Molecular Plant Breeding 2012, Vol.3, No.7, 63-79
http://mpb.sophiapublisher.com

Research Report Open Access

Entomopathogenic Fungi as Microbial Biocontrol Agent

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Molecular Plant Breeding, 2012, Vol.3, No.7 doi: 10.5376/mpb.2012.03.0007
Received: 25 Apr., 2012
Accepted: 11 May, 2012
Published: 20 May, 2012

Preferred citation for this article:
Khan et al., 2012, Entomopathogenic Fungi as Microbial Biocontrol Agent, Molecular Plant Breeding, Vol.3, No.7 63-79 (doi: 10.5376/mpb.2012.03.0007)

Abstract An attractive alternative method to chemical pesticides is the microbial biocontrol (MBCAs) agents. They are the natural enemies devastating the pest population with no hazard effects on human health and the environment. Entomopathogenic fungi has an important position among all the biocontrol agents because of its route of pathogenicity, broad host rang and its ability to control both sap sucking pests such as mosquitoes and aphids as well as pests with chewing mouthparts, yet they only cover a small percentage of the total insecticide market. Improvements are needed to fulfill the requirements for high market share. Entomopathogenic fungi differ from other microorganisms in their infection process: they directly breach the cuticle to enter the insect hemocoel, while other microorganisms enter by ingestion through mouth and then cause disease. Insect cuticle is mainly composed of chitin and protein surrounded by wax, lipid layer or fatty acids. Fungal pathogenesis mainly starts with the secretion of cuticle degrading enzymes. Some important and well known cuticle degrading enzymes are chitinase, protease and lipase which can degrade chitin, protein and lipid of the cuticle, respectively. In this review we collected literatures from different sources and we arranged them in a such a way to better to understand the process of insect pathogenicity of entomopathogenic fungi and to find ways to improve the virulence of wild strain fungi to shorten the killing time of the pest and to commercialize the entomopathogenic fungi. In this way, the market share of the fungal entomopathogenic fungi will increase and a decrease in the usage of synthetic chemical pesticides will automatically follow.

Keywords Entomopathogenic fungi; *Beauveria bassiana*; Biocontrol agents; Enzymes; Pathogenesis

Background

Integrated Pest Management (IPM) involves inspection, identification and treatment of pests. The treatment (when required) is carried out after inspection and identification with an environmentally safe and pest specific pesticide with limited persistence. Therefore biological pest management is considered as an important part of IPM. Biological control is an important part of integrated pest management (IPM). According to Oerke and Dehne (2004), insect pests are responsible for an estimated 42% of all losses in crop production. Extensive use of synthetic chemical pesticides, insecticide resistance to chemical pesticides (Ffrench-Constant et al., 2004), the resulting environmental pollution, adverse effects on human health and other organisms and the demand for reduced chemical inputs in agriculture have provided an impetus to the development of alternative forms of pest control (Wilson and Tisdall, 2001). An attractive alternative method to chemical pesticides is biocontrol (Nicholson, 2007) and the microbial biocontrol (MBCAs) agents as the natural enemies of the pest population devastate pests with no hazard effects on human health and environment. As the microbial biocontrol agents have complex mode of action, it's very difficult for a pest to develop resistance against MBCAs. The present MBCAs are viruses, bacteria, nematodes, and fungi and they are used throughout the world with great advantage and success. But fungal biocontrol agents are the most important among all the MBCAs due to easy delivery, improving formulation, vast number of pathogenic strains known, easy engineering techniques and over-expression of endogenous proteins or exogenous toxins (St. Leger et al., 1996; Butt et al., 2001; Wang and St. Leger, 2007; Federici et al., 2008; St. Leger and Wang, 2010). Similarly, the entomopathogenic fungi are important among all the biological control agents due to its broad host range, route of pathogenicity and its ability to control sap sucking pests such as mosquitoes and aphids (Butt, 2002; Qazi and Khachatourians, 2005; Thomas and Read, 2007; Fan et al., 2007) as well as pests with chewing mouthparts (Hajek and St. Leger, 1994; de Faria and Wraight, 2007).

This review will help us understand entomopathogenic fungal virulence and present the most recent improvements and achievements in the relevant field. This will help us determine how to improve the virulence of entomopathogenic fungi to shorten the killing time of pest.

1 Entomopathogenic Fungi (EPF)

Entomopathogenic fungi (EPF) are widely distributed with both restricted and wide host ranges which have different biocontrol potentials against arthropods insects and plant pathogenic fungi. Entomopathogenic fungi were among the first organisms to be used for the biological control of pests. More than 700 species of fungi from around 90 genera are pathogenic to insects (Khachatourians and Sohail, 2008). Most EPF species are from the fungal divisions *Ascomycota* and *Zygomycota*. The *ascomycete* fungi were previously divided into two groups, the *Ascomycota* and the *Deuteromycota* (Table 1). The Fungi Imperfecti of *Deuteromycota* was known for having no sexual stage was known called as. But later on, cultural and molecular studies have demonstrated that some of these “imperfect fungi” (formally class Hyphomycetes in the Deuteromycota) were in fact anamorphs (asexual forms) of the Ascomycota within the order Hypocreales, and Clavicipitaceae family (Fukatzu, 1997; Hodge, 2003; Krasnoff, 1995; Shimazu, 1998). Within the Zygomycota, the most entomopathogenic species are in the order Entomophthorales (Roy et al., 2006).

These fungi nutritionally may be saprotrophs that colonize the rhizosphere and phyllosphere, endophytic saprotrophs, hemibiotrophic, necrotrophic of plants, entomopathogenic or mycoparasitic and some of them have adopted more than one econutritional mode.

2 Life cycle of entomopathogenic fungi

The life cycle of EPF is composed of the spore which geminates into mycelia and the mycelia in turnproduce spores (spore–mycelia–spore phases). The life cycle of most entomopathogenic fungi consist of two phases: a normal mycelia growth phase mostly outside the host body and a yeast like budding phase mostly in the hemocoel of host. The yeast-like, dimorphic mode of growth in *Beauveria bassiana* was described by Alves et al (2002); and the production of oblong blastospore-like propagules in *M. flavoviride* was described by Fargues et al (2002). The life cycle of *M. anisopliae* under liquid culture conditions has also been described (Uribe and Khachatourians, 2008).

Table 1 Classification of entomopathogenic fungi (Roy et al., 2006)

Division	Class	Order	Family	Genus
Zygomycota	Zygomycetes	Entomophthorales	Entomophthoraceae	*Entomophaga* *Entomophthora* *Erynia* *Eryniopsis* *Furia* *Massospora* *Strongwellsea* *Pandora* *Tarichium* *Zoophthora*
			Neozygitaceae	*Neozygites*
Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	*Beauveriaa* *Cordyceps* *Cordycepioideus* *Lecanicilliuma* *Metarhiziuma* *Nomuraea*

Beauveria bassiana in the absence of a specific insect host grows through an asexual vegetative life cycle consists of germination, filamentous growth and formation of sympoduloconidia (Figure 1B). In the presence of its host insect, *Beauveria* conidiospores germinate on the surface of the cuticle of host and penetrate the insect's integument through the germinated hyphal tubes where the fungus alters its growth morphology to a yeast-like phase and produces hyphal bodies by budding like growth, which circulate in the haemolymph (Figure 1A) resulting in the host death. The fungal growth then reverts back to the typical hyphal form (the saprotrophic stage). The ability to convert to the yeast-like phase may be a prerequisite for pathogenicity.

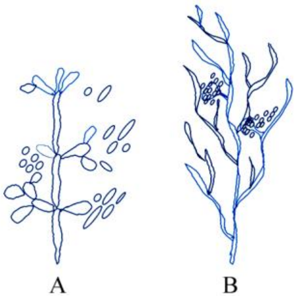

Figure 1 Entomopathogenic dimorphic mode of growth
Note: A: Yeast-like parasitic phase during infection of susceptible species; B: Saprobic phase shows filamentous hyphae

Entomopathogenic fungi, *Verticillium lecanii*, *Beauveria bassiana* and *Metarhizium anisopliae* are intensively studied as common natural enemies and important epizootics of aphids and other agricultural pests (Roberts and St. Leger, 2004; Thomas and Read, 2007; Wang et al., 2004; Milner, 1997; Li and Sheng, 2007). *Beauveria bassiana* (Balsamo) Vuilemin is one of the major fungal entomopathogens infecting nearly 95% of migratory alate aphids, especially *M. persicae* (Chen et al., 2008). *Beauveria bassiana* and *Verticillium lecanii* have dual biological control properties, i.e. they are natural enemies of pests and also plant pathogens (Bonnie et al., 2010; Goettel et al., 2008). Koppert Biological Systems currently use *Verticillium lecanii* (Zimm. Viegas) as an insect pathogen which has been commercialized for controlling aphids (Faria and Wraight, 2007) and it is effective in controlling plant pathogenic fungi like powdery mildews (Askary et al., 1998; Dik et al., 1998; Miller et al., 2004), rusts (Spencer and Atkey, 1981), green molds (Benhamou and Brodeur, 2000), *Fusarium* (Koike et al., 2007), *Verticillium dahliae* (Kusunoki et al., 2006) and *Pythium ultimum* (Benhamou and Brodeur, 2001). *Beauveria bassiana* is reported to limit the growth of plant pathogenic fungi in vitro, colonize endophytically in numerous plants and induce systemic resistance when pathogen infect the plant as well as reducing the diseases caused by soil born plant pathogens like *Pythium*, *Rhizoctonia*, and *Fusarium* (Ownley et al., 2010). Mitosporic fungi are generally environmentally friendly with negligible or low mammalian toxicity, have no residual toxicity (Copping, 2004) and are successful as mycoinsecticides against aphids (Faria and Wraight, 2007; Milner, 1997; Shah and Pell, 2003).

To date, several mycopesticides have been developed and used in several countries including the United Kingdom and the United States (Table 2) (Goettel et al., 2005; Kiss, 2003). These include Vertalec® based on *Lecanicillium longisporum* (Petch) Zare & Gams (formerly known as *Verticillium lecanii* (Zimm.) Viegas) against aphids, Botanigard® based on *Beauveria bassiana* (Bals.) Vuill. against aphids and whitefly; however, they remain a small percentage of the total insecticides. The major reason for the small market share of these fungi as mycoinsecticides is its slow killing rate and an increase in market share is directly proportional to killing speed (St Leger and Wang, 2009).

Although these products have the advantage of a restricted host range, this specificity is also one of the limiting factors for their commercial use (Ownley et al., 2004). Therefore, a mycopesticide with a wider host range but with little to no influence on other natural enemies of pests or beneficial organisms may have a commercial advantage if it simultaneously controls various pests and/or plant diseases (Wraight and Carruthers, 1999).

Insect pathogenic fungi are different in pathogenicity than bacteria and viruses in that they infect insects by breaching the host cuticle. The cuticle is composed of chitin fibrils embedded in a matrix of proteins, lipids, pigments and N-acylcatecholamines (Richard et al., 2010). They secrete extracellular enzymes proteases, chitinases and lipases to degrade the major constituents

Table 2 Entomopathogenic fungi produced commercially and experimentally (Butt et al., 2001; Wraight et al., 2001; Copping, 2004; Kabaluk and Gazdik, 2005; Zimmermann, 2007; Khachatourians, 1986)

Fungus	Product/Trade name	Company/Producer	Country/Origin	Target pests
Culicinomyces clavisporus			Austria, Belgium, Czech	Mosquito larvae
Hirsutella thompsonii	Mycar		Austria, Belgium	Citrus rust mite
Metarhizium anisopliae	Meta-Sin®			Spittle bug; Sugarcane frog hopper
Nomuraea rileyi				Lepidopteran larvae
Verticillium lecanii	Vertalec			Aphids; Coffee green bug; Greenhouse whitefly thrips
Beauveria bassiana	Bio-Power	Stanes	India	Mite; Coffee green bug
	BotaniGard ES; BotaniGard 22WP	Laverlam International (formerly Emerald BioAgriculture)	USA	
	Boverol	Fytovita	Czech Republic	
	Conidia	LST	Columbia	
	Mycotrol ES; Mycotrol-O	Laverlam International (formerly Emerald BioAgriculture)	USA	
	Naturalis	Intrachem	Italy	Aphids Spittle bug; Sugarcane
	Naturalis-L Andermatt Biocontrol	Troy Biosciences Inc	Switzerland USA	
	Ostrinil	Arysta (formerly NPP, Calliope)	France	
	Proecol	Probioagro	Venezuela	
	Racer BB	SOM Phytopharma	India	
	Trichobass-L; Trichobass-P	AMC Chemical/Trichodex	Spain	
B. brongniartii (_*B. tenella*)	Beauveria Schweizer	Lbu (formerly Eric Schweizer Seeds)	Switzerland	Greenhouse whitefly thrips Mosquito larvae
	Betel	Arysta (formerly NPP, Calliope)	France	
	Biolisa-Kamikiri	Nitto Denko	Japan	
	Engerlingspilz	Andermatt Biocontrol AG	Switzerland	
	Melocont-Pilzgerste	Agrifutur-Kwizda	Italy, Austria	

of the cuticle (i.e. protein, chitin and lipids) and allow hyphal penetration (Wang et al., 2005; Cho et al., 2006). Extracellular lipases are also involved in microbial virulence and play different roles in the infection process (Stehr et al., 2003). The successfulness of infection was directly proportional to secretion of exoenzymes (Khachatourians, 1996). It is believed that both mechanical force and enzymatic action are involved in the penetration of fungus to the hemocoel of the insect. In this review, we take an overview of the chitinase, protease and lipase with their importance in the process of pathogenesis.

Besides exoenzymes, the entomopathogenic fungi are reported to secrete toxin proteins and metabolites in vitro and sometime in vivo as well. There are a number of toxic compounds in the filtrate of entomopathogenic fungi such as small secondary metabolites, cyclic peptides and macromolecular proteins. *Beauveria bassiana* is reported to produce low molecular weight cyclic peptides and Cyclosporins A and C with insecticidal properties such as beauvericin, enniatins, bassianolide, (Roberts, 1981; Vey et al., 2001), Oosporein (a red-colored dibenzoquinone with antibiotic activity against gram-positive bacteria), andcyclic peptides with immunosuppressive activities. There are also some insecticidal cyclic peptides like beauvericin and bassianolide isolated from *Beauveria bassiana*. Some of the strains of *Beauveria bassiana* are reported to produce high molecular weight compounds with toxic activity against pest, like hirsutellin A (Enrique and Alain, 2004). However, the major obstacle limiting the market share of these fungi as mycoinsecticides is their slow killing speed and increase in market share is directly proportional to killing speed (St Leger and Wang, 2009).

A large number of studies were conducted to potentiate and improve the virulence of entomopathogenic fungi to a greater extent than their individual activities and thus increasing the market share. The extensive transcriptomic and genetic study of entomopathogenic fungal infection process revealed that a number of different genes were involved in the pathogenicity (Freimoser et al., 2003; Wang et al., 2005; Cho et al., 2006a, 2006b, 2007) such as chitinases (Bagga et al., 2004; Fang et al., 2005), guanine nucleotide-binding proteins and its regulator (Fang et al., 2007, 2008), adhesin which helps in attachment of spore (Wang and St. Leger, 2007a), a perilipin-like protein that regulates appressorium turgor pressure and differentiation (Wang and St. Leger, 2007b) and a cell protective coat protein helping in escaping the pathogen from the host immunity recognition (Wang and St. Leger, 2006). Similarly, an increased virulence of the entomopathogenic fungi was observed with over-expression of virulence genes such as subtilisin protease PR1A (St. Leger et al., 1996), subtilisin protease PII gene (Ahman et al., 2002), and hybrid chitinase containing a chitin binding domain (Fan et al., 2007). Wang and St. Leger (2007c) modified scorpion neurotoxin peptide, AAIT using *Metarhizium's* codon preferences under the control of mc11 gene promoter for fungal transformation. The LC50 of the transgenic strain AaIT-Ma549 was reduced 22-fold against the *Manduca sexta* and nine fold against the *Aedes aegypti* (Wang and St. Leger, 2007c). AaIT-Ma549 was also tested against the coleopteran coffee pest, *Hypothenemus hampei*, reducing LC50 by 15.7-fold and the average survival time by 20% (Pava-Ripoll et al., 2008).

3 *Beauveria bassiana* (Clavicipitaceae)

The entomopathogenic mitosporic ascomycete, *Beauveria bassiana* (Bals.) Vuill. is an important natural pathogen of insects and it has been developed as a microbial insecticide for use against many major arthropod pests in agricultural, urban, forest, livestock and aquatic environments (Charnley and Collins, 2007; Faria and Wraight, 2007). It has been developed as a microbial insecticide for use against many major pests, including *lepidopterans* and *orthopterans*. About 33.9% of the mycoinsecticides is based on *B. bassiana*, followed by *Metarhizium anisopliae* (33.9%), *Isaria fumosorosea* (5.8%) and *Beauveria brongniartii* (4.1%) (Faria and Wraight, 2007); however, to increase the market share of *B. bassiana*, the killing speed which is the major hindrance limiting their use as mycoinsecticides should be accelerated, (St Leger and Wang, 2009). As natural strains of these fungi often lack sufficient virulence or tolerance to adversity (Braga et al., 2001a, 2001b; Ying and Feng, 2004; Rangel et al., 2005), genetic manipulation is necessary to improve their efficacy and ecological fitness (Roberts and St. Leger, 2004; Fang et al., 2005). The importance of the *B. bassiana* and *B. brongniartii* can be briefly understood from table 3 below.

Research studies are carried out to optimize the preparation and application of fungal inoculums (Wraight et al., 2001) and (St Leger and Wang, 2009) to improve the virulence of mycoinsecticides by genetic modification. Based on this approach, most studies on the virulence factors of entomopathogenic fungi have been directed to elucidate the most relevant cuticle degrading enzymes (Griesch and Vilcinskas, 1998; Khachatourians, 1996; St Leger et al., 1996), because their over-expression in engineered strains results in more fungi that are more deadly toward insects (Fang et al., 2005; St Leger et al., 1996). Similarly, over-expression of a chitinase gene (Bbchit1) was found to enhance the virulence of *Beauveria bassiana* against aphids (*Myzus persicae*) (Fang et al., 2005). Importantly, different genes involved in pathogenicity have been characterized from *M. anisopliae* or *B. bassiana* and some of these genes include cuticle degrading enzymes (Bagga et al., 2004; Fang et al., 2005), G protein and its regulator (Fang et al., 2007), adhesin that mediates spore attachment and fungal differentiation (Wang and St. Leger, 2007a), a perilipin-like protein that regulates appressorium turgor pressure and differentiation (Wang and St. Leger, 2007b), and a cell protective coat protein

Table 3 Examples of effects of *B. bassiana* and *B. brongniartii* (strains and formulations) on beneficial and non-target organisms (this table was taken from Zimmermann, 2007)

Beneficial organism	Fungus (Strain/Formulation)	Lab./Field Trials (L/F)	Results/Observations	References
Amblyseius cucumeris	*B. bassiana* (Naturalis-L, BotaniGard WP)	L/F	No detrimental effect when sprayed onto excised cucumber leaves	Jacobson et al., 2001
Aphidius colemani; Orius insidiosus; Phytoseiulus Persimilis Encarsia formosa	*B. bassiana* (commercial formulation, strain JW−1)	L	Highly susceptible under laboratory conditions, lower infection rates in greenhouse	Ludwig and Oetting, 2001
Apis mellifera	*B. bassiana*	F	Conidia were applied in bee hives: low mortality and no noticeable effect on behavior, larvae and colony characteristics	Alves et al., 1996
Apis mellifera	*B. bassiana* (unformulated Spore preparation)	L	*B. bassiana* reduced bee longevity at the two highest concentrations tested and caused mycosis at 10^6–10^8 spores per bee	Vandenberg, 1990
Apis mellifera	*B. bassiana* (Naturalis-L, Bio-Power)	L	30−day dietary and contact studies had no significant effect; LC50 (23 days, ingestion) 9 285 μg/bee	Copping, 2004
Apis mellifera	*B. brongniartii*	F	No negative effects noticed	Wallner, 1988
Arthropod and nematode populations	*B. bassiana* (Naturalis-L)	F	Chlorpyrifos had a stronger negative impact than the microbial treatment	Wang et al., 2001
Bembidion lampros; Agonum dorsale	*B. bassiana*	F/L	A negligible number was infected; low susceptibility of both species	Riedel and Steenberg, 1998
Bombus terrestris	*B. bassiana*	L/F	Able to infect bumblebees; it appears that there are no risks if the fungus is incorporated into soil or sprayed onto plants that are not attractive to bumblebees	Hokkanen et al., 2003
Carabidae; Calanthus micropterus; Calanthus piceus; Carabus violaceus; Cychrus caraboides; Leistus ruefescens; Nebria brevicollis; Pterostichus oblongopunctatus; P. niger	*B. bassiana*	L	No adverse effects noticed	Hicks et al., 2001
Carabidae; Staphylinidae	*B. bassiana*	F	Infection levels in adult ground beetles and rove beetles were low (Carabidae max. 7.6% and Staphylinidae max. 7.0%); an epizootic in the staphylinid *Anotylus rugosus* (67%) and *Gyrohypnus angustatus* (37%) was observed	Steenberg et al., 1995
Cephalonomia tarsalis	*B. bassiana*	F	3 h exposure to 100 and 500 mg/kg wheat resulted in 52.5 and 68.6% mortality	Lord, 2001
Chrysoperla carnea	*B. bassiana*	L	Temperature, starvation and nutrition stresses significantly affected the susceptibility; nutrition stress caused the most increase in adult and larval mortality	Donegan and Lighthart, 1989
Coleomegilla maculate	*B. bassiana* (isolate ARSEF 3113)	L/F	No mortality was observed	Pingel and Lewis, 1996
Coleomegilla maculate Eriopis connexa	*B. bassiana* (isolate ARSEF 731)		Mortality after direct application of spores; exposure via sprayed leaf surfaces resulted in no infection	Magalhaes et al., 1988
Coleomegilla maculate	*B. bassiana* (10 isolates)	L	6 isolates were highly virulent, 3 isolates caused low mortality	Todorova et al., 2000
Diadegma semiclausum	*B. bassiana*	L	Detrimental effects on cocoon production and emergence depending on concentration	Furlong, 2004
Formica polyctena	*B. brongniartii*	F	No negative effects noticed	Dombrow, 1988

Continuing table 3

Beneficial organism	Fungus (Strain/Formulation)	Lab./Field Trials (L/F)	Results/Observations	References
Earthworms: *Lumbricus terrestris* and others	*B. brongniartii* (commercial product of barley grains)	L/F	No effect in lab and in field noticed	Hozzank et al., 2003
Earthworms: *Lumbricus terrestris*	*B. brongniartii*	L	No effect on earth worms noticed	Arregger-Zavadil, 1992
Earthworms: *Aporrectodea caliginosa*	*B. bassiana* (Bb64)	L	No effect on hatching rate of cocoons	Nuutinen et al., 1991
Lysiphlebus testaceipe *Aphidius colmani*	*B. bassiana*	F	No significant impacts on both parasitoids	Murphy et al., 1999
Megachile rotundata	*B. bassiana* (strain for grasshopper control)	L	Spray-application of flowering alfalfa in pots: female and male mortality averaged 9%; no difference in treatment and control; however *B. bassiana* grew out from dead bees	Goettel and Johnson, 1992
Nontarget arthropods (forests)	*B. brongniartii*	F	Only 1.1% of 10 165 collected insects and spiders were infected	Baltensweiler and Cerutti, 1986
Nontarget arthropods (forests)	*B. brongniartii*	F	1 671 nontarget specimens were collected: 3.4% of them were infected, mainly species from Araneae, Thysanoptera, Homoptera, Coleoptera and Lepidoptera	Back et al., 1988
Nontarget arthropods (major predators, parasitoids and pollinators on rangeland)	*B. bassiana* (strain GHA)	F	No statistical differences in the abundance of aerial insects	Brinkman and Fuller, 1999
Nontarget arthropods (forests)	*B. bassiana* (emulsifiable concentrate)	F	From 3 615 invertebrates collected, only 2.8% became infected; *B. bassiana* could be applied to forest soil without a significant negative impact on forest-dwelling invertebrate population	Parker et al., 1997
Non-target beetle communities	*B. bassiana* (strain SP 16)	F	No detectable effects	Ivie et al., 2002
Perillus bioculatus	*B. bassiana* (six isolates)	L	5 isolates were highly pathogenic, isolate IPP46 showed low pathogenicity	Todorova et al., 2002
Pimelia senegalensis, Trachyderma hispida, Bracon hebetor, Apoanagyrus lopezi	*B. bassiana*	L	No infection in *P. senegalensis* and T. hispida ; 100% mortality in the parasitoids *B. hebetor* and *A. lopezi*	Danfa et al., 1999
Poecilus versicolor	*B. brongniartii* (Melocont-Pilzgerste, Melocont-WP, and Melocont-WG)	L	No significant negative effects on *P. versicolor* could be observed	Traugott et al., 2005
Predatory mites: *O. insidiosus, A. colemani, Dacnusa sibiria, Parasites: Encarsia Formosa, Eretmocerus Eremicus, Aphidoletes aphidimyza*	*B. bassiana* (Botanigard ES)	F	Can be used, not recommended during application of *B. bassiana* used with caution during application of *B. bassiana*	Shipp et al., 2003
Prorops nasuta	*B. bassiana* (3 isolates)	L	Strain 25 caused the lowest infection level	De La Rosa et al., 2000
Serangium parcesetosum	*B. bassiana*	L	The predator had significantly lower survivorship when sprayed with *B. bassiana* than with *P. fumosoroseus*; feeding on *B. bassiana* contaminated prey caused 86% mortality	Poprawski et al., 1998

involved in evading host immune responses (Wang and St. Leger, 2006).

4 Entomopathogenic fungal virulence enzymes

The initial interaction in the pathogenesis is mediated by mechanical force, enzymatic processes and perhaps certain metabolic acids. The enzymes responsible for successful interaction with the host and environment are listed in Table 4 (Khachatourians and Qazi, 2008). The enzymes involved in pathogenesis of insects are generally grouped in to proteases and peptidases, chitinases and lipases.

4.1 Proteases and peptidases

Insect cuticle mainly is composed of chitin and protein; hence proteases and peptidases of EPF are important for the degradation of the insect cuticle, saprophytic growth of the fungi, activation of the prophenol oxidase in the hemolymph, and they act as virulence factor. The fungi from which protein degrading enzymes proteases, collagenases, and chymoleastases have been identified and characterized are *A. aleyrodis*, *B. bassiana*, *B. brongniartii*, *E. coronata*, *Erynia* spp., *Lagenidium giganteum*, *Nomuraea rileyi*, *M. anisopliae* and *V. lecanii* (Charnley and St Leger 1991; Khachatourians, 1991, 1996; Sheng et al., 2006). Joshi et al., (1995) cloned extracellular subtilisin-like serine endoprotease (Pr1) from *B. bassiana* and subtilisin-like protease (Pr1B) (Joshi et al., 1997) with 54% sequence homology to Pr1A. Screen and St Leger (2000) found chymotrypsin (CHY1) of 374 amino acids with pI of 5.07 and MW38279 from *M. anisopliae*. Similarly, Freimoser et al (2005) identified some overlapped gene responses with unique expression patterns in response to cuticles from *Lymantria dispar*, *Blaberus giganteus* and *Popilla japonica* and measured gene expression responses to a number of insect cuticles by using cDNA microarrays constructed from an expressed sequence tags (EST) clone collection of 837 genes.

Small and Bidochka (2005) identified the sequence of seven conidiation associated genes (*cag*) using subtractive hybridization in *M. anisopliae*. Out of which, cag7 was found to be essential for cuticle degradation having encoded an extracellular subtilisin-like proteinase (Pr1). Kim et al (1999) described the gene structure and expression of a novel *B. bassiana* protease (bassianin I) which is 1 137 bp and 379 amino acids long. Bidochka and Melzer (2000) reported genetic polymorphisms in three

Table 4 Entomopathogenic fungal protein encoding genes isolated and sequenced (Khachatourians and Qazi, 2008)

Fungus	Gene	Enzyme	References
Metarhizium anisopliae	*sod*	Superoxide dismutase	Shrank et al., 1993
	Pr1B	Subtilisin like protease	Joshi et al., 1997
	Pr1 (A-K)	Protease	Bagga et al., 2004
	CRR1	DNA binding protein	Screen et al., 1997
	nrr1	Nitrogen response regulator	Screen et al., 1998
	chit1	Chitinase	Bogo et al., 1998
		Chitinase	Kang et al., 1998
		Chitin synthase	Nam et al., 1998
	chi2	Chitinase	Baratto et al., 2003; 2006; Screen et al., 2001
	MeCPAA	Zinc carboxypeptidase	Joshi and St Leger, 1999
	*ssg*A	Hydrophobin	Bidochka et al., 2001
		Trehalase	Zhao et al., 2006
		Peptide synthetase	Bailey et al., 1996
	trp1	Tryptophan synthetase	Staats et al., 2004
Beauveria bassiana	*prt1*	Protease	Joshi et al., 1995
		Bassianin I	Kim et al., 1999
	prt1−like	Serine endoprotease	Fang et al., 2002
	chit	Chitinase	Fang et al., 2005
		Endonuclease	Yokoyama et al., 2002
	buv1	UV repair	Chelico et al., 2006
B. brongniartii	*buv1*	UV repair	Chelico et al., 2006

subtilisin-like protease isoforms (Pr1A, Pr1B, Pr1C) from isolates of *M. anisopliae* and an extracellular protease from *B. bassiana* (BBP) has also been purified and characterized by Urtiz and Rice (2000). The isoelectric point of BBP was 7.5 and it is 0.5 kDa smaller than Pr1. Fang et al (2002) reported a cuticle-degrading protease (CDEP-1) from *B. bassiana* predicting a protein of 377 amino acids (Mr 38 616, pI 8.302) with 1 134 bp. Southern analysis indicated that CDEP-1 is a single-copy gene. St Leger et al (1996) constructed an engineered mycoinsecticides based on *M. anisopliae* by over-expressing the toxic protease Pr1 from *M. anisopliae* genome to accelerate the killing speed of *M. anisopliae*. The over-expression of Pr1 in the hemolymph of *M. sexta* activates the phenoloxidase system which causes 25% reduction in the time of death and 40% reduction in food consumption.

4.2 Chitinases

The major component of insect cuticle is chitin, therefore both endo and exo-chitinases play critical roles in the cleavage of *N*-Acetylglucosamine (NAGA) polymer of the insect cuticle into smaller units or monomers. Khachatourians (1991) demonstrated that the extracellular chitinases are virulence determinant factors. Chitinolytic enzymes (*N*-acetyl-β-D-glucosaminidases and endochitinases were present in the broth culture supplemented with insect cuticles from *M. anisopliae*, *M. flavoviride*, and *B. bassiana* (St Leger et al., 1996). The chitinase from *M. anisopliae* consists of acidic (pI 4.8) proteins with molecular masses 43.5 kDa and 45 kDa. The identified N-terminal sequences of both bands were similar to an endochitinase from *Trichoderma harzianum*. Valadares-Inglis and Peberdy (1997) located chitinolytic enzymes in enzymatically produced protoplasts and whole cells (mycelia) of *M. anisopliae*. No significant induction was observed from mycelia, yet protoplasts were found to induce these enzymes significantly. The majority of chitinolytic activity was cell-bound in both whole cells and protoplast preparations, and the activity was mainly located in the membrane fraction.

Kang et al (1998, 1999) reported a chitinase with molecular mass of 60 kD from *M. anisopliae* grown in a medium containing chitin as the sole carbon source with an optimum pH of 5.0, which is different from the chitinases values previously reported by St Leger et al (1996) for endo-chitinases of 33.0, 43.5, and 45 kDa and exo-chitinases of 110 kDa. Screen et al (2001) cloned the chitinase gene (*Chit1*) from *M. anisopliae* sf. *acridum* ARSEF strain 324 and *M. anisopliae* sf. *anisopliae* ARSEF strain 2575 (*Chit1*) using the promoter of *Aspergillus* (*gpd*) for constitutive expression.

A 42 kD chitinase of *M. anisopliae* was expressed and characterized in *Escherichia coli* by Baratto et al (2003) using a bacteriophage T7- based promoter expression vector. Baratto et al (2006) performed transcriptional analysis of the chitinase *chi2* gene of *M. anisopliae* var. *anisopliae* and showed that it has 1 542 bp encoding for a deduced 419 amino acids. Nahar et al (2004) reported that the extracellular constitutive chitin deacetylase (CDA) secreted by *M. anisopliae* converts chitin (a β-1, 4-linked N-acetyl-glucosamine polymer) into its deacetylated form chitosan (a glucosamine polymer). This CDA was not inhibited by solubilized melanin.

Fang et al (2005) purified an endochitinase from liquid cultures of *B. bassiana* supplemented with chitin. Bbchit1 was 33 kD (pI 5.4) and the encoding gene, *Bbchit1*, and its upstream regulatory sequences were cloned based on N-terminal amino acid sequence. *Bbchit1* contains no introns and it is present as a single copy in the *B. bassiana* genome. The amino acid sequence of Bbchit1 is similar to that of the endochitinase of *Streptomyces avermitilis, S. coelicolor* and *T. harzianum* (Chit36Y), but not to EPFs that reflect novel chitinases. Fang and co-worker (2005) constructed a *B. bassiana* transformants (*gpd-Bbchit1*), which overproduced *Bbchit1* and had enhanced virulence.

4.3 Lipases

Although the major bulk components of the insect cuticle are protein and chitin, the outermost epicuticular surface layer are made up of a complex mixture of non-polar lipids. Epicuticular lipids play a role in chemical communication events (Blomquist and Vogt, 2003), and in keeping the cuticular surface dry which affects insecticide and chemicals penetration (Hadley, 1981; Blomquist et al., 1987; Juárez, 1994). They exhibit certain unique structural characteristics such as relatively high molecular mass and chemical stability, which is mainly due to specific physicochemical properties such as length and branching of the carbon chains (usually between 20 to more than 40 carbons), as well as the position and the kind of functional groups and double bonds. The most abundant components are long chain HC, wax esters, fatty alcohols and free or sterified fatty acids.

The insect epicuticle contains lipoproteins, fats, and waxy layers which would be barriers to EPF without the action of lipases and lipoxygenases, as some of these structures have anti-fungal activities, and EFP can't use them as substrates (Khachatourians, 1996). In addition, some chains of saturated fatty acids even inhibit the growth of some EPF. Lord et al (2002) showed a role for the lipoxygenase pathway through eicosanoid-mediated cellular immune response to the *B. bassiana*. James et al (2003) demonstrated that conidial germination of *B. bassiana* and *P. fumosoroseus* are affected by cuticular lipids and silverleaf whitefly (*B. argentifolii*). The whitefly nymphs produce thick coating of long-chain wax esters affecting spore germination.

4.4 The function of phospholipases in cuticle penetration

Since lipids represent major chemical constituents of the insect cuticle, enzymes capable of hydrolyzing these compounds, such as phospholipases, could be expected to be involved in the cuticle disruption processes that occur during host invasion. Phospholipases are a heterogeneous group of enzymes that are able to hydrolyze one or more ester linkages in glycerophospholipids. The action of phospholipases can result in the destabilization of membranes, cell lysis and the release of lipid second messengers (Ghannoum, 2000). These enzymes are categorized according to the location of the ester link that is cleaved (Figure 2). Although phospholipase B (PLB) refers to an enzyme that can remove both *sn−1* and *sn−2* fatty acids, this enzyme also has lysophospholipase-transacylase activity.

Extracellular phospholipases have been implicated as pathogenicity factors for bacteria, rickettsiae and protozoa. The type of phospholipase involved in virulence varies with the organism. For example, *C. perfringens* (Alape-Giron, 2000) secretes a phospholipase C (PLC), whereas *T. gondii* secretes a phosphorlipase A (PLA). The importance of these enzymes, especially PLB, for virulence has so far only been verified in medically important fungi. PLB was secreted by different clinically important fungal species such as *Candida albicans* (Mukherjee, 2001), *Aspergillus fumigatus* and *Cryptococcus neoformans*. The role of PLB in the pathogenicity of entomopathogenic fungi remains to be determined, even in the best-studied species *M. anisopliae*.

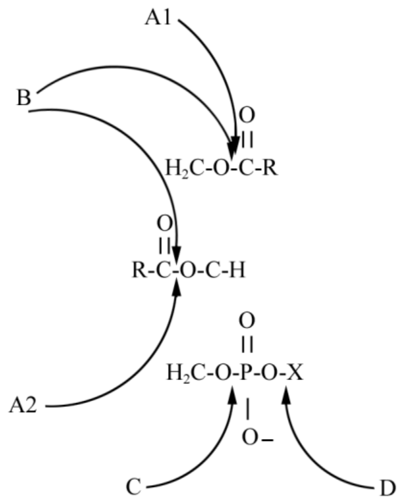

Figure 2 Sites of action of phospholipases
Note: A1, A2, B, C and D indicate cleavage sites of the corresponding phospholipases (PLA1, PLA2, PLB, PLC and PLD)

5 Advantages of using fungi as insecticides

The advantages of using fungi as insecticides are: (1) They have high degree of specificity for controlling pest without affecting beneficial insect predators and non-harmful parasites. (2) They have no hazard effects on environment or the health of mammals which is normally affected by chemical insecticide applications. (3) They have different ways of infection; hence insect resistance cannot be developed and they can be used as prolonged pest control. (4) They have genes for secretion of insect toxins; hence they have high potentials for further development by biotechnological research. (5) Some of them have endophytic capability; hence they can play important roles in the activation of immune system. (6) High persistence in the environment provides long-term suppression effects of entomopathogenic fungi on pest.

6 Disadvantages of the fungi as biocontrol agents

(1) They have very slow killing rate: normally 2−3 weeks are required to kill the insects whereas chemical insecticides may need only 2−3 hours. (2) As the pathogenesis process of the fungi is bioprocess, it requires specific conditions to be carried out, such as specific temperature, humidity and period of light. (3) They have high specificity in killing pests making them a narrow host killer while a broad range killer pesticide is required for commercialization, hence additional control agents are needed for other pests. (4) Their production is relatively expensive and the short shelf life of spores necessitates cold storage. (5) The persistence and efficacy of entomopathogenic fungi in the host population vary in different insect species, thus insect-specific application techniques need to be optimized to retain long-term impacts. (6) They also present potential risks to immunodepressive people.

Conclusion

Since centuries ago, fungi have always been used for medicinal and other beneficial proposes and they are just as important nowadays. In this review, we summarized the advantages and applications of fungi as a biopesticides, attempted and collected the knowledge about the entomopathogenic fungi as biocontrol agents. We collected knowledge of the past and present about entomopathogenic fungi to explore ways to improve the abilities of entomopathogenic fungi as a biocontrol agent. In this way, new ideas and hypothesis will emerge which will farther help developing the fungi's capabilities as biocontrol agents. New techniques will be developed which will help manage the pest in a better way as the present pathogenesis mechanism of fungi is slow and needs improvement. Genetic and proteomic studies are expected to be the main tools for the future development of the entomopathogenic fungi as biocontrol agents; however, in the near future, there will be awider array of techniques become available to biologists which will enable us to take full advantage of entomopathogenic fungi.

Acknowledgements

This work was supported by International Cooperation Fund (No. 2012DFR30810). We also acknowledge the China Scholarship Council for the PhD scholarship.

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