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Fungal parasites of invertebrates: multimodal biocontrol agents?

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Introduction

Nematophagous and entomopathogenic fungi (NEF) comprise an important group of fungal parasites of invertebrates (FPI). NEF belong to a wide range of fungal taxa, but most of them are anamorphic fungi and facultative parasites. These fungi can infect, kill and digest nematodes and insects, respectively, which we will call their canonical, or normal, hosts. These hosts have barriers to the environment (eggshells and cuticles) that have common structural features. Therefore, the infection cycles share common strategies (e.g. adhesion to the host) or metabolites (e.g. proteases and chitinases for host penetration). Some species (e.g. *Lecanicillium lecanii*) can even be isolated from both infected nematodes and insects. The NEF may also infect other organisms (other fungi and plants) apart from their canonical hosts in a similar or different mode. We will use the term multimodal to describe the mode of action of these biological activities (Fig. 17.1). However, to date, the main emphasis in research has covered their mode of action on their canonical hosts (e.g. nematodes for nematophagous fungi). Many of these fungi are used for biological control of plant-parasitic organisms.

In this review we will describe the NEF and their hosts in general terms (both canonical or non-canonical) at biological, ecological and physiological-molecular levels. We will also analyze the reasons for this multitrophic behaviour, trying to use a comparative approach of both types of hosts (canonical and non-canonical) and pathogens (nematophagous and entomopathogenic fungi) under an evolutionary perspective. For recent reviews of the NEF see Charnley (2003); Morton, Hirsh & Kerry (2004); Jansson & Lopez-Llorca (2004).

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Fungal parasites of invertebrates

Most entomopathogenic and nematophagous fungi are facultative parasites. This way of life usually implies 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. This versatile behaviour allows FPI to colonize (and eventually infect) hosts other than their canonical.

Entomopathogenic and nematophagous fungi bear multiple similarities. The most important species of both fungal groups have been described as soil inhabitants (Domsch, Gams & Anderson, 1993) where they spend most of the saprophytic phase of their life-cycles. Soil is – anyway – a reservoir of both organic matter and dead canonical hosts. It is also the environment of nearly all plant-parasitic nematodes and of soil dwelling insects, such as pests of roots or other underground plant organs. The main parasitic phase of NEF on their canonical hosts will be briefly described, as an introduction to the multimodal behaviour.

Nematophagous fungi

Living stages of nematodes can be infected by several types of nematophagous fungi (Jansson & Lopez-Llorca, 2001). Dead nematodes may also be invaded saprophytically by nematophagous as well as other (saprophytic) fungi, but these latter are not regarded as proper nematode-destroying fungi. For instance, dead vermiform nematodes may be

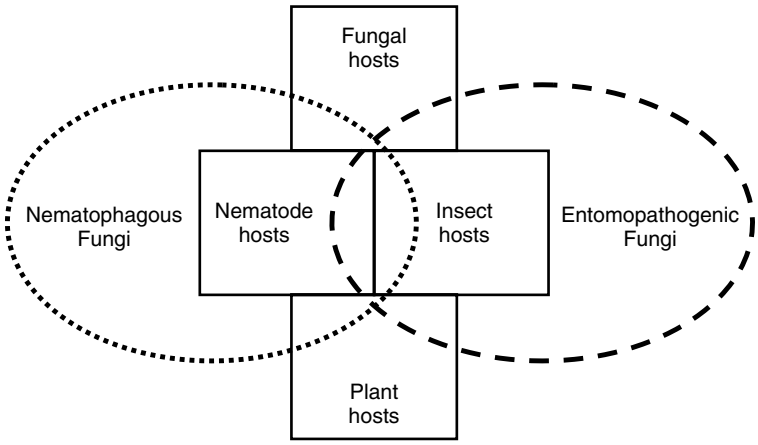

Figure 17.1 Multimodal behaviour of nematophagous and entomopathogenic fungi (NEF) on different hosts. Dashed ovals indicate host range of nematophagous and entomopathogenic fungi, respectively.

invaded by nematode-trapping fungi, which enter the natural openings (mouth, anus, etc.) of the nematodes but never penetrate their cuticles (Nordbring-Hertz & Stålhammar-Carlemalm, 1978). Most nematophagous fungi are facultative parasites and exist in both saprophytic and parasitic stages induced by external and/or internal signals.

The nematophagous fungi are divided into groups depending on their mode of infecting nematodes: nematode-trapping, endoparasitic, egg- and female-parasitic, and toxin-producing fungi (Jansson, Tunlid & Nordbring-Hertz, 1997). Nematode-trapping fungi capture vermiform nematodes in special trapping organs formed on the hyphae (Barron, 1977). The traps can have either an adhesive function, where the nematodes stick to the trap, or a mechanical function. These fungi, e.g. *Arthrobotrys oligospora*, are characterized by low host specificity and lower parasitic ability. The endoparasites, e.g. *Drechmeria coniospora*, use their spores to infect the nematode hosts. These fungi have high host specificity and are obligate parasites. The toxin-producing fungi, e.g. *Pleurotus ostreatus*, immobilize their victims using toxins prior to penetration. These three groups of fungi all attack vermiform nematode stages. Females of sedentary endoparasitic nematodes, as well as their eggs, can be infected by facultative egg-parasites, e.g. *Pochonia chlamydosporia* (syn. *Verticillium chlamydosporium*). As has been shown, the nematophagous habit has examples in the main taxa of the Fungal Kingdom. This has been explained as a multiple origin of the nematophagous habit in fungal evolutionary terms (Barron, 1992).

In practical terms, although some species of nematophagous fungi (mainly egg-parasites and trapping fungi) have experimentally been used for biocontrol of plant parasitic nematodes, no widely available biocontrol products based on these organisms are yet available.

Entomopathogenic fungi

Nearly 1000 fungal species are pathogenic on insects. Entomopathogenic fungi, like nematophagous fungi, belong to most taxonomic groups within the Fungal Kingdom (Charnley, 1997). This fact, shared to some extent with nematophagous fungi, gives entomopathogenic fungi a big biological potential due to the multiplicity of modes of reproduction/multiplication, characteristic of the taxa they belong to. This biological plasticity allows them to infect insects under different ecological conditions (Charnley, 1997).

Entomopathogenic fungi include specialist and generalist groups. The former includes mostly Entomophthorales, which are not commercialized

for insect control, although they may cause epizootics in insects under natural conditions. The generalists are mostly anamorphic facultative parasites and they have been (and are being) developed as biological control agents of insects (Butt, 2002).

Chytridiomycetes include genera such as *Coelomomyces*, with species parasitic on mosquitoes. Within Ascomycetes, *Cordyceps* includes more than 300 species parasitizing most insect orders. Most entomopathogenic fungi belong to the anamorphic fungi. Amongst the most common we have the following genera: *Aschersonia, Beauveria, Culicinomyces, Hirsutella, Metarhizium, Nomuraea, Paecilomyces, Tolypocladium* y *Lecanicillium* (= *Verticillium*). (Charnley, 1997; Gams & Zare, 2001).

Some of these species are currently being exploited or developed for crop or forest protection. Failures or inconsistencies in biocontrol suggest that increased knowledge on the biology of the fungal species used is of paramount importance for future improvement (Bidochka, Kamp & de Croos, 2000). Commercialization of entomopathogenic and nematophagous fungi for pest and pathogen control requires better understanding of physiological aspects of growth, metabolism or genetic basis of virulence as well as their ecological performance. Some tools derived from biotechnology, and recently from genomics and proteomics approaches, may facilitate such studies (Burges, 1998; Tunlid & Talbot, 2002).

Physical barriers for FPI to overcome

Fungal pathogens obtain nutritional or other resources from damaging another organism (its host). Potential hosts have evolved barriers to shield themselves from the harsh environment and from pathogens. Host barriers are to be breached by attacking pathogens. As will be described in this chapter, the multimodal action of FPI implies that these fungi may infect (naturally or artificially) very diverse hosts: nematodes, insects, other fungi or plants (Fig. 17.2). Although diverse, the external barriers (egg shells, cuticles, cell walls, etc.) share structural features. They have a matrix, mostly formed by proteins, embedding structural microfibrils consisting of common or different materials: collagen in nematode cuticles; chitin in nematode eggshells, insect cuticles and fungal cell walls; cellulose in plant cell walls. To further strengthen the structure, the matrix proteins are often cross-linked and contain phenolic and other compounds, such as melanin and DOPA.

Canonical hosts

In spite of their differences, both insects and nematodes present a similar organization regarding composition and structure of their main barriers: cuticle and eggshell. The insect cuticle is formed by several layers (Andersen, 1979). The epicuticle is mainly made of lipoproteins and waxes, whereas the procuticle is mainly made of proteins and chitin. Other compounds (lipids, pigments and other small organic molecules and inorganic materials) are minoritary, athough they may affect the performance of entomopathogenic fungi. Chitin microfibrils (20–50% of procuticle composition) are embedded in a protein matrix (resilin) in different amounts, depending on the insect groups.

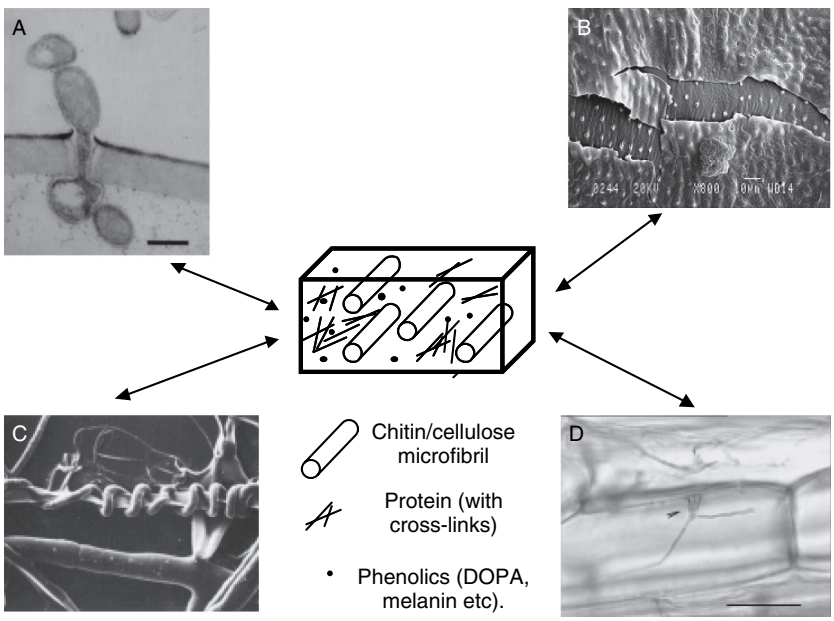

Figure 17.2 Similarities of external barriers of canonical and non-canonical hosts of NEF. (A) *Pochonia rubescens* hyphae penetrating eggshell of the cyst nematode *Globodera rostochiensis* (Lopez-Llorca & Robertson, 1992a, courtesy of *Nematologica*). (B) Degradation of insect (*Galleria mellonella*) cuticle by culture filtrate with proteolytic activity of the entomopathogen *Paecilomyces farinosus* (Lopez-Llorca, Carbonell & Gomez-Vidal, 2002, courtesy of *Mycological Progress*). (C) The nematode-trapping fungus *Arthrobotrys oligospora* coiling around its fungal host *Rhizoctonia solani* (courtesy of *FEMS, Yvonne Persson*). (D) *Pochonia chlamydospora* colonizing root cells of barley (Bordallo *et al*., 2002, courtesy of *New Phytologist*).

The nematode surface, the cuticle, consists of several layers containing proteins (mainly collagen), lipids and carbohydrates (Bird & Bird, 1991). Externally to the cuticle a surface coat (or glycocalyx) consisting of glycoproteins is found (Bird & Bird, 1991). The surface coat is probably the part of the nematode surface most relevant to recognition and adhesion of nematophagous fungi, since proteolytic removal of this structure results in reduced adhesion of microorganisms (Bird, 1985; Jansson, 1993). The eggshell of plant parasitic nematodes is also a multilayered structure (Wharton, 1980; Bird & Bird, 1991). The thickest and most important is the chitinous layer, which like the insect cuticle is also made of chitin and proteins. Just as for insects, structure and composition varies with species. Regarding nematodes, chitin is, for instance, more abundant in *Meloidogyne* spp. eggshells, whereas protein is present in a higher degree in *Globodera* spp. Structurally, *Globodera* spp., have thicker egg-shells than those of *Heterodera* spp. (Lopez-Llorca & Robertson, 1992a). Molecular components of insect and nematode barriers suffer several types of modifications, e.g. protein cross-linking and melanization (Andersen, 1979; Lopez-Llorca & Fry, 1989). These facts can be highly relevant in the susceptibility of both nematodes and insects to FPI.

Non-canonical hosts

From the information given above, it becomes clear that regarding composition and structure the barriers of insects and nematodes bear striking similarities. In fact, the main barrier (cell wall) of plants (the most important hosts in nature of fungal pathogens) and also that of fungi shows some similarities too (Fig. 17.2). The plant cell wall contains cellulose microfibrils as the structural element. These are embedded in a matrix consisting of approximately 90% (dry weight) polysaccharides (mainly pectins, hemicelluloses and cellulose) and 10% (glyco)proteins (Fry, 2001). The cell wall components are strengthened by covalent and non-covalent cross-links, involvement of phenolics and, with age, an increasing lignification of the tissues. The cell wall of true fungi contains chitin microfibrils embedded in a matrix of proteins, also with cross-links, and carbohydrates, mainly various glucans (De Nobel *et al*., 2001). Some authors (Bidochka *et al*., 1999; Barron, 1992) suggest that FPI may have arisen from plant pathogens to escape competition via further specialization in different hosts. The capabilities of changing hosts between invertebrates (insects or nematodes), endophytism or mycoparasitism could then be the consequences of this evolutionary process favoured by some trends that hosts have developed in their barriers to defend

themselves against environmental threats, such as the presence of parasites and pathogens.

Mode vs. multimodes of action: theoretical and practical implications

The diversity of modes of action and the subsequent extended host range of FPI merits some consideration before getting into the topic in more detail. Putting aside the fact of how frequent or relevant the non-canonical hosts are in nature, the fact that they exist has some theoretical and practical implications (Fig. 17.3). From the basic point of view, varying the host (even swapping them) in two groups of FPI and using biological tools at different scales will help in answering key questions: (i) how can this organism infect these hosts? or (ii) where did this capacity came from? It is clear that we are talking about the origin and evolution of pathogenicity, a question that even has implications in the medical field. The multimodal behaviour of FPI also has practical implications, both positive and negative (Fig. 17.3, (+) or (−) signs) regarding the use of FPI as biocontrol agents (BCA) of pests and diseases. Excluding plants as hosts, a potential expansion of the host range of FPI will increase the market of BCAs (a positive economic point). Conversely, it may affect their safety if non-targets (e.g. beneficial insects) are affected. This synergistic behaviour has, however, been used in chemical control where some insecticidal compounds are sold with claimed nematicidal action. The colonization of living plants (the hosts of pests and diseases) by FPI (and other BCA) has a wide array of benefits. FPI may modulate host defences and therefore add a new component in their canonical mode of action (infection of an insect/nematode). This may be positive if the modulation is to the right extent (i.e. not so high that it compromises the survival of the FPI, or so low that the FPI/BCA takes over the plant and becomes a pathogen).

How nematophagous fungi infect nematodes

The interactions between nematophagous fungi and their hosts involve several steps from recognition (attraction phenomena, contact), production of adhesives and lytic enzymes, differentiation of infection structures (appressoria and trapping organs) to host penetration and digestion (Tunlid, Jansson & Nordbring-Hertz, 1992). The nematode-trapping fungus *Arthrobotrys oligospora* forms so-called adhesive network traps on which vermiform nematodes are captured. Traps are essential for infection of living nematodes, and increase fungal attraction of nematodes (Jansson & Nordbring-Hertz, 1979).

After contact between the fungal trap and the nematode cuticle a possible contact recognition step occurs involving a fungal lectin binding to N-acetylgalactosamine (Gal-NAc) on the nematode surface (Nordbring-Hertz & Mattiasson, 1979). The trapping organ of *A. oligospora* contains

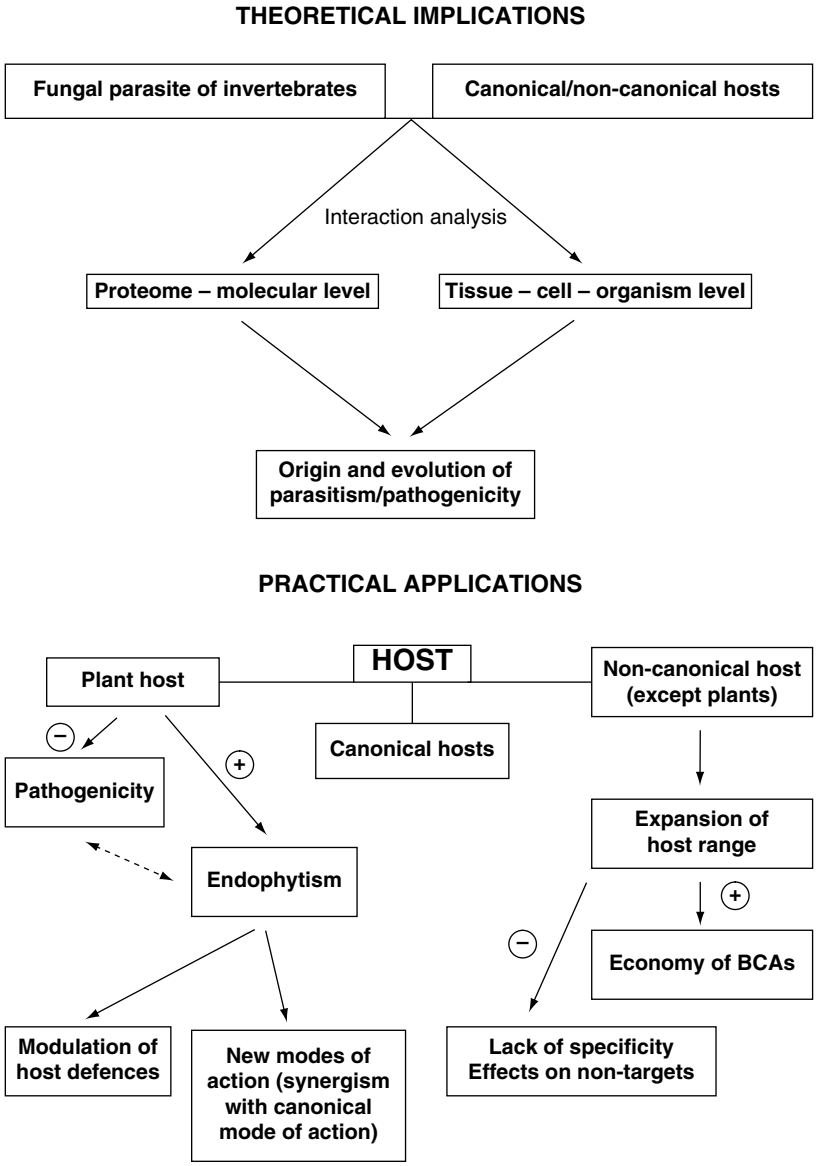

Figure 17.3 Mode vs. multimodes of action of FPI.

an adhesive material. Upon contact with the nematode surface a recognition step, possibly mediated, at least partly, by lectin binding, induces changes in the structure of the adhesive leading to capture of the nematode (Veenhuis, Nordbring-Hertz & Harder, 1985). The adhesive changes from amorphous material to fibrillar structure perpendicular to the nematode surface. This may anchor the nematode to the trap, thus facilitating infection. In contrast, the adhesive of the endoparasitic fungus *D. coniospora* always has a fibrillar structure even in the absence of nematodes (Jansson & Nordbring-Hertz, 1988). After attachment, *A. oligospora* penetrates the nematode cuticle and forms an infection bulb, from which trophic hyphae grow out to digest the nematode contents (Veenhuis *et al*., 1985).

As in many other instances of fungal penetration of their hosts' surfaces, nematophagous fungi use both enzymatic and physical means. The nematode cuticle mainly contains proteins (Bird & Bird, 1991) and therefore the action of proteolytic enzymes may be important for penetration. A serine protease, PII, from *A. oligospora*, has been characterized, cloned and sequenced (Åhman *et al*., 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 (for review see Jansson *et al*., 1997).

Egg-destroying fungi, e.g. *Pochonia rubescens* and *P. chlamydosporia*, act on nematode eggs at two levels: directly as true parasites by penetrating and infecting eggs, and indirectly by causing distortions in the larvae or embryos they contain (Morgan-Jones & Rodriguez-Kábana, 1988). The former mode of action is well documented and is largely responsible for cases of soil supressiveness to nematodes.

Upon growth of germ tubes the hyphal tips swell and differentiate into appressoria on contact with nematode eggs (Lopez-Llorca & Claugher, 1990), as well as on artificial, especially hydrophobic, surfaces (Lopez-Llorca *et al*., 2002b). An extracellular material (ECM) probably functions as adhesive, but possibly also seals the hole caused by the penetration hypha. This extracellular material can be labelled with the lectin Concanavalin A, indicating that the ECM contains mannose/glucose moieties probably on side chains of glycoproteins (Lopez-Llorca *et al*., 2002b). Most ECMs of fungal hyphae consist of proteins and carbohydrates (Nicholson, 1996).

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, 1990a). Involvement of the enzyme in pathogenesis was suggested by quick in vitro degradation of *Globodera pallida* eggshell proteins (Lopez-Llorca, 1990a), but most of all by its immunolocalization 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 extracellular proteases (VcP1) (Segers *et al.*, 1994) which are immunologically related to P32 and similar enzymes from entomopathogenic fungi (Segers *et al.*, 1995). VcP1-treated eggs were more infected than untreated eggs, suggesting a role of the enzyme in eggshell penetration by egg-parasitic fungi. Recently, several chitinolytic enzymes of *Pochonia* spp. were detected. One of those accounting for most of the activity was a 43 kDa endochitiase (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 eggshells (Tikhonov *et al.*, 2002).

How entomopathogenic fungi infect insects

Infection of insects starts with the adhesion of spores to the surface of the cuticle. In this process extracellular polysaccharides, lectins and some extracellular enzymes play an important role (Clarkson *et al.*, 1998). For some fungal isolates, adhesion of spores to insect tegument is host specific. Some enzymes, including esterases and proteases, have been found in ungerminated conidia. Their role could be the modification of the surface of the insect cuticle (the infection court) before germination, to supply nutrients and help with adhesion. Therefore adhesion is not a mere union, but a complex process that modifies the surface of the host to allow further fungal development (St. Leger, 1993).

The germination process requires adequate moisture and nutrient availability for the production of the germ tube. Apart from these requirements, cuticle topography plays an important role in adhesion, germination and differentiation of appressoria (Butt, 2002). Failures in germination of spores of entomopathogens can be attributed to the presence of inhibitory compounds, such as free short-chain fatty acids, quinones and phenolics in the insect cuticle.

Penetration of the insect cuticle may take place in different ways. Germ tubes of *Verticillium lecanii* (= *Lecanicillium lecanii*, Zare & Gams, 2001) can directly penetrate the insect cuticle. On the contrary, *Metarhizium anisopliae* and *Beauveria bassiana* usually form special penetration structures (appressoria) (Bidochka *et al*., 2000). The appressorium firmly links the fungus to the epicuticle of the insect and allows cuticle penetration through mechanical and enzymatic processes (Leucona, 1996; Clarkson *et al*., 1998). In many fungi the appressorium secretes mucilage, thus helping the anchorage of the fungus (Butt, 2002). Penetration of the host cuticle is favoured mainly by extracellular proteases and other enzymes (e.g. lipases and chitinases) secreted by entomopathogenic fungi (Charnley, 1997; Butt, 2002).

Once the fungus has succesfully penetrated the insect cuticle and reached the subcuticular epidermis, it then invades the insect's haemocoel (Clarkson *et al*., 1998). The fungus then produces spherical-ovoid bodies named blastoconidia or blastospores. These propagules have a cell wall different from that of mycelium, to avoid the immune system of the insect. Furthermore, some secondary metabolites produced by entomopathogenic fungi interfere with cell and humoral insect defences (Bidochka *et al*., 2000; Butt, 2002).

Insect death occurs as a result of the invasion of vital organs, massive tissue destruction, the shortage of nutrients, or by the production of toxins by the fungus (Bidochka *et al*., 2000; Butt, 2002). The insect death marks the end of the parasitic phase. The fungus then continues to grow saprotrophically in all insect tissues (Lecuona, 1996).

The life-cycle is completed when the fungus sporulates over the insect cadaver. Under adequate conditions, high relative moisture in particular, the fungus breaks the insect cuticle producing aerial spores. These are dispersed by the wind, rain or by other arthropods. The fungus may produce resistant spores within the dead insect that allow long survival periods under adverse conditions (Charnley, 1997; Butt, 2002).

Antibiosis phenomena of the pathogenic fungus in the dying insect, limits the growth of saprotrophic microorganisms of the insect. These are, in general, potential competitors of the entomopathogenic fungus. After total colonization, the insect cadaver is transformed into a mummy, which serves as a fungus reservoir to withstand adverse climatic conditions (Lecuona, 1996).

Finally, many traits of the host plant may influence, directly or indirectly, the survival of entomopathogenic fungi and its efficacy in biological

control programmes. Plant growth, morphology and especially chemical composition are highly relevant. Plant chemical compounds, depending on their nature and concentration, may influence conidia viability and/or insect susceptibility to biocontrol fungi (Butt, 2002). Crucifers influence germination of conidia of *M. anisopliae* through the presence of stimulating or inhibitory compounds in epicuticular leaf waxes (Inyang *et al.*, 1999).

Mycoparasitic NEF

Several species of nematode-trapping fungi, including *A. oligospora*, can attack other (mostly plant-pathogenic) fungi, e.g. *Rhizoctonia solani* (Persson, Veenhuis & Nordbring-Hertz, 1985). This mycoparasitic behaviour takes place by coiling of the hyphae of the nematode-trapping fungi around the host hyphae (Fig. 17.2C), which results in disintegration of the host cell cytoplasm without penetration of the host. Although this phenomenon has never been observed in soil, it may increase the fitness of the nematode-trapping fungi in soil by reducing competition and providing nutrients. Moreover, it may extend the biocontrol capability of nematophagous fungi as biological control agents to fungal parasites as well as nematodes. Furthermore, *P. chlamydosporia* has been described infecting propagules of important plant pathogens, such as uredospores of rust fungi (Leinhos & Buchenauer 1992), and oospores of *Phytophthora* and other oomycetes (Sneh, Humble & Lockwood, 1977).

We have found that *P. chlamydosporia* growing endophytically in roots (see NEF as endophytes) could reduce growth of the plant-pathogenic fungus *Gaeumannomyces graminis* var. *tritici* (take-all fungus, Ggt) in dual culture Petri dishes 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).

The entomopathogen *Paecilomyces fumosoroseus* has been reported to infect the powdery mildew fungus *Sphaerotheca fuliginea* by colonizing conidia and hyphae (Kavkova & Curn, 2005). Furthermore, *Verticillium lecanii* has been reported as mycopathogen of *Penicillium digitatum* and *S. fuliginea* (Benhamou, 2004; Verhaar, Hijwegen & Zadoks, 1998). *Beauveria bassiana* has recently been shown to produce a fungitoxic compound active against the palm pathogen *Penicillium vermoesenii*. (Asensio, 2004).

NEF as endophytes

Nematophagous fungi as root endophytes

Endophytic microorganisms colonize the interior of plants, especially leaves, branches, stems and roots, showing no apparent harm to the host, and the interaction involves metabolic exchange between endophyte and host. They may play an important role in host protection against pests and pathogens (Lodge, Fisher & Sutton, 1996; Azevedo *et al*., 2000; Schulz *et al*., 2002).

All groups of nematophagous fungi, apart from endoparasitic fungi (*Hirsutella rhossiliensis* and *Nematoctonus pachysporus*), have the ability to grow endophytically in roots (Lopez-Llorca *et al*., 2006). *P. chlamydosporia* or *A. oligospora* colonized epidermis and root cortex of barley and the epidermis of tomato. (Lopez-Llorca *et al*., 2002a; Bordallo *et al*., 2002). These fungi grew inter- and intracellularly and formed appressoria when penetrating plant cell walls of epidermis and cortex root cells, but never entered vascular tissues. In contrast to *Pochonia* spp., appressoria had previously never been observed in *A. oligospora*. Plant defence reactions (e.g. cell wall appositions) induced by nematophagous fungi could be detected. *P. chlamydosporia* grew extensively, especially in monocotyledonous plants, producing abundant mycelia and chlamydospores. Necrotic areas of the roots were observed at initial stages of colonization by the nematode-trapping *A. oligospora* and the toxin producing *P. ostreatus*, indicating a different behaviour of the fungi. Roots colonized by *P. chlamydosporia* displayed higher proteolytic activity than non-inoculated control roots using immunochemical techniques (Monfort, 2004). The significance of this fact for biological control of root pathogens is under investigation in our laboratory.

Arthrobotrys dactyloides and *P. chlamydosporia* formed coiling structures in barley root cells and extensively colonized the roots. Such structures are also formed by other root endophytes, e.g. *Piriformospora indica* (Varma *et al*., 1999), and presumably improve the exchange of metabolites.

Whether the root endophytic behaviour of nematophagous fungi is functional in their nematophagous habit is not known, although structures resembling trapping organs were observed in epidermal cells colonized by *A. oligospora* (Bordallo *et al*., 2002). These traps may serve to capture nematodes in the roots. The internal root colonization by egg-parasitic fungi, e.g. *Pochonia* spp., may help egg infection of economically important endoparasitic nematodes, e.g. *Meloidogyne* spp., thus reducing their spread and root damage. 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. Root inoculation of nematophagous fungi may help us to circumvent the lack of receptivity of soil (even sand) to inoculum of nematophagous fungi (Monfort, 2004), which prevents *P. chlamydosporia* from controlling *Meloidogyne* spp. (Verdejo-Lucas *et al*., 2003). This, in spite of the fact that the nematode is naturally found to be infected by *P. chlamydosporia* and other egg-parasitic fungi in similar Mediterranean agroecosystems (Verdejo-Lucas *et al*., 2002, Olivares-Bernabeu & Lopez-Llorca, 2002).

Entomopathogenic fungi as leaf and stem endophytes

Insect pests are more abundant in aerial plant organs than in roots. Therefore endophytic behaviour of entomopathogenic fungi in these tissues seems a desirable trait for these FPI. To this respect, *Beauveria bassiana* inoculated in corn leaves and stems (Bing & Lewis, 1991) grew endophytically and controlled the European corn borer, *Ostrinia nubilalis* (Wagner & Lewis, 2000). The control of *Ostrinia nubilalis* achieved was due to the fungus' capacity to penetrate corn leafs, follow the apoplast pathway in all directions, and even reach the xylem (Wagner & Lewis, 2000). Perhaps, fungal movement within corn could be attributed to passive movement of *B. bassiana* within the xylem or to active development within the plant through mycelial growth (Bing & Lewis, 1991; Wagner & Lewis, 2000). Although *B. bassiana* is a ubiquitous entomopathogenic fungus in soil, certain isolates have been found colonizing plant tissue (Bing & Lewis, 1993; Boucias & Pendland, 1998). The capability of EF to colonize plant tissues is also documented by the fact that inoculum of entomopathogenic fungi can be efficiently produced on plant wastes. We have used almond mesocarp (Lopez-Llorca & Carbonell, 1998) or palm seed and leaves (Lopez-Lorca, Carbonell & Salinas, 1999) for production of entomopathogenic fungi, e.g. *B. bassiana* and *L. lecanii*. This endophytic behaviour of entomopathogenic fungi could be a choice for targeting armoured sap-sucking insects such as scale insects. *Phoenicoccus marlattii*, the red scale-insect of palms, could be a good example (Asensio *et al*., 2005). We are currently investigating the endophytic behaviour of entomopathogenic fungi in date palm (*Phoenix dactylifera*) leaf tissue.

Beauveria bassiana, *Lecanicillium dimorphum* and *L.* c.f. *psalliotae* were inoculated into *P. dactylifera* leaf petioles. The entomopathogenic fungi colonized leaf petioles endophytically and were recovered up to 2 cm from the inoculation site. Fungi were detected inside the parenchymatic tissue and rarely within vascular tissue. The fungi survived for at least 30 days

and colonized date palm tissues both in bioassays in laboratory and field experimental conditions, with no evidence of significant damage to date palm tissues (Gomez *et al*., personal communications). *B. bassiana* was located in intercellular spaces and inside parenchymatic cells and was also occasionally found inside vascular bundles, mostly close to the inoculation site. In rare occasions, hyphal tip swellings resembling appressoria were found. We have found that the structures of fungal antagonists colonizing plant waste substrates (including leaves) by entomopathogenic and mycoparasitic fungi (Lopez-Llorca *et al*., 1999) are very similar to those produced for canonical host penetration.

Hu and St. Leger (2002), using a gfp-transformed *Metarhizium anisopliae* strain, showed that the fungus can survive for over a year in soil, especially in the inner rhizosphere of cabbage plants. The authors concluded that rhizosphere soils are potential reservoirs for the fungus.

Similarities and differences between nematophagous and entomopathogenic fungi

Nematophagous and entomopathogenic fungi must breach the barriers of their hosts to get inside them and resume their infection process. These structures have been briefly described and compared earlier in this chapter (see Physical barriers for FPI to overcome). Some striking similarities have been stressed. The first step for a successful infection by FPI is the adhesion of the infective structures (conidia, hyphae) to the host barrier (cuticle, eggshell). In general, both groups of fungi produce adhesive substances for that purpose. For entomopathogenic fungi, with the best known adhesion process, the adhesion of conidia to the insect cuticle comprises three major steps (St. Leger, 1993; Hajek & St. Leger, 1994): adsorption (passive stage), consolidation of adhesion/germination, and fungus growth on the cuticle surface until penetration (active stage). For *Metarhizium anisopliae*, these stages are favoured by hydrophobicity of the conidium and cuticle. The presence and role of adhesives in the pathogenesis of nematophagous fungi (endoparasites, trapping fungi and egg parasites has already been discussed (see How nematophagous fungi infect nematodes). Once germination is over, the germ tube secretes enzymes that facilitate adhesion. Germlings and ungerminated conidia of nematode egg parasites secrete several proteases (Lopez-Llorca *et al*., 2002b). This has also been found for entomopathogenic fungi (Charnley, 2003). Lectins and other glycoproteins plus other proteins (including hydrophobins) also play a role in the early pathogenesis of FPI (Charnley, 1997;

Jansson & Lopez-Llorca, 2001). The FPI penetrate host barriers by means of appressoria (St. Leger *et al*., 1989; Lopez-Llorca & Claugher, 1990), which concentrate both mechanical pressure and enzymatic degradation in a small area of the host. However, there are reports of direct penetration (without appressoria) for entomopathogenic fungi (Altre & Vandenberg, 2001) and nematode-trapping fungi form appressoria only when colonizing plants roots, but never when infecting nematodes (Bordallo *et al*., 2002).

The fungal pathogenesis of insects and nematodes is facilitated by the secretion of similar extracellular enzymes, which degrade the main components of the host barriers. Entomopathogenic and nematophagous fungi secrete proteases then chitinases (Bidochka *et al*., 1999). This is highly adaptative, since the protein component in insect and nematode eggshell barriers is amorphous and shields chitin microfibrils (Andersen, 1979; St. Leger, 1993; Wharton, 1980; Bird & Bird, 1991). The proteases involved in the infection process are generally serine-proteases class II, and are extracellular and of subtilisin-type (Segers *et al*., 1999). Characteristics of subtilisins may vary between species and isolates. Proteases of entomopathogenic fungi are synthesized in large amounts by virulent isolates. The synthesis is very fast and localized in the early infection stages (Charnley & St. Leger, 1991; Charnley, 1997). Regarding similarities between different species, basic proteases of several *B. bassiana* isolates were serologically identical, and the same was true for isolates of the close species *B. brogniartii* (Shimizu, Tsuchitani & Matsumoto, 1993). However, in spite of their similar substrate degradation capacities, *B. bassiana, P. fumosoroseus* and *M. anisopliae* proteases were not serologically identical, although common antigens could be found.

The species of *Pochonia* (formerly *Verticillium*) infecting nematode eggs produce chymoelastase-type proteases involved in the infection of nematode eggs (Jansson & Lopez-Llorca, 2001). *P. chlamydosporia*, *P. rubescens* and *M. anisopliae* proteases are serologically and functionally related (Segers *et al*., 1995). Subtle differences in specificity of these and other enzymes may favour competence for diverse nutrient sources. This may also determine the potential host range. In this respect, the capacity to secrete chitin degrading enzymes distinguishes the invertebrate pathogenic species from the plant pathogenic species in the old *Verticillium* spp. genus. (Bidochka *et al*., 1999). Expression of chitinolytic enzymes is considered a key factor in infection processes of entomopathogenic, nematophagous and mycoparasitic fungi (Dupont, Segers & Coosemans, 2002). *Pochonia* spp. produce a major endochitinase (CHI43) that shares biochemical

features with those entomopathogenic fungi (Tikhonov *et al.*, 2002). These key enzymes (proteases and chitinases) have been located in appressoria of both entomopathogenic and nematophagous fungi infecting their canonical hosts.

Changing hosts: the '*Lecanicillium lecanii case*' and others

We shall provide an overview of the fact that bona fide entomopathogenic and nematophagous fungi are found in nature infecting hosts other than their canonical. A striking example in the changing hosts scenario is the '*Lecanicillium lecanii* case', but it is by no means the only one. *L. lecanii* has been well documented as an entomopathogen (Inglis *et al.*, 2001) infecting mainly aphids, mites and whiteflies (Boucias & Pendland, 1998; Tanada & Kaya, 1993). However, *L. lecanii* has also been described as a mycopathogen (see Mycoparasitic NEF), as a nematophagous fungus (Meyer, Roberts & Wergin, 1998; Olivares-Bernabeu & Lopez-Llorca, 2002), and as a root endophyte (Meyer *et al.*, 1998; Benhamou & Brodeur, 2001).

Pochonia chlamydosporia is usually found infecting eggs and nematode females in agricultural soils of plant parasitic nematodes, where it is the main cause of natural suppressivity (Kerry & Jaffee, 1997). It has also been described as a rust parasite (Leinhos & Buchenauer, 1992) and as an entomopathogen (Bidochka *et al.*, 1999). We have found that it can experimentally colonize roots of both mono- and dicotyledonous plants as an endophyte (Bordallo *et al.*, 2002). *Pochonia* sp. was also retrieved from roots of several cereal crops grown in soils naturally suppressive to the cereal cyst nematode *Heterodera avenae* (Lopez-Llorca & Boag, 1993). However, the root endophytic behaviour of the fungus in nature remains to be clearly established.

Paecilomyces includes important entomopathogenic species such as *P. fumosoroseus* or *P. farinosus*. *Paecilomyces lilacinus* is nematophagous (Chen, Dickson & Mitchell, 1996; Olivares-Bernabeu & Lopez-Llorca, 2002), entomopathogen (Oborník, Klic & Zizka, 2000) and has even been described as an opportunistic human pathogen (Walsh & Groll, 1999). *B. bassiana*, a typical entomopathogen found worldwide, also parasitizes *Heterodera glycines* eggs (Chen *et al.*, 1996) and is an endophyte in corn (Wagner & Lewis, 2000).

These examples illustrate the fact that FPI not only are facultative parasites that can be grown in artificial media in the laboratory and degrade organic matter in soil. They also have a flexible parasitic/mutualistic life style where they can infect their canonical hosts and others, including plants.

We do not know whether, in nature, an isolate infecting a given host is able to infect another one. Such plastic behaviour can, however, be assessed under controlled laboratory conditions. We shall give two extra examples on the issue of 'changing hosts' in FPI discovered in our laboratory. *Drechmeria coniospora* is an endoparasitic nematophagous fungus that infects vermiform nematodes by means of adhesive conidia (Fig. 17.4a). The mode of infection of this fungus is well documented (Jansson, Hofsten & Meckenburg, 1984; Jansson, 1993; Dijksterhuis, Veenhuis & Harder, 1990). We have found that this fungus can – at least in vitro – also infect root-knot nematode eggs (Fig. 17.4b).

Beauveria bassiana, as has already been discussed, is an entomopathogen with a wide host range. It is frequently found infecting coleopterans (Fig. 17.4c) under natural conditions. We have experimentally infected cyst nematode eggs with this fungus (Fig. 17.4d). Just as for nematode egg parasites the entomopathogen formed appressoria on the nematode eggshell (Fig. 17.4d, arrows).

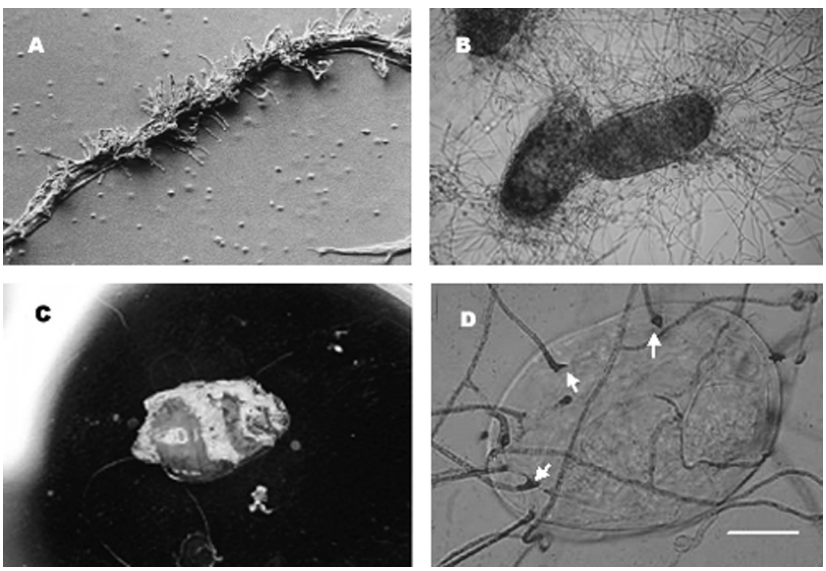

Figure 17.4 Canonical (A and C) and non-canonical mode of action of NEF. (A) *Drechmeria coniospora* infecting larva of the nematode *Panagrellus redivivus* (Jansson, Hofsten & Mecklenburg, 1984, courtesy of *Antonie van Leeuwenhoek*). (B) *D. coniospora* infecting egg of the nematode *Globodera rhosochiensis*. (C) *Beauveria bassiana* infecting adult insect coleopteran (Lopez-Llorca, 1992b, courtesy of *Boletín de la Sociedad Micológica de Madrid*). (D) *B. bassiana* infecting egg of the nematode *Globodera rhosochiensis*.

These examples illustrate the fact that due to the similarities between FPI, changing hosts is easy within the canonical (from nematode larvae to egg) and non-canonical (from insects to nematode eggs). There are, however, limitations. In our laboratory we have found that entomopathogenic fungi may easily infect nematode eggs, but nematode egg parasites are bad entomopathogens (Lopez-Serna, 2004). The reasons for this behaviour are unknown.

Multimodal fungal invertebrate pathogens, evolution and biocontrol – conclusions

The similarities in modes of action (enzymatic capacities and infection mechanisms) of entomopathogenic and nematophagous fungi may indicate that both groups are related phylogenetically. Some hypothesese point towards evolutionary divergence as the process evolved. In fact, these fungi have similar life styles and some of them, as has been discussed in this chapter, may infect hosts other than their own canonical hosts. As we have discussed above, FPI members of the 'old *Verticillium*' such as the entomopathogen *L. lecanii* or the nematophagous *P. chlamydosporia*, are clear examples of multimodal FPI, since these fungi infect (even in nature) non-canonical hosts. Most pathogenic fungi possess so called wide spectrum enzymes (e.g. proteases, polysaccharidases), which can degrade many different substrates. Most of the proteases of FPI studied to date are wide spectrum enzymes, such as the well known Pr1 for *Metarhizium anisopliae* or *Beauveria bassiana* proteases. This does not rule out that the variability that may account for host preference can be found, as for *Pochonia* proteases. According to Bidochka *et al*. (1999), the old *Verticillium* genus may have suffered evolution in the production of enzymes, as an adaptation to the conditions imposed by new hosts. In this sense, entomopathogenic, nematophagous and mycoparasitic species differ from phytopathogenic fungi that they (the former) can produce chitin-degrading enzymes (chitinases and other enzymes). Proteases of nematophagous and entomopathogenic fungi are coded by several genes and can be polymorphic. FPI as facultative pathogens and saprotrophic fungi have adaptative polymorphism (Segers *et al*., 1999). They display more genetic variability (some species such as *P. chlamydosporia* are often considered as species complexes) than obligate parasites or highly specialized pathogens, since they colonize more uniform environments. In this way, fungi may secrete enzymes different that the common ones, which may degrade other compounds, allowing the exploitation of diverse nutrient sources. This may in turn influence the virulence and potential

host range (Segers *et al*, 1995). According to St. Leger, Joshi & Roberts (1997), FPI displayed enzymatic adaptation to polymers present in the barriers of their hosts. As a result, plant pathogenic fungi display enzymes able to degrade polysaccharides, e.g. cellulose, xylan or cutin. In turn, these fungi do not show chitinolytic or proteolytic activity towards elastin or mucin. Entomopathogenic fungi are able to degrade a wide array of proteins, including elastin and mucin, and produce very few polysaccharidases, except chitinase, cellulase (*L. lecanii*) and few cutinase (*B. bassiana*). Saprotrophic fungi and opportunist pathogens produce the widest spectrum of enzymes capable of degrading proteins and polysaccharides, indicating their unspecialized nutritional status. Phylogeny studies indicate that *Paecilomyces* is polyphyletic, and that it is closely related (amongst others) to the old *Verticillium* and *Beauveria* genera (Obroník, Jirku & Dolezel, 2001). Phylogenetic studies of nematode-trapping fungi shows that there is a closer homology between fungi with the same type of trapping organ than traditional spore morphology (Ahrén, Ursing & Tunlid, 1998; Hagedorn & Scholler, 1999). The close relationships between different FPI (especially entomopathogenic fungi) could be due to the presence of a common ancestor, probably meiotic, able to form several mitotic stages. This may explain the capacity of the widespread multimodal behaviour of FPI.

Pleurotus forms basidiocarps and its standard means of living is saprophytic growth on decaying wood. The most common species, *P. ostreatus*, is an edible mushroom commercially cultivated. The fungus compensates for the lack of nitrogen in wood, its natural substrate, with its nematophagous habit (Thorn & Barron, 1984). The strong relation with plant tissues, as a natural wood decomposer, was also confirmed in roots when we found that, just as *N. robustus*, *P. djamor* penetrated early, and extensively colonized barley roots. The fungus was in fact very aggressive since some parts of the root appeared to be decorticated three weeks after inoculation (Lopez-Llorca *et al*., 2006), as was *A. oligospora* in previous experiments (Bordallo *et al*., 2002). *Arthrobotrys* spp. are wood colonizers (Persson, Olsson & Jansson, 2000) and their teleomorphs in *Orbilia* are also known as wood decomposers (Pfister, 1997).

In practical terms, the multimodal behaviour of NEF can widen their usage as biocontrol agents (i.e. broaden host range). Recent studies on the endophytic behaviour of NEF point to new biocontrol activities (e.g. modulation of plant defences) or even new (more targeted) means of delivery in agroecosystems. The latter may increase efficiency of the inoculum in adverse environments such as the rhizosphrere. This multimodal

behaviour may also shed light on the very nature of biocontrol and may help us to understand why a soil can be suppressive to, for instance, plant-parasitic nematodes.

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