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Prof. Tarek Mohamed Abd El-Ghany

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About Author

Dr. Tarek Mohamed AbdelGhany

Prof. of Microbiology, Plant and microbiology Department Faculty of Science Al Azhar University Egypt.

Biology Department Faculty of Science Jazan University KSA

Scientific Employment and Academic Responsibility:

Demonstrator 1996-2000 Plant and microbiology Department Faculty of Science Al Azhar University Egypt

Assist. Lecturer 2000-2004 Plant and microbiology Department Faculty of Science Al Azhar University Egypt

Lecturer 2004-2010 Plant and microbiology Department Al Azhar University

Associate Professor 2010 to now Biology Department Faculty of Science Jazan University KSA.

Professor 2015 to now Biology Department Faculty of Science Jazan University KSA.

He obtained the Ph.D. degree at Faculty of Science Al azhar University , Cairo , Egypt in 2004.

Prof. Tarek M. Abdelghany has published more than 60 articles on topics such as Biocontrol, Biofertilizers, Biodegradation of insecticides and dyes, Control of fungal growth and mycotoxins

Entomopathogenic Fungi and Their Role in Biological Control

Tarek Mohamed Abd El-Ghany

[1]Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt

***Corresponding author:** Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt. E-mail: tabdelghany@yahoo.com

Introduction

Of the nearly one million known species of insects, about 15,000 species are considered pests and about 300 require some form of control. Fortunately, most insect pests have pathogenic microorganisms associated with them. There are two aspects of economic problems caused by insects.One concerns the loss of production that results from damage to crops and to the health of human and domestic animals, the other concerns the cost of attempt to prevent or control such production loses. At the same time with increasing agriculture, insects become more and more important competitors of human food damaging or even destroyingthe crops. Mosquitoes and black flies are a constant threat to health and comfort, yet the chemical pesticides used to control them have created serious ecological problems. Environmental and health concerns about the application of chemical insecticides to reduce large-scale insect pest infestations have led to renewed interest in the development of microbial agents for incorporation into integrated pest management strategies for the control of acridids.

The potential to control insects with fungi dates back to Augostino Bassi's 1835 demonstration that a fungus could cause a deliberately transmissible disease in silkworm [1,2]. In the late 1870s Metschnikoff observed a high proportion of Metarhizium-killed sugarbeet curculio Cleonus punctiventris Germar and proposed the concept of controlling this insect with conidia artificially produced on sterile brewer's mash [3,4]. His work was extended by Krassilstschik, who established a production facility using beer mash to produce considerable amount conidia for distribution [5].

Entomopathogens have been suggested as controlling agents of insect pests for over a century, and belong to species of fungi, viruses, bacteria, and protozoa. Naturally occurring entomopathogens are important regulatory factors in insect populations [6]. There has been an increasing interest in employing fungal pathogens to combat insect pests. New application and production combined with a greater understanding of both fungal and insect ecology have shown that biological insecticides can now complete traditional chemical pesticides much faster. Many species are employed as biological control agents of insect pests in row and glasshouse crops, orchards, ornamentals, range, turf and lawn, stored products, and forestry and for abatement of pest and vector insects of veterinary and medical importance and the efficacy of fungal pathogens in the field depend on biotic and abiotic factors (Figure 1).

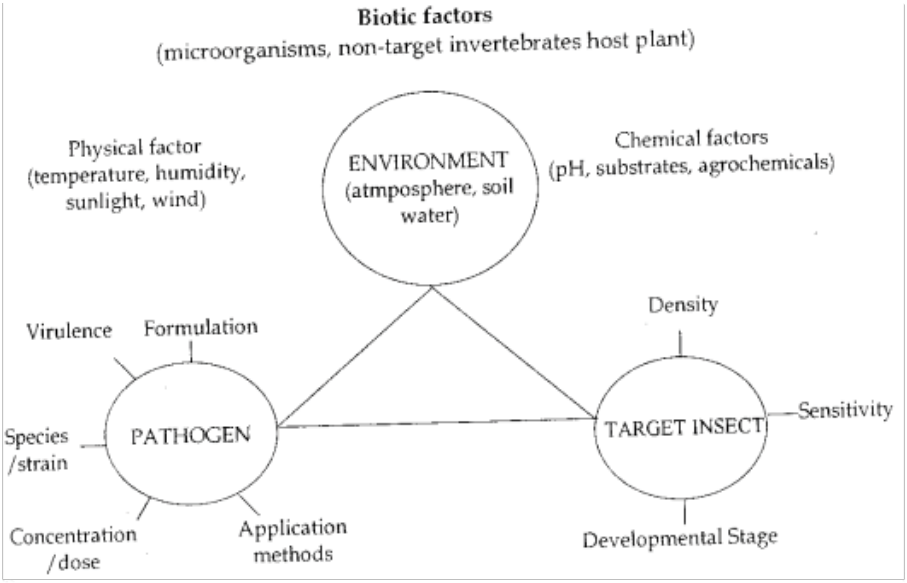

Figure 1: Interaction involved in efficacy of fungal pathogens in the field.

The use of agrochemicals, although decreasing the attack of insects and phytopathogenic microorganisms, still represents a high risk to field workers and consumers. In addition, their use is, in certain cases, economically unviable. The control of pests and diseases by means of biological processes i.e., use of entomopathogenic microorganisms or those that inhibit/antagonise other microorganisms pathogenic to plants, is an alternative that may contribute to reduce or eliminate the use of chemical products in agriculture. Generally, the comparison of entomopathogens with conventional chemical pesticides is usually solely from the perspective of their efficacy and cost. In addition to efficacy, the advantages of use of microbial control agents are numerous. These include safety for humans and other nontarget organisms, reduction of pesticide residues in food, preservation of other natural enemies, and increased biodiversity in managed ecosystems

Thomas and Read [7] stated that recent research has raised the prospect of using insect fungal pathogens for the control of vector-borne diseases such as malaria. In the past, microbial control of insect pests in both medical and agricultural sectors has generally had limited success. Over the last 25 years, chemical pesticides have become less attractive for numerous reasons including increased cost, the development of pesticide-resistant insects and weeds, concerns raised about human health hazards, and deleterious effects upon non-target organisms. During this time there has been a renewed interest in the application of biological control measures to replace or reduce chemical pesticide use. Much of this interest has been focused upon the development and use of microbial biocontrol agents. Humans have been aware of insect fungal diseases for at least 2000 years, since the ancient Chinese used fungus-infected insects in religious ceremonies and for medicinal purposes [8]. Fungi were the first microorganisms shown to cause disease in insects [7]. Insects are known since long ages to become infected with different entomopathogenic microorganisms that form an important factor of the natural mortality.The first successful large scale microbial control application using conidiospores of the fungus *Metarhizium anisopliae* was carried out in the Russian Ukraine against the beet weevil, *Bothynoderes punctiventris,* needed amounts of pure conidiospores were produced in the laboratory for this purpose. In the last 50 years, microbial control of pests and plant diseases showed an amazing development associated

with pronounced good results under optimized laboratory conditions, followed many times by disappointing results in the field applications. It was stated that it is thus surprising that, while research directed to these major targets with a number of common goals, very little attention has previously been given to the integration of research effort.

The Russian scientist Metschnikoff must be credited with the first practical use of a fungal agent to control an insect pest, in 1879 [9,10]. He employed the green muscardine fungus, *Metarhizium anisopliae,* against the wheat cockchafer, *Anisoplia austriaca.* Although the practical use of these and other entomopathogens has been investigated for well over 100 years, their full potential is only beginning to be realized [11]. Numerous laboratory and field studies indicate that Entomo Pathogenic Fungi (EPF) can provide a safe and effective control of many important insect pests [12-14].

There are thought to be about 750 species of fungi that cause infections in insects or mites. As a group, they attack a wide range of insect and mite species, but individual species and strains of fungus are very specific. The fungi produce spores which infect their host by germinating on its surface and then growing into its body. Death takes between 4 and 10 days, depending on the type of fungus and the number of infecting spores. After death, the fungus produces thousands of new spores on the dead body, which disperse and continue their life cycle on new hosts. According to commercial products, there are more than 100 commercial products based on entomopathogenic fungi. De Faria and Wraight [15] conducted a survey in 2006 and identified 129 active mycoinsecticide products; another 42 had been developed since the 1970s but were not commercially available at the time of the survey. In the United States, there are nine mycoinsecticides currently registered by U.S. Environmental Protection Agency; in the European Union (EU), 21 different fungi are registered in the Organisation for Economic Cooperation and Development.

Biological Control of Insects

Nowadays biological control as a practical science is much appreciated and as a solvent for long term usage of chemical pesticides problem is completely notified. Biological control is regarded as a desirable technique for controlling insects, due to its minimal environmental impact and preventing the development of resistance in vectors. Biological control (or biocontrol, which is synonymous) has been defined a number of times. A recent definition by Eilenberg et al., [16] is the use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be'.

Biological control can be divided into four complementary strategies: classical, inoculation, inundation and conservation. The strategies are defined (based on Eilenberg et al., [16] a. It should be mentioned that some authors have preferred to write the prefixes as adjectives for the latter three strategies, thus naming the strategies: classical, inoculative, inundative and conservative [17].

Classical Biological Control

The intentional introduction of an exotic, usually co-evolved, biological control agent for permanent establishment and long-term pest control'.Classical biological control has been a very significant strategy within biological control since the striking success with the introduction of the 'Vedalia beetle' to control scale insects in California in the late 1880's. The early successes were the reason for the term 'classical', which cannot be understood without this historical dimension.

The strategy was (and still is) among the most successful to manage introduced pest species in North America and other parts of the world, while it has never been a significant element in biological control in Europe. This is due to two reasons: first, the major bulk of European pests are native and their natural enemies are already present, and secondly,

classical biological control needs a strong, regional co-ordination of the efforts, which has normally not been the case in Europe.

Inoculation Biological Control

The intentional release of a living organism as a biological control agent with the expectation that it will multiply and control the pest for an extended period, but not permanently. The main principle of inoculation biological control is that a pest population increases in size but in due course, before this population density has reached the potential maximum, a biocontrol agent is inoculated in small to moderate amounts. The goal is that the natural enemy increases in population size and thus controls the pest over a period of time. The inoculated biocontrol organisms will, however, not establish permanently at a sufficiently high population density to prevent damage. New inoculations must be carried out after a period of time. It can be postulated that inoculation biological control represents a reestablishment of a natural balance, temporarily distorted by man. Soil for cropping is inoculated with other additives to enhance growth (mycorrhiza for example) and inoculation with a biocontrol agent can be seen as a moderate help to speed up a natural process.

Inundation Biological Control

The use of fungal biological control agents that have been formulated for inundativeapplication has been extensively studied but only a few products are commercially available [18]. Using of living organisms to control the pests when control is achieved exclusively by the released organisms themselves. The principle is as follows: a pest population increases in size, but at a certain time a biocontrol organism is applied in large amounts (inundated). The pest is quickly controlled and the population density of both the pest and the biocontrol agent decrease over time. The pest population will, after a period of time, increase again and a new application of a biocontrol agent is needed. The term 'biopesticide' is often associated with inundation biological control, linking the concept rather closely to the use of chemical pesticides. Inundation can be hard to separate from inoculation and these two strategies are often together called augmentation. Inundation is probably the strategy for biological control, which most easily can be explained to everybody: the population density of a nasty pest is too high, and a biocontrol agent is applied to control the pest.

Conservation Biological Control

'Modification of the environment or existing practices to protect and enhance specific enemies or other organisms to reduce the effect of pests'. A pest species occurs at high population levels due to insufficient effects of the natural enemies. Natural enemies include all kinds of biological regulation: macro-and microorganisms controlling invertebrates, weeds and plant diseases, including the antagonistic microorganisms responsible for 'suppressive soils'. To perform conservation biocontrol, the environment is modified or the practice is changed in order to enhance the natural enemies, which are already present. They increase in population size and their effect results in a lower pest population. Among the four biological control strategies, conservation biological control can be seen as the one most tightly connected to the main principles of organic farming, which has protection of the existing natural enemies as one of its main principles.

Safety of Fungi as a Biocontrol Agents

Introduction

Entomopathogenic fungi can serve as alternatives to broad-spectrum chemical insecticides.Efficacy and cost are usually the sole perspectives when comparing microbial control agents with conventional chemical pesticides. Numerous advantages can be found in the utilisation of entomopathogens in addition to efficacy. Advantages consist in safety

for humans and other non-target organisms, reduction of pesticide residues in food, preservation of other natural enemies and increased biodiversity in managed ecosystems. However, many factors still limit the acceptance of entomopathogens by growers and general public. In order to increase their utilisation, research needs to concentrate on:

(a) Pathogen virulence and speed of kill,

(b) Pathogen performance under challenging environmental conditions (cool weather, dry conditions etc.),

(c) Efficiency in the production process,

(d) Formulations that enable ease of application, increased environmental persistence and longer shelf-life,

(e) Integration into managed ecosystems and interaction with the environment and other Integrated Pest Management (IPM) components [19].

Resently the safety of entomopathogenic fungi was curently studied [20]. Most of the entomopathogenic fungi developed for commercial use in microbial control of insect pests showed no infectivity to man or other vertebrates [21]. Generally, particular attention is paid to the following aspects by registration of the bioproducts based on microbes:

1) Allergic properties,

2) Risks of toxic metabolites,

3) Genetic recombination and displacement of natural strains, and

4) Effect on biodiversity, i.e., on nontarget organisms Safety tests with *Nomuraea rileyi*, *Hirsutella thompsonii* [22], *Verticillium lecnii* [21] and *Lagenidium giganteum* [23] assured negative findings to different mammalians and birds [24].

On the other hand, *Beauveria bassiana* has been reported to cause allergies in humans and is at least an opportunistic pathogen to man and other mammals. Concerning non-target invertebrates, high mortality appear when they contacted or ingested spores of the entomopathogenic fungi. Larvae of the coccinellid *Cryptolaemus montrouzieri* suffered 50% mortality when fed Boverin-t, a commercial conidiospore preparation of *B. bassiana* [25]. Honey bee workers experienced 29% mortality when fed spores of *H. thompsonii*. Both *B.bassiana* and *Metarhizium anisopliae* infect *B.mori,* and also killed honey bees following field applications [21]. Besides, the parasitized hosts of some species showed increased susceptibility to entomopathogenic fungi and those populations of some over wintering predacious carabid beetles and other invertebrates are killed by fungi showing an increased risk if large amounts of fungal inoculums are added to soils as a consequence of agricultural microbial pest control [25]. Infection of different beneficial natural enemies were recorded [26-28], e.g., adults of the braconid parasitoid *Apanteles* sp., the coccinellid predator *Cydonia vicina isis,* the earwig *Labidura riparia,* and the syrphid fly *Syrphus corollae* in fields of sugar beet treated with conidiospores of both *B. bassiana* and *M. anisopliae.*

Under natural conditions, fungi are a frequent and often important natural mortality factor in insect populations. All groups of insects may be affected and over 700 species of fungi have been recorded as pathogens. Some species are facultative generalist pathogens, such as Aspergillus and Fusarium. However, most species are obligate pathogens, often quite specific and rarely found, e.g. many species of Cordyceps. Entomophthoran fungi are often important in natural control of flies and aphids under warm humid conditions but attempts to use these fungi as biological insecticides have usually failed because they are too difficult to mass produce and are often ineffective under conditions of moderate humidity common in the field. Research has focussed on the relatively easily produced asexual spores (conidia) of the hyphomycete genera *Metarhizium*, *Beauveria*, *Verticillium*

and *Paecilomyces*. These fungi often have a wide host range although there is considerable genetic diversity within species and some clades show a high degree of specificity [29]. For example, *Metarhizium anisopliae* var. *acridum* is only effective against acridid insects (grasshoppers and locusts). Unlike other potential biocontrol agents, fungi do not have to be ingested to infect their hosts but invade directly through the cuticle, and so can, potentially, be used for control of all insects including sucking insects.

Despite the many advantages of entomopathogenic fungi, several abiotic factors detract from their effectiveness, and have contributed to their limited use in agricultural production. The spores of many entomopathogenic genera are damaged or killed by direct exposure to UV-B radiation for only a few hours [30, 31]. UV-A has also been found to inactivate and delay germination of conidia of some fungi [31]. Temperature also influences fungal efficacy. While most entomopathogenic fungi tolerate a wide range of temperatures (commonly 0–40°C), the optimal temperatures for germination, growth and sporulation are generally 20–30°C [30]. Historically, moisture has been considered one of the most significant factors limiting their effectiveness, and low humidity has been implicated in failures of field trials. However, it is now recognized that ambient humidity levels may not accurately reflect moisture conditions in the microhabitat around the insect where the spore germinates. Thus, issues of low moisture can be addressed by timing the application of fungi when humidity levels are naturally higher (e.g. early morning or late afternoon). In addition, oil formulations have been shown to protect spores from the negative impact of low humidity. Rainfall can have a negative effect on fungal efficacy by washing off propagules before they are able to germinate and enter the insect. Research has been done on formulation technology to minimize this problem. Although several environmental factors are known to inhibit fungal efficacy, wide variation in the susceptibility to individual abiotic factors has been observed among and within fungal species and genera. Careful strain selection can minimize these disadvantages.

Survey and Taxonomy of Entomopathogenic Fungi

Entomopathogenic fungi have been found in many diverse habitats and associated with a broad range of insect hosts [10]. These habitats include aquatic, forest and agricultural ecosystems that are of direct importance to insect-vector control, silviculture and crop protection [32,33]. Other habitats where EPF have been observed include weeds, river banks, stream beds and even caves. The identification, isolation and characterization of EPF from these ecosystems are driven by a need to fully understand the roles of these fungi within their natural environments. Only with this knowledge can we attempt to utilize EPF for the control of insect pests.

Entornogenous fungi belong to 12 classes within 6 phyla of the kingdom fungi. Fungi and fungus-like organisms are defined by their heterotrophic, absorptive mode of nutrition and their apical hyphal growth [34]. The classification of fungi has traditionally depended upon morphology (e.g., conidiogenesis) and ultrastructure (e.g., cell wall and septum structure) as primary criteria. The more precise placement of EPF into defined taxonomic groups has been achieved by comparing metabolisms (e.g., nutrient utilization, enzyme and toxin production) and using genetic analyses (e.g., rRNA sequences, karyotyping, mtDNA restriction length polymorphism [35]. True fungi are now placed in four proper phyla (Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota) and one artificial form phylum, Deuteromycota that contains filamentous fungi that exist in asexual forms (anamorph). It is thought that deuteroraycetes are actually ascomycetes or basidiomycetes that have either lost their potential for sexual reproduction or have undescribed sexual forms [36]. Moreover, teleomorphs for numerous entomopathogenic deuteromycete genera have subsequently been identified, and some of these teleomorphs can also be insect pathogens (e.g., *Torrubiella* and *Cordyceps* spp., are teleomorphs of *Paecilomyces* spp., [10]. The oomycetes, which once resided in the subdivision Mastigomycotina, have now been placed in the kingdom Stramenopila, phylum Oomycota [37]. Members of this new kingdom include brown algae, diatoms and labyrinthulids, and all share various features of monophyletic

origin, including a similar flagellum hair structure [38]. The plasmodial slime molds, which are not known to be entomopathogens, have now been assigned to the phylum Myxomycota within the kingdom Protista along with the cellular slime molds [39]. The phylogenetic relationships among the four true fungal phyla have been proposed, based originally on morphological and ultrastructural features, and largely confirmed by subsequent DNA sequence analyses [36]. True fungi are now thought to be more closely related to animals than plants and it is thought that fungi and animals share a choanoflagellate-like ancestor [40]. Chytridiomycetes may be considered the most 'primitive' fungi since the ancestor of this group diverged early and retained centrioles and flagella [37,41]. The zygomycetes diverged after the loss of flagella and before the appearance of a regularly septate mycelium and dikaryotic stage which are characteristic of the ascomycetes and basidiomycetes (i.e., the higher fungi). After the ancestors of ascomycetes and basidiomycetes diverged, the ascomycetes' ancestor developed ascospores (i.e., meiospores usually produced eight per ascus) while the basidiomycetes' ancestor developed basidiospores (meiospores usually produced four per basidium), barrel-shaped septa (dolipores) and clamp connections.

On the other hand fungal systematics, particularlyat the higher levels of classification, is continually being reviewed and restructured, Hibbett et al., [42] propose a system of classification for all groups of fungi based on recent molecular phylogenetic studies. There are over 700 species of insect pathogenicfungi and these can be found within two main groups: phylum Ascomycota (subkingdom Dikarya) and the order Entomophthorales from the subphylum Entomophthoromycotina.The Entomophthorales, obligate pathogens of arthropods, are an extremely important group of insect pathogens historically placed within the Zygomycota. However, the Zygomycota is not an accepted phylum within the revised classification [42]. Therefore, the Entomophthorales have not been assigned a phylum in the current classification but will be pending resolution of clades from the Zygomycota [42]. The taxonomy of the Ascomycota is more clearly defined and contains two major orders: Hypocreales (class: Sordariomycetes; subclass: Hypocreomycetidae) and Laboulbeniales (class: Laboulbeniomycetes). It is worth noting that the ascomycete fungi were previously divided into two groups: Ascomycota and the Deuteromycota.The Deuteromycota were known as the Fungi Imperfecti (Deuteromycota: Hyphomycetes) and were species for which no sexual stage was known. However, morphological and molecular studies have demonstrated that some of these imperfect fungi are anamorphs (asexual forms) of the Ascomycota (order: Hypocreales; family: Clavicipitaceae). Throughout this paper we will refer to the classification proposed by Hibbett et al., [42]. The majority of EPF identified to date belong to the following four classes including: Laboulbeniales and Pyrenomycetes (phylum Ascomycota), Hyphomycetes (form phylum Deuteromycota), and Zygomycetes (phylum Zygomycota) [10]. Naturally, EPF research has been mainly focused upon members of these classes.

Phylum Chytridiomycota

Chytridiomycetes are characterized by their mode of sexual reproduction that involves the fusion of motile gametes and the production of asexual, uniflagellate propagative spores [34]. These propagative spores are highly adapted for movement within liquid media [43]. Two orders, Chytridiales and Blastocladiales, possess EPF species that are important entornopathogens of aquatic insects. Although fungi with motile zoospores are poorly represented in aerial and epigeal habitats, an exception is *Myiophagus ucrainicus* belonging to the class Chytridiales. This fungus is a pathogen of citrus pests, with particular affinity for armoured scale insects. After a heavy rainfall, spores are released onto thesurface of the drenched leaves where they can proceed to find a suitable host. One genus *Coelomomyces* (class Blastocladiales) contains most of the EPF belonging to the phylum Chytridiomycota [9]. This genus contains approximately 70 species that are pathogenic to a variety of aquatic insects including mosquitoes, midges, backswimmers and black flies. *Coelomomyces stegomyiae* is of particular interest, since its hyphae penetrate the ovaries of adult *Aedes*

aegypti and mature to produce sporangia in response to elevated ecdysterone titres that follow a blood meal. An outstanding feature of *Coelomomyces* is that these fungi require an alternate host to complete their life cycle (i.e., heteroecism). The alternate hosts are other aquatic arthropods, usually copepods or ostracods, where the sexual (gametophytic) generation of the fungus is propagated. The asexual (saprophytic) generation is then expressed within the insect host

Phylum Oomycota

Members of the phylum Oomycota resemble fungi in behavior and lifestyle, but this phylum is considered part of the kingdom Stramenopila instead of the kingdom Fungi, since its members have cellulose-based walls and have plant-like biochemical features [36] The fungus like organisms of this phylum reproduce sexually by forming thick-walled oospores from the fusion of sex organs (i.e., a male antheridium with a female oogonium), and asexually by the production of bifiagellate zoospores [34]. The class Oomycetes has two orders containing EPF; Lagenidiales and Saprolegniales. Within the order Lagenidiales, most EPF species belong to the genus Lagenidium. These species are known worldwide as important pathogens of mosquito larvae [9,43]. *Lagenidium giganteutn* can infect mosquito larvae through ingestion or by integument penetration. This fungus is quite tolerant of environmental extremes, being capable of overwintering in addition to surviving the drying and flooding of its habitat. An *L.giganteum* based product, Laginex TM is now being sold as a biological larvicide for the control of many mosquito genera, including: Aedes, Anopheles and Culex (AgraQuest Inc., Davis, CA). Species within the order Saprolegniales have characteristic non-reproductive thalli that are vegetative and unsegmented [43]. These Saprolegniales species are also pathogens of mosquito larvae, in addition to being important pathogens of midge eggs (Díptera, Chironomidae).

Phylum Zygomycota

Members of the phylum Zygomycota are characterized by their multinucleated, aseptate hyphae, and sexual reproductive mechanism that involves the fusion of sex organs (gametangia) to form resilient thick-walled zygospores [9,43]. Asexual reproduction involves the production of non-motile spores by cytoplasmic cleavage within sporangia [34]. Two classes contain EPF, Trichomycetes and Zygomycetes. Trichomycetes are not usually regarded as entomopathogens, though there is evidence that some members can infect aquatic insects such as mosquito larvae [9,10]. The class Zygomycetes contains two orders with EPF, Mucorales and Entomophthorales. The fungi within the class Mucorales mostly appear as opportunistic pathogens that can only infect weakened insects. The order Entomophthorales has received extensive study since its members are important pathogens of both epigeal and soil-inhabiting insect pests approximately 200 entomogenous species have been identified [9]. Members of the Entomophthorales are characterized by a modified sporangium functioning as a singular conidium that is forcibly discharged upon maturity [43]. These conidia are known as ballistospores and have a sticky coating that facilitates substrate (e.g., host integument) adherence. The genus Massospora is unlike other genera of this order, since its members do not produce ballistospores. This genus is of interest since its members are restricted to individual species of cicadas that typically remain underground for many years. Since these fungi are only associated with cicadas whilst in the epigeal environment, their spores must be very resilient to remain viable over the many years of dormancy. Over 200 entomopthoralean species have been identified that infect hosts belonging to at least 7 insect orders [43]. Some of these fungi have wide host ranges, while others are more selective and in some cases infect only a single host species. Many EPF members of this order are known to create dramatic epizootic events within susceptible insect populations. Grasshopper populations are very susceptible to epizootic outbreaks caused by *Entomophaga grylli* [44], while periodic crashes of green peach aphid population have been attributed to *Pandora neoaphidis*. Additionally, *Entomophaga maimaiga* was found to be the causative agent of North American epizootics in populations of the gypsy

moth *Lymantria dispar* in 1989 [45]. These epizootic eliciting fungi are currently being investigated as experimental control agents. *Entomophaga maimaiga* promoted epizootic events in *Lymantria dispar* populations in the third year after its introduction in Michigan [46]. A potential drawback of entomophoralean fungi for insect biocontrol is that they, generally, can not be easily cultured and it is suspected that many members of this order are obligate pathogens, without the means to proliferate saprophytically.

Phylum Basidiomycota

The phylum Basidiomycota is comprised of fungi possessing dolipore septate hyphae, and some yeasts [34]. Sexual reproductive cells called basidia are formed by the fusion of compatible hyphae. On each basidium, basidiospores are produced, usually in groups of four, by nuclear fusion and meiosis [43]. Asexual reproduction is rare, with only a few groups known to produce conidia [34]. Only two genera within the order Septobasidiales are thought to have entomogenous species. Both genera, Septobasidium and Uredinella, are obligate parasites/symbionts of scale insects. It has been very difficult to determine whether the fungi of the genus Septobasidium are actual pathogens. These fungi are always found together with scale insects on host trees and even though fungal hyphae do penetrate into the insect's hemocoel and absorb nutrients, there is no apparent insect immune response. In addition, the fungus provides a highly organized macrocolony where the scale insects are sheltered from the external environment and are protected from avian predators and hymenopteran parasitoids. However, fungi belonging to the genus Uredinella are regarded as parasites. Although these fungi do not kill the host insect, they can be regarded as mortality factors that negatively impact upon population dynamics since parasitized hosts are rendered infertile. Insect death is mostly related to some species of Mucor (Mucorales). The order Entomophthorales, contain above 200 insect infecting species within phylum Zygomycota.Some species are capable of producing secondary spores from primary spores and some produce long lasting restingspores [47].

Phylum Ascomycota

The phylum Ascomycota is comprised of members that form septate haploid hyphae, and yeasts. Sexual reproduction occurs by the fusion of either modified hyphae or yeast-like cells leading to the formation of asci that produce ascospores, usually in groups of eight [9,34]. Asexual reproduction is also quite common for ascomycetes. There are five classes that contain entomopathogens, but most species are found in the classes Laboulbeniales and Pyrenomycetes [43]. The class Hemiascomycetes contains the yeast like entomopathogens that are typically slow acting and cause chronic infections, as is the case with the infection of the biting midge (Dasyhelea obscura) by *Monosporella unicuspidata.* Members of the class Plectomycetes have no unique characteristics although some of its members are responsible for chalkbrood disease in bee colonies. An example is *Ascophaera apis* that preferentially infects drones through the body cavity or by feeding. The class Loculoascomycetes produces bitunicate asci that are released in specialized stomatic compartments (locules). This class has two entomopathogenic orders, Myriangiales and Pleosporales that are considered important pathogens of scale insects. A major class of the phylum Ascomycota is Pyrenomycetes. Members of this class possess distinguishing unitunicate cylindroid asci. All EPF species in this class belong to the order Sphaeriales, and most are contained within the Cordyceps genus that has over 250 species. Members of this genus are pathogens of a broad range of insects belonging to both exo and endopterygote orders, and some are even pathogens of spiders. *Cordyceps spp.,* is especially abundant in tropical forest ecosystems, where they are considered important insect pathogens [10]. They are also important pathogens of numerous soil dwelling insect pests. Over 2000 years ago, Cordyceps infected insects were being used in Ancient China and Indonesia in religious ceremonies. Infected insect larvae were also thought to have medicinal properties and were used to treat ailments such as opium addiction and tuberculosis. Even today Cordyceps-mfecxcd larvae are sold in Chinese markets [43]. The other major class of the phylum Ascomycota is Laboulbeniomycetes. This

class is composed of minute, seemingly inconspicuous fungi that were originally thought to be external commensals of insects [43]. There are an estimated 115 genera of this class [8] and the relatively few entomopathogenic species identified to date have been found in a wide variety of habitats. These EPF are known to be pathogenic to members of at least 11 insect orders, although coleopteran insects appear to be the most common targets. Klich [48] described that thespores of Ascosphaera, in an unusual manner of insect infecting species are ingested by the bee larvae that germinatein gut causing infection. The most important insect infecting species occur in Aspergillus, Metarhizium, Hirsutella, Beauveria, Aschersonia, Culicinomyces, Lecanicillium, Paecil-omyces, Tolypocladium andSorosporella. Mostly, these genera have a linkage with one or many genera that can be verified with biological studies or by the molecularstudies presenting the genetic relationship between telemorphs and anamorphs [49].

Form phylum Deuteromycota

Members of the form phylum Deuteromycotina are also collectively known as the Fungi Imperfecti since most are only known for their asexual conidial form, while accompanying sexual forms are absent or unknown [34]. It is believed that most of these fungi have lost their sexual potential, although some fungi (e.g., Beauveria, Fusarium, Paecilomyces and Verticillium*)* can exchange genetic information by the rare fusion of somatic hyphae (anastomosis) that allows for the exchange and fusion of nuclei creating diploid cells [35,50]. This parasexual reproductive cycle continues with mitotic crossing-over, and the non-raeiotic reduction of the altered nuclei to a haploid state [50]. Both of the deuteromycete classes, Coelomycetes (Sphaeropsidales) and Hyphomycetes (Mondiales), possess entomopathogenic members [43]. Hyphomycete species produce conidia externally on conidiophores of varying complexity that are usually solitary but some species form multicellular structures known as conidiomata. These conidiomata are not enclosed and can be synnematal, consisting of columnar conidiophore aggregations, or sporodochial, where aggregated conidiophores resemble cushion-shaped masses. Coelomycetes species produce conidia within enclosed conidiomata that are either acervular (flat) or pycnidial (flask-shaped) [50]. The class Coelomycetes has two genera, Aschersonia and Tetranacrium, with species that are important pathogens of whiteflies and scale insects [22]. In contrast, the class Hyphomycetes contains over 40 entomopathogenic genera that are found worldwide in many varied habitats including aquatic environments, soil ecosystems, forest and agricultural areas, and even caves (e.g., Hirsutella and Stilbella) [10]. These fungi mainly infect hosts through the exoskeleton, though aquatic EPFs can infect their target insects post ingestion by penetrating the gut epithelia. Many hyphomycetes cause muscardine insect diseases where host cadavers become engulfed by a coat of mycelial growth that is often pigmented. Another feature of these fungi is the production of secondary metabolites that are thought to be intimately involved with pathogenesis. Interesting hyphomycete members include *Verticillium lecanii* and *Culicinomyces clavisporus* [43]. *Verticillium lecanii* is capable of sporulating on live, infected aphids that continue to produce viviparous young. This adaptation undoubtedly helps with the dispersal of spores to new hosts with the immediate vicinity. *Culicinomyces clavisporus* is unusual in that it produces submerged conidia rather than blastospores within its aquatic habitat as with *Tolypocladium cylindrosporunu* the spores of this fungus infect mosquito larvae by penetrating the gut wall, after they are ingested by the insect. Unlike most entomophthoralean EPF, hyphomycetes can be easily cultured on artificial media. This has led to extensive research into the physiology, biochemistry and genetics of this class of fungi. Most importantly, the facile culturing and mass production of these fungi, along with their high virulence, has made hyphomycetes attractive candidates for use as microbial insecticides.

Distribution and Diversity of Entomopathogenic Fungi

Studies of biodiversity in agroecosystems and the delivery of ecosystem services to agricultural production have usually ignored the contribution of entomopathogens in the

regulation of pest populations [51,52]. However, entomopathogens are among the natural enemies of arthropod pests in agroecosystems. An improved understanding of the ecology of indigenous populations of these beneficial organisms is a prerequisite for the evaluation of their contributions to pest control and for predicting the impact of agricultural practices on their populations. The anamorphic entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin from the order Hypocreales (Ascomycota) are natural enemies of a wide range of insects and arachnids and both fungi have a cosmopolitan distribution [53,54]. Much effort has been put into research on the development of *B. bassiana* and *M. anisopliae* as biological control agents (for inundation and inoculation biological control) to be applied in agriculture and forestry in temperate regions. However, this bulk of knowledge is in striking contrast to the lack of research into the fundamental ecology of these fungi in terrestrial ecosystems, including agroecosystems.

Distribution and Diversity of Indigenous Populations

The soil environment is usually the conventional isolation site for hypocrealean entomopathogenic fungi [55,56], and several species can be found in both cultivated and more natural habitats [57-61]. In studies of natural occurrence of entomopathogenic fungi in the soil, susceptible bait insects such as *Galleria mellonella L.* (Lepidoptera: Pyralidae) or *Tenebrio molitor L.* (Coleoptera: Tenebrioidae) are usually added to soil samples in order to recover fungal isolates; in principle the method can be described as the use of selective media. Keller et al., [59] found *M. anisopliae* to be common in both arable fields and adjacent meadows, but the species occurred at higher densities in meadows. In Canada, M. anisopliae was most frequent in agricultural fields compared to forest habitats [58], while *M. anisopliae* in Finland was isolated more often in southern parts of the country and the occurrence was not adversely affected by cultivation of the soil. In Danish soils M. anisopliae was more frequent in sun exposed habitats (i.e., cultivated areas) than in shaded habitats [57], which is similar to the Canadian findings. Furthermore, M. anisopliae was not among the entomopathogenic fungi isolated in the soil of a Danish forest ecosystem Nielsen et al.,. This implies characteristics of *M. anisopliae* as an agricultural species that is most common in exposed and regularly disturbed soil environments. Local and significant differences in natural distribution on one location can, however, occur. Meyling and Eilenberg [61] found M. anisopliae to be locally rare in a Danish agricultural field while B. bassiana was the dominant fungal species. Bidochka et al. [58] found B. bassiana to be affiliated with shaded and uncultivated habitats (i.e., forests) and B. bassiana also occurred frequently in hedgerow soils at a Danish locality [61]. These differences in natural occurrences in soil challenge the CBC strategy. Based on the studies made at a regional scale, indigenous populations of M. anisopliae appeared to be the most suitable candidates for environmental manipulations because M. anisopliae was most associated with agricultural field soil. However, in the case of the Danish agroecosystem described by Meyling and Eilenberg [61], *M. anisopliae* was found to be rare locally and *B. bassiana* would be a more suitable candidate for CBC. Thus the scale of the landscape needs to be considered when evaluating indigenous populations of entomopathogenic fungi in soils.

Insects

Entomopathogenic fungi occur naturally as infections in insect hosts which can be collected in the field and incubated in the laboratory for documentation of the fungus. *B. bassiana* have been documented to occur naturally in >700 species of hosts [62]. Studies on the prevalence of fungi in insects have usually been limited to species that are pests or are important non-target species such as certain predators and parasitoids. However, it is likely that almost any major insect taxon collected intensively will be found to be a natural host for *B. bassiana* in temperate regions. The occurrences of the fungi as infections in hosts are presumably the only part of the fungal life cycle in which the fungi can build up significant population sizes by reducing vast numbers of conidia. Thus contributing to the availability of susceptible hosts for fungal population increase is a key component when considering environmental manipulations in CBC strategies.

Plant Associations

Recent evidence suggests that both *B. bassiana* and *M. anisopliae* have the potential to engage in fungus plant interactions. The large majority of investigated higher vascular plants have been found to host fungal endophytes [63] including species in Clavicipitaceae contained within Hypocreales [64]. B. bassiana has also been included in this spectrum of fungi with endophytic activity by infecting corn (*Zea mays*) [65,66]. Endophytic fungi are often regarded as plant-defending mutualists [67] and the presence of B. bassiana in internal plant tissue has been discussed as an adaptive protection against herbivorous insects [64]. Besides natural occurrence in leaf tissue of corn, *B. bassiana* exhibited endophytic activity in cacao (Theobroma cacao), poppy (Papaver somniferum) [68] and coffee (Coffea spp.,) and tomato (*Lycopersicon esculentum*) (F.E.Vega, personal communication). In temperate regions, inoculum of *B. bassiana* has furthermore been isolated from phylloplanes of various plants in hedgerows in Denmark [60]. This occurrence was hypothesized to be a consequence of deposition from the surroundings but was also suggested to act as a natural infection pathway of endophytic activity [60]. Plant association was also recently documented for *M.anisopliae*, but this association occurred below ground in the rhizosphere [69]. The rhizosphere is the layer of soil immediately surrounding the root and many interactions between plants and other organisms occur in this interface [70]. Factors in the rhizosphere therefore seemed to promote the persistence and biological activity of *M. anisopliae* [69]. Wang et al., [71] further documented that *M. anisopliae* expressed similar genes when growing in exudates from bean roots and on a nutrient rich medium while different genes were expressed by the fungus when growing on insect cuticle and in insect hemolymph. This indicated that *M. anisopliae* has developed different adaptations to function as a pathogen and to grow saprophytically in the rhizosphere [71]. Survival outside the host may thus be critical for the ability of *M. anisopliae* to control insect pests in the soil [53,72].

Genetic Diversity

Biodiversity is usually evaluated by assessment of species diversity. Biodiversity assessment of fungal communities is, however, challenging, because fungal taxa often consist of complexes of cryptic species [73]. Cryptic species are found in *B. bassiana* [74,75] and seemingly also in the morphological species M. anisopliae [76]. Evaluating the biodiversity contribution of *B. bassiana* and *M. anisopliae* in agroecosystems must therefore be based on an assessment of the genetic diversity to discover potential cryptic species. Traditional assessment of fungal species diversity is based on morphological features. Unfortunately, few and sometimes ambiguous characters are used for species separation. Furthermore, many entomopathogenic hypocrealean fungi have probably exclusively anamorphic life cycles in temperate regions, at least outside East Asia, which additionally complicates matters about defining biological species and comparing intraspecific and interspecific genetic diversity. Recent advances in molecular techniques have shed new light on our understanding of species boundaries, especially within the genus Beauveria. Several studies have revealed much genetic diversity of the morphological species *B. bassiana* [77]. However, a recent phylogeny of Beauveria spp. showed that the morphological species *B. bassiana* in fact is paraphyletic and consists of two unrelated clades of which one is more related to *Beauveria brongniartii* (Saccardo) Petch than to the second *B. bassiana* group [74]. This latter group is tentatively referred to as pseudobassiana [75] but it needs a formal description. The existence of two unrelated clades may explain some of the large genetic diversity reported in the morphological species *B. bassiana*. Furthermore, the two groups of *B. bassiana* are themselves assemblages of cryptic species or separate clades [74,75] thus each group also contains much genetic diversity. Both groups infect a wide range of insects and can be isolated from the soil [61,74] but unfortunately no data currently exists on differences in ecological niches between the groups.

Population Dynamics

Population Increase and Infections of Hosts

Entomopathogenic fungi rely on arthropod hosts to build up population levels of infective stages (mitosporic conidia). During the cropping season outbreaks of diseases can regularly be observed in insect populations in the field, referred to as epizootics. Generally, the development of epizootics rely on host population dynamics, the number of infective stages in the pathogen population and the viability of these, infection efficiency and development [78] and a complex set of environmental factors and timing [62]. Considerable information on the biology of the organisms as well as specific environmental parameters (in time and space) is necessary to understand and predict the development of epizootics. Key components of population dynamics of the entomopathogenic fungi are the build up of the population, the infection of hosts, and the survival and dispersal in the environment [78].

Dispersal in the Environment

Dispersal of infective stages of a pathogen is an important factor in disease development [78]. Infective propagules of entomopathogenic fungi in the Hypocreales are passively dispersed, and this is mainly considered to occur through the action of weather components like wind and rain [56,62,79]. In air samples, *B. bassiana* was isolated among a large array of airborne fungi [80-83] and deposition from the air could be one likely source of the newly documented occurrence of *B. bassiana* on phylloplanes of hedgerow plants [60]. However, localized transmission onto plant parts by rain splash has also been shown [84] but rainfall also removed fungus inoculum that had been applied to foliage [62]. In the soil environment the hypocrealen entomopathogenic fungi can persist, but extensive proliferation and dispersal are limited. Population build up relies on the conversion of host cadaver resources into infective conidia that are released from cadavers over time following sporulation [85]. The number of conidia released per host is dependent both on fungus species, host species, and host size. For example, *B. bassiana* released 10-200 times more conidia than *M. anisopliae* from adult pecan weevils [85]. Additionally, *B. bassiana* radiated out from weevil cadavers in the soil by hyphal growth and subsequently infected larvae in neighbouring experimental cells while *M. anisopliae* growth was restricted to the surface of the cadaver [86]. Entomopathogenic fungi are dispersed by living infected hosts which migrate and die in another place than where they became infected [56]. Several aphid species migrate long distances high in the atmosphere and migrating aphids were found to harbour several entomopathogenic fungi (Entomophthorales and *B. bassiana*) [87]. This implies that *B. bassiana* is able to travel over long distances as infections in hosts, which can later lead to new infections and establishment far away from the original site of the fungus. The potential of arthropods to disperse and vector entomopathogenic fungi by their activity has been demonstrated in different terrestrial ecosystems. In the soil, collembolans dispersed conidia of *B. bassiana* and *M. anisopliae* which were not pathogenic to them, both by carrying conidia on the cuticle and by ingesting conidia which, after passage through the digestive tract, could remain viable. Moreover, collembolans were able to vector inoculum to other soil dwelling insects and initiate infections in laboratory experiments [88]. Also soil-dwelling mites were shown to be potential vectors of *B. bassiana* [54].

Effects of Agricultural Practices and Structure of the Agroecosystem

Soil Disturbance and Environmental Factors

Annually cropped agroecosystems are highly disturbed mostly due to tillage regimes and this affects the populations of natural enemies of crop pests. The communities of entomopathogenic fungi in the arable soil environments are different from communities of less disturbed habitats [57,58,61] and less disturbance in the cropping system also affect

the populations of the fungi. In corn fields in the US, soil densities of *B. bassiana* (as measured by colony forming units per g of soil) under different tillage regimes were very variable between years,but were seemingly higher in no-tillage systems compared to systems subjected to ploughing and chiselling [66]. Like wise, conservation tillage regimes, using strip-till and no-till, were more favorable to *B. bassiana* and *M. ansiopliae* populations in the soil than conventional tillage regimes employing ploughing and disking. Furthermore, no-till cultivation in soybean and wheat positively affected the population levels of *B. bassiana* and *M. anisopliae* compared to conventional tillage [89]. These findings of higher fungal densities in reduced tillage and no-till systems could be observations of indirect effects caused by increased levels of host populations of non-pest insects. High population levels of non-pest insects have been observed in reduced tillage systems. Exposed fungal inoculum is usually inactivated by the UV-components of solar radiation [90]. Other abiotic factors affecting entomopathogenic fungi include temperature [62] with strains exhibiting different temperature optima for growth [90]. Indeed, temperature, moisture and UV-radiation seem to be most important for *B. bassiana* survival [91]. Persistence of applied fungus material in soils has been studied for several isolates of different species but the complexity of the soil environment makes it difficult to evaluate single factors determining survival [62]. Factors such as soil texture [92], pH values and moisture contents have been explored and are thoroughly reviewed by Inglis et al., [62] and Klingen and Haukeland [93].

Use of Agrochemicals

Chemical insecticides, herbicides and fungicides are usually applied in conventional farming practices. These compounds, especially fungicides applied against plant pathogens, might also negatively affect the populations of entomopathogenic fungi with reduced pest regulation potential as a consequence. Klingen and Haukeland [93] provided a detailed review of published studies of effects of chemical pesticides on entomopathogenic fungi and nematodes. Their main conclusions were that insecticides and herbicides were not very harmful to fungal growth while fungicides were sometimes harmful [93]. However, most studies were performed in vitro with fungal cultures and extrapolation from studies in laboratory experiments to field conditions may not be straightforward. In the UK, for example, previous field application of the fungicide benomyl correlated with a lower incidence of *B. bassiana* in soil samples [94,95]. *In vitro* experiments further showed that the fungicide triadimefon inhibited the growth of *B. bassiana*, but fields previously treated with this product showed a higher frequency of occurrence of the fungus in soil samples than in samples from untreated control soils [94-95]. The fungicidal product albicarb even increased activity of in vitro cultures of *B. bassiana* [94]. This emphasizes that due to the complex interactions and composition of agroecosystems applications of specific fungicides are not necessarily detrimental to the occurrence of entomopathogenic fungi in the soil. Selected compounds could thus possibly be used in integrated pest management [94].

Hummel et al., [96] found that the application of certain pesticides significantly reduces the occurrence of entomopathogenic fungi in the soil. According to other authors, the discontinuance of the use of chemical plant protection products in organic cultivations can have a positive effect onthe occurrence of these fungi [97-99]. Tkaczuk [100] found that fungus I. fumosorosea was the most resistant to pesticides of the studied species under in vitro conditions. It can be assumed that I. fumosorosea is the best species to be concomitantly used with pesticides in integrated crop protection systems. A more frequent occurrence of *B. bassiana* species in soil from organic fields may be the result of using organic fertilizers, such as manure or green manure, which enrich the soil with organic matter. Using higher doses of manure may favorably affect the efficiency of *B. bassiana* as a biological control agent of soil pests [101].

Crop Diversification

Mixtures of plants within the crop can reduce colonization by pest species and the use of trap crops can lure the pest insects away from the crop by a push-pull strategy [102,103].

Manipula tion of insect behavior may also affect the dispersal of entomopathogenic fungi in agroecosystems because fungal inoculum can be distributed by insect activity.

Reducing the area of bare ground between the crop plants by mulching may reduce the population sizes of pests by enhancing conditions for ground dwelling predators [104,105]. Mulching may be unfavorable for hypocrealean entomopathogenic fungi as increased amounts of organic matter in soil have been shown to increase antagonistic activity against the fungi [106]. Establishment of beetle banks within the fields for CBC targeted at populations of carabid beetles [107] could also promote populations of entomopathogenic fungi. Specifically, populations of certain genetic groups of *B. bassiana* or *M. anisopliae,* which have been documented to be absent from the cultivated soils in agroecosystems, could potentially benefit from beetle banks.

Action Mechanisms of Entomopathogenic Fungi

Large-scale production of entomopathogenic fungi for the control of insects concentrates mainly on three types of propagules:

(a) Vegetative cells named blastospores, which grow in submerged, liquid culture

(b) Vegetative, multicellular mycelium, produced in liquid fermentation either in pellets or in hyphae with a filamentous morphology, which are fragmented afterwards [108],

(c) Conidia as so-called resistant stage, which can be produced in a surface culture on solid medium, in a submerged culture in liquid medium or in a diphasic system which consists of inoculation of solid medium with blastospores produced in liquid fermentation [109,110].

While blastospores and conidia can infect the host directly, mycelium needs to grow and form infectious propagules first. Conidia can be produced easily and are more stablein challenging environmental conditions than blastospores. Additionally, spore germination on artificial media can differ to a great extent from germination oninsect cuticle. The insect cuticle is covered by a waxy layer containing fatty acids, lipids and sterols [111]. The cuticles of most insects contain fungistatic compounds that retard spore germination [112]. Cuticular fatty acids have a profound effect on spore germination and differentiation. They are either toxic, fungistatic, or, occasionally for pathogenic species, stimulatory. The ability of oils to extract substances from insect cuticle was noted by Ibrahim et al., [113]. Those substances were found to have stimulatory or inhibitory effects on conidia of *M. anisopliae*.

Infection of an insect by entomopathogenic fungi occurs by a series of events. Some of the processes are known and understood, but there are many areas that still require clarification or investigation. The process can be divided into three parts: adhesion of the fungal spore, penetration through the cuticle and establishment within the host. Much of the work examining adherence and penetration of entomopathogenic fungal spores has been carried out using *Metarhizium anisopliae* (Metschnikoff) [17], but some studies have also been made with *B. bassiana* [114]. Attachment of several species of entomopathogenic fungi to insect cuticle has been found to be passive and nonspecific [115]. It has been shown that the dry conidia of both *M. anisopliae* and *B. bassiana* are hydrophobic and suggested that hydrophobic interactions are responsible for adherence of the spore [115,116]. Once the conidia have adhered to the cuticle and in response to stimuli the conidia will germinate and may eventually penetrate the cuticle as a result of both mechanical force and enzymatic degradation. Of the various processes that may be involved in determining virulence of an isolate one of the easiest to observe is adherence and germination of the conidia on the insect cuticle

An electron microscope study was undertaken to determine whether differences in adherence and germination of the conidia on the cuticle of *Oryzaephilus surinamensis* and *Tribolium confusum* could explain the apparent differences. This study showed that

quantitative and qualitative differences could be observed between the two species. *O. surinamensis* had a greater number of conidia adhering to the cuticle at each of the post-treatment periods. This species had a much greater number of setae, particularly on the ventral abdomen, and the presence of these may have assisted with adherence of the conidia. Germinating conidia were observed more frequently on the cuticle of *O. surinamensis* than for *T. confusum*. There are several possible reasons for this: 1). The setae of *O.surinamensis* may trap air near the surface of the cuticle which, as a result of cuticular and respiratory transpiration processes, may contain higher levels of moisture Figure 2.

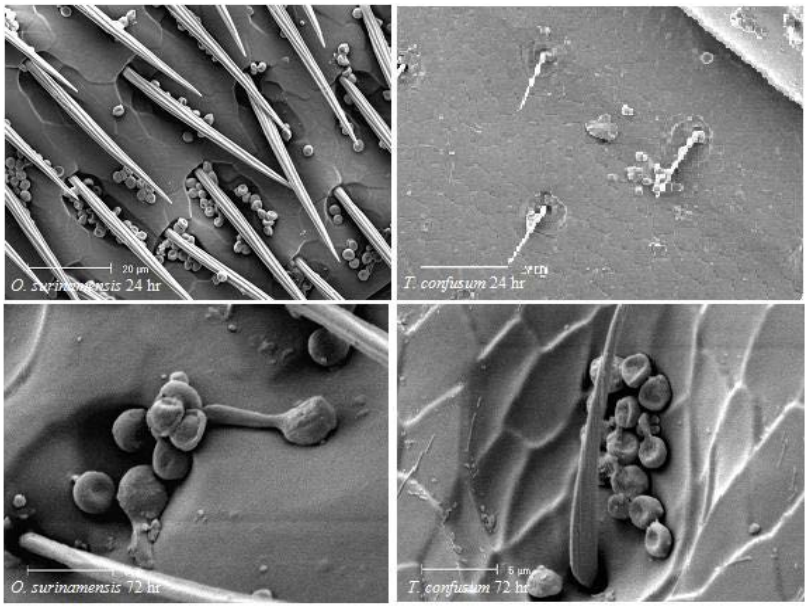

Figure 2: Attachment and germination of conidia on the ventral abdomen of *O. surinamensis* and *T. confusum* [118].

This would provide more favourable conditions for germination of the conidia. 2) The cuticular lipid profiles will differ between the two species. It is possible that the cuticle of *T. confusum* contains a substance that is inhibitory for conidia germination. It has been shown that cuticular hydrocarbons can either promote or inhibit conidial germination [117]. *Tribolium* species are also known to produce defensive quinones and it is possible that these chemicals may also inhibit germination. Inhibition of yeast and bacterial growth by the defensive secretions from *Tribolium* spp., has been shown [118]. Current studies are investigating these possibilities. The number of conidia found on both species decreased over time. This could be as a result of grooming activities. From this it would appear that conidia that have not germinated and penetrated the cuticle within the first 24-48 hours are unlikely to remain on the cuticle and play a role in the infection process.

Penetration through the cuticle was not easily observed using this method. Even at points where penetration may be expected such as intersegmental regions penetration could not clearly be seen. The lack of significant germ tube growth on the cuticle of both species may indicate that penetration occurs directly below the point of attachment. Both *B. bassiana* and *M. anisopliae* have been shown to produce germ tubes that grow over the surface of the insect cuticle until they contact an area of relative weakness where penetration can easily be achieved [119]. Penetration by the fungal conidia is one of the factors that have been linked to virulence of various isolates of *Paecilomyces fumosoroseus* [120]. This study has shown that the early stages of fungal infection may play a key role in the susceptibility of storage beetles to some isolates of *B. bassiana*. The possible mechanisms for this require

further investigation. It may be possible to increase susceptibility through the formulation of the conidia. In particular compounds that are known to act on the cuticle could be incorporated if not detrimental to the conidia.

Biophysical Properties of Spore Surfaces

At the field level, EPF spores initiate their infective action through contact. Biophysical studies have indicated that there are nonspecific- followed by specific, modes of associations, requiring hydrophobic and electrostatic attractions between spores of EPF and insect exoskeleton. Spores then initiate the second and more specific attachment, frequently mediated by lectin-like associa- tions between spore surface sugars and the insect cuticle. The hydrophobicity of EPF spores is in part determined by proteins called hydrophobins [121]. A particular characteristic of insect epicuticle fatty acid and waxes is relevant to the hydrophobic or hydrophilic elements of the spore, favoring or impairing attachment. For example, a heavily waxy cuticle found in some homopterans is unlikely to be a target of EPF species with highly hydrophilic spores. Characterization of spore hydrophobicity by phase partition assay has been cumbersome. As a result, the employment of hydrophobicity tests a key initial step of selection of potentially superior pathogenic EPF has lagged. A new salt-mediated aggregation and Sedimentation Assay (SAS) for the determination of hydrophobicity with corroborating correlation to occurrence of specific proteins among EPF isolates has been developed in our laboratories.

Enzymes, Toxins and Pigments and Pathogenecity or Virulence

The entry into the host and successful growth of EPF needs production of extra-cellular hydrolytic enzymes such as proteases, chitinases and lipases, adhesive mucilaginous substance(s) and appressoria to aid penetration of a peg and spread of hyphal bodies. Also, production of toxins, whether peptides/proteins or secondary metabolites is important in the disease process. In spite of the arsenals of toxins and hydrolytic enzymes, the speed or time with which EPF kill target pests is slow and unlike those of the synthetic chemicals. Chemical pesticides that work by contact mode of action work quickly and visibly in a matter of hours or a day. The performance of the microbial agents in general falls short of the chemicals and has generated a negative perception. As long as EPF survive, spores should be able to germinate after contact with the host cuticle even if it occurs through the tarsi of the target insect as it moves on treated plants or other surfaces in a field environment. The knowledge of the timing of the EPF life cycle should allow the selection of those isolates with fast germination rates [57]. Toxins, pigments and other secondary metabolites that could affect immune cell function and even singularly kill some insects could be detoxified within certain insects. For example, cyclic depsipeptides and linear peptides lower the immune system of the host. In fact, toxic metabolites from *B. bassiana* affect filopodia formation and hemocytic activation [122]. Because the genetic basis for the expression of such peptides is understood, the overproduction of linear or cyclic toxic peptides by fungal mutants in order to enhance pathogenicity should be within the realm of possibility [123]. The entry of entomopathogenic fungi through the relatively massive barrier presented by the insect cuticle is considered to occur by a combination of mechanical pressure and enzymic degradation, but the relative importance of the two mechanisms is not known [124]. Several entomopathogenic fungi such as *Metarhizium anisopliae, Beauveria bassiana* and *Verticillium Iecanii,* when grown in liquid cultures containing locust cuticle as sole carbon source, produce a variety of hydrolytic enzymes with activity against the major components of insect cuticle, namely protein, chitin and lipid. Entomopathogenic fungi exhibit many attributes that determine virulence toward their hosts, including the production of degradative enzymes [125]. Fungal proteases are believed to play an important role in cuticle penetration [126]. The best known determinant of fungal entomopathogenicity is based on subtilisinlike serine protease (designated Pr_1) of *Metarhizium anisopliae*, where its role in host invasion has been clearly demonstrated [127] This enzyme is adapted to extensively degrade insect cuticular protein and has been ultrastructurally located in the host cuticle during the early stages of penetration [13]. A

trypsin-like enzyme (Pr_2) belonging to the serine protease group also occurs during the early stages of cuticle colonization suggesting that it has some role in degrading extracellular proteins complementary to that of Pr_1 [128].

The fungal infection process

The infection process of entomopathogenic fungi is divided into

a) Parasitic phase and

b) Saprophytic phase

The infection process comprises of the following steps:

i. Attachment of the infective units, e.g., conidia or zoospores to the cuticle.

ii. Germination of the infection unit on the cuticle.

iii. Peneration of the cuticle either directly by germ tubes or by infection pegs from appressoria

iv. Multiplication of the yeast phase-hyphal bodies in the haemocoel.

v. Death of the host.

vi. Growth in the mycelial phase with invasion of virtually all the host.

vii. Penetration of hyphae from the interior through the cuticle to the exterior of the insect.

viii. Production of infective units on the exterior of the insect.

The parasitic phase starts with the adhesion of the conidia to the cuticle. The entemopathogenic fungi invade their hosts by direct penetration of the host exoskeleton or cuticle. The penetration of the insect cuticle can be performed in different ways. *Verticillium lecanii* is capable of penetrating the insect cuticle only with its germtube while *Metarhizium anisopliae* and *Beauveria bassiana* produce specific infection hyphae originating at appressoria.After the successful penetration the fungus then distributed into the haemolymph by formation ofblastospores.

The saprophytic phase starts with the death of insect which is performed by a number of Mechanisms Viz.

i) Mechanically by growth of the fungus in the insect body & by retrival of nutrients from the insect body.

ii) Mechanically by growth of the fungus in the insect body & by toxins produced by the fungus [129].

Recognition and Spore Attachment to Host

The process by which a spore recognizes an appropriate host has not yet been fully elucidated. There are a complex signalling apparatus (G-proteins, receptors, kinases, and secondary messengers) in some entomogenous fungi. Attachment of the conidium to host cuticle is mediated through non-specific hydrophobic interaction between conidial rodlets and the waxy surface of the insect cuticle [71].

Germination and Penetration of Integument

Before production of a germ tube can be initiated, the spore must overcome fungistatic or toxic compounds present in the insect cuticle [130]. Successful germination is also dependent upon appropriate humidity, available nutrients and surface topography [131]. Ibrahim et al. [113] determined that *M. anisopliae* conidia require water activity >0.98 (=98% RH) for germination, irrespective of aqueous or oil-based formulations. Wang and St Leger

[71] observed that *M. anisopliae* var. *acridum* conidia germinated on *S. gregaria,* however, germination on beetles or hemipteran bugs was either repressed or occurred with low levels of differentiation.

Penetration through the cuticle is accomplished by production of an appressorium, a specialized structure at the apex of the germ tube [127]. Formation of the appressorium is influenced by surface topography, with preference for hard, smooth surfaces. Penetration pegs produced by the appressorium enter the cuticle, usually at intersegmental folds, with the aid of mechanical pressure and cuticle-degrading enzymes including proteases, chitinases, lipases, esterases and phosphatases [128,132,133].

Growth and Proliferation within Host

Once the fungus has entered the haemocoel, colonization is dependent upon the ability of the fungus to overcome a combination of cellular and humoral responses that comprise the host immune system [134]. Fungal cytotoxic compounds are produced by blastospores, free-floating yeast like cells produced as hyphae bud within the haemocoel. The production of secondary fungal metabolites, primarily destruxins A, B and E, by isolates of *M. flavoviride* has been demonstrated *in vitro* [135]. Destruxins have been shown to possess immunomodulatory ability [136] as well as disrupt normal cell metabolism. Expression of genes involved in stress response, detoxification and transmembrane transport in *M.anisopliae* var. *acridum* [133] is likely induced by the host humoral response. Subsequent to successful suppression of the host immune response, colonization of the haemocoel is completed and the insect succumbs. The reasons for host mortality are not yet fully understood, but may be due to a combination of mechanical damage to internal organs, nutrient depletion and/or toxicosis.

Re-Emergence from the Host and Conidiation

Under favourable environmental conditions, mycelial growth resumes and hyphae emerge soon after host death to colonize the cadaver surface. Hyphal differentiation into conidiogenous cells occurs and concomitant sporulation completes the infection process. At this point, abiotic factors are the main determinants in persistence of the infectious *In vivo* developmental cycle of entomopathogenic fungi

Adhesion of conidea to the host cuticle→Germ tube formation→Penetration of host cuticle→Vegetable growth within host→Vegetable growth within host→Production of conidiophores→Death of the host insect

Behavioral Changes in Infected Insects

Modified Behaviors Induced In Insects by Entomopathogenic Fungi

Behavioral modifications have been observed in many insects as a result of parasitism or pathogens. Altered behaviors can include changes in activities which result in the insect being more conspicuous to predation. Induced behavioral alterations could have arisen for the following reasons .There is many hypotheses as to why parasitized animals would exhibit behavioral changes. The behaviors could be described as (1) beneficial to the parasite, making the intermediate host more susceptible to predation and allowing the parasite to be transmitted to the definitive host. This susceptibility to predation is only advantageous however, if the parasite is in an intermediate host which is preyed upon by the final host, and if the parasite has developed to its infective stage. By far the majority of documented parasite induced changes in host behavior thought to be parasite adaptations are believed to enhance parasite transmission from host to host [137]. The behaviors may also be beneficial to the pathogen. The changes may cause better dispersal of an air-borne pathogen or enhance the pathogens growth rate. (2) The modified behavior may be beneficial to the host. From the intermediate host's perspective behaviors such as choosing different locations or lighting regimes could constitute induced physiological fever (behavioral fever)

and exist as a mechanism to fight off the parasite. Such situations would have arisen by natural selection to benefit the host. Behavioral fever has been observed for several insects infected with protozoans, endotoxins, or bacterial pyrogens. This behavior would be considered host defense [138]. Other explanations for altered behavior due to parasitism include kin selected-host suicide. In host suicide the host behaves in such a way as to increase the probability of death by predation in order to lower the risk of parasite infection for other members of the host species (3) Other modifications of behavior could not be adaptive to the host or the parasite, but rather the response to the pathological effects of the parasite [139]. Some modified behaviors are discussed as following:

Feeding Behavior

(A) Changes in Food Consumption: Many studies have investigated the effects of fungal infection on feeding by host insects. These studies show that although insects killed by entomopathogenic fungi often take longer to die than if treated with chemical pesticides, damage to crops is decreased during the disease incubation period because infected insects eat less than healthy ones. Most studies investigating insect species infected with the hypocrealean fungi, *Metarhizium sp.*, or *Beauveria* sp., have demonstrated a significant reduction in feeding as early as 1 to 4 days after inoculation [140]. Feeding then decreases through time until death [141]. Several studies have shown that the reduction in feeding is associated with dose, e.g., at the highest dose of *Metarhizium anisopliae* var. *acridum* (*M. flavoviride*), *Schistocerca gregaria* had eaten only as much wheat before death on day 5 after treatment as insects that received the lowest dose had eaten by day 3 [11]. Nutritional indices were calculated for lepidopteran larvae infected with *Beauveria bassiana* and *Nomuraea rileyi* to show that weight gain and efficiency of conversion of ingested and digested food decreased at the same time as food consumption. Therefore, as infected insects start eating less, they are less able to digest food. It has been hypothesized that reduction in feeding may be due, at least in part, to toxic substances or mechanical disruption by these hypocrealean fungi.

Feeding reductions documented for *Entomophaga maimaiga*, *Entomophaga aulicae*, and *Zoophthora radicans*, all of which are entomophthoralean fungi, seem to occur later in disease incubation than observed for hypocrealean infection. Gypsy moth, *Lymantria dispar*, larvae infected with *E. maimaiga* reduced feeding only 2 days before death [142]. Spruce budworm larvae, *Choristoneura fumiferana*, infected with *E. aulicae* appeared to assimilate food similarly to healthy insects until 24 h before death and this was similar for diamondback moth, *Plutella xylostella*, larvae infected by *Z. radicans* [143]. The delayed reduction in feeding associated with these entomophthoralean pathogens suggests that these more obligate pathogens affect host behavior only near death, thus allowing hosts to feed and grow for as long as possible, ensuring maximum growth and reproductive potential for these pathogens. There is a direct relationship between cadaver size and the number of conidia produced [9]. Transmission electron microscopy has shown that these fungi do not invade the hosts' vital tissues until late in the infection process [144] and therefore hosts behave normally until shortly before death. Maintaining normal rates of food consumption and digestion in fungal-infected insects for as long as possible clearly benefits the fungal pathogen because this maximizes the amount of food available for the entomopathogen.

(B) Changes in Feeding Location: Few studies have been conducted on shifts in feeding location by infected hosts during disease incubation. In general, the feeding and resting locations of fungal infected insects do not appear to change throughout most of the disease incubation period and distinctive changes in location occur briefly before death when insects are no longer feeding. However, pea aphids, *A. pisum*, infected with *Pandora neoaphidis* were found on the undersides of leaves or had left alfalfa plants and were found in the surrounding habitat more frequently than were healthy aphids [145]. Distribution of infected versus healthy pea aphids at lower densities on bean plants was not significantly different, although there was a trend toward more infected aphids at mid-height and lower

positions on plants (30.0%) compared with healthy aphids (12.5%). The less mobile aphid *Sitobion avenae* on wheat did change feeding height when infected [146]. Changes in location of infected *A. pisum* could be a fungal-induced modification for optimizing spore dispersal and transmission. It could, however, also be considered altruistic behavior (kin selection) moving the aphids away to protect progeny and sisters from infection. Aphids moving off plants could even be attempting suicide [147], which would decrease chances of infection spreading to other colony embers. Infected aphids are alive for several days between infection and death, and it would be interesting to evaluate when during disease incubation these movements begin.

Behavioral Fever

Behavioral fever is the elevation of body temperature in infected insects above that normally occurring in uninfected insects. Infected insects achieve this by seeking out locations in the environment that are at a higher temperature, and the outcome is death or suppression of the pathogen and a delay in the time until death. Fever is a common host response to many pathogens. It is an energetically costly process and is not inevitably beneficial to hosts, but there are many examples in which the onset of fever does suppress pathogens and so reduces or delays host mortality [148]. Observations on fungal-infected grasshoppers and caterpillars demonstrated the benefits of raising body temperature because basking at elevated positions reduced pathogen induced mortality [149]. Such a response has been noted in many taxonomically diverse host-pathogen interactions but it is sometimes difficult to distinguish between active behavioral fever and a benefit that is a side effect of the natural thermoregulatory basking behavior of some insect hosts. Olesen [150] first documented behavioral fever in fungal-infected house flies. In the first few days of infection, house flies seek temperatures in excess of 40°C and benefit from this preference by suppressing the pathogen. Kalsbeek et al., [151] showed that most infected flies captured in cool positions within farmyard barns died within 2 days but infected flies sampled from sun-exposed places took between 6 and 8 days to die after capture, inferring that they were newly infected at the time of sampling. Insect species that naturally thermoregulate can maintain body temperatures that restrict pathogen growth without changing their normal behavior [152,153]. Study [154] demonstrated a direct relationship between behavioral fever and host fitness. Infected locusts, which were allowed to reach body temperatures preferred by healthy hosts but not allowed to fever, took longer to die than infected insects at cooler temperatures.

Reproductive Behavior

(A) Direct Effect on Fecundity: Reduction in fecundity can increase pathogen fitness by diverting host resources such as energy to the pathogen. Studies on both hypocrealean fungi [140,155] and entomophthoralean fungi [155] have shown that infected hosts produce fewer progeny. The total reproductive output of fledgling desert locusts, *S. gregaria*, infected with *M. anisopliae* var. *acridium* (Hypocreales) was lower than that of uninfected individuals, although in the first few days of infection the locusts produced more eggs [156]. From an evolutionary perspective, early onset of reproduction is a sensible strategy because it ensures that individuals realize part of their reproductive potential. In the same study the fecundity of mature infected locusts was also assessed and interestingly the pathogen had no effect on reproductive behavior. The authors suggest that this is due to effects on the synthesis of juvenile hormone. Infection is thought to induce rapid synthesis of juvenile hormone and this likely has a greater effect on newly emerged adults, in which concentrations of the hormone are nil or low, compared with older adults (10 to 12 days), which already have high hormone levels [156]. A reduction in reproductive output was observed for pea aphids, *A. pisum*, infected with *B. bassiana* (Hypocreales) and similarly for pea aphids infected with *P. neoaphidis* (Entomophthorales). In contrast, *B. bassiana* takes approximately 6 days to kill the aphid, often by the production of secondary metabolites; it then enters a saprophytic phase, sporulating a few days post death. It is conceivable that *P. neoaphidis* is inducing the

reduced reproductive rate and therefore diverting host resources for its benefit. However, the reduction in reproductive rate observed for *B. bassiana* infected aphids may simply be a result of the fungus indiscriminately invading the host's tissues and producing secondary metabolites that interfere with nymph production.

In the case of adult carrot flies, *Chamaepsila rosae*, infected with *E.schizophorae* the effect on fecundity is more indirect. Normally, female carrot flies deposit their eggs on the ground near the base of food plants such as carrots, and after hatching the larvae move into the soil, where they eat small roots. However, infected flies seek elevated positions such as the top of trees and shrubs in the hedgerows, and sporulating cadavers are found several meters aboveground [157].

(B) Increase in Sexual Attractiveness of Infected Hosts to Mates: The exposed abdomen, which is commonly seen among adults of some insect species (especially flies) killed by entomophthoralean fungi (Figures 3*a*–*c*), is apparently highly attractive to individuals in the host population seeking a mate for copulation. Male house flies, *M. domestica*, are thus significantly more attracted to fungus-killed females than to uninfected females [158]. A fungus infecting domestic flies manipulates sexual behaviour of its hosts. It is assumed that this increased attractiveness is a result of the increase in size of the swollen abdomen of the infected females. However, a further study has demonstrated that even significantly smaller infected females are more attractive to males than are larger infected female flies. Further more, sex pheromone production by young, infected female house flies is reduced compared with uninfected house flies [159]. Therefore, the occurrence of increased attractiveness of infected female house flies to male flies cannot be explained by size or sex pheromone production alone. Other visual or chemical cues are likely to account for the observed phenomenon; perhaps the fungus is producing a semiochemical that attracts males.

The increased attractiveness is undoubtedly advantageous to the fungus for a number of reasons. First, males may themselves receive infection during physical contact with sporulating cadavers. Second, males trying to copulate with the cadavers may transmit conidia to uninfected females that they subsequently copulate with [160]. For the fly, this behavior is disadvantageous. Not only do more individuals die from infection but females lay fewer eggs after mating with infected males compared with females mating with uninfected males [160].

Figure 3: (*a*) Male house fly (Musca domestica) probing a dead female house fly killed by Entomophthora schizophorae. (b) Live male house fly copulating with dead female house fly killed by E. schizophorae infection. (*c*) Male secondary screwworm fly (Cochliomyia macellaria) attempting to copulate with dead female infected with Erynia bullata. (d) Carrot flies (Chamaepsila rosae) killed by E. schizophorae attached to leaves in a hedge at a height of four meters (Entomol. 2006).

(C) Response to and Production of Sex Pheromones

Female *P. xylostella* moths produce sex pheromone to attract males, which respond by pre-mating wing fanning. When females were infected by *Z. radicans*, release of pheromone was significantly reduced compared with uninfected females, but only within 24 h of death. The response of infected male *P. xylostella* was also significantly reduced compared with uninfected moths 2 days after infection and by 3 days after infection no infected males responded at all and were dead on day 4 [161].

Social behavior: Eusocial insects (all ants and termites; many bees and wasps) are similar to solitary insects in many aspects of life history, but fitness in eusocial insects is the result of cooperative effort [162]. Therefore, any behavioral response to a pathogen will have fitness implications beyond the individual and will be more complex to decipher in terms of costs and benefits to the host and pathogen. Despite this complexity there have been many reports of host-mediated behavior reducing pathogen transmission within colonies. Host behaviors observed include increased grooming, increased nest cleaning, secretion of antibiotics, pathogen avoidance, dispersal of infected individuals, and relocation of the entire colony. Increased grooming in response to fungal pathogens has been documented widely in both solitary and eusocial insects [163,164]. Removal of fungal conidia by allo-grooming and mutual grooming can be highly effective. The number of *B. bassiana* conidia on the integument of both larval and adult red imported fire ants, *Solenopsis invicta*, was significantly reduced by grooming [164]. Similarly, Cordyceps spores were removed by another species of ant, Cephalotes atratus. Although an increase in grooming behavior would appear beneficial to the host in terms of reducing infection, inoculum could be incidentally disseminated to conspecifics within the nest Kramm et al.,. Infective propagules that are removed by grooming with mouthparts are not ingested but are stored in an infrabuccal cavity within the buccal chamber, where they are discharged as a pellet. Infection can occur if inocula germinate and penetrate through the buccal chamber, but it has been hypothesized that the labial gland, which produces secretions rich in chitinases, has a fungistatic function [164].

The presence of *B. bassiana* within the nest correlates with an increase in the concentration of these antimicrobial substances, and so it can be inferred that release of these secretions is induced by the pathogen .In some circumstances social insects avoid areas of high inoculum density within the nest or establish new nest sites [164]. The benefits to the host are clear in terms of reducing contact with the pathogen but there are undoubtedly costs in moving. Leaf-cutting ants living in high densities in colonies had much greater resistance to *Metarhizium anisopliae* infections than would be predicted, and apparently at a low cost Hughes et al.,. Hygienic behaviors and pathogen avoidance are cooperative behaviors that reduce contact and transmission of pathogens, increasing the inclusive fitness of host insects, but the pathogen is not acting directly on the individual eliciting the behavior. However, a well-documented behavioral alteration of social insects infected with fungal pathogens is dispersal from the colony. This has been termed adaptive suicide McAllister et al., but this phrase is contentious not just because of the anthropomorphic connotations it implies but also because the process does not necessarily increase the inclusive fitness of the host (Poulin). Dispersal of infected individuals undoubtedly removes the pathogen from the colony but it is likely that this would happen through nest cleaning anyway. Therefore, unless transmission is so rapid that nest cleaning would be ineffective, the inclusive fitness would be unaffected [164]. Behavioral alterations of social insects can have implications for the fitness of the entire social group. Therefore, the task of unraveling the adaptive nature of host-altered behavior in social insects is challenging. This group of insects presents a unique situation in which to study host-pathogen relationships from a behavioral perspective.

Defensive reactions: Insects employ various defensive strategies in response to attack from predators and parasites ranging from crypsis to dramatic escape behaviors. In general,

defensive reactions of hosts to pathogens are considered from an immunological perspective. However, recent studies indicate that termites (Reticulitermes flavipes and Zootermopsis angusticollis) detect the presence of conidia of the fungal pathogen *M. anisopliae* and exhibit a striking vibratory display [165]. Nestmates detecting the signal through the substrate increase the distance between themselves and spore-exposed termites, thus escaping infection. It is conceivable that the selection pressures to develop such alarm behaviors in response to pathogens are higher in subterranean environments where pathogen density is high and particularly in colony-forming insects where high host densities would be optimal for rapid pathogen transmission.

Host behavioral changes can be detrimental to the pathogen if the host is less able to escape predation or if greater movement makes them more apparent to predators prior to death (Arthurs and Thomas [166] found that *S. gregaria* locusts infected with *M. anisopliae* increased locomotion within 3 days of infection (11days to kill), potentially making them easy targets for predators. Later in infection they became sluggish and less able to escape predation. Whether this is altruistic behavior on the part of the locust or a side effect of infection is unknown but could result in reduced pathogen fitness. Pathogens can benefit from the defensive behaviors of infected individuals but there is also the potential for the pathogen to manipulate defensive behavior to increase the advantage. Pea aphids infected with *P. neoaphidis* become less responsive to alarm pheromone produced by conspecifics [167]. As a result, infected aphids could be more susceptible to attack by predators and this would reduce the pathogen density. However, aphid-specific predators do not generally consume fungal-infected aphids [168,169]. The escape response of pea aphids commonly includes dislodging from the plant, and infected aphids are less able to recolonize plants. Therefore, it is advantageous to the pathogen to prevent infected aphids from responding, ensuring a greater number of infected cadavers remain on the plant and thus benefiting pathogen transmission to other foliar-feeding aphids.Aphids infected with *P. neoaphidis* continue to produce alarm pheromone and elicit a response in neighboring aphids. Again, this is advantageous to pathogen transmission because host movement enhances transmission [168,170,171].

The intimate association of such fungi with their host would seem to drive such adaptations. However, the selection pressure on more generalist fungi, such as *B. bassiana*, to manipulate individual host species is likely to be minimal because such fungi have diffuse relationships with many hosts and can also persist saprotrophically. In contrast, because of the broad host range of *B. bassiana*, it is in the interest of potential hosts to avoid contact with the pathogen. This behavior is seen in termites that recognize and avoid conspecifics contaminated with virulent isolates of *M. anisopliae*, although they show no response to contamination with less virulent isolates of *B. bassiana* [172,173]. It is possible that termites can discriminate fungal species or even isolates that are pathogenic from those that are not. Alternatively, the interactions may represent new associations in evolutionary time and so avoidance behavior has not yet been selected for. This would explain the inability of the parasitoid *Cephalonomia tarsalis*, which attacks grain beetles, to identify and avoid *B. bassiana* conidia or infected beetles. Although the beetle and parasitoid are coevolved, the fungus (*B. bassiana*) is a new addition to the system (introduced as an augmentative biological control agent). *B. bassiana* not only competes with the parasitoid for host resources but can also directly infect the parasitoid [174].

Phototropic and Geotropic Responses

Relations between light and behavior during disease incubation were studied. Results are somewhat equivocal because effects of light (positive phototrophism) are not easily separated from potential effects of gravity (negative geotrophism) and, to some extent, temperature. Hence, we deal with phototropic and geotropic responses. Common armyworm larvae, *Pseudaletia separata*, normally spend the day in the soil, where it is dark, and they

leave the soil to feed at night. However, when infected with the entomophthoralean fungus *E. aulicae*, many larvae spend the day in the light instead and even move vertically upward on plants [175]. Therefore, their normal behavior of avoiding light is completely changed when infected. Such a change in behavior that exposes larvae to visual predators could be risky, but it could also benefit the fungus because larvae die in exposed locations. Similarly, larvae of sciarid flies normally live in the upper soil layers. However, when a disease caused by *Erynia sciariae* is progressing, the larvae move to the top of the soil or growth medium to die after which the fungus sporulates (Figure 4g). This behavior appears to benefit the fungus because infective conidia are dispersed on the soil surface, where they infect small larvae moving into the soil. Numerous studies have documented that entomophthoralean fungi kill insect hosts during the late afternoon or evening [175,176]. Elegant experiments have been conducted to document associations between light cycles and timing of death of fungal-infected insects. Most deaths were associated with photoperiod and occurred during periods of light [176].

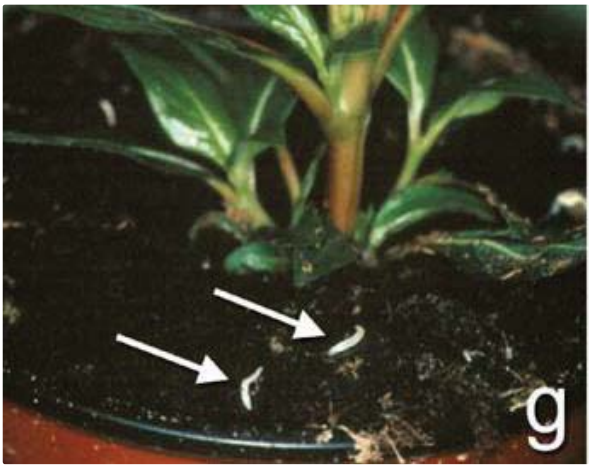

Figure 4g: Sciarid fly larvae dying from infection with *Erynia sciariae* at the top of the growth medium.

Genomics of Entomopathogenic Fungi

The extensive transcriptomic and genetic study of entomopathogenic fungal infection process revealed that a number of different genes were involved in the pathogenicity [177] such as chitinases, guanine nucleotide-binding proteins and its regulator [178], adhesin which helps in attachment of spore, a perilipin-like protein that regulates appressorium turgor pressure and differentiation and a cell protective coat protein helping in escaping the pathogen from the host immunity recognition [179]. Similarly, an increased virulence of the entomopathogenic fungi was observed with over expression of virulence genes such as subtilisin protease PR1A [128], subtilisin protease PII gene, and hybrid chitinase containing a chitin binding domain [178].

Cytological studies of entomopathogenic fungal nuclei have been performed on *Metarhizium anisopliae* and *Beauveria bassiana* showing the nuclei divide immediately before or during spore germination. One can assume the nucleus in these fungi are haploid even though some hyphal cells may contain two or more nuclei or heterokaryons from two nuclei of different types presumably due to anastomosis and nuclear migration. Khachatourians [180] concluded that the nucleus in a conidium divided just before germination and that one of the two daughter nuclei subsequently emerged into the germ tube while the other stayed in the spore. During the germination process each conidium therefore contains two nuclei and during conidial development, both daughter nuclei occasionally moved into the

young conidium. As early as mid 1980's this was attempted through the formation, fusion and regeneration of protoplasts from several EPFs [35]. Cytogenetic evidence was used to indicate relationship between ploidy and nuclear size of EPF [35]. Drummond and Heale [181] demonstrated that complementary parental diauxotrophic strains of Verticillium lecanii could be paired on minimal medium using hyphal anastomosis and protoplast fusion techniques to produce diploid strains. The pathogenicity of *V. lecanii* diploids was demonstrated against the white fly (*Trialeurodas vaporariorum*). From cadavers of white flies infected with heterozygous diploid conidia, prototrophic and auxotrophic progeny haploid strains were reisolated. These results indicated recombination for genes involved in pathogenicity, sporulation and germination. Viaud et al., [182] study of *B. bassiana* and *B. sulfurescens* protoplasts showed that hybrids appeared to be diploid or aneuploid with portions of the genome being heterozygous while mitochondrial molecular marker indicated homoplasmy of the hybrids and inheritance of mitochondria. Also, Leal et al., [183] demonstrated genetic exchange in *M. anisopliae* strains co-infecting Phaedon cochleariae as revealed by molecular markers. The first study of the genome of any EPF was that of Entomophaga aulicae examined by Murrin et al., [184]. This genome was shown to contain 8 x l06 kb DNA per nucleus. The base composition of E. aulicae chromosomal DNA was 38% G+C. There were 15 chromosomes and 11 pairs of kinetocores. The ratio of nuclei to mitochondria in 12 protoplasts was estimated to be 1:25±13. In comparing the nuclei of this fungus with those of the others, Murrin et al., [184] suggest that the genome size of this fungus is two orders of magnitude greater than that traditionally attributed to fungi. The most comprehensive studied description of biophysical and a biochemical characteristic of any EPF chromosomal DNA is that of B. bassiana. Pfeifer and Khachatourians [185] determined the G+C content of this fungus by CsC1 buoyant density centrifugation and thermal denaturation and found to be 56.9 ± 1.9%, a value in line with 53.0% G+C ratio of B. tenella. Demonstrations of differences in the genetic make up of various EPF isolates and strains would be another approach to begin in the dissection of the genetic basis for fungal disease of insects. Restriction fragment length polymorphisms especially between those EPF showing differences in their virulence or host specificity could be a direct way of elucidation of the genetics and the molecular biology of insect pathogenesis. Further, study of RFLP would contribute to the taxonomic status of various EPF isolates and strains. The initial examples of the use of the restriction enzymes to analyze RFLP for EPF were demonstrated for *Entomophaga maimaiga* [45]. RFLP has been demonstrated to show sympatric occurrence of two *E. aulicae* isolates [186]. Differences in RFLP between virulent and less virulent mutant isogenic strains of B. bassiana were reported [187]. In the latter study the RFLP of genomic DNA of two strains of *B. bassiana*, representing strain GK2016, a 'wild type' (virulent) and strain GK2051, a less virulent mutant was demonstrated. The data showed the loss of some DNA sequences from the mutant strain, which may be responsible for loss of virulence. Interstrain and interspecies RFLP comparison of the genus Beauveria by Kosir et al., [187] where the genomic DNA of *B. bassiana*, *B. brongniartii* and *B. cylindrospora* were tested for RFLP banding patterns and the discrimination between Hirsutella longicolla vat. longicolla and Hirsutella longicolla vat. cornuta were performed by [188]. Fegan et al., [189] used RAPD DNA markers to show high degree of diversity between *M. anisopliae* varieties. Thomsen and Beauvais [190] cloned two chitin synthase gene fragments from hyphal bodies of Entomophaga aulicae. Two chitin synthase gene fragments EaCHS 1 and EaCHS2 of 600 bp were obtained using PCR amplification of genomic DNA. Compared with other fungal chitin synthases, they belong to class II. EaCHS1 and EaCHS2 were used to probe total RNA from *E. aulicae* hyphal bodies and protoplasts. A single transcript of 2.4 kb hybridized only with EaCHS1 inprotoplasts and hyphal bodies. Thomsen and Bruun-Jenssen [191] demonstrated nested primers for PCR amplification to resting spores of E. muscae a pathogen of adult Diptera and other insects. Mitochondrial genome size determination was first described for *B. bassiana*, 28.5Kb [192], and *M. anisopliae* with aprox. 32 Kb [193], and *V. lecanii*, 24.5Kb [194]. These mitochondrial genome sizes place them at the small end of the fungal mitochondrial genomes, where we can find ranges between 19Kb (Torulopsis

glabrata) and 176Kb (*Agaricus bitorquis*) [195]. Taken in account the mitochondrial genome of organisms from other kingdoms, the fungal mitochondrial genome has an intermediate complexity which ranges between the small and compacted animal mitochondrial genome which present sizes of 14 kb (*Caenorhabditis elegans*) and 42 kb (*Placopecten magellanicus*) and the larger and more complex plant mitochondrial genomes with sizes between 184Kb (*Marchantia polymorpha*) and 2,400 kb (*Cucumis melo*) [195]

Certain Mycoinsecticides

Beauveria bassiana

An extensive literature search was conducted to evaluate risks related to human exposure to a total of 8 distinct reports naming the genus Beauveria Vuill. As the alleged cause of fungal infections and disease of humans were identified, but only 4 of these reports could be conclusively attributed to species of the genus Beauveria. The most severe human cases of Beauveria infections are two recent reports of disseminated mycoses [196,197]. Both of these infections occurred in severely immuno-compromised patients with acute leukemia. Prior the development of mycoses, one patient underwent 4 full cycles of chemotherapy; the other was in her first cycle of chemotherapy and had been diagnosed with *Streptococcus viridans* in her bloodstream. Despite their poor health, both patients responded well the antimycotic treatments and fully recovered from their mycoses.

While there are some reports of *Beauveria* spp., isolated from patients with corneal keratitis, the *B. bassiana* can certainly not be considered a significant eye pathogen. Of four reports linked to *Beauveria*, only two [198] can be conclusively attributed to *Beauveria bassiana*. Exposure to the fungus *B. bassiana* caused higher mortality rates in malaria-infected mosquitoes, reduced the proportion of surviving mosquitoes carrying sporozoites in their salivary glands, and diminished the likelihood for infected mosquitoes to take subsequent bloodmeals [199]. Similarly effective, the entomopathogenic fungus *Metarhizium anisopliae* reduced the degree of malaria transmission by 75% in independent field experiments [200]. Species of the genus Beauveria have been reported to produce the secondary metabolites bassianin, bassiacridin, beauvericin, bassianolide, beauverolides, tenellin and oosporein [68,201]. Hatting et al., [202] showed that *B. bassiana* could control up to 65% of Duraphis noxia in field condition. Comparison of mortality percentage of this chemicals demonstrated the significant differences and the most percentage (100%) was observed in *A. annua* extract, *B. bassiana* secondary metabolites [203]. Recently Claudio et al., [204] stated that infection of an insect by *B. bassiana* comprises numerous complex developmental transitions, which require intricate genetic regulation. Specific roles of a number of regulatory genes in *B. bassiana* have been established, while the roles of others have yet to be unraveled.

It is important to note that the discovery of a certain metabolite during liquid cultivation of a specific strain cannot be extrapolated to all strains of the species. Moreover, it cannot be assumed that these substances will also be produced under natural conditions in the soil or in the target host. Further, it should be kept in mind that entomopathogenic fungi naturally cause epizootics similar to those resulting from artificial inoculations. There are no reports of metabolites entering the food chain or accumulating in the environment as a result of such natural or artificicial epizootics or natural metabolite secretion [201]. In contrast, numerous studies have documented environmental accumulation and food chain contamination with chemical pesticides and antibiotics used in agricultural production.

Metarhizium anisopliae

Metarhizium anisopliae, one of the most famous soil inhabitant entomopathogens has a virulence potential on plant and animal pests [205-207]. Metarhizium is a genus composed of three species divided into ten clades (or varieties) [29]. The most common form is the

genetically highly diverse *Metarhizium anisopliae* var. *anisopliae* (Metsch) Sorokin. Murad et al., [208] Stated that entomopathogenic fungi, such as *Metarhizium anisopliae*, are able to control insect-pests and are widely applied in biological control. *M. anisopliae* infects susceptible hosts via direct penetration through the cuticle. For description purposes the infection process can be divided as: (1) conidia adherence to the host cuticle through hydrophobic interactions and thin mucilaginous material; (2) conidia germination and development; (3) germ-tube differentiation into apressoria; (4) cuticle penetration; (5) hyphae differentiation into blastospores/hyphal bodies in the haemolymph; (6) host colonization; (7) extrusion to the host cadaver surface and (8) conidiophores formation and conidia production. The soil forms its normal habitat, although it does not grow saprophytically in soil but exists as dormant conidia which infect susceptible hosts on contact. The soil-inhabiting larvae of scarab beetles are typical hosts and coevolution has led to some isolates being specific to one or two genera of scarab. Thus the most virulent strains are usually those which cause natural epizootics in that particular host. This hostspecificity is dose-dependent: a high dose will infect a very wide range of hosts. At present, while the DNA profile is a guide to finding a virulent isolate, bioassay is the only way to find an effective isolate. Besides virulence and host specificity, temperature is an important factor in selecting an isolate for development as a mycoinsecticide. Most isolates grow well between 15°C and 30°C, although some develop at temperatures as low as 5-10°C and others grow even at 35-40°C. Many researchers suspectedthat the rapid kill by *M. anisopliae* on its host could be caused not only through direct physical invasion of the hyphae, but also possible due to some enzymatic mechanisms or toxic metabolites produced by the fungus [209,210]. Some isolates produce one or more members of a family of toxins called destruxins and while production of destruxin does sometimes correlate with virulence, their role in pathogenicity is controversial. Certainly these compounds are toxic to some non-hosts when injected directly into the body cavity and one, destruxin E, is toxic for other insects such as Diptera, leading to speculation that destruxins could be used as insecticides. They can also be antifeedants. These toxins play an important role to weaken the host immune defences, damage the muscular system and the Malpighian tubules, affecting excretion and leading to feeding and mobility difficulties [211]. Infected insects usually seek places with higher temperature in order to increase body temperature and so inhibit the development of the infecting microorganism [154]. Therefore, the action of the destruxins reducing host mobility would also impair this comportamental defence mechanism. Indeed Metarhizium isolates that produce higher quantities of destruxins are more virulent [212]. Recently, both the virulence and insecticidal activities of the crude extracts of *M. anisopliae* were evaluated against the second-instar larvae of *Spodoptera littoralis* (Boisduval) (Lepidoptera, Noctuidae), a very harmful polyphagous insect pest. Topical application of the crude extracts did not cause mortality. Combined treatments with fungal suspensions of the *M. anisopliae* and their extracts caused higher mortality rates than the single *M. anisopliae* and extract, [213] therefore the potential of certain entomopathogenic fungal isolates for use in an integrated *S. littoralis* management strategy targeting larvae, as well as the potential of the combined use of entomopathogenic fungi and their extracts.

Lagenidium giganteum

Only one species of the genus Lagenidium is known to be a facultative parasite of mosquito larvae, namely *Lagenidium giganteum*. It consists of two stages: oospores (sexual), and zoospores (asexual). Fungal reproduction is both asexual (zoospores) and sexual (oospores). In order to infect mosquito larvae, zoospores must be formed. These biflagellate, motile zoospores are the asexual stage of the fungus. They do not have a cell wall, and are therefore too fragile to be used directly for mosquito control. A further disadvantage of the asexual stage is its short shelf life; zoospores survive for only 48 hrs after emerging from an infected larva. Further problems include the need to keep the mycelium completely hydrated, its susceptibility to be overwhelmed by contaminating microorganisms following formulation, lack of stability under extreme temperatures, and special handling required

to keep the formulated product from becoming anaerobic. Oospores, the sexual stage of *Lagenidium giganteum*, can also be used as inoculum. They are dormant propagules, resistant to desiccation and mechanical abrasion and stable for at least seven years, which allows multivoltine persistence of the fungus in some habitats. On a commercial scale, L. giganteum mycelium and oospores were produced in liquid fermentation using media consisting of crude carbon and nitrogen sources, with vegetable or fish oils providing the required sterols and unsaturated fatty acids; the unsaturated fatty acids, primarily triglycerides, were thought to help solubilize the sterols to optimize uptake and provide a higher percentage of fatty acids, thus increasing zoospore production. Another critical component is $CaCl_2 \cdot 2H_2O$. Fermentation output at pilot-scale level was 1–5l fermenter volume per hectare of mosquito habitat, with a production cycle of 3–4 days. Commercial production used this method, with harvesting of the fungus from medium and storage in refrigerated containers; effectiveness of such material lasted 1–3weeks [214]. Kerwin [215] still considered economical scale-up of liquid fermentation a serious challenge. Although mycelium and oospore formulations of the fungus were registered and commercialized by Agraquest Inc. in the United States, the company abandoned the continued sale of the fungus. As an alternative, L. giganteum could be produced using wheat bran as a solid fermentation substrate [214]. The fungus retained its efficacy for 4 weeks. Glucose and wheat germ oil could increase the shelf life of the fungus and the whole culture could be efficacious against larval mosquitoes, at least in laboratory assays.

Leptolegnia chapmani

Leptolegnia chapmani Seymour (Straminipila: Peronosporomycetes) has been under study for a number of years as an alternative mosquito control agent. Much less work has been done regarding production of L. chapmani, primarily because the fungus remains in an experimental mode. Pelizza et al., [216] evaluated a series of agar-based media for zoospore production. Although they observed that most media supported mycelial growth equally, zoospore production in an agar medium that contained 10% Fortisip (Nutricia, Wiltshire, UK), a complex human nutritional supplement, was 10-fold greater than any of the other media. The sterol requirement of *L. giganteum* does not seem to exist for L. chapmani.

Pythium

Most species belonging to the genus *P*ythium are pathogens of vascular plants, other fungi, and algae [217]. Some species however, have been found to be mildly, to highly pathogenic to insects. A *Pythium* sp., caused a high level of mortality in a field collection of the tree hole mosquito *Ochlerotatus sierrensis* (Ludlow) [218]. In 1988, Saunders et al. [219] isolated *Pythium flevoense* Van der Plaats-Niterink from wild populations of *Ochlerotatus sierrensis* in California, occurring in 42% of the sampled tree holes, although this fungus caused infections in only 14% of larvae during 21 weeks of exposure in laboratory bioassays. The fact that this fungus infected mechanically injured larvae rather than healthy larvae indicates that the fungus is opportunistic rather than strictly entomopathogenic. Su et al., [220] isolated *P. carolinianum* Matthews from Guizhou province, China, in 1994. In outdoor bioassays the authors found infection levels of 13.3-100% in *Culex quinquefasciatus* larvae, and mentioned that a population of *Aedes albopictus* (Skuse) was 'markedly controlled', but no infection percentages were given. Notwithstanding the pathogenicity of some *Pythium* species to mosquitoes, on the whole they are not considered suitable for biocontrol of mosquitoes.

Crypticola

Crypticola clavulifera Humber, Frances and Sweeney has been isolated from the midge *Forcipomyia marksae* Tokunaga (Ceratopogonidae) in Queensland, Australia, in 1984 Frances et al.,. Its biology is similar to that of *Lagenidium giganteum*. In the laboratory the fungus successfully infected *Aedes notoscriptus* (Skuse), *Anopheles farauti* Laveran, *Culex*

annulirostris Skuse, *Culex quinquefasciatus*, and *Aedes aegypti* [221]. *Aedes kochi* (Dönitz) was not susceptible [221]. Despite its pathogenicity to several mosquito species no further studies have been published on this fungus.

Coelomomyces

The genus Coelomomyces consists of more than seventy species of obligatory parasitic aquatic fungi that undergo a complex life cycle involving alternating sexual (gametophytic) and asexual (sporophytic) generations [222] The life cycle of Coelomomyces is complex and includes obligatory development in an intermediate microcrustacean host (cyclopoid copepods, harpacticoid copepods, or ostracods) and two mosquito generations for completion [223,224]. The fungus survives unfavorable environmental conditions, such as cold or dry periods, as Resting Sporangia (RS) [225] that develop from diploid hyphae in infected mosquito larvae.

Verticillium Lecanii

Verticillium lecanii is a very common fungal species; it was capable of infecting a wide range of insect hosts from board geographical and climatic locations [12]. *Verticillium lecanii*, or the white halo fungus, is a fungal species which belongs to the class Deuteromycetes, and the order Moniliales. The *V.lecanii* species contains a complex of several fungal strains, which differ little in appearance but rather in their host range *Verticillium lecanii* is a commonly occurring fungus that can, among others, affect arthropods. The fungus has been observed on several kinds of insects, but particularly on aphids, scale insects and on whitefly. It has also been found as a saprophyte, which is an organism living or dead, organic material. *Verticillium lecanii* also occurs as a hyperparasite on rusts and other plant pathogens. The fungus can easily be isolated from soil. The mode of action of V.lecanii is based on a direct contact between fungal spores and insects. After applying, the spores land on the target insects and germinate. Mobile insects, such as thrips, can also pick up the spores while moving within the crop. *V. lecanii*, under the right environmental conditions, kills the insects after 7-10 days. After spraying, the spores germinate and grow producing hyphae that penetrate into the body cavity where they proliferate destroying the tissues. The fungus then grows through the insect cuticle and under high humidity conditions spores are produced on the outside of the insect body which may spread the infection to other insects [226].

Paecilomyces

Paecilomyces fumosoroseus (Moniliales: Moniliaceae) has been isolated from most regions of the world and has been reported to infect several insects belonging to many different orders [227,228]. *P. fumosoroseus* was reported to be one of the most common fungi attacking nymph and adult Bemisia in fields and glasshouses and *T. vaporariorum* in glasshouses [229]. Several isolates of the fungus were screened against whiteflies and successful isolates were tested in the field and glasshouses [230]. *Paecilomyces lilacinus* (Thom) Samson (Moniliales: Moniliaceae) is a typical soil-borne fungus that appears to be common in the tropics and subtropics [231].

Hirsutella thompsonii

Hirsutella thompsonii Fisher has undergone several commercialization efforts, primarily in the United States in the 1970s and Cuba in the 1980s. Only one product made from this fungus was identified by De Faria and Wraight [15] in their survey. The fungus was marketed for the control of eriophyid mites in citrus, but sales were terminated in the 1980s for a number of technical reasons. In 1988, Latge et al., [232] were also able to obtain microcycle conidia from submerged fermentation, but from a strain unique in this regard.

Lecanicillium

Lecanicillium muscarium Zare and Gams and L. longisporum Zare and Gams (both formerly classified as Verticillium lecanii (Zimmerman) have attracted some attention

as biocontrol agents of Homoptera and spider mites. In their 2007 survey, De Faria and Wraight [15] noted 16 commercial products in existence. Two have been in commercial use in Europe since the 1980s. This genus is notable in that conidia are borne in slime balls and rarely in dry chains, unlike the other Hyprocreales fungi, which produce hydrophobic conidia. Derakhshan et al., [233] reported that molasses-yeast broth was the best liquid medium while rice yielded the highest conidial production. A wheat bransugarbeet pulp mixture (9:1 w/w) has also been touted as an excellent solid substrate by Grajek [234]. Feng et al., [235] identified rice bran to be the best medium, better than cooked rice; a bran: husk ratio of 1:1 was almost as good. Optimal temperatures for growth and sporulation in both systems are typically 20–25°C.

Culicinomyces

Culicinomyces clavisporus Couch was investigated beginning in the 1980s as a biocontrol agent for control of larval mosquitoes [236]. Since then, however, interest in this fungus seem to have waned, probably because of the commercial success of Bacillus thuringiensis israelensis Berliner (Bti) and B. sphaericus Meyer and Neide for mosquito control. One exception was a small resurgence regarding its potential to control biting midges (Culicoides Latreille), reported by Unkles et al., [237], but there is little published literature about the topic. Conidia have been produced on wheat-bran solid substrate or in liquid media (corn meal extract, corn steep liquor, or standard nutrient broths), but yields were very low in comparison to efficacious field rates. According to Roberts et al., [238] mycelia were produced in liquid peptone-yeast extract-glucose medium, harvested by filtration treated with 10% sucrose, air dried to 13% moisture, and then granulated. Although freshly dried and granulated marcescent mycelium produced abundant conidia that could be stored at room temperature or 4°C, the product lost viability within 2 weeks. Mycelium stored at−20°C did retain viability at least for 63 days.

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