plant species tested (Persmark & Jansson, 1997). In an investigation on chemotropic growth of nematophagous fungi towards roots of several plants, only isolates of *A. oligospora* were attracted (Bordallo et al., 2002). In a 3-month pot experiment, *Dactylellina ellipsospora* (syn. *Monacrosporium ellipsosporum*) and *D. dactyloides* were especially competent in colonising tomato roots (Persson & Jansson, 1999).

Several nematode-trapping fungi are able to form so-called conidial traps in response to roots and root exudates (Persmark & Nordbring-Hertz, 1997). The external root colonisation by the egg-parasite *Pochonia chlamydosporia* also varied with plant species and was increased when plants were infected with the root-knot nematode *Meloidogyne incognita* (Bourne et al., 1996). This effect is possibly due to increased leakage of root exudates after damage to the root surface by the nematodes.

In recent investigations we studied the endophytic root colonization of the four groups of nematophagous species. The nematode-trapping species *A. oligospora, D. dactyloides* (Figs. 6a, b), and *N. robustus* (Figs. 6b, c) were all capable of endophytic colonization of barley roots. Similar root colonization was also detected for the egg-parasite *P. chlamydosporia* (Figs. 6e, f) and the toxin-producing *P. djamor*. The only fungi which did not show root colonization were the endoparasitic fungi *H. rhossiliensis* and *N. pachysporus* (Lopez-Llorca, Bordallo, Salinas, Monfort, & Lopez-Serna., 2002a; Bordallo et al., 2002; Lopez-Llorca, Jansson, Macia Vicente, & Salinas, 2006). The fungi grew inter- and intracellularly, formed appressoria when penetrating plant cell walls of epidermis and cortex cells, but never entered vascular tissues (Lopez-Llorca et al., 2002a; Bordallo et al., 2002). In contrast to *Pochonia* spp., appressoria had never been observed previously in *A. oligospora.*

Using histochemical stains it was possible to reveal the plant defence reactions, e.g. papillae and other cell wall appositions induced by nematophagous fungi, but these never prevented root colonization. Nematophagous fungi grew extensively especially in monocotyledon plants producing abundant mycelia, conidia and chlamydospores. Necrotic areas of the roots were observed at initial stages of colonization by the nematode-trapping and toxin-producing fungi tested, but were never seen at later stages, even when the fungi proliferated in epidermal and cortical cells.

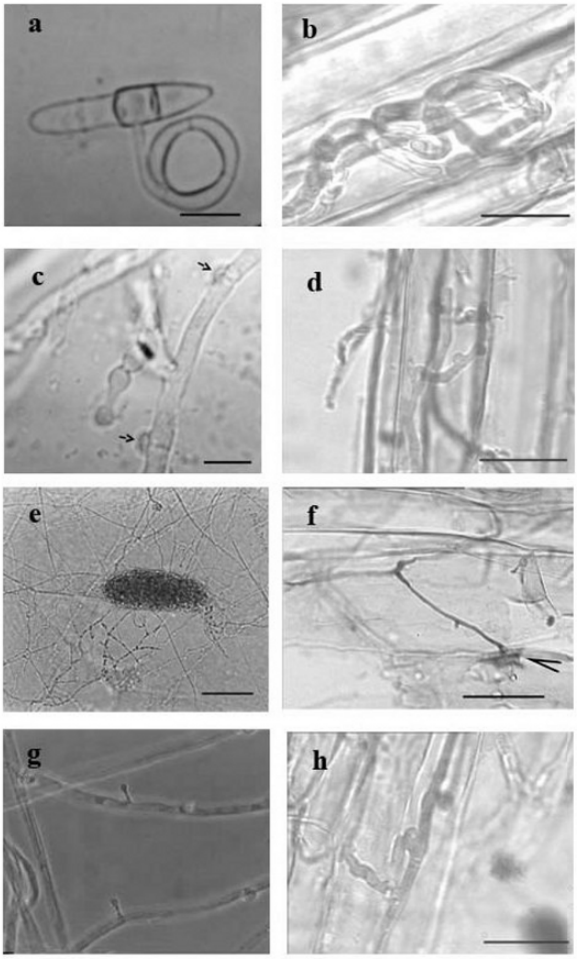

Figure 6. Parasitic (a, c, e, g) vs. endophytic (b, d, f, h) behaviour of nematophagous fungi. (a) Conidial trap of Drechslerella *sp. (c) Mycelia of a* Nematoctonus *sp. showing an "hour glass" trapping device and clamp connections (arrows). (e) Nematode egg infected by* Pochonia *sp. (g) Hyphae and toxin-producing organ of* Pleurotus *sp. (b, d, f, h). Display of endophytic colonisation of barley cortex cells by the nematophagous fungi displayed on the left hand side of each picture. Scale bars: a = 25 µm; b, d, h = 15 µm; c = 2 µm; e = 10 µm; f = 30 µm; g = 1 µm. (a and c: C. Olivares-Bernabéu, unpublished; b, d, h: from Lopez-Llorca* et al., *2006, courtesy of Springer; e: from Lopez-Llorca* et al., *2002b, courtesy of Elesevier; f: from Bordallo* et al., *2002, courtesy of the New Phytologist Trust; g: from Nordbring-Hertz* et al., *1995, courtesy of IWF Wissen und Medien, Göttingen).*

In cereal roots proceeding from soils naturally infested with the cereal cyst nematode *Heterodera avenae* and *Pochonia* spp., either the syncytia induced by the nematode and fungal hyphae could be detected inside the roots (Fig. 7c). Abundant sporulation of *Pochonia* spp. was also observed on the root surface (Fig. 7 a, b). The results at least indicate the possibility that nematode infection by the fungus may occurr inside roots, although so far this event has not been observed.

Actually, it is unknown whether endophytic colonization induces systemic resistance to nematodes and/or plant pathogens in plants. We have found that *P. chlamydosporia* could reduce growth of the plant pathogenic fungus *Gaeumannomyces graminis* var. *tritici* (take-all fungus, Ggt) in dual culture Petri dish and in growth tube experiments. In pot experiments *P. chlamydosporia* increased plant growth whether Ggt was present in the roots or not, suggesting a growth promoting effect by *P. chlamydosporia* (Monfort et al., 2005).

Endophytic rhizobacteria reducing plant-parasitic nematodes have been described (Hallmann, Quadt-Hallmann, Miller, Sikora, & Lindow, 2001), as well as the reduction of root knot nematodes by arbuscular mycorrhizal fungi (Waecke, Waudo, & Sikora, 2001). If this is true also in nematophagous fungi this will open up a new area of biocontrol using these fungi. The endophytic root colonization by egg-parasitic fungi, e.g. *Pochonia* spp., may provide them an opportunity to infect eggs of economically important endoparasitic nematodes (e.g. cyst and root-knot species) inside the roots and to reduce subsequent spread and roots infection by the second generation of juveniles.

Structures resembling trapping organs were observed in epidermal cells colonized by *A. oligospora*, and these may serve the purpose of trapping newly hatched juveniles escaping the roots. The ability to colonize plant roots may also be a survival strategy of these fungi and could explain soil suppressiveness to plant-parasitic nematodes in nature. The colonization of plant roots by nematophagous fungi is a new area of research that deserves in-depth investigations, not the least for biocontrol purposes and is presently underway in our laboratory.

4.3. Rhizosphere Dynamics and Biocontrol

The rhizosphere is a microecosystem in which roots release nutrients which in turn will affect microbes and their grazers. The former will modify these nutrients and could affect root and plant development. In this complex scenario, nematophagous fungi are both “hunters” and “hunted” since they predate on nematodes and can be affected, for instance, by myceliophagous species. It is tempting to use a combination of current non-destructive methods to analyse dynamics of the biotic component of the rhizosphere. Modification, or engineering, of the rhizosphere resource exchange could be vital for modifying the endophytic behaviour of nematophagous fungi. This may in turn affect their capability to control root diseases. Recently, microbiosensors, i.e. hybrids of soil sensors and

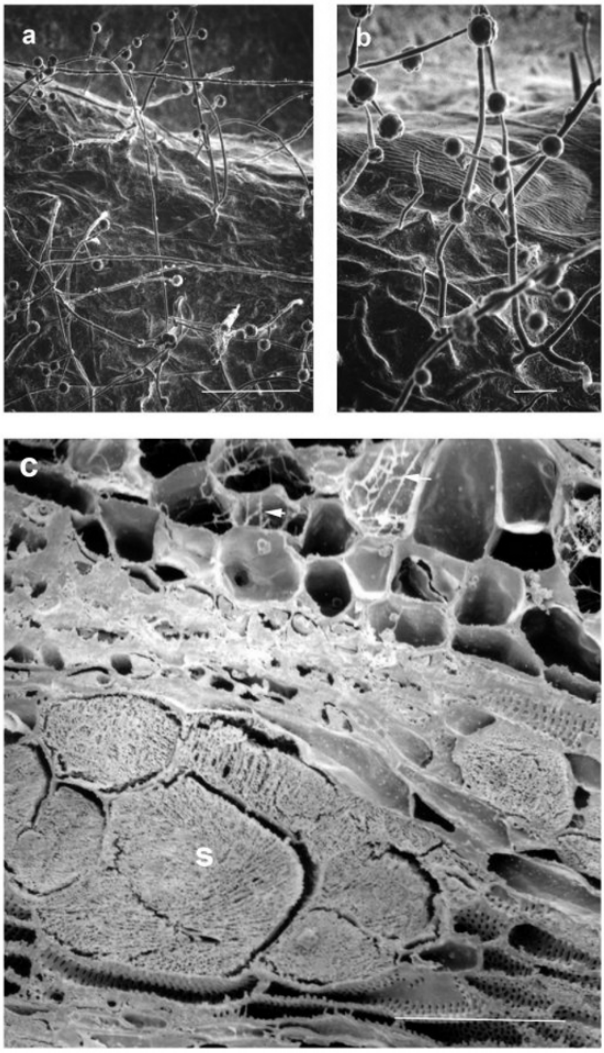

Figure 7. Rhizosphere colonization by fungal egg parasites in nematode suppressive soils. (a) Profuse hyphal growth and sporulation (LTSEM) in oat rhizosphere. (b) Close-up of phialides and slimy conidia of Pochonia *spp. (c) Field emission scanning electron microscopy (FESEM) of longitudinal section through a cereal root infected by the nematode* Heterodera avenae*, showing syncytia (S) and fungal colonization (arrowheads) in root cortex cells (a: Lopez-Llorca & Duncan, 1988; b,c: Lopez-Llorca & Claugher, unpublished).*

molecular methods for rhizosphere studies, have been devised (Cardon & Gage, 2006). These are genetically engineered bioreporter bacteria which join reporter genes, e.g. GFP and Lux, with promoters induced by several rhizosphere conditions (starvation, contaminants, quorum sensing). These are timely approaches for global studies on general rhizosphere function in ecosystems. Some of these bioreporters are biocontrol bacteria. Biocontrol fungi, e.g. nematophagous, are next on the list.

4.4. Root Exudates

To this point it is clear that the biocontrol scenario of plant-parasitic nematodes by nematophagous fungi relies on a multitrophic interaction in which plant roots play an important role. There is also abundant scientific evidence that roots produce compounds (exudates) which mediate plant-plant and plant-microbe interactions (Bais, Weir, Perry, Gilroy, & Vivanco, 2006). The latter would also include plant-nematode (and other micro- and meso-fauna) interactions.

Root exudates are very diverse structurally and chemically, and vary among plant species, but above all they may influence a wide array of processes relevant to the biocontrol action of nematophagous fungi. Leaving aside the effect of root exudates on nematode feeding and colonization, these compounds can influence nutrient availability in the rhizosphere (e.g. siderophores). Root exudates can also elicit release of compounds which could act in root defence or mediate signalling processes.

Root exudates also mediate plant-microbe interactions. The role of flavonoids on the specificity of rhizobia-*Leguminosae* interactions is well established (Perret, Staehelin, & Broughton, 2000). These root exudates induce the expression of rhizobia Nod genes, which are then involved in the synthesis of Nod factors (lipochitino-oligosaccharides with diverse chemical modifications) that are recognized by the appropriate host plant.

Closer to nematophagous fungi, arbuscular mycorrhizal fungi (AMF) recognize the presence of a compatible host plant through root exudates. A sesquiterpene has been identified as a branch-inducing factor for AMF in legumes (Akiyama, Matsuzaki, & Hayashi, 2005). Hyphal morphogenesis is vital for successful AMF-root colonization. This aspect may also be important in nematophagous fungi.

Root exudates affect nematodes, especially microbivorous species. On the other hand, plant-parasitic nematodes increase production of root exudates (rhizodeposition). The quality of root exudates is also changed. C/N-ratio in particular can alter the trophic stage of the fungus *Rhizoctonia solani* and turn it into a root pathogen (Van Gundy, Kirkpatrick, & Golden, 1977). These effects of root exudation on nematophagous fungi remain largely unknown, but are worth investigating.

There are new evidences that tri-trophic webs can be established in the rhizosphere leading to benefits for the plant host. Plant roots produce exudates which attract nematodes (Green, 1971). These can act as vectors of rhizobia that are thus transferred to roots (Horiuchi, Prithiviraj, Bais, Kimball, & Vivanco,

2005). It is also known that nematodes are attracted to nematophagous fungi to various extents (Jansson & Nordbring-Hertz, 1979; Jansson, 1982a). The role of non-parasitic nematodes as vectors to inoculate nematophagous fungi or root endophytes in nature has not yet been investigated.

4.5. Detection and Quantification

It is vital to be able to detect and quantify biocontrol agents, e.g. nematophagous fungi, in soil and rhizospheres, in the period following their addition. Many techniques for this purpose have been too unspecific or difficult to perform (Jansson, 2001). Antibodies have been tried with little success due to cross-reactions with other fungi (Eren & Pramer, 1966). Molecular markers such as the GUS gene have been transformed to *A. oligospora* (Persmark, Persson, & Jansson, 1996b; Tunlid, Åhman, & Oliver, 1999) and the GFP gene has been transformed to *P. chlamydosporia* (Atkins, Mauchline, Kerry, & Hirsch, 2004). In the former case it was not possible to quantify the growth of the fungus in soil at sufficiently low levels (Persmark et al., 1996b). The problem with *P. chlamydosporia* was to obtain stable transformants. A possible solution could be to try *Agrobacterium*-mediated transformation (Michielse, Arentshorst, Ram, & Van den Hondel, 2005).

Another promising approach is to use PCR-based techniques in combination with fluorogenic probes (e.g. scorpions and beacons). Such methods using real-time PCR and primers based on ITS sequences of *P. chlamydosporia* and *Paecilomyces lilacinus* have recently been presented (Ciancio, Loffredo, Paradies, Turturo, & Finetti Sialer, 2005; Atkins, Clark, Pande, Hirsch, & Kerry, 2005).

5. NEMATOPHAGOUS FUNGI AND BIOCONTROL

Nematophagous fungi have been tested for biological control of plant-parasitic nematodes for many years but, so far, met with little success, partly due to lack of knowledge on the ecology of these organisms (Stirling, 1991). One of these factors may be the soil receptivity to nematophagous fungi, which varies as discussed above. This receptivity will need to be part of a screening for possible biocontrol agents. Another important factor is the endophytic colonization of plant roots. This may protect the plants from nematode and fungal diseases through induced resistance or production of antibiotic secondary metabolites.

Nematophagous fungi (as endophytes or not) may also increase plant growth by participation in nutrient uptake, or by modification of plant growth regulators (hormones and related compounds). Therefore, in the search for nematophagous fungi as biocontrol agents, endophytic colonization also needs to be included.

The combination of several types of nematophagous fungi, e.g. egg-parasitic and nematode-trapping, which destroy nematodes at their different life stages may also be an important criterion. Interactions with other soil fungi, including both

plant-parasitic and biocontrol agents, is also an important consideration when selecting the proper fungi for biological control of plant-parasitic nematodes.

6. CONCLUSIONS

Nematophagous fungi are ubiquitous organisms with the capacity to attack, infect and digest living nematodes at all stages, adults, juveniles and eggs. They may use trapping organs, spores and appressoria to initiate infection of their nematode hosts. The nematophagous fungi may not only infect nematodes, but may also infect other fungi as mycoparasites, and colonize plant roots endophytically. These various capabilities of nematophagous fungi, the latter in particular, may render them good candidates for biological control of plant root diseases.

ACKNOWLEDGEMENTS

In Memoriam: This chapter is dedicated to the memory of Mr. D. Claugher, a close friend and a fantastic microscopist at the Natural Science Museum in London, that we gratefully acknowledge and thank for his help in scanning "the secret life of nematophagous fungi". His contribution to the understanding of their mode of action is partly revealed in some of the wonderful images obtained with his Field Emission Scanning Electron Microscope. This work was financed by a grant (AGL2004-05808/AGR) from the Spanish Ministry of Education and Science. We also thank Dr. C. Olivares-Bernabéu and Mr W. Robertson for supplying unpublished images and data.

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