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ENTOMOPATHOGENIC FUNGI AS BIOLOGICAL CONTROLLERS: NEW INSIGHTS INTO THEIR VIRULENCE AND PATHOGENICITY

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Abstract - Entomopathogenic fungi vary considerably in their mode of action and virulence. Successful infection depends primarily on the adherence and penetration ability of a fungus to the host integuments. A variety of extracellular enzymes is produced during the degradation of insect integument. The attempts to control insects have changed over time from chemicals to natural control methods. This is why the development of natural methods of insect control or biopesticides, is preferred. By the use of fungal entomopathogens, insect pests can be controlled. There is no doubt that insects have been used for many years, but their effective use in the field remains elusive. However, their additional role in nature has also been discovered. Comparison of entomopathogens with conventional chemical pesticides depends on their efficiency and cost. In addition to efficiency, there are advantages in using microbial control agents, such as human safety and other non-target organisms; pesticide residues are minimized in food and biodiversity increased in managed ecosystems. In the present review the pathogenicity and virulence of entomopathogenic fungi and their role as biological control agents using biotechnology will be discussed.

Key words: Entomopathogenic fungi, pathogenicity, virulence, proteinases, biocontrol and biopesticides.

INTRODUCTION

Fungi, which induce disease symptoms in insects, include fungi from quick killers to absolute parasites that provide disease symptoms in the host. Absolute parasites are considered to be fungi which live in association with a host and benefit at the host's expenses (Smith et al., 1981). Entomopathogenic fungi cause lethal infections and regulate insect and mite population in nature by epizootics (Burges, 1981; Carruthers and Soper, 1987; McCoy et al., 1988). They are host specific with a very low risk of attacking non-target organisms or beneficial insects. They are reported to infect a very wide range of insects including lepidopterous larvae, aphids and thrips, which are of great concern in agriculture worldwide (Roberts and Humber, 1981).

The virulence of fungal entomopathogens involves four steps: adhesion, germination, differentiation and penetration. Each step is influenced by a range of integrated intrinsic and external factors, which ultimately determine the pathogenicity. A successful infection is achieved by the attachment or adhesion of spores to the host. Adhesion is necessary and normally achieved through the secretion of mucilage. However, enzymes, lectins, as well as hydrophobic and electrostatic forces also play a role (Boucias et al., 1998). The virulence of an entomopathogenic fungus is recognized first by adhesion to an insect body. The failure of a pathogen to adhere with the epidermis is considered a feature of virulent strains (Al-Aidroos and Roberts, 1978). After adhesion, the next factor for the virulence of a strain is the enzymes that hydrolyze the epidermis of

the insect. The most important enzymes secreted by entomopathogenic fungi are lipases, proteases and chitinases, which are produced sequentially, reflecting the order of the substrates they encounter (Smith et al., 1981).

A wide range of factors such as water, ions, fatty acids and nutrients on the cuticle surface and the physiological state of the host, influence spore germination and behavior (Hassan et al., 1989). Successful germination requires the assimilation of utilizable nutrients and a tolerance to any toxic compound present on the surface (Latge et al., 1987). After germination, appresoria appear at the end of short germ tubes, subterminally or on the side branches. Appressorium morphology is reported to be found either in swollen clavate or spherical structures to slightly swollen terminal hyphae (St. Leger et al., 1988).

Penetration of the cuticle is accomplished by the germ tube itself or by the formation of an appressorium that attaches to the cuticle and gives rise to a narrow penetration peg (Boucias and Pendland, 1982; Roberts and Humber, 1981; Wraight et al., 1998; Zacharuk, 1973). Penetration is both a mechanical and an enzymatic process (Charnley, 1984; McCoy et al., 1988; St. Leger et al., 1988). Most terrestrial pathogens are known to penetrate directly, rarely via wounds, sense organs or spiracles. The penetration process is considered to be a combination of enzymatic and mechanical forces. The exact mechanism for entry is usually peculiar to the species. A range of cuticle-degrading enzymes is produced during penetration into the host (Gillespie et al., 1998).

In forestry, horticulture and agriculture, entomopathogens are considered as primary candidates for mycoinsecticides. Fungal insect pathogens are important natural control agents for many insects and other arthropods, which significantly reduces host insect populations (Burges, 1981; Carruthers and Soper, 1987; McCoy et al., 1988). The significance of fungi in regulating insect population was noted early in recorded history by the ancient Chinese (Roberts and Humber, 1981). Approximately 750 species of entomopathogenic fungi are known from 85 genera found throughout the classes of fungi (Gillespie and Moorhouse, 1989; McCoy et al., 1988).

Chemical pesticides have been the practical method used by growers for the control of economically important pest insects for many decades, but their effects on non-target organisms, groundwater contamination, residues on food crops and the development of insect resistance to chemicals have forced the industry and scientists to focus on the development of alternative control measures. Alternatives, including biological control with reference to entomopathogenic fungi in the genera of *Metarhizium,* have been sought. Biological control is a well-recognized success story, and was initiated in 1762 with the introduction of the Mynah bird (*Acridotheres tristis)* from India into Mauritius for the control of the sugar cane red locust *Nomadacris septemfasciata*. Control of insect pests, particularly by their natural enemies comprising parasitoids, predators and pathogens in agro-ecosystems, is a continuous process. In the search for new avenues in biological control, the importance of entomopathogens has been highlighted as an environmentally friendly pest control method.

Fungi constitute a large group of more than 500 species that can parasitize insects. Most of the taxonomic groups contain entomopathogenic genera, such as *Metarhizium, Beauveria, Verticillium, Nomuraea, Entomophthora, and Neozygites,* to name a few (Desphande, 1999). Fungal biocontrol agents are promising because they act by contact and do not require ingestation, they can be mass-produced very easily and are quite host specific.

Metarhizium anisopliae is an entomopathogenic fungus that is present in soils throughout the world. It was first found to be a biocontrol agent in the 1880s. Four types of insect pests (beetles, termites, spittlebugs and locusts) are presently being used for control by *this fungus* (Zimmermann, 1993). Various formulations of the spores or mycelia of *M. anisopliae* are applied. Once the induction of a fungal epizootic control is achieved, the new spores and vegetative cells produced in the infected insects are spread to healthy population of insects. *It* is a ubiquitous spe-

cies but strain selection is vital, since a high level of variation exists among isolates in relation to pathogenicity, optimal temperature and viability (Moutia, 1936).

In the early days of biological control and especially microbial control, there was no concern for the possible side effects or safety considerations of entomopathogens. Steinhaus (1957) was possibly the first to raise questions on the safety of microbial control products for man, other vertebrates and even crops. He very carefully discussed the different aspects of the scientific knowledge at that time. Although he concluded that microorganisms pathogenic to insects are in general harmless to man, animals and plants, he recommended that such products be subjected to appropriate State and Federal regulations. A few years later, Müller-Kögler (1965) published a book on the fungal diseases of insects, their practical use for biological control and the basics of insect mycology, in which some sections on the side effects of entomopathogenic fungi on humans and other warm-blooded animals as well as on beneficial insects, were already included. In 1971, Heimpel summarized the knowledge on the safety of insect pathogens, i.e. bacteria, viruses, protozoa, fungi and rickettsiae, for man and vertebrates. He also emphasized the necessity to test the safety of insect pathogens and said that 'it seems incredible that so many good scientists have worked so long with insect pathogens without testing them for safety'. He also mentioned the registration guidelines of the USA and other countries at that time. A similar review was published two years later by Ignoffo (1973).

With the increasing interest in pest insects as biological control between 1980 and 1990, safety aspects were discussed in more detail (e.g. Austwick 1980; Burges 1981; Hall et al., 1982; Laird et al., 1990). Burges (1981) outlined the main principles and guidelines for testing the safety of insect pathogens and stated 'that a pathogen should be registered as safe when there is reasonable evidence that it is so and in the absence of concrete evidence that it is not'. The first guidelines for the registration of entomopathogenic fungi were published by Hall et al. (1982). The first book dedicated to the safety of microbial insecticides was published by Laird et al. (1990), and included sections on the safety to domestic animals and wildlife (Saik et al. 1990), and to vertebrates and humans (Siegel and Shadduck 1990). In 1996, Cook and coauthors published an interesting work about pest and plant disease control related to scientific safety evaluations of microorganisms. The intention was to identify and discuss safety issues linked to microbial control agents that should stimulate and improve discussions on possible risks and risk management. Later reviews on the safety of entomopathogenic fungi, and especially on *Beauveria* spp., were published by Goettel and Jaronski (1997), Goettel et al. (2001), Vestergaard et al. (2003) and Copping (2004). This review article will describe the biology, classification, virulence and pathogenicity of entomopathogenic fungi. Their use as biological control agents and safety issues will also be discussed.

BIOLOGY OF ENTOMOPATHOGENIC FUNGI

The entomopathogenic fungi have life cycles that synchronize with insect host stages and environmental conditions. In broad terms, the differences between Hyphomycetes and Zygomycetes, particularly Entomophthorales, have been described by Shah and Pell (2003). However, as with all general principles, there are exceptions and features of the two groups form a continuum. Species, and sometimes isolates within a species can behave very differently. For example, insect host range, infection levels, germination rates and temperature optima can vary between species and isolates (Sierotzki et al. 2000; Pell et al., 2001; Shaw et al., 2002). Members of the Hyphomycetes are generally considered to be opportunistic pathogens infecting many species in a range of insect orders, and host death is commonly associated with toxin production which overwhelms the host defense responses (Roberts, 1981; Samson et al., 1988). In contrast, other groups of fungi are thought to have evolved into higher parasitic forms. For example, infection and host death by Entomophthorales tends to occur due to tissue colonization with little or no use of toxins (Humber, 1984). One of the best examples of a highly evolved insect pathogen-

ic fungus is *Strongwellsea castrans* that infects flies. Infection does not interfere with insect feeding and movement but conidia are discharged and thereby dispersed from a cavity in the abdomen of infected insects over a long period of time prior to death (Pell et al., 2001). In general, Entomophthorales have biotrophic relationships with their insect hosts with little or no saprophytism, while Hyphomycetes can be hemibiotrophic with well-defined parasitic phases within insect hosts and saprophytic phases on the death of their hosts.

The origin of the entomopathogenic lifestyle may have arisen several times from a common saprophytic ancestor inhabiting soil and leaf litter (Humber, 1984; Samson, et al., 1988; Evans, 1989; Spatafora and Blackwell, 1993). The greatest radiation into different host groups occurred within Clavicipitaceae (Ascomycotina), and involved multiple inter-kingdom jumps between animals (e.g. insects), fungi and plants (Nikoh and Fukatsu, 2000; Artjariyasripong et al., 2001; Spatafora et al., 2007). Hyphomycete species exist as separate asexual (anamorph) and sexual (teleomorph) forms. All known genera of entomopathogenic Hyphomycetes, now proven teleomorphs in the Clavicipitales, and life cycle stages for Hyphomycetes may have become simplified in agricultural situations because of a superabundance of insect hosts (Evans 2003).

CLASSIFICATION OF ENTOMOPATHOGENIC FUNGI

Entomopathogenic fungi are found in the divisions Zygomycota, Ascomycota and Deuteromycota (Samson et al., 1988), as well as Chytridiomycota and Oomycota, which were previously classified within Fungi. Many of the genera of entomopathogenic fungi currently under research belong either to the class Entomophthorales in the Zygomycota or to the class Hyphomycetes in the Deuteromycota. It is important to mention that fungal infections occur in other arthropods as well as insects and/or species that are not pests of cultivated crops. For example, *Gibellula* species infect spiders and several species of *Cordyceps* and *Erynia* infect ants. Further information on the biology and ecology of entomopathogenic fungi can be obtained from Steinhaus (1949, 1964), Samson et al. (1988), Evans (1989), Bałazy (1993) and Eilenberg (2002).

FUNGAL INFECTION AND TRANSMISSION

Asexually produced fungal spores or conidia are generally responsible for infection and are dispersed throughout the environment in which the insect hosts are present. When conidia land on the cuticle of a suitable host, they attach and germinate, initiating cascades of recognition and enzyme activation reactions, by both the host and the fungal parasite (Samson et al., 1988). Invasion of the insect body and circulatory system (hemolymph) occurs once the fungus has passed through the cuticle of the external insect skeleton. Structures and processes for the invasion of insect tissues are similar to plant pathogens, including the formation of germ tubes, appresoria and penetration pegs (Samson et al., 1988).

With entomophthoralean fungi, unicellular yeast-like cells with chitinuous walls (hyphal bodies) spread throughout the insect obtaining nutrients, leading to the death of the host by physiological starvation 3-7 days after infection. Some entomophthoralean species initially produce rounded protoplasts that either lack sugar-rich residues in the outer cell layers or mask their presence in order to avoid detection by insect hemocytes (Samson et al., 1988; Glare and Milner, 1991; Pell et al., 2001). With hyphomycete fungi, circulation within the insect hemolymph and toxin production is carried out by yeast-like cells that resemble hyphal bodies but are termed blastospores with this fungal group (Samson et al., 1988).

On the death of the insect host, the fungus appears from the dead host and sporulation occurs on the outside of the cadaver. Sporulation can occur internally when ambient humidity precludes external sporulation. For example, West African grasshoppers infected with *Metarhizium anisopliae* var. *acridum* (*M. flavoviride*) exhibited sporulation on the internal surfaces of the desiccated hosts. With entomophthoralean species, cadavers are attached and

grow through the ventral surface or mouthparts of the cadaver. The attachment structures indicate that the fungus remains in the environment of new hosts for further transmission.

CONIDIA DISPERSAL

Conidia of Hyphomycetes such as *Metarhizium* and *Beauveria* spp. are hydrophobic and passively spread from infected cadavers. Entomophthoralean conidia are actively discharged under hydrostatic pressure with the exception of *Massospora* spp. After discharge, entomophthoralean conidia are carried on wind or by co-occurring insects (Hemmati et al., 2001; Roy et al., 2001). If the primary conidia of entomophthoralean species fail to find suitable host substrates on which to germinate, then most form higher order conidia which can either be actively discharged, e.g. *Erynia* or *Pandora* spp., or form passively held secondary conidia (capilliconidia) borne on long stalks (capilliconidiophores), e.g. *Zoophthora* spp. With *Neozygites* spp., the primary conidia are not infective but serve to disperse the infective secondary conidia produced on capilliconidiophores. The conidia of some entomophthoralean species are discharged while the host insect is still alive, e.g. flies infected with *S. castrans* and thrips infected with *Entomophthora thripidum* (Steinhaus, 1964; Pell et al., 2001).

OCCURRENCE

Entomopathogenic fungi naturally occur in insect hosts as infections; they can be collected in the field and grown in the laboratory for the documentation of the fungus. *B. bassiana* have been reported to occur naturally in more than 700 species of hosts (Inglis et al., 2001). 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. Infections in hosts are considered the only part of the fungal life cycle in which the fungi can build up significant population sizes by producing vast numbers of conidia. By considering environmental manipulation strategies, the contribution to the availability of susceptible hosts for fungal population increase is a key component.

FUNGUS-PLANT INTERACTIONS

Recent evidence suggests that entomopathogens have the potential to engage in fungus-plant interactions. The large majority of investigated higher vascular plants have been found to host fungal endophytes (Saikkonen et al., 1998; Arnold and Lewis, 2005) including species in Clavicipitaceae contained within Hypocreales (White et al., 2002). *B. bassiana* has also been included in this spectrum of fungi with endophytic activity by infecting corn (*Zea mays*) (Bing and Lewis, 1991, 1992, 1993). Endophytic fungi are often regarded as plant-defending mutualists (Saikkonen et al., 2004) and the presence of *B. bassiana* in internal plant tissue has been discussed as an adaptive protection against herbivorous insects (Elliot et al., 2000; White et al., 2002). Besides its natural occurrence in the leaf tissue of corn, *B. bassiana* exhibited endophytic activity in cacao (*Theobroma cacao*) (Posada and Vega, 2005), poppy (*Papaver somniferum*) (Quesada-Moraga et al., 2006), coffee (*Coffea* spp.) and tomato (*Lycopersicon esculentum*). In temperate regions the inoculum of *B. bassiana* has been isolated from the phylloplanes of various plants in hedgerows in Denmark (Meyling and Eilenberg, 2006a). 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 (Meyling and Eilenberg, 2006b). These new findings open exciting perspectives for the understanding of the ecology of *B. bassiana*. However, no knowledge is currently available about the natural endophytic activity and host plant range of *B. bassiana* in temperate regions or of the significance of *8* as an endophyte for fungus or plant fitness.

Plant association has also recently been documented for *M. anisopliae*, but this association occurred below ground in the rhizosphere (Hu and St. Leger, 2002). The rhizosphere is the layer of soil immediately surrounding the root and many interactions between plants and other organisms occur

in this interface (Bais et al., 2006). By releasing a recombinant isolate of *M. anisopliae* to the soil in an experimental cabbage field in Maryland, USA, Hu and St. Leger (2002) were able to demonstrate that the released isolate persisted better in the soil immediately surrounding the cabbage roots as compared to the bulk soil. Factors in the rhizosphere seemed to promote the persistence and biological activity of *M. anisopliae* (Hu and St. Leger, 2002). Wang et al. (2005) further documented that *M. anisopliae* expressed similar genes when growing in exudates from bean roots and on a nutrient rich medium, while the fungus when growing on insect cuticle and in insect hemolymph expressed different genes. This indicates that *M. anisopliae* has developed different adaptations to function as a pathogen and to grow saprophytically in the rhizosphere (Wang et al., 2005). The implication for biological control with *M. anisopliae* exploring the rhizosphere competence was investigated by Bruck (2005). Inoculated conidia of *M. anisopliae* persisted significantly better (up to one year) in the rhizosphere of *Picea abies* compared to the bulk soil (Bruck, 2005). Survival outside the host may thus be critical for the ability of *M. anisopliae* to control insect pests in the soil (Roberts and St. Leger, 2004; Bruck, 2005). Whether the rhizosphere of plants generally provides a "refuge" (where the fungus can survive outside insect hosts) for *M. anisopliae* in the soil, remains to be investigated. Perhaps associations with plants are important in the life cycle of both *B. bassiana* and *M. anisopliae* in temperate regions.

USE OF FUNGAL PATHOGENS

Fungal pathogens are important natural biological control agents of many insects and other arthropods and frequently cause epizootics that significantly reduce host populations (Burges, 1981; Carruthers and Soper, 1987; MacLeod, 1963; McCoy et al., 1988). The significance of fungi in regulating insect populations was noted early in recorded history by the ancient Chinese (Roberts and Humber, 1981) due to the frequency of natural epizootics and the conspicuous symptoms associated with fungus-induced mortality (McCoy et al., 1988; Steinhaus, 1963).

Approximately 750 species of entomopathogenic fungi are known from 85 genera found throughout the classes of fungi (Gillespie and Moorhouse, 1989; McCoy et al., 1988; Roberts and Humber, 1981). The majority of the entomopathogenic species are classified in the classes Hyphomycetes, Zygomycetes (order Entomophthorales), and Ascomycetes (in particular, the genera *Cordyceps* and *Torrubiella*). These pathogens cause mycoses in many different taxa of Arthropods and in almost every order of the Insecta (Bell, 1974, Gillespie and Moorhouse, 1989). They are known to infect all life stage of insects and are commonly found in aquatic, terrestrial, and subterranean habitats (Ferron, 1978).

Although fungal pathogens have much in common with viruses, bacteria, and other insect-pathogenic microbes, they are unique in many ways (Ferron, 1978). Perhaps the most significant difference lies in the mode of infection; whereas most entomopathogens infect their hosts through the gut following consumption, fungi typically penetrate the insect cuticle and thus are the only major pathogens known to infect insects with sucking mouthparts, orders Hemiptera and Homoptera (Roberts and Humber, 1981).

LIFE CYCLE

The life cycle of entomopathogenic fungi on the insect cuticle begins with spore germination and penetration, followed by a rapid proliferation of fungal cells which ultimately results in the death of the host. Host death may be followed by the production of infective spores that can insert immediately to repeat the cycle, or by the production of resting spores or other resistant structures that requires a period of dormancy.

FUNGAL INFECTION PROCESS

It is reported that after contact with a potential host infective spores of entomopathogenic fungi adhere to the insect cuticle if host recognition is positive (Al-Aidroos and Roberts, 1978). Adhesive processes involve both physical and chemical interactions and

are probably important (Fargues, 1984). Epicuticular compounds such as fatty acids, amino acids, and glucosamines are thought to play a significant role in determining the specificity and pathogenicity of entomopathogenic fungi (Boucias and Pendland, 1984; Kerwin, 1983; Smith and Grula, 1982; Woods and Grula, 1984).

Spore germination is highly dependent on moisture, and probably requires free water (Kramer, 1980; Newman and Carner, 1975; Roberts and Campbell, 1977; Shimazu, 1977), but this requirement may be met by moisture conditions of the microclimate in the absence of measureable precipitation (Kramer, 1980; Mullens et al., 1987). The penetration of entomopathogenic fungi to the cuticle is accomplished by the germ tube itself or by the formation of an appressorium that attaches to the cuticle and gives rise to a narrow penetration peg (Boucias and Pendland, 1982; Roberts and Humber, 1981). Penetration is both a mechanical and an enzymatic process (Charnley, 1984; McCoy et al., 1988; St. Leger et al., 1987a, 1988b).

Vegetative growth in the insect hemocoel is common to most entomopathogenic fungi (Roberts and Humber, 1981) and is usually described by discrete yeast-like structures or hyphal bodies. This form of growth, in contrast of the typical filamentous fungal mycelium, allows the entomopathogen to disperse rapidly and colonize the insect's circulatory system and increases the fungal surface area that is in contact with the nutrient medium. Several species of the order Entomophthorales produce vegetative protoplasts (cells without cell walls) within the haemocoel (Butt et al., 1988; Latge et al., 1987; Nolan, 1985) which may help the pathogen to escape detection by the host's immune responses. The length of the incubation period varies among species; however, disease development during the vegetation stage is typically temperature-dependent (Carruthers and Soper, 1987; Carruthers et al., 1988; Hall, 1981). Fungi have been observed to elicit insect immune responses, but it is not known what role they play in preventing or slowing the development of mycoses (Butt et al., 1988; Gupta, 1986; St. Leger et al., 1988a).

REQUIREMENT OF ENZYMES DURING PENETRATION

Entomopathogenic fungi penetrate the host cuticle shortly after germination or after limited hyphal growth (Butt et al., 1988; Wraight et al., 1990; St. Leger, 1993). This can occur between 24 to 48 h under ideal conditions (Wraright et al., 1990). Most often, pathogens produce penetration hyphae (or pegs) from appresoria, but occasionally hyphae may penetrate the cuticle directly (Schreiter et al., 1994). Penetration sites were often observed as dark, melanotic lesions in the epicuticle that indicate degradation of the epicuticle, known to be the result of enzymes and mechanical pressure (Zacharuk, 1973).

Shortly after host death, the fungal hyphae penetrate the cuticle from within and terminate in the formation of sporophores (usually conidiophores) that yield asexual spores (conidia) which function as dispersive and infective units. In many species of fungi, the production of conidia is highly dependent on moisture (Millstein et al., 1983; Wilding, 1969). Conidia are the infective propagules of secondary infection, determining disease development and spreading within a season. Environmental factors that control conidial production, survival, and germination are critical to the rate of epizootic development (Carruthers and Soper, 1987). Proteins are major components of insect cuticle and a recyclable resource for the insect. Therefore, both insects and entomopathogenic fungi produce a variety of cuticle-degrading proteases (Samuels and Paterson, 1995). A number of cuticle-degrading enzymes are produced during penetration of the host, including proteases, lipases and chitinases (Smith et al., 1982). Proteases have been shown to play a key role in the penetration process and a wide range has been identified, including trypsin, chymotrypsin, elastases, collagenase and chymoelastase (St. Leger et al., 1988b; Butt et al., 1990; Bidochcka and Knachatourians, 1988).

In enzymatic degradation, the chymoelastase serine protease Pr1 serves as a major cuticle-degrading enzyme because 70% of the cuticle comprises of protein (Hepburn, 1985). Pr1 is reported

to possess considerable ability to degrade cuticle (St. Leger et al., 1987b), by its high concentration at the site of penetration peg (Goettel et al., 1989; St. Leger et al., 1987a). This leads to the notion that treatments of Pr1 inhibitor prevented the infection of *Manduca sexta* larvae. The major role of Pr1 is to degrade cuticle proteins, making them available for fungal nutrition (St. Leger et al., 1988a). Pathogenicity of *V. lecanii* related to various growth characteristics and enzymatic activities indicates that high germination and sporulation rates as well as the production of extra cellular chitinases, are correlated with the virulence of *V. lecanii* to the aphid *Macrosiphoniella sanborni* (Jackson et al., 1997). *Verticillium lecanii* pathogenesis also involves the production and diffusion of toxins with insecticidal properties (Claydon and Grove, 1982; Gindin et al., 1984). Several extracellular cuticle-degrading proteinases are produced by the insect pathogenic fungus Metarhizium anisopliae such as PR1, a determinant of pathogenicity. Paterson et al. (1994) reported that the proteinaceous component(s) of insect cuticle was capable of inducing PR1 production.

In our previous studies (Shahid et al., 2003), locally isolated entomopathogenic fungi were grown on sabourd dextrose medium, which showed high pathogenicity on *Heliothus armigera* and cotton aphids in laboratory bioassay. The experiments were designed to check similarities at the level of virulence-determining factors. The partially purified proteins exhibited different banding patterns under similar growth conditions when studied through silver staining. The results of the study showed that the presence of VCP1 serine-like proteases were not the only virulence factors, as some of the isolates did not show any homology in immunodetection studies. One of the isolates, showing pathogenicity towards *Heliothus* and aphids with strong homology for VCP1 protease, was grown large-scale in liquid medium. Isoelectric focusing (IEF) purification of secretary proteins was carried out through chromatography. The combined effect of fungal spores with selected fractions on cotton aphids reduced the lethal time and increased the mortality percentage.

Sattar (2002) studied three local strains of entomopathogenic fungi belonging to *Metarhizium, Verticillium* and *Beauveria* for the presence of virulence factors pathogenic towards sucking insects of cotton. *Metarhizium* strain v245 exhibited a 60 kD protease, while the reported molecular weights of Pr1 and VCP1 proteases were 33 kD and 40 kD, respectively (St.Leger et al., 1993). The other two species, *Verticillium* and *Beauveria,* expressed 45 kD and 70 kD proteases, respectively, which exhibit homology with VCP1 and Pr1 proteases. The data indicates that the proteases obtained from the local environment can differ in their interaction with the host. Biotoxicity assays were performed to determine the pathogenicity and level of virulence of the specific proteases. Maximum mortality was observed in *Verticillium* spp against aphids (Sattar, 2002).

THE EFFECT OF COMBINING ENTOMOPATHOGENS WITH OTHER MICROORGANISMS

Public concern about chemical residues on fruits, vegetables and other crops has led to a progressive increase of interest in alternative strategies for the control of diseases and pests. The application of biological control is increasing largely because of greater environment awareness and food safety concerns, as well as because of the failure of conventional chemicals due to an increased number of insecticide resistant species (Dent, 1993). Synergistic effects resulting from the combination of entomopathogenic nematodes with other entomopathogens have been reported in a number of studies (Koppenhöfer and Kaya, 1997; Koppenhöfer et al., 1999; Thurston et al., 1993, 1994).

Nematodes

Combining entomopathogens with other entomopathogenic nematodes would result in synergistic interactions that enhance the potential for biological control. In studies targeting other insect pests, synergistic interactions have been observed from certain combinations of entomopathogenic nematodes with other pathogens. For example, synergistic virulence

to *Cyclocephala* spp. was observed in combinations of entomopathogenic nematodes with *Paenibacillus popilliae* (Thurston et al., 1993, 1994), or with *Bacillus thuringiensis* Berliner subspecies *japonensis* (Koppenhöfer and Kaya, 1997; Koppenhöfer et al., 1999). However, interactions between entomopathogenic nematodes and other entomopathogens can also be antagonistic (Baur et al., 1998; Brinkman and Gardner, 2000; Koppenhöfer and Kaya, 1997). In addition to entomopathogenic nematodes, several other entomopathogens have been reported to occur naturally including the entomopathogenic fungi *Beauveria bassiana* (Balsamo), Vuillemin and *Metarhizium anisopliae* (Metschnikoff), Sorokin (Sri-Arunotai et al., 1975; Swingle and Seal 1931), and *Paecilomyces* sp. (Sikorowski, 1985), and the bacterium *Serratia marcescens* Bizio (Sri-Arunotai et al., 1975). Field trials with *B. bassiana* and *M. anisopliae* caused significant reductions in *Curculio caryae* larval populations, but the levels of control were generally low (Gottwald and Tedders, 1983; Harrison et al., 1993; Shapiro-Ilan, 2003). Aside from the entomopathogenic nematodes *B. bassiana* and *M. anisopliae*, no other pathogens that naturally occur in *C. caryae* have been tested as microbial control agents for C. caryae suppression (Shapiro-Ilan, 2003).

Shapiro-Ilan et al. (2004) studied combining entomopathogenic fungi with entomopathogenic nematodes on the larvae of *Curculio caryae* and pecan weevil. The experiments were conducted in the laboratory by applying the nematodes *Steinernema carpocapsae* or *Heterorhabditis indica* with the fungi *Metarhizium anisopliae*, *Beauveria bassiana*, or *Paecilomyces fumosoroseus*, or the bacterium *Serratia marcescens*. After 14 days of application, the mortality of *C. caryae* was determined. In all pathogens, combination antagonism was determined. Additive effects were seen in *M. anisopliae* with *H. indica*. Depending on the application rate, *S. carpocapsae* combined with *B. bassiana* or *S. marcescens* also resulted in additivity. *S. carpocapsae* generally caused greater *C. caryae* mortality than other pathogens. *P. fumosoroseus* and *S. marcescens* were not pathogenic to *C. caryae* when applied alone. It was concluded that the pathogen combinations are not likely to improve the suppression of *C. caryae* larvae beyond what is expected from a single application of the pathogen with greatest virulence (Shapiro-Ilan et al., 2004).

Bacteria

Biopesticides based on the fungus *Metarhizium anisopliae* and bacteria *Bacillus thuringiensis* (Bt) were used by Shahid et al., (2003) on the stem borer and leaf folder of rice, which have reduced the population of these pests effectively both in laboratory and in the field. In the laboratory, bioassay biopesticides were very useful on the larvae of *Helicoverpa armigera*. By using the combination of fungal and bacterial formulation the increase in mortality was studied and was established to be 100% after 96 h. In the field trials, a significant effect on the stem borer and leaf folder of rice was observed (Shahid et al., 2003). The field trials of biopesticides based on fungus and bacteria were performed for the control of cotton pests (Shahid et al., 2004). When the cotton field was attacked with bollworms, the biopesticides were sprayed onto the field. The criterion for the evaluation of the effect was based on pre- and post-spray pest scouting. The number of larvae was counted randomly in each replicate. The maximum larvae were observed in all the treatments. Due to the effect of the spray, the population of larvae was reduced and maximum reduction was observed with the combination of Bt and a fungus spray. Based on these results, the biopesticide can safely be recommended for the control of rice and cotton pests with no harmful effect on its predators as in the case of chemical pesticides.

ENTOMOPATHOGENIC FUNGI AS BIOPESTICIDES

Entomopathogenic fungi are usually identified based on the growth on insect cadavers. Most research on entomopathogenic fungi has been aimed at developing them as inundative biological control agents of insects, mites and ticks, despite the great potential for use in conservation and classical biocontrol strategies (Butt et al. 2001; Goettel et al. 2005; Vincent et al. 2007). This is normally achieved through a strategy in which pest control relies on the action of the

released agent but not on successive generations of the fungus. Under this paradigm, over 170 products have been developed based on at least 12 species of fungi (Faria and Wraight, 2007). Despite there being an estimated 700 species of entomopathogenic fungi in approximately 90 genera (Roberts and Humber, 1981), most of the commercially produced fungi are species of *Beauveria, Metarhizium, Lecanicillium* and *Isaria* that are relatively easy to mass produce. Attention has focused predominantly on the technical aspects of biopesticide development, such as mass production and formulation, and the selection of strains with rapid kill. Production requirements include reasonable cost, long-term stability, and, most importantly, consistent efficacy under field conditions. The prevalent methods involve the production of diaspores (dispersal units) by induction of aerial conidiation on solid growth media, production of blastospores by yeast-like growth in liquid media or growth of hyphal biomass in liquid or solid media (Faria and Wraight, 2007).

For control of insect pests in phylloplane, suspensions of aerial conidia including blastospores are applied in spray applications, e.g. *M. anisopliae* var. *acridum* for locust control in Africa (Langewald and Kooyman, 2007). The numerous, discrete, infective propagules provided by spore forms satisfy the requirement for complete coverage of the foliar surface to ensure contact and infection of the insect host. To improve dispersion, hydrophobic conidia are often formulated in oil or added to spray mixes containing wetting agents as adjuvants. Spray preparations of hydrophilic blastospores can include wetting agents as adjuvants usually formulated as wettable powders or water-dispersible granules. Since propagule persistence of fungi on the foliar surface is affected by solar radiation, considerable effort has focused on the protection of these entomopathogens by incorporating solar blockers and sunscreens (Inglis et al., 2001). However, to open up a wider array of biocontrol strategies there is a need to significantly improve our understanding of the ecology of entomopathogenic fungi outside of the insect host, especially fungal life-history strategies and their role in the ecosystem.

A number of recent discoveries suggest that current approaches to insect control with pathogenic fungi require revision. For example, rhizosphere competence by strains of *M. anisopliae* is dependent on the plant community and not necessarily on the presence of an insect host (Hu and St. Leger, 2002) while strains of *B. bassiana* exist as endophytes in various plant species and exhibit the potential for insect and plant disease suppression (Vega, 2008; Ownley et al., 2008).

RELATIONSHIP BETWEEN FUNGUS AND HOST

Entomopathogenic fungi infect their insect hosts by penetrating through the cuticle or through body openings (Tanada and Kaya 1993). They have evolved specialized mechanisms for the enzymatic degradation of the integument and for overcoming insect defense compounds. The relationships by which different fungal species obtain energy from their insect hosts (i.e., their econutritional mode) include biotrophy (nutrition derived only from living cells, which ceases once the cell has died), necrotrophy (utilization of dead tissues), and hemibiotrophy (initially biotrophic and then becoming necrotrophic). Recent phylogenetic studies indicate that the ability to utilize insects as a source of nutrition has arisen more than once among fungi (Spatafora et al., 2007). Scale insects, particularly Coccidae and Aleyrodidae, have the greatest diversity of fungal pathogens documented (Humber, 2008); these insects occur in dense and mainly immobile populations feeding on plants. The sustained proximity between these insects, fungi and other potential hosts may provide pathogenic fungi with the opportunity to move from plant to insect and beyond. Indeed, scale insects and their pathogenic fungi provide model systems for studying the fundamental aspects of host-fungal pathogen interactions. Fungi within the genus *Hypocrella* (Clavicipitaceae) form small stromata utilizing the nutrients available from one to a few scale insects under each stroma. However, a few *Hypocrella* species produce gigantic stromata (Hywel-Jones and Samuels, 1998) and these can only form with sustained nutrition

from the plant after the insect host is destroyed. This is an extreme example of the nutritional adaptability that some insect pathogenic fungi exhibit. This also highlights the diversity of nutritional modes and the ability of entomopathogenic fungi to switch between them.

A critical question is whether species of *Metarhizium*, *Beauveria*, *Lecanicillium* and *Isaria* (Luangsa-ard et al., 2005; Sung et al., 2007) function in nature as ecologically obligate insect parasites or make use of additional sources of nutrition. Meyling and Eilenberg (2007) considered *Beauveria* and *Metarhizium* to function primarily as insect parasites but did not discount the possibility of additional nutritional modes. Insect parasitism by these species is common in nature (Ormond et al., 2006; Meyling and Eilenberg, 2007). However, there is increasing evidence that they exhibit a more dynamic life-history pattern than previously thought. Based on the abundance of entomopathogenic fungi obtained from the surface of 1,700 individual arthropods captured in aspen-dominated woodlands in western Canada (*B. bassiana* represented one-quarter of all isolates), it appears that entomopathogens are common components of the surface mycota of arthropods and are not necessarily restricted to diseased insects (Greif and Currah, 2007). There is also increasing evidence that *Beauveria*, *Metarhizium* and related genera can act as mycoparasites and plant endophytes, as well as interact with plant roots. Entomopathogenic fungi exhibit a diverse array of adaptations to insect parasitism. These include the general ability to overcome insect immune defenses and obtain nutrition from insects, but also less well-studied behavioral responses (Roy et al., 2006). Host-altered behavior by some fungi has been demonstrated (e.g. summit disease in which infected insects exhibit climbing behavior), but there are considerably fewer examples with hypocrealean-infected insects than in entomophthoralean-infected ones (Roy et al., 2006). However, we would caution against concluding that the scarcity of these adaptations in the entomopathogenic Hypocreales is evidence that these fungi are not highly specialized insect parasites, but much more basic ecological research is required. Behavioral avoidance of entomopathogenic fungi has also been reported for various insects: *B. bassiana* is avoided by *Anthocoris nemorum* (Meyling and Pell, 2006), while *Coptotermes lacteus* avoids *M. anisopliae* (Staples and Milner, 2000). Avoidance indicates recognition of the fungus by the insect, although the specific mechanism for avoidance is not known.

A major handicap in the understanding of the ecology of entomopathogenic fungi has been a lack of phylogenetic information to explain the history of the interactions. Phylogenetic classifications based on DNA analysis have helped to improve and stabilize our understanding of fungal relationships (Blackwell et al., 2006). Phylogenetic studies have been important to understand insect fungi. For example, asexual fungi can now be placed among their nearest sexual relatives, and previously used terms such as Deuteromycota as a taxon have been abandoned completely. Insect parasites in the Hypocreales have been discovered to have convergent morphologies and moreover, different histories as symbionts.

PLANT DISEASE ANTAGONISTS

In plant pathology, biological control most often refers to the use of natural or modified fungi or bacteria that are antagonists of plant pathogens. The term antagonism refers to a generalized mechanism by which the survival or disease-causing activity of a pathogen is reduced. Several mechanisms of antagonism against plant pathogens have been identified. These include production of various metabolites, such as antibiotics, bioactive volatile compounds (e.g., ammonia, hydrogen cyanide, alkyl pyrones, alcohols, acids, esters, ketones and lipids) and enzymes. Other mechanisms are competition (for niche or infection site, carbon, nitrogen or various minerals), parasitism, hypovirulence, induced systemic resistance and increased plant growth response (Ownley and Windham, 2007).

In addition to activity against insects, there is substantial evidence that some entomopathogenic fungi, including *Beauveria bassiana* (Ownley et al., 2004; Ownley et al., 2008) and species of *Lecanicil-*

lium (Askary et al., 1998; Benhamou and Brodeur 2000, 2001; Kim et al., 2007, 2008) are also antagonistic to plant pathogens. The mechanisms of antagonism utilized by *B. bassiana* may include antibiosis (Renwick et al., 1991; Reisenzein and Tiefenbrunner, 1997; Bark et al., 1996; Veseley and Koubova, 1994; Lee et al., 1999), competition (Ownley et al., 2004) and induced systemic resistance (Griffin et al., 2006; Ownley et al., 2008).

POSSIBLE ROLES OF ENTOMOPATHOGENIC FUNGI IN NATURE

Various unexpected roles have been reported for fungal entomopathogens, including their presence as fungal endophytes, plant disease antagonists, rhizosphere colonizers and plant growth promoting fungi (Vega et al., 2009).

Fungal endophytes

Endophytes infect above ground internal plant tissues without causing symptoms. They are garnering increased attention because they are ubiquitous and have immense diversity and varied roles (Saikkonen et al., 2006; Arnold and Lutzoni, 2007). Some fungal endophytes protect host plants against pathogens and herbivores (Arnold et al., 2003; Schulz and Boyle, 2005; Arnold and Lewis, 2005; Rudgers et al., 2007), and many fungi traditionally known as insect pathogens have been isolated as endophytes, including species of *Acremonium*, *Beauveria*, *Cladosporium*, *Clonostachys* and *Isaria* (Vega, 2008; Vega et al., 2008).

Rhizosphere colonizers and plant growth-promoting fungi

Entomopathogenic fungi in the Hypocreales are ubiquitous members of the soil microbiota. The entomopathogenic fungal species most frequently isolated from soils in temperate regions belong to the genera *Beauveria*, *Isaria* (Cordycipitaceae) and *Metarhizium* (Clavicipitaceae) (Meyling and Eilenberg, 2007). As an environment, soil presents opportunities and challenges to entomopathogenic fungi. It protects from damaging solar radiation and acts as a buffer against extremes of temperature and water availability (Gaugler et al., 1989; Inglis et al., 2001; Roberts and Campbell, 1977; Rangel et al., 2005). Furthermore, it is a habitat for many potential insect hosts, some of which occur at high densities. Continuity in proximity to potential hosts is a factor in the evolution of fungal entomopathogenicity (Humber, 2008). However, soil is also infused with antimicrobial metabolites secreted by microbes that can impair the ability of entomopathogenic fungi to infect their hosts. For example, Groden and Lockwood (1991) identified a significant trend of lower mortality of the Colorado potato beetle by *B. bassiana* with increased soil fungistasis levels. A dead or dying insect infected by an entomopathogenic fungus represents a potential source of energy for other opportunistic soil microorganisms. Some species of hypocrealean entomopathogens produce secondary metabolites within their insect hosts that are postulated to help the fungus outcompete opportunists during the saprotrophic phase of insect utilization (Strasser et al., 2000).

Species of *Beauveria* and *Metarhizium* that have infected and killed an insect in soil produce only limited somatic growth from the fungus-infected cadaver. This has been taken as evidence that these fungi rely predominantly on the insect rather than on the soil for carbon (Inglis et al., 2001; Pereira et al,. 1993; Gottwald and Tedders 1984). However, in the rhizosphere, free carbon is abundant and there is evidence that entomopathogenic fungi interact with plant roots for growth or survival (St. Leger, 2008). Between 10 and 40% of the carbon that is assimilated by a plant is transferred into the soil in the form of exudates, mucilage, sloughed root cells and lysates (Andrews and Harris, 2000; Bardgett, 2005). This carbon is exploited by a diversity of saprotrophic microorganisms in the rhizosphere (Cooke and Whipps 1993; Whipps, 2001). In most cases, it is still not clear whether this is purely a one-way interaction benefiting only microbial saprotrophs, or whether a mutualistic interaction has evolved in which the plant also benefits from the provision of mineral nutrients or protection from parasites and herbivores (Singh

et al., 2004). Studies on plant parasitic nematodes and their microbial antagonists have demonstrated that nematode control is greatest on roots that support the highest rhizosphere colonization of *Pochonia chlamydosporia*, a facultative fungal pathogen of nematodes. The extent of rhizosphere colonization by *P. chlamydosporia* varies on different plant cultivars and between different isolates of the fungus (De Leij and Kerry, 1991; Bourne et al., 1996; Kerry, 2000). These studies clearly demonstrate a relationship between rhizosphere competence and a functional role such as biological control. *Metarhizium anisopliae* increased stand density and the fresh weight of field corn after conidia were applied to corn seeds prior to planting, in an attempt to reduce damage caused by wireworms (Kabaluk and Ericsson, 2007). The mechanism for this effect on yield remains unknown.

A NEW PARADIGM FOR ENTOMOPATHOGENIC FUNGI AND FUTURE RESEARCH

Despite the publication of approximately 7,000 papers on topics related to entomopathogenic fungi since 1983 (Vega et al,. 2009), there is still limited success in solving agricultural problems with entomopathogenic fungi. The following proposed research areas should lead to a new paradigm for entomopathogenic fungi that should refocus our efforts and hopefully lead to exciting new findings that will bring success to the field.

Antagonist studies

Although the potential for biological control of plant pathogens has been clearly demonstrated with certain entomopathogenic fungi, the key to a successful exploitation of these organisms in agriculture is identifying and understanding the operative mechanisms of biocontrol activity. New evidence suggests that *B. bassiana* and *Lecanicillium* species employ multiple mechanisms that vary with plant pathogen, but may also vary with plant host species or cultivar. In addition, efficacy will be affected by a myriad of abiotic and biotic environmental factors. Using model plant systems, profiles of global gene expression in response to endophytic or rhizosphere colonization can be examined in the absence of other variables. In addition to expected changes in expression profiles of recognized plant defense response genes, genome-wide expression arrays could reveal novel plant genes that respond to colonization by entomopathogenic fungi. Naturally occurring nonpathogenic epiphytic and endophytic microorganisms will also influence the efficacy of entomopathogenic fungi against plant pathogens. Identifying beneficial and deleterious relationships with other microorganisms may allow for the manipulation of agricultural systems to enhance the positive influences. Likewise, identification of abiotic factors, such as soil characteristics that enhance or inhibit biological control of soil-borne plant pathogens, would allow manipulation of these factors and improvements in efficacy. Such abiotic factors may include minerals needed as co-factors for the production of bioactive compounds involved in the biological control mechanisms of entomopathogenic fungi. By gaining a greater understanding of all of the interacting factors related to mechanism, significant improvements in efficacy against plant pathogens with entomopathogenic fungi should be possible.

Production technology for fungal biocontrol agents

Both the rhizosphere and the phylloplane present unique challenges to biological control with living fungal agents. The environmental and ecological variations within agro-ecosystems have made consistent insect pest management with fungal pathogens difficult to achieve at a commercial level. The use of a generalized approach to the formulation and application of microbial biocontrol agents has in part, led to this inconsistency in control. A more detailed understanding of the pathogen-insect ecology as well as other environmental and ecological interactions is needed to improve the consistency of control for these living microbial pest control agents. For insect pests of the rhizosphere, fungal biological control agents are typically applied as granules containing hyphae or spore-hyphae preparations. Granules may contain infective conidia or rely on primary growth and *in situ* secondary sporulation for the formation of infective conidia. The conidia-

containing granules must be adequately dispersed and remain viable in the soil to insure contact with foraging insect pests.

Recently, *M. anisopliae* was shown to be capable of producing sclerotia in liquid culture fermentation (Jaronski and Jackson, 2008). Sclerotia are overwintering structures formed by many plant pathogenic fungi that sporulate to produce infective conidia when environmental conditions are suitable for infection of their host plant. The ability of *M. anisopliae* to form sclerotia may be important for rhizosphere competence following a pattern seen in phytopathogenic fungi. The use of sclerotial preparations for granular application of *M. anisopliae* in soil and the use of conidia or blastospores in foliar applications for phylloplane insects are examples of how the ecology of the fungus-insect interaction directs the production and use of appropriate infective propagules. Likewise, the use of endophytic entomopathogenic fungi for insect control will require an understanding of the ecological factors that enhance the ability of the fungus to become endophytic. Awareness of these ecological factors will guide the development of production and formulation technologies that deliver optimally infective fungal propagules.

Formulation of fungal propagules

The formulation of propagules of fungal entomopathogenic fungi for use in biocontrol has been guided by the need to improve product shelf-life, biocontrol efficacy, and/or the physical characteristics of the product for application (Wraight et al., 2001). Undoubtedly, these goals are often conflicted with ecological considerations. For control of insect pests of the phylloplane, spore suspensions are applied in spray applications. The numerous, discrete, infective propagules provided by spore forms satisfy the requirement for complete coverage of the foliar surface to ensure contact and infection of the insect host. Formulations that improve spore desiccation tolerance or shelf-life such as cryoprotectants or oils may inhibit spore germination or intimate contact of the spore with the insect host, resulting in reduced biocontrol efficacy.

Recently, research was initiated to analyze the surface chemistry of the spores of entomopathogenic fungi, an important contribution towards understanding their ecology. For example, *Isaria fumosorosea* blastospores were found to have a basic, monopolar, hydrophilic surface with an isoelectric point of 3.4 (Dunlap et al., 2005). The isoelectric point is the pH at which a surface or compound has a neutral charge. At a pH higher than 3.4, the surface charge of *I. fumosorosea* is negative, and at a more acidic pH the surface is positively charged. Therefore, the pH of the environment or of the formulation can affect the charge of the spore surface and its ability to adhere to the insect cuticle or other surfaces. Similar work on the characterization of the surface chemistry of *Beauveria bassiana* spore forms has also been reported (Holder et al., 2007). A directed approach to the formulation for improved biocontrol efficacy should include an understanding of the fungal spore–insect surface chemistries and how they interact to enhance adhesion and fungal infection. Understanding how the insect pest or the microbial pathogen interacts or survives in a given ecological environment is critical in directing the use of appropriate formulations.

ROLE OF BIOTECHNOLOGY

Changes in the conventional crop protection system and in the prescribed application of chemicals are underway. Biotechnology provides exciting opportunities for improving fungi for pest control and this technology is more valuable in elucidating the mechanisms of pathogenicity. By using *Aspergillus nidulans,* the transformation of *M. anisopliae* has been reported. Progress in understanding the mechanisms of pathogenicity is now being made, particularly in the area of cuticular penetration where the key enzyme is probably an endoprotease (St. Leger et al., 1986). To increase the speed of insect kill, it may be possible in the future to insert toxin genes from *Bacillus thuringiensis* into fungi. However, improvement in strains achieved by genetic manipulation could be more useful. Future research should concentrate on the development of formulation and the targeting of the pests of economically valuable crops. If these strategies are followed, the understanding of

entomopathogenic fungi will increase and mycoinsecticides may become commercially available. By achieving this aim, agriculture will benefit from prolonged pest control, reduced risk of resistance and a high degree of safety to non-target organisms without disturbing the agro-ecosystem and the quality of environment. We believe that insights gained from biotechnology will result in the effective use of these promising organisms as an integral part of agricultural systems throughout the world.

CONCLUSIONS

The application of entomopathogenic fungi in biological control is increasing largely because of greater environmental awareness, food safety concerns and the failure of conventional chemicals due to an increasing number of insecticide resistant species. In determining whether the use of entomopathogenic fungi has been successful in pest management, it is necessary to consider each case individually, and direct comparisons with chemical insecticides are usually inappropriate. Gelernter and Lomer (2000) concluded that for any microbial control agent to be successful, technical efficacy was essential but had to be combined with at least two other criteria from the following: practical efficacy (easy and cheap uptake), commercial viability (profitability), sustainability (long-term control) and/or public benefit (safety).

The safety of entomopathogenic fungi for humans, the environment and non-target organisms is clearly an important criterion for consideration and each insect–fungus system must again be considered on a case-by-case basis. However, existing research suggests that there are minimal effects of entomopathogenic fungi on non-targets, and they offer a safer alternative for use in integrated pest management (IPM) than chemical insecticides (Goettel and Hajek, 2000; Pell et al., 2001). Successes with these fungi are often based on considerable, multidisciplinary financial investments in research and development from industry, aid agencies and governments. When commercial interests are absent, especially in the development of classical, inoculative and conservation strategies, then long-term government support is essential (Gelernter and Lomer, 2000). Most entomopathogenic fungi are best used when total eradication of a pest is not required; instead, insect populations are controlled below an economic threshold, with some crop damage considered as acceptable. In addition, entomopathogenic fungi have an essential role in IPM if they can be used in conjunction with other strategies for sustainable pest control.

REFERENCES

Al-Aidoors, K., and *D. W. Roberts* (1978). Mutants of *Metarhizium anisopliae* with increased virulence toward mosquito larvae. *Canadian Journal of Genetics and Cytology*. **11**, 211-219.

Andrews, J. H., and *R.F. Harris* (2000). The ecology and biogeography of microorganisms on plant surfaces. *Annual Review of Phytopathology,* **38**, 145-180.

Arnold, A. E., and *F. Lutzoni* (2007). Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? *Ecology*, **88**, 541-549.

Arnold, A. E., and *L. C. Lewis* (2005). Ecology and evolution of fungal endophytes, and their roles against insects. In: Vega FE, Blackwell M (Eds.), *Insect-Fungal Associations: Ecology and Evolution*. Oxford University Press, New York, pp.74-96.

Arnold, A. E., Mejía, L. C., Kyllo, D., Rojas, E. I., Maynard, Z., Robbins, N., and *E.A. Herre* (2003). Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences,* **100**, 15649-15654.

Artjariyasripong, S., Mitchell, J. I., Hywel-Jones, N., L., and *E.B.G. Jones* (2001). Relationship of the genus *Cordyceps* and related genera based on parsimony and spectral analysis of partial 18S and 28S ribosomal gene sequences. *Mycoscience*, **42**, 503–517.

Askary, H., Carrière, Y., Bélanger, R. R., and *J. Brodeur* (1998). Pathogenicity of the fungus *Verticillium lecanii* to aphids and powdery mildew. *Biocontrol Science and Technology,* **8**, 23-32.

Austwick, P.K.C. (1980). The pathogenic aspects of the use of fungi: The need for risk analysis and registration of fungi. *Ecological Bulletin*, **31**, 91-102.

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and *J.M. Vivanco* (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Ann. Rev. Plant Biology*, **57**, 233–266.

Bałazy, S. (1993). Entomophthorales. In: Flora of Poland, Fungi (Mycota), Instytut Botaniki, Krakow, Poland, vol. **24**.

Bardgett, R. D. (2005). The Biology of Soil: A Community and Ecosystem Approach. Oxford University Press, New York.

Bark, Y. G., Lee, D. G., Kim, Y. H., and *S.C. Kang*. Antibiotic properties of an entomopathogenic fungus, *Beauveria bassiana*, on *Fusarium oxysporum* and *Botrytis cinerea*. *Korean Journal of Plant Pathology*, **12**, 245-250.

Baur, M.E., Kaya, H.K., Tabashnik, B.E., and *C.E. Chilcutt* (1998). Suppression of diamondback moth (Lepidoptera: Plutellidae) with an entomopathogenic nematode (Rhabditida: Steinernematidae) and *Bacillus thuringinensis* Berliner. *J. Econ. Entomol,* **91**, 1089-1095.

Bell, J. V. (1974). *Verticillium lecanii* on the bean rust fungus, *Uromyces appendiculatus. Trans. Br. Mycol. Soc,* **79**, 362-364.

Benhamou, N., and *J. Brodeur* (2001). Pre-inoculation of Ri T-DNA transformed cucumber roots with the mycoparasite, *Verticillium lecanii,* induces host defense reactions against *Pythium ultimum* infection. *Physiological and Molecular Plant Pathology*, **58**, 133-146.

Benhamou, N., and *J. Brodeur* (2000). Evidence for antibiosis and induced host defense reactions in the interaction between *Verticillium lecanii* and *Penicillium digitatum*, the causal agent of green mold. *Phytopathology*, **90**, 932-943.

Bidochka, M. J., and *G.G. Khachatourians* (1988). Regulation of extracellular protease in the entomopathogenic fungus *Beauveria bassiana. Experimental mycology,* **12**, 161-168.

Bing, L. A., and *L.C. Lewis* (1993). Occurrence of the entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin in different tillage regimes and in *Zea mays* and virulence towards *Ostrinia nubilalis* (Hubner). *Agr. Ecosyst. Environ*., **45**,147–156.

Bing, L. A., and *L.C. Lewis* (1992). Endophytic Beauveria bassiana (Balsamo) Vuillemin in corn: the influence of the plant growth stage and *Ostria nubialis* (Hu¨bner). *Biocontrol Sci. Technology*, **2**, 39–47.

Bing, L. A., and *L.C. Lewis* (1991). Suppression of *Ostrinia nubilalis* (Hubner) (Lepidoptera, Pyralidae) by endophytic *Beauveria bassiana* (Balsamo) Vuillemin. Environ. *Entomology*, **20**, 1207–1211.

Blackwell, M., Hibbett, D. S., Taylor, J. W., and *J.W. Spatafora* (2006). Research coordination networks: a phylogeny of the kingdom Fungi (Deep Hypha). *Mycologia*, **98**, 829-837.

Boucias, D. G., Farmerie, W. G., and *J.C. Pendland* (1998). Cloning and sequencing the cDNA of the insecticidal toxin, Hirsutellin A. *J. Invertebr. Pathol*, **72**, 258-261.

Boucias, D. G., and *J.C. Pendland* (1991). Attachment of mycopathogens to cuticle: The initial event of mycoses in arthropod hosts. In "The Fungal Spore and Disease Initiation in Plants and Animals" (G. T. Cole and H. C. Hoch, Eds.), Plenum, New York, pp.101-128.

Boucias, D. G., and *J.C. Pendland* (1984). Host recognition and specificity of entomopathogenic fungi. In "Infection Processes of Fungi" (D.W. Roberts and J. R. Aist, Eds.), pp. 185-196. Rockefeller Foundation, New York.

Boucias, D. G., and *J.C. Pendland* (1982). Ultrastructural studies on the fungus, *Nomuraea rileyi,* infecting the velvetbean caterpillar, *Anticarsia gemmatalis. Journal of Invertebrate Pathology*, **39**, 338.

Bourne, J. M., Kerry, B. R., and *F. de Leij* (1966). The importance of the host plant in the interaction between root-knot nematodes (*Meloidogyne* spp.) and the nematophagous fungus *Verticillium chlamydosporium* Goddard. *Biocontrol Science and Technology*, **6**, 539-548.

Brinkman, M. A., and *W.A. Gardner* (2000). Possible antagonistic activity of two entomopathogens infecting workers of the red imported fire ant (Hymenoptera: Formicidae). *J. Entomol. Science*, **35**, 205–207.

Bruck, D. J. (2005). Ecology of *Metarhizium anisopliae* in soilless potting media and the rhizosphere: implications for pest management. *Biol. Control*, 32, 155–163.

Burges, H. D. (1981). Safety, safety testing and quality control of microbial pesticides. In: Burges HD, editor. Microbial control of pests and plant diseases 1970-1980. London: Academic Press, pp.737-767.

Butt, T. M., Jackson, C., and *N. Magan* (2001) (Eds.). *Fungi as Biocontrol Agents: Progress, Problems, and Potential.* CABI Publishing, Wallingford, United Kingdom.

Butt, T. M., and *P. Burrows* (1990). Brookes CP. *Mycological resources*, **120**, 211-219.

Butt, T. M., Wraight S.P., Galaini-Wraight, S., Humber, R A., Roberts, D. W. and *R.S. Soper* (1988). Humoral encapsulation of the fungus *Erynia radicans* by the potato leafhopper. *Journal of Invertebrate Pathology*, **52**, 49-56.

Carruthers, R. I., Larkin, T. L., and *R.S. Soper* (1988). Simulation of insect disease dynamics: An application of SERB to a rangeland ecosystem. *Simulation*, **51**, 101-109.

Carruthers, R. I., and *R.S. Soper* (1987). Fungal diseases. In Epizootiology of Insect Diseases, J. R. Fuxa and Y. Tanada, eds. New York: John Wiley and Sons.

Charnley, A. K. (1984). Physiological aspects of destructive pathogenesis in insects by fungi: A speculative review. In "Invertebrate-Microbial Interactions" (J. M. Andersonn, A. DM, Rayner, and DWH Walton etc.). British Mycological Society Symposium 6, Cambridge Univ. Press, London, pp. 229-271.

Claydon, N., and *J.F. Grove* (1982). Insecticidal secondary metabolic products from the entomopathogenous fungus *Verticillium lecanii. J. Invertebr. Pathol.,* **40**, 413-418.

Cook, R. J., Bruckart, W. L., Coulson, J. R., Goettel, M. S., Humber, R. A., Lumsden, R. D., Maddox, J. V., McManus, M. L., Moore, L., Meyer, S. F., Quimby, P.C., Jr., Stack, J. P., and *J.L. Vaughn* (1986). Safety of microorganisms intended for pest and plant disease control: A framework for scientific evaluation. *Biological Control,* **7**, 333-351.

Cooke, R.C., and *J.M. Whipps* (1993). *Ecophysiology of Fungi*. Wiley-Blackwell, Boston.

Copping, L. G (2004). editor. The manual of biocontrol agents. 3rd ed. Alton: British Crop Protection Council, pp. 702.

De Leij, F. and *B.R. Kerry* (1991). The nematophagous fungus, *Verticillium chlamydosporium* Goddard, as a potential biological control agent for *Meloidogyne arenarea* (Neal) Chitwood. *Revue de Nématologie*, **14**, 157-164.

Dent, D. R. (1993). The use of *Bacillus thuringiensis* as an insecticide. In: Exploitation of Microorganisms (Ed.Gareth jones) Chapman and Hall, London, pp.19-44.

Deshpande, M. V. (1999). Mycopesticide production by fermentation: Potential and challenges. *Crit. Rev. Microbiology*, **25**,229-243.

Dunlap, C. A., Biresaw, G., and *M.A. Jackson* (2005). Hydrophobic and electrostatic cell surface properties of blastospores of the entomopathogenic fungus *Paecilomyces fumosoroseus*. *Colloids and Surfaces B: Biointerfaces*, **46**, 261-266.

Eilenberg, J. (2002). Biology of fungi from the order Entomophthorales, with emphasis on the genera *Entomophthora*, *Strongwellsea* and *Eryniopsis*. The Royal Veterinary and Agricultural University, Copenhagen, Denmark.

Elliot, S. L., Sabelis, M.W., and *A. Janssen* (2000). van der Geest LPS, Beerling EAM, Fransen J. Can plants use entomopathogens as bodyguards? Ecol. Lett. **3**, 228–235.

Evans, H. C. (2003). Use of Clavicipitalean fungi for the biological control of arthropod pests. In: White JF, et al. (eds) Clavicipitalean fungi. Marcel Dekker, New York.

Evans, H. C. (1989). Mycopathogens of insects of epigeal and aerial habitats. In: Wilding N, Collins NM, Hammond PM, Weber JF (eds) Insect–fungus interactions. Academic Press, London, pp. 205–238.

Fargues, J. (1984). Adhesion of the fungal spore to the insect cuticle in relation to pathogenicity. In Infection Processes of Fungi, A Bellagio Conference, D.W. Roberts and J. R. Aist eds. New York: The Rockefeller Foundation.

Faria, M. R., and *S.P. Wraight* (2007). Mycoinsecticides and mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. *Biological Control,* **43**, 237-256.

Ferron, P. (1997). Biological control of insect pests by entomogenous fungi. Annual Review of Entomology 1978; 23,409. fungi of the genera *Fusarium*, *Armillaria* and *Rosellinia*. *Pflanzenschutzberichte*, **57**, 15-24.

Gaugler, R., Costa, S. D., and *J. Lashomb* (1989). Stability and efficacy of *Beauveria bassiana* soil inoculations. *Environmental Entomology*, **18**, 412-417.

Gelernter, W. D., and *C.J. Lomer* (2000). Success in biological control of above-ground insects by pathogens. In: Gurr, G., Wratten, S. (eds). Biological control: measures of success. Kluwer Academic, Dordrecht, The Netherlands, pp.297–322.

Gillespie, A. T., and *E.R. Moorhouse* (1989). The use of fungi to control pest of agricultural and horticultural importance. In Biotechnology of Fungi for Improvement of Plant Growth, J. M. Whipps and R. D. Lumsdon, eds. London: Cambridge University Press.

Gillespie, J. P., Bateman, R., and *A.K. Charnley* (1988). Role of cuticle-degrading proteases in the virulence of *Metarhizium* spp. for the desert locust, *Schistocerca gregaria*. *Journal of Invertebrate Pathology*, **71**, 128-137.

Gindin, G., Barash, I., Harari, N., and *B. Raccah* (1994). Effect of endotoxic compounds isolated from *Verticillium lecanii* on the sweet potato whitefly, *Bemisia tabaci*. *Phytoparasitica*, 22, 189-196.

Glare, T.R., and *R.J. Milner* (1991). Ecology of entomopathogenic fungi. In: Arora DK, Ajello L, Mukerji KG (eds) Handbook of applied mycology, vol 2: humans, animals, and insects. Marcel Dekker, New York, pp.547-612.

Goettel, M. S., Eilenberg, J., and *T.R. Glare* (2005). Entomopathogenic fungi and their role in regulation of insect populations. In: Gilbert L, Iatrou K, Gill S (Eds.), *Comprehensive Molecular Insect Science*, *Vol. 6.* Elsevier, Boston, pp.361-406.

Goettel, M. S., Hajek, A. E., Siegel, J. P., and *H.C. Evans* (2001). Safety of fungal biocontrol agents. In: Butt TM, Jackson C, Magan N, editors. Fungi as biocontrol agents: progress, problems and potential. Wallingford: CAB International, pp.347-376.

Goettel, M. S., and *A.E. Hajek* (2000). Evaluation of non-target effects of pathogens used for management of arthropods. In: Wajnberg E, Scott JK, Quimby PC (eds) Evaluating indirect ecological effects of biological control. CABI Publishing, Wallingford, pp.81–97.

Goettel ,M. S., and *S. T. Jaronski* (1997). Safety and registration of microbial agents for control of grasshoppers and locusts. Memoirs of the Entomological Society Canada, **171**, 83-99.

Goettel, M. S., St. Leger, R. J., Rizzo, R. I., Staples, R. C., and *D.W. Roberts* (1989). Ultrastructural localization of a cuticle degrading protease produced by the entomopathogenic fungus *Metarhizium anisopliae* during penetration of host cuticle. *Jnl. Gen. Microbiol.*, **135**, 2233-2239.

Gottwald, T. R., and *W.L. Tedders* (1984). Colonization, transmission and longevity of *Beauveria bassiana* and *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) on pecan weevil larvae (Coleoptera: Curculionidae) in the soil. *Environmental Entomology*, **13**, 557-560.

Gottwald, T. R. and *W.L. Tedders* (1983). Suppression of pecan weevil (Coleoptera: Curculionidae) populations with entomopathogenic fungi. *Environ. Entomology,* **12**, 471–474.

Greif, M. D., and *R.S. Currah* (2007). Patterns in the occurrence of saprophytic fungi carried by arthropods caught in traps baited with rotted wood and dung. *Mycologia*, **99**, 7-19.

Griffin, M.R., Ownley, B. H., Klingeman, W. E., and *R.M. Pereira* (2006). Evidence of induced systemic resistance with *Beauveria bassiana* against *Xanthomonas* in cotton. *Phytopathology*, **96**, S64.

Groden, E., and *J.L. Lockwood* (1991). Effects of soil fungistasis on *Beauveria bassiana* and its relationship to disease incidence in the Colorado potato beetle, *Leptinotarsa decemlineata*, in Michigan and Rhode Island soils. *Journal of Invertebrate Pathology*, **57**, 7-16.

Gupta, A. P. (1986). Hemocytic and Humoral Immunity in Arthropods. New York: John Wiley and Sons.

Hall, R. A., Zimmermann, G., and *A. Vey* (1982). Guidelines for the registration of entomogenous fungi as insecticides. Entomophaga, **27**, 121-127.

Hall, R. A. (1981). The fungus *Verticillium lecanii* as a microbial insecticide against aphids and scales. In Microbial Control of Pests and Plant Diseases, 1970-1980, H. D. Burges, ed. London: Academic Press.

Harrison, R. D., Gardner, W. A., and *D.J. Kinard* (1993). Relative susceptibility of pecan weevil fourth instars and adults to selected isolates of Beauveria bassiana. *Biol. Control,* **3**, 34–38.

Hassan, A. E. M., Dillion, R. J., and *A.K. Charnley* (1989). *J Invert. Pathol*, **54**, 227-279.

Heimpel, A. M. (1971). Safety of insect pathogens for man and vertebrates. In: Burges, H. D., Hussey, N. W., editors. Microbial control of insects and mites. London: Academic Press, pp. 469-489.

Hemmati, F., Pell, J. K., McCartney, H. A., and *M.L. Deadman* (2001). Airborne concentrations of conidia of *Erynia neoaphidis* above cereal fields. *Mycol Res*, **105**, 485–489.

Hepburn, H. R (1985). Structure of the integument. *In* Comprehensive insect physiology, biochemistry, and pharmacology. Vol. 3. Edited by G. A. Kerkut and L. I. Gilbert. Pergamon, Oxford, pp.1-58.

Holder, D. J., Kirkland, B. H., Lewis, M. W., and *N.O. Keyhani* (2007). Surface characteristics of the entomopathogenic fungus *Beauveria* (*Cordyceps*) *bassiana*. *Microbiology*, **153**, 3448-3457.

Hu, G., and *R.J. St. Leger* (2002). Field studies using recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Applied and Environmental Microbiology,* **68**, 6383-6387.

Humber, R. A. (2008). Evolution of entomopathogenicity in fungi. *Journal of Invertebrate Pathology*, **98**, 262-266.

Humber, R. A. (1984). Foundations for an evolutionary classification of the Entomophthorales (Zygomycetes). In: Wheeler Q, Blackwell M (eds) Fungus–insect relationships. Columbia University Press, New York, pp.166–183.

Hywel-Jones, N. L., and *G.J. Samuels* (1998). Three species of *Hypocrella* with large stromata pathogenic on scale insects. *Mycologia,* **90**, 36-46.

Ignoffo, C. M. (1973). Effects of entomopathogens on vertebrates. *Annals New York Academy Sciences,* **217**, 141-172.

Inglis, G. D., Goettel, M. S., Butt, T. M., and *H. Strasser* (2001). Use of hyphomycetous fungi for managing insect pests. In: Butt TM, Jackson CW, Magan N (Eds.), *Fungi as Biocontrol Agents: Progress, Problems and Potential.* CABI International/AAFC, Wallingford, United Kingdom, pp.23-69.

Jackson, M. A., McGuire, M. R., Lacey, L. A., and *S.P. Wraight* (1997). Liquid culture production of desiccation tolerant blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*. *Mycol. Res.,* **101**, 35-41.

Jaronski, S. T., and *M.A. Jackson* (2008). Efficacy of *Metarhizium anisopliae* microsclerotial granules. *Biocontrol Science and Technology*, **18**, 849-863.

Kabaluk, J. T., and *J.D. Ericsson* (2007). *Metarhizium anisopliae* seed treatment increases yield of field corn when applied for wireworm control. *Agronomy Journal*, **99**, 377-1381.

Kerry, B. R. (2000). Rhizosphere interactions and the exploitation of microbial agents for the biological control of plant-parasitic nematodes. *Annual Review of Phytopathology*, **38**, 423-441.

Kerwin, J. L. (1983). Fatty acid regulation of the germination of *Erynia variabilis* conidia on adults and puuparia of the lesser housefly, *Fannia canicularis*. *Canadian Journal of Microbiology*, **30**, 158.

Kim, J.J., Goettel, M. S., and *D.R. Gillespie* (2008). Evaluation of *Lecanicillium longisporum,* Vertalec® for simultaneous

suppression of cotton aphid, *Aphis gossypii,* and cucumber powdery mildew, *Sphaerotheca fuliginea,* on potted cucumbers. *Biological Control*, **45**, 404-409.

Kim, J. J., Goettel, M. S., and *D.R. Gillespie* (2007). Potential of *Lecanicillium* species for dual microbial control of aphids and the cucumber powdery mildew fungus, *Sphaerotheca fuliginea*. *Biological Control*, **40**, 327-332.

Koppenhöfer, A. M., Choo, H. Y., Kaya, H. K., Lee, D. W., and *W.D. Gelernter* (1999). Increased field and greenhouse efficacy against scarab grubs with a combination of an entomopathogenic nematode and *Bacillus thuringiensis*. *Biol. Control.* ***14****,* 37–44.

Koppenhöfer, A. M., and *H.K. Kaya* (1997). Additive and Synergistic interactions between entomopathogenic nematodes and *Bacillus thuringiensis* for scarab grub control. *Biol. Control,* **8**, 131–137.

Kramer, J. P., 1990. The house-fly mycosis caused by *Entomophthora muscae* 1980.

Laird, M., Lacey, L.A., Davidson E.W., editors. Safety of microbial insecticides. Boca Raton, FL: CRC Press, 259.

Langewald, J., and *C. Kooyman* (2007). Green MuscleTM, a fungal biopesticide for control of grasshoppers and locusts in Africa. In: Vincent C, Goettel MS, Lazarovits G (Eds.), Biological Control: *A Global Perspective.* CABI International/AAFC, Wallingford, United Kingdom 2007; p.311-318.

Latge, J. P., Sampeoro, L., Brey, P., and *M. Diaquin* (1987). *J. Gen. Microbiol*, **133**, 1987-1997.

Lee, S. M., Yeo, W. H., Jee, H. J., Shin, S. C., and *Y.S. Moon* (1999). Effect of entomopathogenic fungi on growth of cucumber and *Rhizoctonia solani*. *Journal of Forest Science*, **62**, 118-125.

Luangsa-ard, J. J., Hywel-Jones, N. L., Manoch, L. and *Samson, R. A.,* 2005. On the relationships of *Paecilomyces* sect. Isarioidea species. *Mycological Research,* **109**, 581-589.

MacLead, D. M. (1963). Entomophthorales infections. In Insect Pathology: An Advanced Treatise, E. A. Steinhaus ed. New York: Academic Press, Vol. 2.

McCoy, C. W., Samson, R. A., and *D.G. Boucias* (1988). Entomogenous fungi. In Handbook of Natural Pesticides, Boca, Raton, Fla: *Mr ic* Press. Vol. 5, Microbial Insecticides, Part A, Entomogenous Protozoa and Fungi, C. M. Ignoffo and N. B. Mandava, eds.

Meyling, N., and *J. Eilenberg* (2007). Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: Potential for conservation biological control. *Biological Control*, **43**, 145-155.

Meyling, N., and *J.K. Pell* (2006a). Detection and avoidance of an entomopathogenic fungus by a generalist insect predator. *Ecological Entomology*, **31**, 162-171.

Meyling, N. V., and *J. Eilenberg* (2006b). Isolation and characterisation of *Beauveria bassiana* isolates from phylloplanes of hedgerow vegetation. *Mycol. Research,* **110**, 188–195.

Millstein, A., Brown, G. C., and *G.L. Nordin* (1983). Microclimatic moisture and conidial production in *Erynia* sp.: In vivo moisture balance and conditional phenology. *Environmental Entomology,* **12**, 13-39.

Moutia, L. A (1936). The sugar cane white grub *Lachnosterna (Phytalus) smithi* Arrow in Mauritius. *Proceedings International Society of Sugar Cane Technologists*, **5**, 436-445.

Müller-Kögler, G. E., and *B.I. Pilzkrankheiten* (1965). Anwendung zur biologischen Scha¨dlingsbeka¨mpfung und Grundlagen der Insektenmykologie. Berlin: P. Parey Verlag, 444.

Mullens, B. A., Rodriguez, J. L., and *J.A. Meyer* (1987). An epizootiological study of *Entomophthora muscae* in muscoid fly populations on southern California poultry facilities, with emphasis on *Musca domestica.* Hilgardia, **55**, 1.

Newman, G. G., and *G.R. Carner* (1975). Environmental factors affecting conidial sporulation and germination of *Entomophthora gammae. Environmental Entomology*, **4**, 615-618.

Nikoh, N., and *T. Fukatsu* (2000). Interkingdom host jumping underground: phylogenetic analysis of entomoparasitic fungi of the genus *Cordyceps*. *Mol Biol Evol*, **17**, 629–638.

Nolan, R. A. (1985). Protoplasts from Entomophthorales. In Fungal protoplasts, Applications in Biochemistry and Genetics, J. F. Peberdy and L. Ferenczy, eds. New York: Marcel Dekker.

Ormond, E. L., Thomas, A. P. M., Pell, J. K. and *H.E. Roy* (2006). Overwintering ecology of *Coccinella septempunctata*, *Beauveria bassiana* and *Dinocampus coccinellae*. *IOBC wprs Bulletin*, **29**, 85-88.

Ownley, B. H., Dee, M. M., and *K.D. Gwinn* (2008). Effect of conidial seed treatment rate of entomopathogenic *Beauveria bassiana* 11-98 on endophytic colonization of tomato seedlings and control of *Rhizoctonia* disease. *Phytopathology*, **98**, S118.

Ownley, B. H., Griffin, M. R., Klingeman, W. E. Gwinn, K. D., Moulton, J. K.., and *R.M. Pereira* (2008). *Beauveria bassiana*: endophytic colonization and plant disease control. *Journal of Invertebrate Pathology*, **98**, 267-270.

Ownley, B. H., and *M. Windham* (2007). Biological control of plant pathogens. In: Trigiano RN, Windham MT, Windham AS, (Eds.), *Plant Pathology: Concepts and Laboratory Exercises*, 2nd ed. CRC Press, Boca Raton, Florida, pp.423-436.

Ownley, B. H., Pereira, R. M., Klingeman, W. E., Quigley, N. B., and *B.M. Leckie* (2004). *Beauveria bassiana*, a dual purpose biocontrol organism, with activity against insect pests and plant pathogens, In: Lartey RT, Caesar AJ, (Eds.), *Emerging Concepts in Plant Health Management*, Research Signpost, India, pp.255-269.

Paterson, I. C., Charnley, A. K, Cooper, R. M., and *J.M. Clarkson* (1994). Partial characterization of specific inducers of a cuticle-degrading protease from the insect pathogenic fungus *Metarhizium anisopliae*. *Microbiology*, **140**, 3153-3159.

Pell, J. K., Eilenberg, J., Hajek, A. E., and *D.C. Steinkraus* (2001). Biology, ecology and pest management potential of Entomophthorales. In: Butt TM, Jackson C, Magan N (eds) Fungi as biocontrol agents: progress, problems and potential. CAB International, Wallingford, pp.71–153.

Pereira, R. M., Stimac, J. L., and *S.B. Alves* (1993). Soil antagonism affecting the dose response of workers of the red imported fire ant, *Solenopsis invicta*, to *Beauveria bassiana* conidia. *Journal of Invertebrate Pathology*, **61**, 156-161.

Posada, F., and *F.E. Vega* (2005). Establishment of the fungal entomopathogen Beauveria bassiana (Ascomycota : Hypocreales) as an endophyte in cocoa seedlings (Theobroma cacao). *Mycologia,* **97**, 1195–1200.

Quesada-Moraga, E., Landa, B. B., Munoz-Ledesma, J., Jimenez-Diaz, R. M., and *C. Santiago-Alvarez* (2006). Endophytic colonisation of opium poppy, Papaver somniferum, by an entomopathogenic Beauveria bassiana strain. *Mycopathologia,* **161**, 323–329.

Rangel, D. E. N., Braga, G. U. L., Anderson, A. J., and *D.W. Roberts* (2005). Variability in conidial thermotolerance of *Metarhizium anisopliae* isolates from different geographic origins. *Journal of Invertebrate Pathology*, **88**, 116-125.

Reisenzein, H., and *W. Tiefenbrunner* (1997). Growth inhibiting effect of different isolates of the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill. to the plant parasitic fungi of the genera *Fusarium*, *Armillaria* and *Rosellinia*, *Pflanzenschutzberichte,* **57**, 15–24.

Renwick, A., Campbell, R., and *S. Coe* (1991). Assessment of *in vivo* screening systems for potential biocontrol agents of *Gaeumannomyces graminis. Plant Pathology*, **40**, 524-532.

Roberts, D. W., and *R.J. St. Leger* (2004). *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: Mycological aspects. *Adv. Appl. Microbiology*, **54**, 1–70.

Roberts, D. W., and *R.A. Humber* (1981). Entomogenous fungi. In: Cole GT, Kendrick B, (Eds.), *Biology of Conidial Fungi*. Academic Press, New York, pp.201-236.

Roberts, D. R. (1981). Toxins of entomopathogenic fungi. In: Burges HD (ed) Microbial control of pests and diseases, 1970–1980. Academic Press, London, pp.441–464.

Roberts, D. W., and *A.S. Campbell* (1977). Stability of entomopathogenic fungi. *Miscellaneous Publications of the Entomological Society of America*, 10, 19-76.

Roy, H. E., Pell, J. K., and *P.G. Alderson* (2001). Targeted dispersal of the aphid pathogenic fungus *Erynia neoaphidis* by the aphid predator *Coccinella septempunctata*. *Biocontrol Sci Technol,* **11**, 99–110.

Roy, H. E., Steinkraus, D. C., Eilenberg, J., Hajek, A. E., and *J.K. Pell* (2006). Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts. *Annual Review of Entomology*, **51**, 331-357.

Rudgers, J. A., Holah, J., Orr. S. P., and *K. Clay* (2007). Forest succession suppressed by an introduced plant-fungal symbiosis. *Ecology,* **88**, 18-25.

Saik, J. E., Lacey, L. A., and *C.M. Lacey* (1990). Safety of microbial insecticides to vertebrates idomestic animals and wildlife. In *Safet. of microbial insecticides* (ed. M. Laird, L. A. Lacey and E. W. Davidson), pp.115-132. Boca Raton: CRC Press.

Saikkonen, K., Lehtonen, P., Helander, M., Koricheva, J., and *S.H. Faeth* (2006). Model systems in ecology: dissecting the endophyte-grass literature. *Trends in Plant Science*, **11**, 428-433.

Saikkonen, K., Wali, P., Helander, M., and *S.H. Faeth*, 2004. Evolution of endophyte-plant symbioses. *Trend Plant Science,* **9**, 275–280.

Saikkonen, K., Faeth, S. H., Helander, M., and *T.J. Sullivan* (1998). Fungal endophytes: a continuum of interactions with host plants. *Ann. Rev. Ecol. Syst.,* **29**, 319–343.

Samson, R. A., Evans, H. C., and *J.P. Latg* (1988). Atlas of entomopathogenic fungi. Springer, Berlin Heidelberg New York.

Samuels, R. I., and *I.C. Paterson* (1995). Cuticle-degrading proteinase from insect moulting fluid and culture filtrates of entomopathogenic fungi. *Comparative Biochemistry and Physiology,* **110**, 661–669

Sattar, A., 2002. Isolation and identification of virulent factors from entomopathogenic fungi. CEMB, University of the Punjab, Lahore, M. Phil thesis, pp.1-63.

Scheriter, G., Butt, T. M., Beckett, A., Vestergaard, S., and *G. Moritz* (1994). Invasion and development of *Verticillium lecanii* in the western flower thrips, *Frankliniella occidentalis*. *Mycol. Research*, **98**, 1025-1034.

Schulz, B., and *C. Boyle* (2005). The endophytic continuum. *Mycological Research*, **109**, 661-686.

Shah, P. A., and *J.K. Pell* (2003). Entomopathogenic fungi as biological control agents. *Appl Microbiol Biotechnol,* **61**,413–423.

Shahid, A. A., Nasir, I. A., Zafar, A. U., Sumrin, A., Chaudhry, B., and *S. Riazuddin* (2004). The Camb biopesticides: Preparation, uses and comparative studies with chemical pesticides. In: ed. G. R. Pathade and P. K. Goel, Biotechnological Methods in Environmental Management, ABD Publishers Jaipur, India, pp.260-282.

Shahid, A. A., Nasir, I. A., Zafar, A. U., Sumrin, A., Chaudhry, B., and *S. Riazuddin* (2003). The use of camb biopesticides to control pests of rice (*Oryza sativa*). *Asian J. Plant Sci,* **2**, 1079-1082.

Shahid, A. A., Sattar, A., Chaudhry, B., and *S. Riazuddin* (2003). Determination of protein virulence factors and pathogenicity of entomopathogenic fungi. *Pak. J. Biochem and Mol. Biology,* **36**, 100-107.

Shapiro-Ilan, D. I., Jackson, M., Reilly, C. C., and *M.H. Hotchkiss* (2004). Effects of combining an entomopathogenic fungi or bacterium with entomopathogenic nematodes on mortality of *Curculio caryae* (Coleoptera: Curculionidae). *Biological Control,* **30**, 119–126.

Shapiro-Ilan, D. I. (2003). Microbial control of the pecan weevil, Curculio caryae. In: Dutcher, J. D., Harris, M. K., Dean, D. A. (Eds.), Integration of Chemical and Biological Insect Control in Native, Seedling, and Improved Pecan Production. *Southwest. Entomol. Supplement*, **27**, 100–114.

Shaw, K. E., Davidson, G., Clark, S. J., Ball, B. V., Pell, J. K., Chandler, D., and *K.D. Sunderland* (2002). Laboratory bioassays to assess the pathogenicity of mitosporic fungi to *Varroa destructor* (Acari: Mesostigmata), an ectoparasitic mite of the honeybee, *Apis mellifera*. *Biol Control,* **24**, 266–276.

Shimazu, M., 1977. Factors affecting conidial germination of *Entomophthora del phacis* Hori. *Applied Entomology and Zoology*, **12**, 260.

Siegel, J. P. (1990). Shadduck JA. Safety of microbial insecticides to vertebrates: humans. In: Laird M, Lacey LA, Davidson EW, editors. Safety of microbial insecticides. Boca Raton, FL: CRC Press, pp.101-113.

Sierotzki, H., Camastral, F., Shah, P. A., Aebi, M., and *U. Tuor* (2000). Biological characteristics of selected Erynia neoaphidis isolates. *Mycol Res,* **104**, 213–219.

Sikorowski, P. P. (1985). Pecan weevil pathology. In: Neel, W.W. (Ed.), Pecan Weevil: Research Perspective. Quail Ridge Press, Brandon, MS, pp.87–101.

Singh, B. K., Millard, P., Whiteley, A. S., and *J.C. Murrell* (2004). Unravelling rhizosphere – microbial interactions: opportunities and limitations. *Trends in Microbiology*, **12**, 386-393.

Smith, R., and *E.A. Grula* (1982). Toxic components on the larvae surface of the corn earworm *(Heliothis zea)* and their effects on germination and growth of *Beauveria bassiana*. *Journal of Invertebrate Pathology*, 36, 15-22.

Smith, R. J., Pekrul, S., and *E.A. Grula* (1981). Requirement for sequential enzymatic activities for penetration of the integument of the corn earworm. *Journal of invertebrate pathology,* **38**, 335-344.

Spatafora, J. W., Sung, G. H., Sung, J. M., Hywel, J. N. L., and *J.F. White Jr.* (2007). Phylogenetic evidence for an animal pathogen origin of ergot and the grass endophytes. *Molecular Ecology*, **16**, 1701-1711.

Spatafora, J. W., and *M. Blackwell* (1993). Molecular systematics of unitunicate perithecia ascomycetes: the Clavicipitales-Hypocreales connection. *Mycologia,* **85**, 912–922.

Sri-Arunotai, S., Sikorowski, P. P., and *W.W. Neel* (1975). Study of the pathogens of the pecan weevil larvae. *Environ. Entomology,* **4**, 790–792.

St. Leger, R. J. (2008). Studies on adaptation of *Metarhizium anisopliae* to life in the soil. *Journal of Invertebrate Pathology*, **98**, 271-276.

St. Leger, R. J. (1993). Biology and mechanism of insect cuticle invasion by deutromycete fungal pathogen. In" Parasites and pathogens of insects. Vol. 2: pathogens". Academic press, San Deido, pp.211-229.

St. Leger, R. J., Durrands, P. K., Chamely, A. K., and *R.M. Cooper* (1988a). Role of extracellular chymoelastase in the virulence of *Metarhizium anisopliae* for *Manduca sexta. Journal of Invertebrate Pathology*, **52**, 285-293..

St. Leger, R. J., Durrands, P. K., Charnnley, A. K.., and *R.M. Cooper* (1988b). Role of extracellular chemoelastases in the virulence of *M. anisopliae. Arch. Biochem. Biophys*, **253**, 21-232.

St. Leger, R. J., Charnley, A. K., and *R.M. Cooper* (1987a). Characterization of cuticle-degrading Proteases produced by the entomopathogen *Metarhizium anisopliae. Arch. Biochem. Biophys*, **253**, 221-232.

St. Leger, R. J., Cooper, R. M. and *A.K. Charnley* (1987b). Production of cuticle degrading enzymes by the entomopathogenic *Metarhizium anisopliae* during infection of cuticles from *Calliphora vomitoria* and *Manduca sexta. Journal of General Microbiology,* **133**, 1371.

St. Leger, R. J., Cooper, R. M., and *A.K. Charnley* (1986). Cuticle degrading enzymes of entomopathogenic fungi: Cuticle degradation in vitro by enzymes from entomopathogens. *Journal of invertebrate Pathology*, **47**,167-177.

Staples, J. A., and *R.J. Milner* (2000). A laboratory evaluation of the repellency of *Metarhizium anisopliae* conidia to *Coptotermes lacteus* (Isoptera: Rhinotermitidae). *Sociobiology*, **36**, 133-148.

Steinhaus, E. A., (1964). Microbial diseases of insects. In: DeBach P (ed) Biological control of insect pests and weeds. Chapman and Hall, London, pp.515–547.

Steinhaus, E. A., 1963. Principles of insect pathology. McGraw-Hill, New York 1949.

Steinhaus, E. A., ed. Insect Pathology: An Advanced Treatise, New York: Academic Press. Vol. 2.

Steinhaus, E. (1957). Concerning the harmlessness of insect pathogens and the standardization of microbial control products. *Journal of Economic Entomology*, **50**, 715-720.

Strasser, H., Abendstein, D., Stuppner, H., and *T.M. Butt* (2000). Monitoring the distribution of secondary metabolites produced by the entomogenous fungus *Beauveria brongniartii* with particular reference to oosporein. *Mycological Research*, **104**, 1227-1233.

Sung, G. H., Hywel, J. N. L., Sung, J. M., Luangsa-ard, J. J., Shrestha, B., and *J.W. Spatafora* (2007). Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. Studies in Mycology, **57**, 5-59.

Swingle, H. S., and *J.L. Seal* (1931). Some fungus and bacterial diseases of pecan weevil larvae. *J. Econ. Entomology*, **24**, 917.

Tanada, Y., and *H.K. Kaya* (1993). *Insect Pathology*. Academic Press, Inc., San Diego, USA, 1993.

Thurston, G. S., Kaya, H. K., and *R. Gaugler* (1994). Characterizing the enhanced susceptibility of milky disease-infected scarabaeid grubs to entomopathogenic nematodes. *Biol. Control,* **4**, 67–73.

Thurston, G. S., Kaya, H. K., Burlando, T. M., and *R.F. Harrison* (1993). Milky disease bacterium as a stressor to increase susceptibility of scarabaeid larvae to an entomopathogenic nematode. *J. Invertebr. Pathol*, **61**, 167–172.

Vega, F. E., Goettel, M. S., Blackwell, M., Chandler, D., Jackson, M. A., Keller, S., Koike, M., Maniania, N. K., Monzon, A., Ownley, B.H., Pell, J. K., Rangel, D. E. N., and *H. E. Roy* (2009). Fungal entomopathogens: new insights on their ecology. http://dx.doi.org/10.1016/j.funeco.2009.05.001.

Vega, F. E., Posada, F., Aime, M. C., Pava-Ripoll, M., Infante, F., and *S.A. Rehner* (2008). Entomopathogenic fungal endophytes. *Biological Control,* **46**, 72-82.

Vega, F. E. (2008). Insect pathology and fungal endophytes. *Journal of Invertebrate Pathology*, **98**, 277-279.

Vesely, D., and *D. Koubova* (1994). In vitro effect of entomopathogenic fungi *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Beauveria brongniartii* (Sacc.) Petch on phytopathogenic fungi. *Ochrana Rostlin*, **30**,113-120.

Vestergaard, S., Cherry, A., Keller, S., and *M. Goettel* (2003). Safety of hyphomycete fungi as microbial control agents. In: Hokkanen HMT, Hajek AE, editors. Environmental impacts of microbial insecticides.Dordrecht: Kluwer Academic Publishers, pp.35-62.

Vincent, C., Goettel, M. S., Lazarovits, G. (Eds.)., 2007. *Biological Control: A Global Perspective*. CAB International/AAFC, Wallingford, United Kingdom.

Wang, C. S., Hu, G., and *R.J. St. Leger* (2005). Differential gene expression by *Metarhizium anisopliae* growing in root exudate and host (*Manduca sexta*) cuticle or hemolymph reveals mechanisms of physiological adaptation. *Fungal Gen. Bio.,* **42**, 704–718.

Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany*, **52**, 487-511.

White, J. F., Belanger, F., Meyer, W., Sullivan, R. F., Bischoff, J.F., and *E.A. Lewis* (2002). Clavicipitalean fungal epibionts and endophytes—development of symbiotic interactions with plants. *Symbiosis*, **33**, 201–213.

Wilding, N., 1969. Effect of humidity on the sporulation of *Entomophthora aphidis* and *Entomophthora thaxteriana. Transactions of the British Mycology Society*, **53**, 126-130.

Woods, S. P., and *E.A. Grula* (1984). Utilizable surface nutrients on *Heliothis zea* available for growth of *Beauveria bassiana. Journal of Invertebrate Pathology*, **43**, 359-369.

Wraight, S. P., Jackson, M. A., and *S.L. de Kock* (2001). Production, stabilization, and formulation of fungal biocontrol agents. In: Butt TM, Jackson CW, Magan N (Eds.), *Fungi as Biocontrol Agents: Progress, Problems and Potential.* CABI Publishing, Wallingford, United Kingdom, 253-288.

Wraight, S. P., Carruthers, R. I., Bradely, C. A., Jaronski, S. T., Lacey, L. A., Wood, P., and *W.S.Z. Galaini* (1998). Pathogenicity of entomopathogenic fungi *Paecilomyces* spp. and *Beauveria bassiana* against silver whitefly, *Bemisia argentifolii. J. Inver. Pathology*, **71**,217-226.

Wraight, S. P., Carruthers, R., Jaronski, S. T., and *C.A. Bradley* (1990). Development and field testing of *Beauveria bassiana* and *Paecilomyces fumosoroseus* for biological control of whiteflies. IN: Silverleaf whitefly: 1995 supplement to the five-year national research and action plan - third annual review.

Zacharuk, R. Y. (1973). Electron-microscope studies of the histopathology of fungal infections by *Metarhizium anisopliae. Miscellaneous Publication of the Entomological Society of America*, **9**, 112.

Zimmermann, G. (1993). The entomopathogenic fungus *Metarhizium anisopliae* and its potential as a biocontrol agent. *Pestic. Sci,* **37**, 375-379.