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Chapter 5
Unraveling the Importance of Metabolites from Entomopathogenic Fungi in Insect Pest Management

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Abstract More than 750 species of entomopathogenic fungi (EPF) belonging to 85 genera are reported to date infecting more than 1000 species of insect pests. The typical EPF mode of action by direct penetration through the insect cuticle and establishment in host haemocoel makes them successful biocontrol agents. However, this process requires a biochemical artillery like the production of enzymes, toxins and other metabolites that facilitates host infection and invasion. Enzymes like chitinase, proteinase and lipase are directly involved in degradation of the host cuticle, the first and foremost barrier towards EPF infection. Secondary metabolites such as destruxins of *Metarhizium*, beauvericins of *Beauveria*, hirsutellides of *Hirsutella*, isarolides of *Isaria*, cordyols of *Cordyceps*, vertihemipterins of *Verticillium* etc., directly and indirectly disable the defence mechanism of insect hosts and accelerate the EPF infection process. The chemical nature of these secondary metabolites range from simple non-peptide pigments like oosporine to highly complex piperazine derivatives, like vertihemiptellides. These structural distinctions imply multiple modes of action which are yet to be deciphered along with their synthesis and regulatory mechanisms. In this chapter we focus on a few important issues related to the utilization of metabolites by EPF for insect host invasion. The major focus is given to enzymes, toxins and other metabolites synthesised by a few important EPF species, and their mode of action to counteract the host cellular and humoral defence mechanisms. Some strategies to enhance the infection efficiency of EPF, their regulatory mechanism and genetic basis behind production are detailed.

Keywords Entomopathogens · Chemical pesticides · Joint action · Compatibility · Synergism

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5.1 Introduction

Environmental and human hazards associated with preponderant use of chemical pesticides lead to the use of safe pest management methods, especially the entomopathogenic organisms. In the mounting knowledge of these pest suppressive agents, entomopathogenic fungi (EPF) occupy a great niche with respect to number of studies and wide commercial application. They infect a wide range of insect pests and play an important role in agricultural ecosystems by causing natural epizootics. They can be hence considered as eco-friendly alternatives to chemical pest management (Rohlfs and Churchill 2011). For example, the two predominant species of EPF, *Beauveria bassiana* and *Metarrhizium anisopliae* have a host range of over 1000 insect species, covering more than 50 insect families (Jaber and Ownley 2018). The distinct symptomatology associated with fungal infection and prevalence of epizootics lead to their early identification as pathogenic organisms (Mondal et al. 2016). To date, more than 750 species of entomopathogenic fungi belonging to 85 genera and throughout the major lineage of class fungi are known to infect insects. The largest numbers of fungal species that are pathogenic to insects belong to the order Hypocreales (Dikarya, Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae) (Mondal et al. 2016; Shah and Pell 2003).

Properties like the vast biodiversity in species and strains, their ubiquitous availability, target specificity and environmental competency, as well as amenability to mass production and facultative saprotrophic survival etc. are the major backing attributes for the wide spread of EPFs use (Thomas and Read 2007). Most importantly, their typical biology, especially pathogenicity by direct penetration through insect cuticle, makes them one of the widely used biocontrol agents (Shah and Pell 2003). To achieve this task EPFs have developed much biochemical tools that facilitate host infection and invasion. These evolutionarily-gained resources can be broadly categorized into enzymes, toxins and metabolites. Enzymes like chitinases and proteases are directly involved in degradation of target substrates thereby enabling the destruction of host physical barriers. Besides, they also facilitate interkingdom host switching, nutrient scavenging, saprotrophic survival etc. (Molnár et al. 2010). Whereas, the proteinaceous toxins (for example: lipase, chitinase, protease, etc.) and metabolites (for example: destruxins, beauvericins, beauverolides, isariolides, etc.) are mostly involved in host invasion by inhibiting host immune/defense responses that ultimately kill the host. They are also involved in defence from other competing pathogens and saprotrophs that exploit host resources. This antagonistic interaction with co-occurring organisms (con- and heterospecific and prokaryotic microorganisms) also decides evolutionary fitness of a given EPF (Rohlfs and Churchill 2011). Above all, utilization of trehalose, the blood sugar of insects, is an important evolutionary adaptation by entomopathogenic fungi to utilize insects as source of nutrients (Jaber and Ownley 2018).

Although the role of metabolites is evidenced largely in EPFs, their permutations and combinations are largely governed by EPF species or strains and target hosts (Shah and Pell 2003). During the co-evolution, both insect hosts and

pathogenic fungi exhibited much plasticity in tolerating, as well as simultaneous evolution of counter machineries (Rohlfs and Churchill 2011). This occurs because the majority of EPFs are either generalists or group specific (especially Hypocrealean species). Some EPFs have also non-insect hosts. In order to explore these diverse hosts, EPFs should have both generalized as well as specialized arm machineries acquired through their evolutionary adaptations. Many studies reported an impressive array of components involved in insect host exploitation. Moreover, entomophthoralean fungi are obligate pathogens with very narrow host range. The mounting pathogenecity exhibited by these fungi against host insects is governed by specialized biochemical adaptations. The arm race between these specialized and generalized EPFs led to the selection of fascinating metabolites that are presented in this chapter. The isolation, identification and commercial application of these metabolites in agricultural pest management is also discussed.

5.2 Biology and Ecology of Entomopathogenic Fungi

In general, EPFs are considered as opportunistic because of their wide host range, saprotrophic survival and adaptation to varied environmental conditions. They belong to families of Zygomycota and Ascomycota, in the class Hyphomycetes in Deuteromycota, as well as in the families of Chytridiomycota and Oomycota (Mondal et al. 2016). Besides infecting insect pests, EPF members may also be found infecting other arthropod hosts and arachnids. For example, *Beauveria bassiana* has been reported to infest more than 700 species and is the most common pathogen associated with almost all major insect taxa found in temperate regions (Jaber and Ownley 2018). However, life cycle of any given species or strain of EPF depend on host factors like species, accessibility, population numbers, life stage of infection etc. Pathogen-related factors include strain, environmental competency, virulence, etc. Above all, environmental factors, most importantly temperature and humidity, decides the infection levels and spread of pathogenic fungi. The knowledge about these ecological factors is important in conserving them, predicting their pest management potential and receiving the maximum ecosystem services to capitalize pest management.

Studies by Tscharntke et al. (2005) showed that the agricultural landscape may directly impact agro-ecosystem diversity, including EPFs and their deliverables. Many studies used soil as a conventional site for EPF isolation. Although great variability in species composition was observed amongst different soil habitats (agricultural, forest, meadows, barren lands etc), *M. anisopliae* was found to be common in regularly disturbed agricultural fields (Gibson et al. 2014). This suggests native isolates of *M. anisopliae* are suitable candidates for conventional biological control programs, even though, some studies reported successful biocontrol with *B. bassiana* (Patočka 2016). Infected host cadavers under field conditions are highly conspicuous and are considered as the main objects for isolation of efficient native strains. Despite of their saprotrophic survival, this is the only stage in the lifecycle

of any given EPF that facilitates multiplication and population build-up in the ecosystem, as they are poor competitors for organic resources compared to other opportunistic saprotrophs. Thus, availability of susceptible host population is one of the primary requirements to sustain EPF populations. In some instances, EPF are associated with plants as endophytes or plant defending mutualists, reviewed by Jaber and Ownley (2018).

Generally, conidia (a resting asexual stage) are the infective form and share the same environment with the potential insect host. The infection process begins once conidia encounter cuticle of a susceptible host. For description purpose the infection process can be divided into several steps: (1) adherence of fungal conidia to the host cuticle through hydrophobic interactions and or by secretion of mucilaginous material (2) germination; (3) apressoria formation through germ tube differentiation; (4) penetration into the cuticle; (5) formation of blastospores/hyphal bodies in the insect haemolymph through hyphal differentiation; (6) host colonization; (7) formation of conidiophores (8) production and extrusion of conidia onto the host cadaver surface.

Conidia adhesion followed by germination is pivotal to the infection process that involves hydrophobic interactions between the spore surface proteins (hydrophobins) and the lipid layer that covers the insect cuticle (Fang et al. 2007). Lipases produced by EPFs are involved in degradation of lipid layer. This is a primary step in host recognition and production of nutrients supports conidia germination. Further breaching the cuticle layer involves a variety of enzymes including proteases, chitinases and lipases that degrade cuticular constituents (proteins, chitin and lipids) (Lubeck et al. 2008; Sbaraini et al. 2016). After penetration, the hyphae in the haemolymph differentiate either into blastospores (unicellular yeast-like cells) or grow as hyphae, causing generalized infection by utilization of host nutrients (Sbaraini et al. 2016). As the host colonization proceeds the nutrients become exhausted and the fungi produce hyphae that will emerge and yield conidia on the surface of the dead host (Gibson et al. 2014).

5.3 Metabolite Involved by EPFs in Infection

EPFs are a group of phylogenetically diverse, heterotrophic, eukaryotic, unicellular or multicellular microorganisms. Of the estimated 1.5 to 5.1 million species of fungi in the world approximately 750 to 1000 are fungal entomopathogens, placed in over 100 genera (Khan et al. 2016). EPF evolved highly specialised mechanisms to produce secondary metabolites and enzymes with immunosuppressive or otherwise toxic functions, that help them in the invasion of the insect hosts by overcoming cellular and humoral defence systems (Rohlfs and Churchill 2011). There are thousands of reported secondary metabolites from hundreds of EPFs, but their exact role is unknown in the host infection process. In this section we will briefly mention about the major enzymes and secondary metabolites produced by few EPF that have potential to be exploited as biocontrol agents.

5.3.1 Enzymes Involved in the Infection Process

The major enzymes produced by EPF to infect and overcome host immunity for a successful infection of insect host include lipases, proteases, chitinases, β-galactosidase, catalase and L-glutaminase. In this section we cover in brief the different enzymes involved in infection, their role and synthesis by EPF. A compilation of enzymes produced by EPF and their modes of action is presented in Table 5.1.

Lipases Serine hydrolases (EC 3.1.1.3) (triacylglycerol acylhydrolases) catalizing the hydrolysis of ester bonds of lipoproteins, fats and waxes that are found in the interior part of the insect integument (Haque et al. 2013). Their activities are triggered only when absorbed to an oil-water interface like the epicuticle of insects (Anguita et al. 1993). The epicuticle, the external layer of insect cuticle, is hydrophobic in nature and acts as the first barrier against microbial attack (Da Silva et al. 2010). In insects it is a heterogeneous mix of lipids, long-chain alkenes, esters and fatty acids. Lipases are responsible for penetration of the cuticle and initiate nutrient release by breaking down the epicuticle. As a counterstrategy defence mechanism,

Table 5.1 List of entomopathogenic fungi, enzymes produced and their mode of action

Enzymes	Mode of action	Entomopathogenic fungi
Lipases	Hydrolysis of ester bonds of lipoproteins, fats and waxes found in the interior part of the insect integument	*Fusarium oxysporum*, *Metarhizium anisopliae, Aspergillus flavus, Beauveria bassiana*
Proteases	Degrades the proteinaceous material of the cuticle	*Metarhizium anisopliae, Beauveria bassiana, Verticillium lecanii, Paecilomyces fumsoroseus, Isaria fumsoroseus, Tolypocladium niveum*
Chitinases	Hydrolysis the β-1,4 bonds of chitin polymer, remodeling of cell walls during hyphal growth, branching, hyphae fusion, protection from other fungi	*Trichoderma atroviridae, Trichoderma harzianum, Trichoderma virens, Metarhizium anisopliae, Beauveria bassiana, Nomuraea rileyi, Aschersonia aleyrodis, Verticillium lecanii, Isaria fumsoroseus*
β-galactosidase	Determination of blastospores permeabilisation in the haemolymph	*Aspergillus spp., Aspergillus foelidis, Beauveria bassiana, Aspergillus fonsecaeus, Aspergillus oryzae, Auerobasidium pullulans, Curvularia inequalis, Fusarium moniliformae, Metarhizium anisopliae, Metarhizium roberstii*
Catalase	Faster germination and increased toxicity, ELimation of ROS (reactive oxygen species) produced by host insect,	*Lecanicillium muscarium, Fusarium oxysporum, Verticillium dahlia, Aspergillus phoenicis*
L-glutaminate	Salt tolerance and heat stability to EPF	*Beauveria bassiana, Trichoderma koningii, Aspergillus flavus, Acremonium forcatum, Aspergillus wentii* MTCC1901, *Trichoderma harzianum*

the insect host secretes lactone B, which is responsible for the inhibition of lipolytic activity, impeding subsequent entomopathogenic infection (Da Silva et al. 2010). However, adhesion of the fungal spores to the epicuticle with the help of lipase is a mandatory pre-step that initiates the degradation of fatty acids and alkenes of the cuticle waxy surface. In studies carried out by Supakdamrongkul et al. (2010) germination of *Nomuraea rileyi* conidia was extensively enhanced when it was coupled with a lipase of 81.3 kDa, secreted by *N. rileyi*, thus increasing the mortality of *Spodoptera litura* larvae.

Proteases Proteases (EC 3.4) form a large group of hydrolytic enzymes that cleave the peptide bonds of proteins and break them into small peptides and amino acids. Proteases are considered as the most important enzymes for the EPF infective process. After the epicuticle has been broken down by lipases, the invading fungi produce great quantities of Pr1 (Serine protease), which degrades the proteinaceous material of the cuticle. Further degradation of solubilised proteins in to amino acids by aminopeptidases and exopeptidases in the haemolymph provides nutrients for fungal development (Qu and Wang 2018). Subtilisin like serine-protease Pr1 and trypsin-like protease Pr2 are the most frequently studied proteolytic enzymes in EPF. The activities of Pr1 and Pr2 have been determined in *B. bassiana, M. anisopliae, Lecanicillium lecanii, Nomuraea rileyi* and *M. flavoviridae*.

Chitinase Chitin is a polymer of β-1,4-acetyl-D-glucosamine and is the most abundant polymer after cellulose (Tharanathan and Kittur 2003). It forms the main structural component of fungal cell and exoskeleton of insects (Haque et al. 2013). Chitinases (EC 3.2.2.14) hydrolyze the β-1,4 bonds of the chitin polymer, producing dominant N-N′-diacetylchitobiose. This is done by breakdown of N-acetyl glucosamine (GlcNac) monomer by chitobiose. Chitinases collaborate with proteases to degrade insects cuticle (Joop and Vilcinskas 2016) and have a role in different stages of the EPF life cycle (germination, hyphal growth, morphogenesis, nutrition and defense against competitors) (Sumarah et al. 2010). Chitinases are also known for their role in various physiological functions including: i) chitin degradation in fungal cell wall and exoskeleton of arthropods, used as nutrient source; ii) remodeling of cell walls during hyphal growth, branching, hyphae fusion, autolysis and competence; iii) protection from other fungi located in the same ecological niche (Sumarah et al. 2010). These chitinolytic enzymes are divided into N-acetylglucosaminidases and chitinases, which differ in their breakdown patterns. The former catalyses the breakage of terminal non-reducing N-acetylglucosamine (GlcNac) residues from chitin. Whereas, the latter catalyse the hydrolysis of β-1,4 linkages of chitin and chitooligomers, resulting in release of short-chain chitooligomers or monomers (Horsch et al. 1997; Mondal et al. 2016). See also Chap. 1 in this Volume for more details.

β-Galactosidase They play a certain role in whole-cell permeabilisation and mainly in determination of blastospores permeabilisation in the haemolymph of

host insect. However, the exact role of the enzyme is yet to be deciphered (Resquín-Romero et al. 2016).

Catalase This enzyme is encoded by the catE7 gene in *B. bassiana* and is activated by stress and detoxification. The transformed strains of *B. bassiana* over-expressed catE7 germinated faster than wild-type and insect bioassays revealed increased virulence and mortality of *Spodoptera exigua* (Chantasingh et al. 2013). It may be assumed that catalase activity eliminates reactive oxygen species (Qu and Wang 2018), hydrogen peroxide and other host derived toxins present in the haemocoel (Vierstraete et al. 2004). Catalase activity might also reduce insect defence capabilities such as melanisation.

L-Glutaminase The enzyme was isolated from an alkophilic and salt tolerant fungus *Beauveria* sp., from marine sediment and is assumed to have a role in salt tolerance and heat stability. But the exact role in entomopathogenicity is yet to be deciphered (Jaber and Ownley 2018).

5.3.2 Toxins and Other Metabolites Involved in Infection Process

In this section we will concentrate on the secondary metabolites produced by major entomopathogenic fungi, *Beauveria*, *Metarhizium*, *Hirsutella*, *Isaria*, *Cordyceps*, *Paecilomyces*, *Verticillium*/*Lecanicillium* and few minor EPF. As a whole the toxins and secondary metabolites produced by EPFs are inseparable as both have similar types of action. They were hence discussed in this section with respect to the associated fungal species. A brief detail about the metabolites discussed is also presented in Table 5.2.

Beauveria **spp.** members of this genus are well known for producing large array of biologically active metabolites (Khan et al. 2016). There are mainly volatile organic compounds, alkaloids (tennelin, bassianin, pyridovericin, pyridomacrolidin), non-peptide pigments (oosporein), non-ribosomally synthesized cyclodepsipeptides (beauvericins and allobeauvericins, bassianolides) and cyclopeptides (beauveriolides), as well as other metabolites involved in pathogenesis and virulence (BbL lectin), that have potential or realized industrial, pharmaceutical and agricultural uses.

(a) **Volatile organic compounds (VOCs):** EPF has to penetrate through the cuticle lipid layers that are composed of mixture of very long chain hydrocarbons with different fatty alcohols and fatty acids (Patočka 2016). Volatile organic compounds released by fungi can overcome this protective layer. Approximately 300 known VOCs are emitted by fungi (Morath et al. 2012). Amongst those released by *B. bassiana*, di-isopropyl naphthalenes (>57%) (2,3- and

Table 5.2 List of EPF, their metabolites and chemical nature

EPF species	Secondary metabolite	Chemical nature	Mode of action	References
Chaetomium sp.	Oosporein	Non-reduced polyketide	Mycotoxin against insects	Pegram and Wyatt (1981)
Gnomonia erythrostoma	Erythrostominones	Octaketide naphthoquinones	Antimalarial and moderate to weak cytotoxic activity	Unagul et al. (2005)
Aspergillus nidulans	Emodin	Octaketide naphthoquinones	Mutagenic, cytotoxic and apoptotic activity	Srinivas et al. 2007
Cephalosporium aphidicola	Cephalosporolides	Pentaketide	Insecticidal against aphids	Ackland et al. (1985)
Trichoderma sp.	Peptaibols	Linear lipopeptide	Dissipate membrane potential and disturb osmotic balance	Toniolo et al. (2001)
Paecilomyces lilacinus, P. Marquardii, Acremonium sp.	Leucinostatins (Paecilotoxins)	Linear nine-residue peptaibiotics	Potent uncouplers of oxidative phosphorylation in mitochondria	Lucero et al. (1976)
Torrubiella cylindrosporum	Efrapeptins	Linear pentadecapeptides	Specific inhibitors of F0F1 ATPase of mitochondria	Gledhill and Walker (2006)
Aschersonia inseperata	Destruxins A4 and A5	Cyclic depsipeptides	Insecticidal, antibiotic and cytotoxic	Pedras et al. (2002)
Tolypocladium niveum	Cyclosporins	Cyclic undecapeptides	Used in immunosupressant therapy	Wenger et al. (1986)
Paecilomyces militaris	Militarinones	Tyrosine containing heptaketide acyltetramic acids	Neuritogenic activity and immediate onset of apoptosis	Schmidt et al. (2003)
Akanthomyces gracilis	Akanthomycin	C-methylated pentaketide	Antibacterial and antimalarial activity	Wagenaar et al. 2002
Torrubiella luteurostrata	Torrubiellutins A-C	Macrocyclic lactones	Cytotoxic and activity against neoplastic cell lines	Pittayakhajonwut et al. (2009)
Paecilomyces tenuipes	Paecilomycines A, B and C, Tenuipesine A	Sesquiterpenoids with trichothecene skeleton	Cytotoxic activity	Kikuchi et al. (2004)

(continued)

Table 5.2 (continued)

EPF species	Secondary metabolite	Chemical nature	Mode of action	References
Paecilomyces cinnamomeus	Dustatin, zeorin	Triterpenoid hopanes	Moderate antimycobacterial activity	Isaka et al. (2005)
Paecilomyces fumosoroseus	Dipicolinic acid	Intermediate of lysine biosynthesis	Inhibits prophenoloxidase system during melanin biosynthesis	Paterson (2008)
Verticillium lecanii	Vertilecanin A	Methyl-esters of phenopicolinic acid	Sublethal activity against *Helicoverpa zea*	Soman et al. (2001)

2,6-isomers), ethanol (10.2%) and sesquiterpenes (6.4%) have been detected. Minor amounts of benzeneacetaldehyde, straight even-chain saturated hydrocarbons of 10–12 and 16 carbons (mainly n-decane), 1-pentadecene, alkylbenzene derivatives, and methyl-alkyl ketones are also detected (Patočka 2016).

(b) **Alkaloids:** They are the derivatives of 2-pyridine. As of now tennelin, bassianin (Gibson et al. 2014), pyridovericin and pyridomacrolidin have been found (Bode 2009). But, their exact role in EPF interaction with insect hosts is not yet clarified. Many of these compounds are shown to possess neuritogenic activity in cell and animal models (Patočka 2016).

(c) **Pigments:** *Beauveria bassiana* produces yellow pigmented substance, tennelin and bassianin and a red pigment oosporein, a dibenzoquinone derivative. Oosporein has antibiotic and cytotoxic properties (Alurappa et al. 2014). Tenellin and bassianin inhibit haemocyte membrane ATPase activity. Whereas all the three pigments inhibit Ca^{2+}-ATPase to a greater extent than Na^{+}/K^{+}-ATPase (Patočka 2016).

(d) **Cyclopeptides and cyclodepsipeptides:** a series of cyclic, biologically active, non-ribosomally synthesised depsipeptides like beauvericins and allobeauvericins, bassianolides and beauveriolides, that have cytotoxic activity, are produced by *B. bassiana*. Currently, seven different beauvericins are known: beauvericin**,** beauvericins A, B, and C and allobeauvericins A, B and C (Brahmachari 2015). Beauvericins induce apoptosis through the mitochondrial pathway, including decrease of relative oxygen species generation, loss of mitochondrial membrane potential, release of cytochrome c, activation of Caspase-9 and -3, and cleavage of poly (ADP-ribose) polymerase (PARP) (Tao et al. 2015). They also inhibit cell proliferation by arresting cells in G0/G1 and increasing apoptosis. Bassianolid is a cyclotetradepsipeptide isolated from cultured mycelia of *B. bassiana* and is pathogenic to insects. Beauveriolides are cyclopeptides with oral activity on acyl-coenzyme A, and cholesterol-acyltransferase inhibitors.

Metarhizium **spp.** *Metarhizium anisopliae* is the best characterized and most widely used EPF in biological control. It has a broad host range including insects and ticks. *Metarhizium* spp. produce a wide range of secondary metabolites that are insecticidal, anti-viral and phytotoxic in nature.

(a) **Destruxins:** these are the most prevalent metabolites produced by *M. anisopliae* and by far the most exhaustively investigated EPF toxins. They are characterized as important virulent factors accelerating the deaths of infected insects. Structurally, the destruxins are cyclic depsipeptides composed of five amino acids and α-hydroxycarboxylic acid moiety, also studied for their toxicity against cancer cells. A total of 38 destruxins and their analogues are reported, divided into five chemical basic groups labelled destruxin A to E (Cavelier et al. 1998; Ravindran et al. 2016). Destruxins A, B and E showed insecticidal property (Li et al. 2017). These toxins weaken the host immune defences, damage the muscular system and malpighian tubules, affecting excretion and leading to feeding and mobility difficulties (Pal et al. 2007). Therefore, the action of destruxins reduces host immunity, mobility and defence mechanism. The *Metarhizium* isolates that produce higher amounts of destruxins are also the most virulent (Ravindran et al. 2016).
(b) **Aurovertins**: Aureovertins are chemically nonaketide polyene pyrones, that resemble the destruxin D analogue of *M. anisopliae*. These compounds are selective inhibitors of the mitochondrial F1F0-ATPase, which catalyses the terminal step of oxidative phosphorylation (Gledhill and Walker 2006).
(c) **Helvolic acid (Fumigacin)**: this is a 1,2-dihydro analogue of helvolic acid, isolated from *M. anisopliae* with antibiotic activity (Rachmawati et al. 2017). Fumigacin did not alter the cellular immune response of insects but has a cytotoxic activity (Sbaraini et al. 2016).
(d) **Serinocyclins**: cyclic heptapeptides isolated from conidia of *M. anisopliae* cultured on agar. Serinocyclin A features several non-proteinogenic amino acids like 1′-aminocyclopropane-1-carboxylic acid and (2R,4S)-hydroxylysine. Serinocyclin B contains D-lysine instead of hydroxylysine. Serinocyclin A showed no antifungal or antibacterial activity, but exposed mosquito larvae exhibited abnormal swimming, characterized by an inability to stabilize the head (Gibson et al. 2014).
(e) **Metarhizins A and B:** these diterpene pyrone derivatives were recently isolated from *M. flavoviridae* as antiproliferative agents against both insects and cancer cell lines (Gibson et al. 2014). They closely resemble viridotoxins which are cytotoxic, antimalarial and anti-inflammatory, with strong inhibition of cytochrome oxidase-2.

Hirsutella **spp.** *Hirsutella* is a genus of asexually reproducing fungi that are pathogens of insects, mites and nematodes. The teleomorphs of *Hirsutella* species belong to the genus *Ophiocordyceps*. *Hirsutella* is known to produce a wide range of secondary metabolites with insecticidal, acaricidal and antibiotic activity.

(a) **Phomalactone**: this is a tetraketide pyrone with antimicrobial, phytotoxic and cytotoxic activity, isolated from *H. thompsonii* var. *synnematosa.* It was found to inhibit fungal germination of filamentous fungi and showed mild toxicity to apple maggot, *Rhagoletis pomonella* (Molnár et al. 2010).
(b) **Hirsutellic acid:** the linear tetrapeptide hirsutellic acid A was isolated from *Hirsutella* sp. BCC1528, featured by a C-terminal anthranilic acid moiety. It showed activity against the malaria parasite *Plasmodium falciparum*, but no significant toxicity to Vero cells and human cancer cell lines (Sbaraini et al. 2016).
(c) **Hirsutellide A:** it is a cyclic hexadepsipeptide which was isolated from *H. kobayasii.* It is a cyclic dimer of the tripeptidol (R)-2-hydroxy-3-phenylpropanoic acid-L-allo-isoleucine-N-methylglycine. These metabolites displayed anti-mycobacterial and weak antimalarial activities (Vongvanich et al. 2002).
(d) **Cytochalasins**: Phenylalanine containing cytostatic cytochalasins are produced by *Hirsutella* sp. and *M. anisopliae* (Cytochalasin D is also known as Zygosporin A) (Vilcinskas et al. 1997). Cytochalasins are a large family of fungal PKS-NRPS (Polyketide synthases – nonribosomal peptide synthases), hybrid metabolites characterized by a tricyclic ring structure with an isoindolone ring fused to the macrocycle. Various members of the cytochalasin family displayed antibiotic, antiviral, anti-inflammatory and cytotoxic activities (Vilcinskas et al. 1997). They specifically bind to actin filaments, thus inhibiting cytokinesis (Singh et al. 2017).

Isaria **spp. (***Paecilomyces* **spp.)** *Isaria* is an entomopathogenic fungal genus with more than 100 species which play an important role in agriculture. The anamorphic stage of *Isaria* is the genus *Cordyceps* whose members mostly infect and kill insects in nature. *Isaria* spp., produce numerous secondary metabolites which have antifungal, antiviral and insecticidal activity. In this section only the SMs produced by *Isaria* will be included and those produced by *Codyceps* will be examined in the next section.

(a) **Cicadapeptins:** they were isolated from *I. sinclairii* and are shown to inhibit the acetylcholine induced secretion of catecholamines in bovine adrenal chromaffin cells (Gibson et al. 2014). Cicadapeptins I and II are unique linear fungal peptides that show moderate antibacterial activity against *Bacillus* sp. and *Escherichia coli*.
(b) **Lateritin:** it is a cyclic non-ribosomal depsipeptide. Lateritin is a diastereoisomer of diketopiperazines, that are frequent microbial metabolites formed by the intramolecular cyclization of dipeptides and dipeptidols. Lateritin was isolated from
I. japonica and was identified as an inhibitor of acyl-CoA:cholesterol acytransferase (ACAT) (Hasumi et al. 1993).
(c) **Isarolides:** they are a family of cyclic tetradepsipeptides featuring 3-hydroxy-4-methylalkanoic acid units, isolated from *I. fumosorosea* (Joop and Vilcinskas 2016). They are identical to the beauverolides isolated from *B. bassiana*. The

isarolides exhibited moderate insecticidal activity against *Spodoptera litura* and *Callosobruchus chinensis* (Mochizuki et al. 1993). More intriguingly, isarolides reduced lipid droplet accumulation in mouse macrophages by inhibiting ACAT and thereby blocking cholesterol ester biosynthesis.

(d) **Isaridins A and B:** An *Isaria* strain isolated from rat dung was found to produce isaridins or pseudodestruxins, featuring a second phenylalanine acylating the β-alanine. These isaridins display a wide range of interesting biological properties including insecticidal, cytotoxic and moderate antibiotic activity (Ravindran et al. 2016).

(e) **Beauvericin:** The cyclooligomer depsipeptide beauvericin is a cyclic trimer of the dipeptidol monomer D-hydroxyisovalric acid. Beauvericin is widely produced by *B. bassiana* and other *Beauveria* spp., as well as by *I. fumosorosea*, *I. japonica*, *I. tenuipes* and *I. cicadae.* They have moderate antibacterial, antifungal and insecticidal activities (Gibson et al. 2014). It transports mono and divalent cations across biological membranes as a freely diffusing sandwich. Acting as an ionophore, beauvericin increases cytoplasmic Ca^{2+} concentration, causes ATP depletion and activates calcium sensitive cell apoptosis pathways (Gibson et al. 2014; Li et al. 2017).

(f) **Isariotins A-F**: The cytotoxic alkaloids isariotins were isolated from *I. tenuipes* and *I. japonica.* The isariotins appear to be derived from fatty acid or polyketide biosynthesis. The actual role of isariotins in insect infection mechanism is not yet deciphered.

(g) **Hanasanagin (XI)**: It was isolated from the entomogenous hanasanagitake mushroom (*I. japonica*) based on its activity as a potent antioxidant. Hanasanagin is a pseudo-dipeptide containing a DOPA moiety originating from L-Tyr and 3,4-diguanidinonutanoyl moiety of unknown biosynthetic origin (Sakakura and Kohno 2009; Sumarah et al. 2010).

Cordyceps **spp.** *Cordyceps* is a genus of ascomycetes that includes about 400 species. Most *Cordyceps* are endoparasitoids or parasitic mainly on insects and other arthropods. *Cordyceps* are abundant in humid temperate and tropical forests. They are extensively used in traditional Chinese medicine and known for a wide range of secondary metabolites production, which have role in pharmacology and biocontrol of insect pests. The following are some metabolites isolated from *Cordyceps* species.

(a) **Cordyol C:** It is a fungal non-reduced polyketide and chemically diphenyl ether, isolated from *Cordyceps* sp. BCC 1816. Cordyol C showed moderate antimalarial activity and cytotoxic activity *in vitro* (Li et al. 2017).

(b) **Cordytropolone:** The bicyclic tropolone was isolated from *Cordyceps* sp. BCC 1681 and showed moderate antimalarial activity and cytotoxic activity (Seephonkai et al. 2001).

(c) **Cordyanhydrides A and B (XII):** These are novel maleic anhydrides that are linear dimers or trimers, of C9 anhydride units, analogous to the cyclic nonadrides (Barton and Sutherland 1965), isolated from *C. pseudomilitaris*. They show moderate cytotoxic activity (Sulikowski and Pongdee 2006).

(d) **Cordyheptapeptides A and B:** A group of cyclic heptapeptides isolated from *Cordyceps* sp. showed antimalarial and cytotoxic activity (Rukachaisirikul et al. 2006)
(e) **Codycepins:** The nucleoside analogues cordycepin (3′-deoxyadenosine) was isolated from *C. militaris* and *C. sinensis*. Cordycepin inhibits DNA and RNA biosynthesis, showed antibiotic activity against *Clostridium* sp. and displayed insecticidal and cytotoxic effects (Mondal et al. 2016).

Verticillium/Lecanicillium **spp.** Anamorphic forms of members from the family Plectosphaerellaceae (Ascomycota). They include saprotrophs and parasites of higher plants, insects, nematodes, mollusc eggs, and other fungi. The genus includes a wide group of taxa characterized by simple but ill-defined characters. The genus, currently thought to contain 51 species, undergone recent revisions into which most entomopathogenic and mycopathogenic isolates fall within a new lineage called *Lecanicillium* (Barbara and Clewes 2003). Few *Lecanicillium* spp. are potent EPF that can infect insect pests and counteract their defense mechanism through the production of secondary metabolites.

(a) **Vertihemiptellides (XIII):** The diketopiperazines and their dimeric derivatives linked by dithio bridges were isolated from *V. hemipterigenum.* They are moderately cytotoxic and anti-mycobacterial constituents (Resquín-Romero et al. 2016).
(b) **Enniatins:** The cyclooligomer hexadepsipeptides enniatins are a group of cyclic trimeric esters of dipeptidol monomer. They are frequent metabolites of *Fusarium* spp. but are also produced by *V. hemipterigenum.* Enniatins display activities similar to those of beauvericin (Nilanonta et al. 2003). They form vertically stacked sandwich complexes with mono and divalent cations that are freely diffusible in biological membranes and thereby, disrupt transmembrane potential. They also display antibiotic, antifungal, ACAT inhibitory, cytostatic and cytotoxic activities and show antihelminthic and phytotoxic properties (Firakova et al. 2007; Gibson et al. 2014; Singh et al. 2017).
(c) **Balanol:** Balanol is a metabolite with a polyketide/fatty acyl and amino acid derived moiety whose production is thought to involve convergent pathways instead of linear biochemical routes utilizing integrated PKS-NRPS enzymes. It is one of the most potent ATP competitive inhibitors of protein kinase C (PKC) and protein kinase A (PKA).
(d) **Vertihemipterin** A: Chemically a sesquiterpinoid resorcylic acid and analogue of ascochlorin glycoside, isolated from *V. hemipterigenum*. It is a potent and selective inhibitor of bacterial respiratory quinol oxidase cytochrome b and of the trypanosome alternative oxidase, and showed promising antibiotic and anti-parasitic activity *in vitro*.

Apart from the above mentioned EPF and their secondary metabolites, there are also a group of minor entomopathogenic fungi that can infect insects and counteract the host defence mechanism to establish a successful infection. A list of such EPF, their metabolites with chemical nature is mentioned in Table 5.2 and Fig. 5.1.

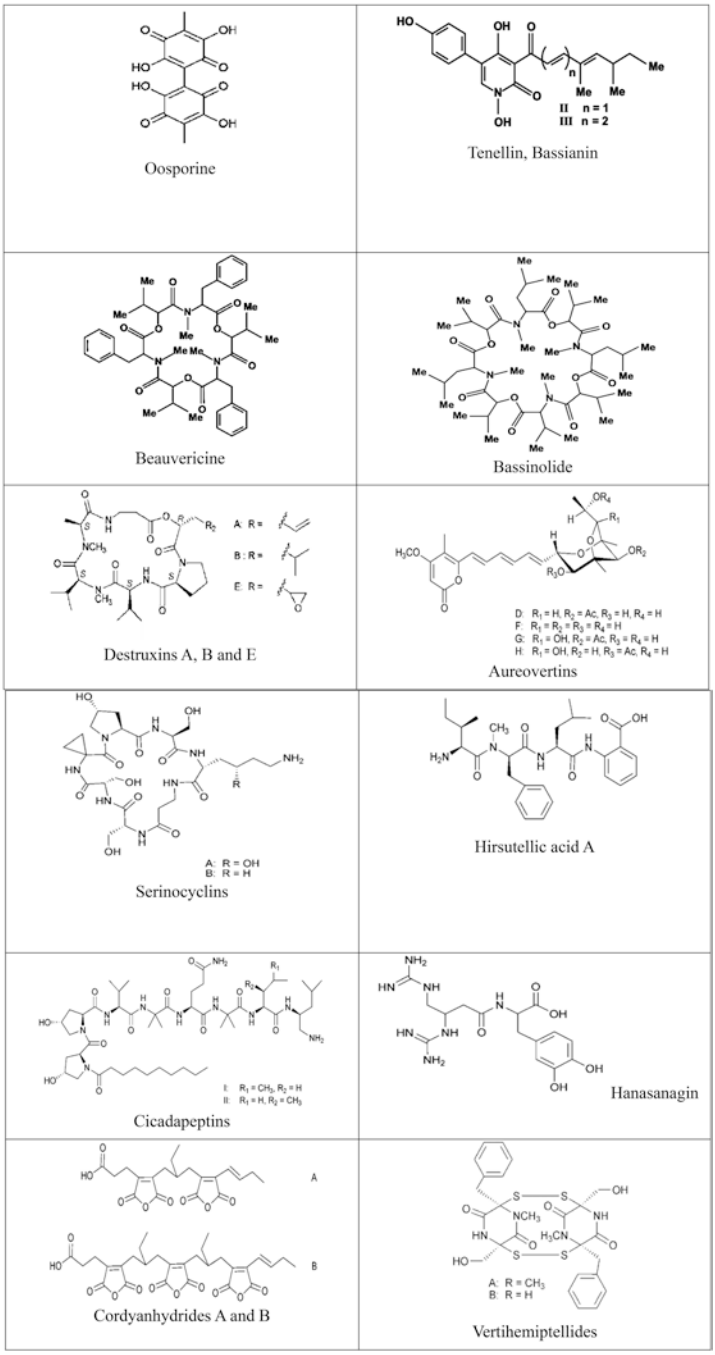

Fig. 5.1 Chemical structure of some important secondary metabolites (Molnar et al. 2010; Patocka 2016)

5.4 Isolation of Secondary Metabolites

Despite substantial developments in extraction and separation techniques, isolation of secondary metabolites from microorganisms is still a challenging task. Hybrid methods i.e. LC-NMR or LC-MS made online structure elucidation possible, without prior isolation. However, in many cases the need to get the purified compounds is still an important requirement (Sturm and Seger 2012). Extracting the compounds of interest from the non-soluble matrix in which they are embedded needs several issues to be taken into account. In fungal cultures, the secondary metabolites are usually intracellular, thus grinding of the culture and breaking tissue and cell integrity before extraction increases the yield. The most important methods for extraction of secondary metabolites from fungal culture in laboratory scale are explained briefly.

(a) **Classical solvent extraction method:** the majority of isolation procedures still utilize simple extraction procedures with organic solvents of different polarity, water and their mixtures (Sticher 2008; Seidel 2012; Haque et al. 2013). The method includes maceration, percolation, soxhlet extraction, ultrasound assisted extraction and turbo-extraction. These methods are mostly used for isolation of thermo-stable compounds.

(b) **Ultrasound-assisted extraction (UAE):** the fungal cultures are placed in a glass container, covered by the extraction solvent and then put into an ultrasonic bath. This method decreases extraction time and improves extraction yields due to mechanical stress which induces cavitation and cellular breakdown, and gained increasing popularity. The method is helpful in extraction of flavonoids and phenolic acid compounds (Bucar et al. 2013).

(c) **Microwave-assisted extraction (MAE):** the extraction is based on either diffused microwaves in closed systems or focused microwaves in open systems. MAE has been modified in different ways leading to Vacuum microwave assisted extraction (VMAE), Nitrogen protected microwave assisted extraction (NPMAE), Ultrasonic microwave assisted extraction (UMAE) and Dynamic microwave assisted extraction (DMAE) (Haque et al. 2013). Principles of these technologies, their pros and cons as well as extraction protocol have been reviewed in detail by Sticher (2008).

(d) **Extraction with ionic liquids:** Application of ionic liquids (ILs) for UAE, MAE or simple batch extraction of plant metabolites at room temperature or elevated temperature has gained increasing attention and has been used extensively (Li et al. 2017). These ILs, also named as "designer solvents", are organic salts in liquid state consisting of an organic cation and an organic or inorganic anion. ILs are able to dissolve a wide range of polar and non-polar compounds, have a low vapour pressure, show a high thermal stability and low combustibility, and a few are also biodegradable.

(e) **Accelerated (Pressurised) solvent extraction (ASE):** The advantage of ASE over other extraction systems is that the additional step for separation of remaining non-soluble matter from liquid extract is omitted. The atomized accelerated

extraction process is conjugated within on-line filtration. The methodology is applicable to solid and semi-solid samples using common solvents at elevated temperature and pressure.

(f) **Supercritical fluid extraction (SFE):** In this method supercritical CO_2 is used. The method can replace other extraction methods that are dependent on organic solvents because, it is less detrimental to environment and meets regulatory requirements, certainly considered as a driving force for the increasing application of SFE. The utilization of organic solvents as modifiers for supercritical CO_2 (to increase its solvating capabilities to medium polar and non-polar compounds) has broadened the spectrum of metabolites accessible to SFE (Sticher 2008; Nahar and Sarker 2012).

(g) **Extraction on solid phases:** The extraction process, with the advantage of adsorption of the unwanted impurities on a solid phase, has gained attention recently. In solid phase extraction a wide range of stationary phases are used, with diverse chemistry, i.e. silica gel, reversed phase material, ion-exchange resins or mixed-model material and HILIC stationary phases in pre-packed glass or plastic columns. Either adsorbing impurities or analytes of interest on solid phase can be done in this method. Elution of analytes of interest in the former can be done through vacuum liquid chromatography.

(h) **Distillation methods:** The distillation technique usually involves working at elevated temperatures and thermo-stable compounds like terpenes and terpinoids can be isolated through this method. Recent developments in distillation methodology includes the use of microwave steam distillation, which increases disruption of cells and the final product yield (Farhat et al. 2011; Sahraoui et al. 2011).

(i) **Liquid-solid chromatography techniques:** a wide range of liquid chromatographic methods with solid as stationary phases, either as planar or column chromatography, are available for further metabolite fractionation and purification. The choice largely depends on the stage of purity of the extract or fraction and the purpose of the final product. High sample capacity combined with relatively low costs made low pressure liquid chromatography (LPLC), Vacuum liquid chromatography (VLC), Flash chromatography (FC) popular for fractionation of crude extracts, and in rare cases even pure compounds can be obtained in single fractionation step. However, in several cases medium pressure liquid chromatography (MPLC), or semi preparative and preparative HPLC with higher peak resolution power, are applied for final purification (Cheng et al. 2012; Hattori et al. 2012; Sherma 2012).

5.5 Mode of Action of Fungal Secondary Metabolites on Insects

5.5.1 Humoral and Biochemical Alterations

In response to fungal infection, insects have evolved behavioural avoidance and physical barriers against pathogens, creating inhospitable physiological body environment that contains chemical compounds (e.g., antimicrobial peptides and reactive oxygen species), which inhibit fungal growth. In addition, innate immune responses, including cellular immunity and humoral immunity, play a critical role in preventing fungal infection. However, pathogenic fungi have evolved a series of sophisticated strategies to overcome insect immune defences by the production of wide variety of enzymes, toxins and secondary metabolites.

Behavioral defenses to eliminate fungal pathogens are common amongst insect hosts, especially in social insects such as termites and honeybees. They involve self-grooming (Tragust et al. 2013), grooming nest members (Qu and Wang 2018), removal of dead or infected nest mates (Swanson et al. 2009) and intake or production of compounds with antipathogenic properties (formic acid, antimicrobial peptides and proteinaceous salivary deposits) (Christe et al. 2003; Tragust et al. 2013; Gene 2019).

EPF typically exert contact toxicity and infect their hosts by direct penetration of the cuticle. However, the multilayered hydrophobic insect cuticle is a hostile structure containing tanned proteins, chitin, antimicrobial compounds, reactive oxygen species and is low in nutrients and water as well (Qu and Wang 2018). In some instances, conidia adhesion or germination is affected by cuticle harboring a native microbial community, microbicidal secretions (Fernandez-Marin et al. 2006) and other defensive compounds (Pedrini 2018). In view of this, an insect cuticle is considered as the first and foremost physical barrier for pathogen infection. Besides, the epidermal basement membrane is also involved in production of antimicrobial compounds such as protease inhibitors, melanin and others (Vilcinskas 2010). They are chiefly involved in early detection of pathogen infection, restricting their growth and the cuticle degrading activity of the invaders' enzymes (Yassine et al. 2012).

To counteract this inhospitable cuticle, adhesion of fungal spores, the crucial step of the infection process, is achieved by secretion of some mucilaginous or adhesive proteins (Holder et al. 2007; Wang and St Leger 2007; Zhang et al. 2011; Sevim et al. 2012). Similarly, other stresses like thermal, oxidative, non-hydrophobicity etc. are also effectively tackled by other cell wall proteins (Li et al. 2013). Additionally, many virulent strains of fungal pathogens exhibited fast conidia germination upon adhesion. Further penetration through chitin and protein rich cuticle is achieved by production of variety of enzymes viz., proteases, chitinases, lipases, esterase, phospholipase C and catalase (Santi et al. 2010; Wang et al. 2011; Beys da Silva et al. 2014; Wei et al. 2017) and volatile organic compounds (Crespo et al. 2008). The permutation and combination of these molecules underpin the virulence of a given strain. Comparative genomic studies also revealed the existence of more

enzyme related genes in EPFs than in plant pathogens (Zheng et al. 2013; Gao et al. 2012; Xiao et al. 2012; Hu et al. 2014). Qu and Wang (2018) proposed that this relative high abundance of enzymes in EPFs is an evolutionary advantage reflecting the association with insect hosts. Similarly, Keyhani (2018) opined that the lipid assimilation is also a co-evolutionary trait associated with insect cuticle degradation, due to its content of an endogenous lipid layer. Above all, ecdysis is one of the physiological mechanisms that eliminates growing pathogens along with the old cuticle, ultimately improving the likelihood of host survival.

Upon access to the hemocoel the invading pathogen should strike the host immune system that includes both cellular and humoral responses (Vilcinskas and Götz 1999). Most cellular responses include coagulation, nodulation, phagocytosis, multicellular encapsulation and nodule formation (Strand 2008). These involve haemocytes and plasmatocytes. The humoral response includes production of antifungal peptides, lectins, protease inhibitors and/or pro-phenoloxidase system (Molnar et al. 2010). The primary recognition of invading pathogen is done via pattern recognition receptors (PRRs) including peptidoglycan recognition proteins (PGRPs), Gram-negative-binding proteins (GNBPs), β-glucan-binding proteins (βGRPs), C-type lectins and others (Stokes et al. 2015). This triggers hemostatic responses in host insects which involve clotting of proteins such as lipophorins, vitellogenin-like proteins, and calcium-dependent transglutaminases containing a cysteine-rich domain homologous to the von Willebrand factor of mammals (Vilmos and Kurucz 1998). The non-self carbohydrate recognition by the host is also an important strategy in detection of invading pathogen (Wanchoo et al. 2009).

Multiple strategies coevolved in EPFs to counter the insect immune components. Fungal propagating in the haemocoel, mostly the hyphae, have fewer carbohydrate epitopes which are unrecognizable by the host immune system (Pendland et al. 1993; Wanchoo et al. 2009). In addition, secretion of immunomodulators and protease repressors are common mechanisms by which the invading pathogen overtakes the host immune responses (Wang and St Leger 2006, 2007). For example, *Metarhizium anisopliae* expresses MCL1, a collagen-like immune evasion protein acting as an anti-adhesive protective coat, to mask antigenic cell wall *β*-glucans and preventing haemocytes from recognising the hyphal bodies (Wang and St Leger 2006, 2007). As discussed earlier, the pathogenic fungi produce either species and/or host specific virulence metabolites viz., beauvericins, allobeauvericins, bassianolides, beauveriolides, bassianin, bassiacridin, oosporeins, cyclosporine, and destruxins (Molnar et al. 2010; Wang et al. 2013; Gibson et al. 2014). They are biologically active cyclopeptides and cyclodepsipeptides with direct cytotoxicity (Valencia et al. 2011).

These metabolites are also involved in down-regulating the production of antimicrobial peptides, resisting phagocytosis etc. However, each metabolite has a specific function. For example, destruxins from *Metarhizium* are involved in induction of oxidative stress that ravages many of the host antioxidant enzymes, whereas beauverolide L from *Beauveria* induces production of antibacterial proteins (Molnar et al. 2010). Similarly, morphological alterations in plasmatocytes (swollen nuclei with clumped chromatin and blebbing) are also a common symptom of mycosis

(Cohen 1993; Vilcinskas et al. 1997). Further nutrient uptake by fungi is also facilitated by the production of metabolites that compete with host metabolism. For example, Zhao et al. (2016) reported production of large amounts of acid trehalase by *Metarhizium* in host haemolymph to utilise trehalose, a major insect carbohydrate, thereby reducing its availability for host nutrition, leading to a physiological starvation. It is important to note that the majority of purified secondary metabolites upon host treatment (either injection or oral) neither cause significant mortality nor macroscopic pathological symptoms. They all together are involved in pathogenicity and successful host invasion or death.

The secondary metabolites produced by EPFs are not only concerned with immune-suppression and further killing of the host. They are also involved in antibiosis interactions (antimicrobials and nematicides) with other invading pathogens and saprotrophs, mediating trophic interactions, growth and development (Molnar et al. 2010). These unrelated bioactivities of the fungal secondary metabolites evolved due to inevitable competition and coevolution with other microbes and plants, respectively.

5.5.2 Cellular Immunity Alterations

Insect cellular response relies on the circulating haemocytes, which are divided into different types based on morphological characteristics and functional features (Price and Ratcliffe 1974). The major types are prohaemocytes, plasmatocytes, lamellocytes, crystal cells, etc. (Evans and Banerjee 2003). Insect haemocytes are involved in a series of cellular defences including nodulation, phagocytosis and encapsulation (Strand 2008). Plasmatocytes recognise pathogens through phagocytic receptors like Eater and Dscam (Kocks et al. 2005; Watson et al. 2005). Moreover, a class of secreted thioester-containing proteins enhance phagocytosis by binding to the invading pathogens (Blandin et al. 2004). Interestingly, few studies indicate that plasmatocytes trigger expression of antimicrobial peptides in *Drosphila* and play a role in humoral immunity (Strand 2008; Shia et al. 2009). In addition, the complex proteolyitc cascades like the prophenoloxidase (PPO) pathway, which induces melanisation, can be activated in response to fungal infection (Cerenius et al. 2008). During EPF invasion, fungal cell walls pathogen-associated molecular patterns (PAMPs) are recognised by pathogen related receptors (PRRs) of the host, inducing maturation of PPO to phenoloxidase (PO) through a series of enzymatic reactions, ultimately leading to the formation of toxic reactive quinines and melanin (Cerenius et al. 2008). These toxic substrates can aid in killing microbial pathogens and are effective against range of fungal infections (Yassine et al. 2012; Binggeli et al. 2014).

Once EPF reach the insect haemolymph, they face a series of potent cellular immune responses from their hosts. EPF have evolved to circumvent these defences through multiple strategies. These involve, masking of the immunogenic carbohydrates from the fungal cell surface, that are recognised by PRRs of the host to trigger immune signalling cascades. *Metarhizium anisopliae* expresses MCL1, a

collagen-like immune evasion protein acting as an anti-adhesive protective coat, to mask antigenic cell wall β-glucans and prevent haemocytes from recognising the hyphal bodies (Wang and St Leger 2006, 2007).

EPF secrete a wide range of secondary metabolites during invasion including bassilin, bassiacridin, oosporeins, cyclosporine and destruxins, which are known to suppress the host immune response (Gibson et al. 2014). Secondary metabolites like destruxins inhibit expression of genes encoding AMPs and block phagocytosis by inhibiting V-ATPase (Chen et al. 2013). Oosporein produced by *B. bassiana* inhibits ProPO activity and down-regulates expression of gallerimycin, an antifungal toxin of the wax moth larvae (Feng et al. 2015). In a case study, Vilcinskas et al. (1997) showed that injection of *M. anisopliae* destruxins into *Galleria mellonella* resulted in morphological alterations of plasmatocytes during mycosis. The majority of plasmatocytes (more than 90%) from infected larvae showed no filopodia formation, remained in a round shape and blebbing occurred upon their surface. The nuclei also appeared swollen and pycnotic, which represented clumped chromatin which are the typical features of cells which undergo programmed cell death (Cohen 1993). Destruxins and cytochalasin D also inhibited the attachment of plasmatocytes to mycelia. Morphological and cytoskeleton alterations suggest strongly that the plasmatocytes ability to participate in cellular defence reactions is predominantly impaired by destruxins liberated by the fungus during mycosis.

5.6 Strategies to Increase Infection Efficacy of EPF Using Metabolites

The bottlenecks in exploiting the full potential of the fungi, despite of their high virulence, include slow mode of action, high dependence on environmental conditions, location specificity, species-specificity of strains. In order to overcome these barriers approaches like genetic engineering and protease recombination have been employed to enhance the virulence of EPF. For example, Wang and St Leger (2007) developed a technique to increase the killing efficacy by modifying *M. anisopliae* to express a neurotoxin from the scorpion *Androctonus australis*. The genetically modified fungus showed an increased pathogenicity and virulence in tobacco hornworm compared to the wild type, even at 22-folds lower doses. Similarly, overexpression of CHI2 chitinase of *M. anisopliae* increased the efficiency of killing *Dysdercus peruvianus* (Boldo et al. 2009), as the LT50 and LT90 of wild strain were 156 h and 209 h respectively, whereas for the CHI2 overexpressed T33 strain they were 125 h and 154 h, respectively. Few possible strategies to enhance the EPF efficacy are:

(a) Genetic transformation of fungi by inserting insect virulent genes, like aaIT.
(b) Gene pyramiding with two or more virulent genes.
(c) Combined use of compatible insecticides and secondary metabolites.

(d) Use of synthetic analogues of natural secondary metabolites for pest control.
(e) Isolation of more virulent strains of EPF

Combining enzymes and toxins for pest management.

5.7 Secondary Metabolites Produced by Fungal Endophytes and Their Role in Pest Management

Endophytes are ubiquitously found in all plant species and contribute to their host plants by producing secondary metabolites that provide protection and have proven to be potential source for exploitation in modern agriculture and industry. It is believed that screening for insecticidal compounds isolated from endophytes is a promising way to overcome the threats posed by insecticide resistant insect pests. These newly emerging, but not yet fully understood, endophytic behavior of EPF hint the possibility of their use as inundative biopesticides against insect and other arthropod pests. Endophytic fungi produce various secondary metabolites, and are a rich source of biomolecules with diverse structural features and potential applications in insect pest management. In Table 5.3 endophytes, their host plants, secondary metabolites produced and their ability to infect insect hosts, are mentioned.

5.8 Regulatory Mechanism and Genomic Basis Behind Metabolite Production by EPF

Based on their chemical structure, the metabolites obtained from fungi can be broadly grouped into three classes: polyketides (obtained from acylCoAs), terpenes (from acyl-CoAs) and peptides. Synthesis of bioactive metabolites is the result of polymerization of primary metabolites by core enzyme groups such as polyketides, which are produced by polyketide synthases and non-ribosomal peptides by non-ribosomal peptide synthetases (NRPSs) (Keller et al. 2005). The regulatory mechanism behind SM production by fungi is a very complex process and performs at various layers, including global and pathway specific regulation, signal transduction and epigenetic control through transcription factors. Global regulators promote synthesis of metabolites upon receiving stimuli from the external environment in the form of abiotic factors. In general, half of the meatbolite producing gene clusters are controlled by global regulators (transcription factors) responsive to abiotic factors such as *PacC* for the pH, *CCAAT* for iron, *AreA* for nitrogen, *velvet complex* for light and *CreA* for carbon (Dowzer and Kelly 1991; Hortschansky et al. 2007; Caddick and Dobson 2007 and Bayram and Braus 2012). In order to recognize and adapt to this challenging environmental conditions such as haemolymph, cadaver, nutrient scarcity and host immunity, EPF possess signalling pathways, relying on their translation for response via ca ascade of events to regulate gene expression.

Table 5.3 Endophytes, their host plant, secondary metabolites produced and insect host

Antimicrobial compound	Host plant	Endophyte	Target pest	References
3-epiisopetasol	*Picea rubens*	CBS 121944 (Not characterized)	*Choristoneura fumiferana*	Sumarah et al. (2010)
Vermiculin	*Picea glauca*	DAOM 221611 (Not characterized)	*C. fumiferana*	Findlay et al. (2003)
7α,8β,11-trihydroxydrimane	*P. glauca*	DAOM 221611 (Not characterized)	*C. fumiferana*	Findlay et al. (2003)
Trans-3-methyldodec-cis-6--en-4-olide	*P. glauca*	DAOM 221611 (Not characterized)	*C. fumiferana*	Findlay et al. (2003)
Trans-8-hydroxy-3-methyldodec-cis-6-en-4-olide	*P. glauca*	DAOM 221611 (Not characterized)	*C. fumiferana*	Findlay et al. (2003)
Trans-9-hydroxy-8-oxo-3-ethyldodecan-4-olide	*P. glauca*	DAOM 221611 (Not characterized)	*C. fumiferana*	Findlay et al. (2003)
Trans-7,9-dihydroxy-3-methyl-8- oxo-dodecan-4-olide	*P. glauca*	DAOM 221611 (Not characterized)	*C. fumiferana*	Findlay et al. (2003)
Trans-6-hydroxymethyl-3-methyl-7-oxo-undecan-4-olide	*P. glauca*	DAOM 221611 (Not characterized)	*C. fumiferana*	Findlay et al. (2003)
Cordyanhydrides A	*P. rubens*	*Dwayaangam colodena*	*C. fumiferana*	Sumarah et al. (2010)
Cordyanhydrides B	*P. rubens*	*Dwayaangam colodena*	*C. fumiferana*	Sumarah et al. (2010)
Ethyl acetate extract (unknown)	*Azardiricta indica*	*Alternaria alternata*	*Spodoptera litura*	Kaur et al. (2015)
3,4-dihydroxyiso- coumarin derivatives	*P. glauca*	CBS 120381 (Not characterized)	*C. fumiferana*	Sumarah et al. (2010)

These pathways are widely known to be conserved across fungal group. Most studied pathways are cAMP/protein kinase A (PKA), calmoudlin and (MAPK) (Rispail et al. 2009). Deletion of important genes (*GpaB, PkaC)* involved in the cAMP pathways has been found to influence metabolite production significantly. Metabolite production mediated by MAPK signalling pathways involves biosynthesis and repairing of cell wall, osmostress response and pheromone pathways. Signals received at membrane level are translated via GTPases to MAPKs and further activated through phosphorylation into the nucleus, where activation of transcription factors takes place (Jain 2011; Macheleidt et al. 2016). Genetically, EPFs biosynthetic pathways of metabolites are co-regulated by clustered genes popularly known as BGCs (biosynthetic gene clusters) containing PKS (Polyketide synthase), NRPS (Non ribosomal peptide synthetase), TCs (terpene cyclases), PTs

(prenyltransferases), hybrids of PKS-NRPS and various regulatory genes assisting in packaging of nucleosome, transport and trimming of metabolites (Inglis et al. 2013; Lazarus et al. 2014). Genomic data availability and prediction software tools suggested presence of numerous BGCs in EPFs and their acquisition via horizontal gene transfer events during their evolution (Khaldi et al. 2008; Slot and Rokas 2011; Dhillon et al. 2015). Bioinformatic analysis suggested that BGCs always differ among the host generalist and host specialist species of EPFs (Hu et al. 2014).

An insight into the genome of *M. anisopliae* revealed presence of a large number of core genes encoding metabolite production, including polyketides, nonribosomal peptides and genes which encode for methyltransferases, dehydrogenases, and CYPs prenyl transferases. In total, the genome of *M. anisopliae* revealed 14 NRPS, 24 PKS, 5 hybrids of NRPS-PKS gene clusters which are very potent in bringing virulence in EPFs. The species also possesses a putative NRPS-like antibiotic synthetase, that plays a role in limiting other microbial community to grow on the host cadaver. The genome of *M. anisopliae* also revealed homologues of bassianolide synthetase (a prominent virulence factor in *B. bassiana*), *HTS1*-like NRPS for synthesis of host selective HC toxin, *ACE1* (PKS/NRPS hybrid) having a role as virulence factor in *Magnaporthe grisea*. Both *M. anisopliae* and *M. acridum* have 54 and 40 putative *PTH11*-like G-Protein couple receptors (GPCRs), respectively, the largest number of GPCRs known so far in fungi. Signal transduction invokes various physiological responses that are regulated by distinguished transcription factors. *Metarhizium anisopliae* has 510 TFs involved in regulation of primary and secondary metabolism. The presence of CREB protein (cAMP response element binding) in cAMP/PKA pathways is the most intriguing feature of *M. anisopliae* as it has not been known in any fungi, but in mammals (Gao et al. 2011).

In the last decade numerous gene clusters of fungal metabolites have been identified through genome sequencing approaches, with prediction of various orphan pathways and activation of silent genes in SM synthesis pathway. In order to activate silent gene cluster and new SM synthesis, in depth studies and knowledge about SM regulating pathways in EPFs are needed. In future the availability of EPFs genomes will unravel the putative gene clusters and specific enzymes involved in mechanism of SM production.

5.9 Commercial Application of Secondary Metabolites

To the best of our knowledge, no commercial product with a fungal metabolite as active ingredient is available for pest management. However, the commercial applications of fungal metabolites have importance, since the discovery of penicillin, a metabolite from *Penicillium chrysogenum.* Similarly, the discovery and commercial application of bacterial metabolites, avermectins and spinosad are noteworthy in agricultural pest management. In particular, for plant growth promotion, gibberellic acid (a terpenoid from *Gibberella fujikuroi*) is extensively exploited. Whereas, regarding EPF metabolites many studies reported use of crude extracts as well as

purified metabolites (Amiri et al. 1999; Quesada-Moraga et al. 2009; Sabbour 2019) for successful management of pests under field conditions. Additionally, some symbiotic fungi like *Epichloe* spp. are reported to confer nematode and aphid resistance in the host plants by the production of metabolite called loline alkaloids (Wilkinson et al. 2000). In view of this, the metabolites alone have great potential in pest management and can be viewed as alternatives to whole organism formulations, which warrant research efforts.

5.10 Conclusion

The complex interactions between EPF and their insect hosts involves dynamic co-evolutionary arms race (Wertheim et al. 2011). Insects exert strong selection pressure on the fungi through production of different and distinct immune molecules (Juneja and Lazzaro 2009) that play a crucial role against the invading pathogen. In turn fungi produce a wide variety of enzymes and secondary metabolites that take part in suppressing the hosts physiological processes, including morphogenesis, pathogenesis, parasitism, growth regulation and immunity. As described in the above sections, metabolites have multiple roles to play in establishing a successful infection in an insect host, but the conclusive knowledge about their ecological role, regulatory mechanism, biosynthetic pathways and mode of action in insect body is still lacking. Recent genomic studies on *Metarrhizium* and *Beauveria* (Gao et al. 2011; Xiao et al. 2012) also suggest existence of unique and vast arrays of gene pools associated with metabolites production. Understanding these interactions between pathogen and host also results in designing of improved pest management tactics that effectively tackles the increasing pest problems.

Before embarking upon the use of secondary metabolites as chemical weapons in pest management, based on large scale production through bioengineering, genetic modification of fungi to improve their activity as biocontrol agents and isolation of novel metabolites from various fungal species, we need to answer the following questions. A better understanding must be achieved about the role of secondary metabolites in suppressing host defence mechanism and counteracting the host immune system.

(a) Whether fungi display induced responses to their natural enemies or hosts?
(b) Do fungi signal their unprofitability or toxicity by the release of volatile organic compounds (VOCs) that differentially affect insect behaviour and lead to enhanced protection against antagonist (direct protection)?
(c) Do insects display adaptive strategies along with innate immune response, how do insects cope with increased production of toxic chemicals by fungal pathogens and to what extent will the detoxification and repair mechanisms in different insect orders evolve?
(d) Is there a cross-talk between fungal and arthropod signalling molecules that mediates host susceptibility?

(e) If insects are capable of displaying an induced response to toxic fungi, what are the relevant fungal signals and how are they perceived and transmitted?
(f) Do fungi withstand the insect resistance and overcome host immunity through genetic variation?
(g) Do insects select for enhanced production of deterrent or harmful fungal compounds or do they favour growth of fungal variants that synthesize qualitatively and quantitatively different compositions of chemicals with stronger synergistic effects?

Given the increasing knowledge on molecular genetic mechanisms underlying the regulation of secondary metabolite biosynthesis and the EPF huge diversity in nature, there is still a wide scope to understand the role of fungal metabolites and develop their use in insect pest management.

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