Sustainability in Plant and Crop Protection 17

Md. Aslam Khan
Wasim Ahmad *Editors*

Microbes for Sustainable Insect Pest Management

Hydrolytic Enzyme & Secondary Metabolite – Volume 2

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Foreword

The exponential growth of human population, especially during the last century, is expected to increase the actual social pressure on the earth resources. Evolution and growth of agriculture sustained in the past the human population growth, as well as industrialization and prosperity. However, these achievements have now rendered agriculture often unsustainable and environment unsupportive. The global demand for food, fibre, fuel, medicinal plants and other agro-generated plant/animal-based commodities increased not only in direct proportion to the population but also due to changes in preferences or demand for greater variety and better quality products. All this means growing pressures leading to depletion and deterioration of our land, natural resources and environment. We must understand that humans and some animals, chosen by humans, are not the only inhabitants of this planet. A further aspect to understand is the complexity of the food-webs underpinning crop production, driven by various kinds of associations based on processes such as commensalism, symbiosis, competition and antagonism that act as natural environmental regulators. Many organisms including bacteria, fungi, plants, nematodes, molluscs, mites, insects, birds, rodents etc., coexisted until the resource depletion put many of them at risk of extinction. The expansion of human interests induced the classification of some species as 'pests and weeds', with a need to restrict or eliminate them. The discoveries of various toxic natural or synthetic chemicals provided pesticide tools to eliminate the above said co-inhabitants, when considered pests.

One positive attribute of our species is that we soon realized the negative fallouts produced, shown in various publications, starting from *Silent Spring* by Rachel Carson (1962). The need for safer methods of managing (not controlling) pests was realized and greater emphasis has been given to the various physical, cultural and biological alternatives to chemical pesticides. Natural antagonists have been recognized and used as biological control agents against the vast range of insects and other serious organisms that have been considered major pests. Many exogenous and endogenous bacteria, fungi and viruses have been identified as biocontrol agents, directly in their living state or as generators of repelling, paralyzing, pathogenic or lethal biomolecules in the form of secondary metabolites and enzymes.

This is an area with much scope and hope for development of eco-friendly, technically sound and practically feasible, low-cost biopesticides, with a minor impact on the environment at large.

I am glad that the very learned and experienced team of authors brought out the eleven chapters of this book. The contributions have been very appropriately selected and edited by Editors Dr. Md. Aslam Khan and Prof. Wasim Ahmad, well known for their valuable contributions to scientific literature. These chapters not only compile the up-to-date information on various metabolites, enzymes and entomopathogenic biomolecules of microbial origin, but also provide elaborated analyses and critical thoughts that are clearly presented. The microorganisms and the biomolecules they produce are amenable to mass-production, as well as to modifications using various abiotic, biotic and biotechnological interventions. This aspect has also been included in this volume to discuss available DNA-based technologies, in the direction of sustainable productions.

This book will serve as a very useful text and reference for the students, teachers, researchers, industry and other stakeholders. I appreciate the effort and presentation given by the authors and editors and take this opportunity to heartily congratulate them for this valuable and well-timed scientific contribution.

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Hari S. Gaur

Preface

A continued need for sustainable pest management became evident as the pressure to efficiently produce more food using less land increased in the last decade. With the intent to manage and control insect pests in field crops each year, a huge amount of resources is spent worldwide. A high amount of crop yields is, however, still lost due to insect pests, particularly in developing countries.

Synthetic organic pesticides applied in crop pest management programs pose several adverse impacts to the environment, along with resistance development in target pests, direct and indirect deleterious effects on non-target organisms, etc. This situation requires the development of more eco-friendly control practices. In regards, biocontrol-based pest management practices allow more environment-friendly and acceptable alternatives to traditional chemical control measures, which are host specific, benign to the environment and mankind.

For eco-friendly pest management purposes, entomopathogenic microorganisms have been successfully isolated from natural sources. They also encompass different types of molecules, usually produced by microbial biosynthesis or the microorganisms themselves, and are widely used for control of pests. Hydrolytic enzymes and secondary metabolites of entomopathogens are toxic to many pests and act synergistically to control their attacks. They became a promising alternative to synthetic organic insecticides. All notorious insect pests are susceptible to these molecules. It is therefore imperative to study the pesticidal activity of microbial hydrolytic enzymes and other metabolites in achieving a sustainable pest management goal.

This volume comprises 11 chapters in an attempt to bring available information on safe use of microorganisms and their bioactive molecules for pest management. Microbial hydrolytic enzymes act as weapons against insect pests. Chapters dealing with bacterial and fungal hydrolytic enzymes provide a review and most updated information about their safe use in integrated pest management. Secondary metabolites from entomopathogens play in fact a key role in biological control programs. In other chapters, the effect of metabolites produced by entomopathogenic bacteria and fungi is also examined and explored.

Nanotechnology and recombinant DNA technology emerged as highly attractive alternative approaches to chemical pesticides. Microbial-based nanoparticles for insect pest management and recombinant DNA technology, applied to improve efficacy of microbial insecticides, represent an important contribution. Use of predators and parasitoids in presence of entomopathogens is effective, economic, and eco-friendly. Understanding their synergistic and antagonistic interactions will certainly promote their use, as shown in the chapters reviewing the effects of entomopathogens on insect predators and parasitoids, and their safe bio-management on vegetable crops.

We hope that this volume will be helpful to students, teachers, researchers, and industry technicians. We are highly grateful to all the contributors for providing their expertise in the form of stimulating contributions. Thanks are due to the head of the Department of Biology and dean of the Faculty of Science at Jazan University, Jazan, for their moral support. We are grateful to Dr. Aurelio Ciancio, CNR, Bari, Italy, for including this volume in the Springer Series Sustainability in Plant and Crop Protection. We extend our thanks to the Springer International team for their generous cooperation at every stage of the book production.

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Chapter 1
Microbial Hydrolytic Enzymes: Powerful Weapons Against Insect Pests

F. C. Lopes, A. H. S. Martinelli, E. B. O. John, and R. Ligabue-Braun

Abstract During its history, humankind has been affected by three factors: food deficiency, health problems, and environmental issues. With world's population increasing at a high rate, our requirement for food is increasing. Consequently, agricultural practices that maximize crop productivity are necessary. These include the development of new agronomic technologies and new plant varieties, the use of fertilizers, pesticides and herbicides, in order to minimize losses due to plant predators and weeds, respectively. Thus, a continued need for pest management in agriculture became evident, with pressure to efficiently produce more food using less land. To solve this issue, conventional chemical pesticides have been widely used in agriculture despite presenting risks to human health, hazards to the environment as well as affecting non-target species. Therefore, the use of biopesticides is desired due to their target specificity and low environmental damage. They encompass different types of molecules, usually produced by microbial biosynthesis, and are widely used for pest control. Biocontrol, which depends on microorganisms or their products such as hydrolytic enzymes, became a promising alternative to conventional pest control. Microbial hydrolytic enzymes such as proteases, chitinases, lipases, and glucanases are attractive for this purpose, since they present toxic properties, acting synergistically to control pest attacks. Proteases act on the insect cuticles, since proteins constitute the majority of this structure. These enzymes also can act in the insect midgut and hemocoel. Proteases can also be used in the biological

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control of other noxious agents, such as bacteria, fungi, and nematodes. Chitinases can degrade the peritrophic matrix and cuticle of insects, as well as the fungal cell wall. Lipases hydrolyze lipoproteins, waxes and fats present in the insect integument, causing its disruption. Glucanases affect fungal cell wall development, differentiation, and mycoparasitism, because glucan is a major cell wall component. In this chapter we cover details about enzymes structure, biochemistry, mechanisms of action, applications, and perspectives in this field.

Keywords Hydrolytic enzymes · Insect pests · Chitinase · Protease · Lipase · Glucanase

1.1 Introduction

The natural plant environment is characterized by a complex set of abiotic stress sources, such as cold, drought, presence of salts and heavy metals, and biotic stresses caused by living organisms. Regarding biotic stresses, plants are constantly challenged by various pathogenic microorganisms, such as fungi, oomycetes, bacteria, viruses, protozoa besides other pests, as arthropods, particularly insects, and also nematodes. These phytopathogens can cause considerable losses in domestic crop species as a result of reductions in yields or aesthetic value, also shortening the storage life of products. The classical disease control techniques used such as crop rotation, chemical control, and resistant varieties are not efficient nowadays, because pathogens continue evolving and developing new mechanisms to attack and damage plants (Pereira et al. 2007; Cramer et al. 2011).

According to World Health Organization (WHO), "*pesticides are chemical compounds that are used to kill pests, including insects, rodents, fungi and unwanted plants, more specifically, weeds. Pesticides are used in public health to kill vectors of disease, such as mosquitoes, and in agriculture, to kill pests that damage crops*" (WHO 2018). Chemical pesticides have been used for a long time around the world, and their use is still a prevalent approach to contain many pathogens. Actually, they cause problems to the environment (non-biodegradable compounds which can contaminate ground water and are highly toxic to non-target organisms such as beneficial insects, amphibians, fishes and birds), as well as public health, mainly farmers, and also consumers. The search for safer molecules and new strategies of pest control are currently of great importance and interest (da Silva et al. 2012). In addition, many synthetic pesticides i.e. organochlorines, organophosphates, carbamates and organophthalides have been banned because of their hazardous risks to the environment and non-target organisms (Ntalli and Menkissoglu-Spiroudi 2011). It is also important to highlight that the indiscriminate use of chemical pesticides can select for pest populations with resistant traits (Devine and Furlong 2007). The basis of this resistance is mostly caused by mutations in single genes, that explains why the use of insecticide alone is now an unsustainable solution (Valero-jiménez et al. 2016)

Microorganisms as biocontrol agents present high potential to control phytopathogens with no adverse effect on the environment or other non-target organisms. This is clearly an advantage compared to the use of synthetic pesticides (Khamna et al. 2009). In order to control plant diseases, the study of antagonistic microorganisms known as biocontrol agents or biological control agents (BCA) is receiving increasing attention. The mechanisms that BCA usually employ are antibiosis, hyperparasitism, enzyme production, induction of plant resistance mechanisms and competition for essential nutrients and space, as well as plant growth promotion (Khasa et al. 2017). Microorganisms and their products are considered biopesticides, meaning that they can be used for management of pests that are injurious to crop plants. The most commonly used biopesticides are living organisms, which are specifically pathogenic for a pest of interest. These include biofungicides (*Trichoderma* spp.), bioherbicides (*Phytophthora* sp.) and bioinsecticides (*Bacillus thuringiensis*, *B. sphaericus*) (Gupta and Dikshit 2010). Biopesticides have an important role in crop protection, although most commonly in combination with other tools, including chemical pesticides, as part of an Integrated Pest Management (IPM) (Usta 2013). IPM refers to "*a decision support system for the selection and use of pest control tactics, singly or harmoniously coordinated into a management strategy based on cost/benefit analyses that take into account the interests of and impacts on producers, society, and the environment*" (Kogan 1998).

Recently, recombinant microorganisms have been developed with enhanced biocontrol capabilities. Several commercially available BCA are currently being used for the efficient control of plant diseases, with improved productivity reported in many crops (Khasa et al. 2017). Another approach used to control pests is plant genetic engineering. This technology is used to develop disease resistant transgenic crops using different defense genes, usually from other species, including microorganisms (Ali et al. 2018). Most commercially available transgenic plants express genes coding for *B. thuringiensis* (Bt) toxins that negatively affect the survival and development of a target herbivore (Aronson and Shai 2001; Chen et al. 2008). Other genes coding lectins (Sadeghi et al. 2008), protease inhibitors (Bi et al. 2006), α-amylases (Sarmah et al. 2004) and other insecticidal products have also been successfully engineered into plants, to combat insect pests.

Besides the use of microorganisms, it is possible to use their metabolites for pest control. These metabolites also include biochemical pesticides, i.e. naturally occurring compounds produced by microorganisms or their synthetic analogues. This category includes microbial secondary metabolites and hydrolytic enzymes as lipases, proteases, chitinases and glucanases (Berini et al. 2018). Hydrolytic enzymes produced by microorganisms inhibit the growth of phytopathogens through hydrolysis of their cell wall, cuticle, proteins and/or DNA (Jadhav and Sayyed 2016). In this chapter, we will focus on plant pest control using microbial hydrolytic enzymes to combat pathogenic insects and fungi. We will review mainly works that studied these hydrolytic enzymes from 2008 to 2018, considering the vast literature about this topic.

1.2 General View of Hydrolytic Enzymes: Definition and Substrates

1.2.1 Lipases

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis of fats and oils to glycerol and free fatty acids, being popular biocatalysts with remarkable applications in different industrial segments. These enzymes also exhibit high enantioselectivity (selectivity towards one enantiomer of a pair) and a broad substrate specificity (Jaeger and Reetz 1998; Sarmah et al. 2018). The hydrolysis reaction is carried out at a biphasic interface, constituted by an immiscible organic solvent and water, but in certain conditions, such as in a medium with a low water content, lipases can also catalyze synthesis reactions, i.e. esterifications and transesterifications (Casas-Godoy et al. 2018).

Many different organisms are able to produce lipases, including animals, plants and microorganisms, the latter being notorious sources, considering their abundance and ease of maintenance (Casas-Godoy et al. 2018). Microorganisms produce extracellular lipases mainly in response to environmental factors, i.e. in presence of lipids in a medium deprived of other nutrients. Furthermore, the expression of this enzyme also constitutes a virulence factor for many fungi and bacteria (Gupta et al. 2015; Nascimento et al. 2016; Nguyen et al. 2018). Fungi are somewhat preferred sources for industrial applications because their enzymes are usually secreted to the extracellular medium, facilitating their separation from the fermentation media (Silva et al. 2005). Aside from traditional techniques for molecule screening and isolation, high-throughput sequencing also has been applied to the discovery of new lipases and other enzymes of biotechnological significance (Fernández-Arrojo et al. 2010; Ferrer et al. 2015).

Lipases belong to the serine hydrolase family, and present: (i) a catalytic triad comprised of a nucleophilic serine (for most lipases it is arranged in the consensus motif G-X_1-S-X_2-G), (ii) an acidic residue (glutamate or aspartate), and (iii) an histidine. Concerning their tridimensional structure, these proteins present a conserved α/β hydrolase fold, with a twisted central β sheet surrounded by a number of α helices. Lipases also form generally stable structures due to the presence of one to four disulfide bonds between cysteine residues. Some structural components need to be distinguished: (i) an α helix that acts as a lid and covers the active site, undergoing conformational changes in the presence of a lipid-water interface, in order to reveal the catalytic residues; (ii) the substrate-binding site, located in a pocket on top of the central β sheet system, that has hydrophobic residues interacting with lipid targets; and (iii) the oxyanion hole, which is situated in the catalytic cavity and is comprised of two residues (one is the X_2 amino acid in the consensus motif and the other is a residue located in the N-terminal domain), that form hydrogen bonds with the intermediate molecule during the catalytic process (Mondal et al. 2016; Casas-Godoy et al. 2018; Sarmah et al. 2018; Bassegoda et al. 2012; Infanzón et al. 2018).

1.2.2 Proteases

Proteases (E.C. 3.4) are hydrolytic enzymes that cleave proteins and break them into small peptides and amino acids. Generally, proteases are subdivided into two major groups: exopeptidases (E.C. 3.4.11-19), that cleave the peptide bond next to the amino or carboxy termini of the substrate, and endopeptidases (E.C. 3.4.21-25), that cleave peptide bonds distant from the termini of the substrate (Rao et al. 1998). Based on the functional group present at the active site, proteases are further classified into: serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Hartley 1960).

These enzymes play many roles in almost all cellular functions, being physiologically necessary for living organisms and may be found in a wide range of sources such as plants, animals, and microorganisms (Rao et al. 1998; Mondal et al. 2016). Microorganisms represent an excellent source of proteases due to their biochemical diversity, rapid growth, and susceptibility to genetic manipulation, all desired characteristics for biotechnological applications (Rao et al. 1998).

Proteases from a variety of organisms including bacteria, fungi, plants, insects and also viruses present toxicity towards insects. Some of them show insecticidal activity being venom components, herbivore resistance factors, or microbial pathogenicity factors, whereas other proteases act in insect development or digestion (Harrison and Bonning 2010). As proteins are the main component of the insect cuticle (55–80%), the proteases attack is followed by the action of chitinases and lipases (Petrisor and Stoian 2017). The main proteins found in cuticle include resilin, an elastic tissue unique to invertebrates, and collagen. These proteins are very susceptible to proteolytic degradation (Bidochka and Khachatourians 1987). Many proteases are cysteine proteases. The sites of protease activity are insect midgut to the hemocoel (body cavity) and the cuticle (Harrison and Bonning 2010).

1.2.3 Chitinases

Chitinases hydrolize chitin, an insoluble linear homopolymer of N- acetylglucosamine (GlcNAc) (Berini et al. 2018). Chitin is the second most abundant polysaccharide on Earth and consists of a polymer of N-acetylglucosamine linked by β-(1,4) bonds (Adrangi and Faramarzi 2013). It is broadly distributed in nature, as a structural polysaccharide in the exoskeleton of arthropods, in fungal cell walls, as well as in the shell of crustaceans and nematode cuticle (Fig. 1.1).

Chitin is arranged in antiparallel form, occurring in three polymorphic forms: α-, β-, and γ-chitins (Dahiya et al. 2006). The major form found in nature is α-chitin, the mainly structural element found in fungal cell walls and invertebrate exoskeletons (Gooday 1990; Van Dyken and Locksley 2018). This polymer exerts a fundamental structural role in fungi, being located in the inner layers of the cell wall in association with carbohydrates and proteins, where it may comprise up to 45% of the fungal dry

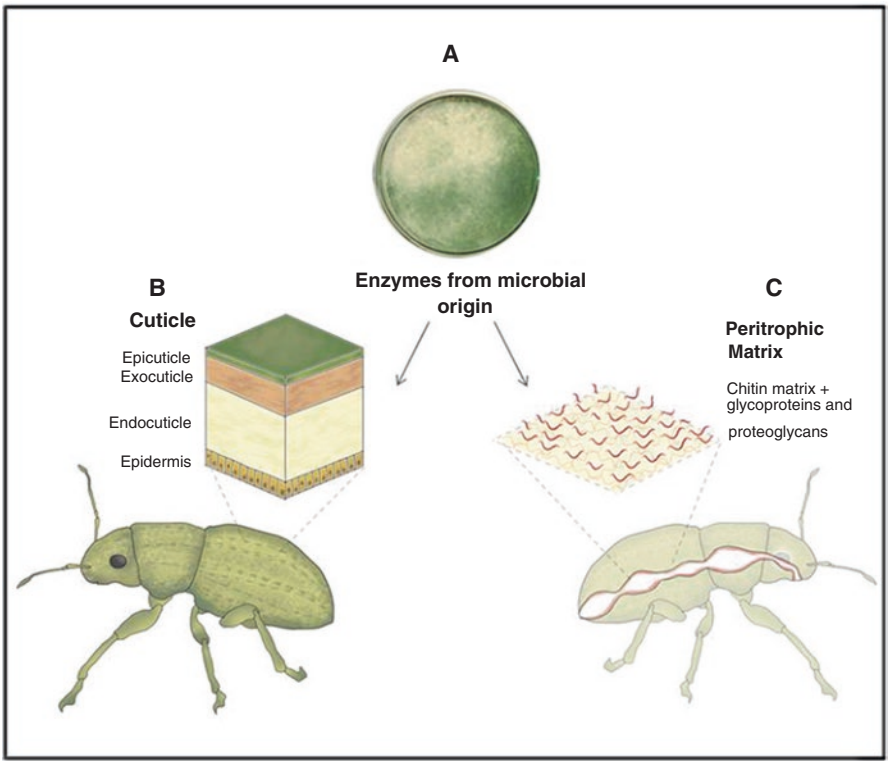

Fig. 1.1 Schematic illustration of cuticle and peritrophic matrix of insects. Microbial hydrolytic enzymes such as chitinase, protease, glucanase and lipase (**a**) interact and damage the insect cuticle (**b**) formed by layers of epicuticle, exocuticle, endocuticle and epidermis, as well as the peritrophic matrix (**c**), composed by layers of chitin, proteoglycan and glycoproteins

weight (Hartl et al. 2012). Insects possess a cuticle that covers all tissues exposed to the outside world, being a multifunctional device that protects the body from dehydration and predators, acting as a physical barrier to prevent the entry of pathogens, also serving as an exoskeleton allowing locomotion (Moussian 2010).

Chitinases are widely distributed, mainly in microorganisms such as bacteria and fungi. These are the main degraders of chitin in nature and are involved in re-cycling of carbon and nitrogen through its hydrolysis (Hartl et al. 2012). These enzymes are also synthesized by insects, plants, and animals for different purposes such as nutrition, morphogenesis and defense (Adrangi and Faramarzi 2013). Bacteria, plants and insects have large families of chitinases with distinct functions, including digestion, cuticle turnover, and cell differentiation. Several genes encode chitinolytic enzymes, i.e. in filamentous fungi that present 10 to 20 different chitinases (Hartl et al. 2012). On the other hand, many plants, invertebrates and animals express genes that encode so-called chitinase-like lectins, lacking the catalytic site (Arakane

and Muthukrishnan 2010). Despite being devoid of chitinolytic activity, they keep the ability to bind chitin (Adrangi and Faramarzi 2013).

Chitinolytic enzymes can be grouped based on their mode of action: endochitinases (EC 3.2.1.14) cleave the chitin chain randomly at internal sites, whereas exochitinases catalyze β-N-acetylhexosaminidases (EC 3.2.1.52) that remove successively GlcNAc from the non-reducing end (Adrangi et al. 2010; Adrangi and Faramarzi 2013; Van Dyken and Locksley 2018). Based on amino acid sequence similarity the chitinases can be grouped into the glycosyl hydrolase families (GH) (Khoushab and Yamabhai 2012). Endochitinases occur mainly in glycosyl hydrolase (GH) families 18, 19, 23 and 48, while exochitinases belong to GH families 3, 18, 20 and 84 (Adrangi and Faramarzi 2013; Berini et al. 2018). The database CAZy (http://www.cazy.org) provides a continuous update access to enzymes like chitinases that modify and breakdown polysaccharides. The classification of enzyme families is based on sequence and 3D structure (Lombard et al. 2014). In this review we will focus only on chitinases belonging to GH families 18 and 19.

1.2.4 Glucanases

β-glucans are the most abundant class of polysaccharides. They are produced by microorganisms and higher plants as structural components of the cell wall, as reserve materials, as well as extracellular substances. Enzymes capable of hydrolyzing β-glucans are produced by different microorganisms (Bielecki and Galas 1991). β-glucanases catalyze the hydrolysis of the β-glucan and four types are described: β-1,3-1,4-glucanase (lichenase, EC 3.2.1.73), β-1,4-glucanase (cellulase, EC 3.2.1.4), β-1,3-glucanase (laminarinase, EC 3.2.1.39), and β-1,3(4)-glucanase (EC 3.2.1.6) (Luo et al. 2010). In this chapter, we will focus only on β-1,3-glucanases.

β-1,3-glucanases are widely produced by bacteria, fungi, viruses, invertebrates (e.g. molluscs) and higher plants. These enzymes are generally classified into two types according to the region of hydrolysis: exo-β-1,3-glucanases (EC 3.2.1.58) and endo-β-1,3-glucanases (EC 3.2.1.39). They hydrolyze terminal or internal glycoside linkages, respectively. In recent years, β-1,3-glucanases have attracted attention due to their potential use in biotechnology, agriculture and pharmaceutics, including vinification (improving organoleptic characteristics of wine), medical applications (as bioactive oligosaccharides) and animal feed and defense against parasites (Cantarel et al. 2009; Papageorgiou et al. 2017).

According to the GH classification system, endo-β-1,3-glucanases are classified into GH families 5, 16, 17, 55, 64, 81, 128 and 152 and exo-β-1,3-glucanases into families 3, 5, 16, 17 and 55, based on amino acid sequence similarity (http://www.cazy.org/Glycoside-Hydrolases.html).

β-1,3-glucanases act in substrates containing linear sequences of glucose units bound by β-1,3 glycosidic linkage type, containing a non-reducing end. However, a moderate degree of substitutions is possible, then the enzymes can act on β-1,3–1,6 glycosidic linkages found in β-1,3–1,6 glucans, found for example, in the laminarin

(10% of ramification degree) produced by the alga *Laminaria digitata.* An interesting characteristic of β-1,3-glucanases is related to their substrate specificity, as they are not specific to only one substrate (Bauermeister et al. 2010).

1.3 Insect Control

Insect pests are those that cause harm to humans and/or their agricultural resources. Insect infestations have a direct impact on agricultural food production and stored products. They can cause 20–30% production losses and in severe cases, total yield loss. Insects damage field crops by sucking, chewing or boring into different parts of the plants. Considering damage to stored products, they directly feed, bore and ruin grains and accelerate the process of decay (de Geyter et al. 2007; Mills 2014). Moreover, as climate warming advances, insect pests are predicted to substantially impact crop production, since higher temperatures increase their metabolic rate, increasing, consequently, their nutrient consumption and population growth rate (Deutsch et al. 2018). Some biological approaches to the management of insect pests have at times been considered to be biological control tactics. These include host plant resistance, transgenic insecticidal crops, insect growth regulators (IGRs), botanical insecticides, pheromone disruption techniques, and sterile insect techniques (Mills 2014).

Entomopathogenic bacteria have been commercially developed for control of insect pests. Some examples include several *B. thuringiensis* sub-species, *Lysinibacillus* (*Bacillus*) *sphaericus*, *Paenibacillus* spp. and *Serratia entomophila*. *Bacillus thuringiensis* sub-species *kurstaki* is the most widely used for control of pest insects in crops and forests, whereas *B. thuringiensis* sub-species *israelensis* and *L. sphaericus* are the primary pathogens used for control of medically important pests, including dipteran vectors (Lacey et al. 2015).

Entomopathogenic fungi are a feasible system for insect control in agriculture with a growing market and also an important model for studies of host-pathogen interactions (Schrank and Vainstein 2010). An increasing number of studies showed that entomopathogenic fungi, often solely considered as insect pathogens, play ecological roles in nature, including endophytism (colonization of the internal plant tissues without causing apparent symptoms or harm to their plant host), disease antagonism, plant growth promotion, and rhizosphere colonization. Such additional roles provide opportunities for the multiple use of these fungi in IPM strategies against insect pests and other arthropods in horticulture, forestry and agriculture (Petrisor and Stoian 2017; Jaber and Ownley 2018). Virulence factors of different entomopathogenic fungi, i.e. *Beauveria bassiana*, *B. brogniarti*, *Metarhizium anisopliae*, *Isaria fumosoroseus* and *Lecanicillium lecanii*, have been mostly associated with the production of cuticle-degrading enzymes . They have an important role in the infection process, by hydrolyzing proteins (proteases), chitin (chitinases) and lipid complexes (lipases), the major components of the insect cuticle (Petrisor and Stoian 2017) (Fig. 1.1). The cuticle is degraded both to obtain nutrients and to

weaken the host structural barrier, enabling invasion (Valero-jiménez et al. 2016). Most insects, although not all, also contain a peritrophic matrix (PM), that lines the insect midgut and is formed by chitin fibrils, glycoproteins and proteoglycans (Lehane 1997; Berini et al. 2018), that is also a target of these enzymes. The PM plays roles in insect digestion, separating the luminal content in two compartments (endo and ectoperitrophic space), selecting the passage of nutrients from the lumen to the epithelial cells and as a barrier of protection against pathogens and toxins (Terra and Ferreira 1994). It is important to emphasize that the gut is the major interface between the insect and its environment. Consequently, an understanding of gut function is essential to develop methods of controling pests such as the phytophagous insects (Terra and Ferreira 1994). Here we will focus on studies that showed toxic effects of hydrolytic enzymes against insects.

1.3.1 Lipases

The insect epicuticle is composed by proteins and a complex mixture of different types of lipids and hydrocarbons. It is an important target for insect control, as this layer constitutes the first protective barrier against chemical agents and pathogens. It is also a site for chemical communication that can be modulated in order to disrupt the pest endurance (Wang and Wang 2017; Balabanidou et al. 2018; Otte et al. 2018). Microbial lipases are used alongside other hydrolytic enzymes to disturb this protective layer, and are intensively studied in order to understand and optimize biocontrol strategies.

1.3.1.1 Fungal Lipases

The presence of lipolytic enzymes as virulent factors in entomopathogenic fungi has been acknowledged since the early biocontrol studies (Ferron 1978). These enzymes are critical mainly during the first steps of cuticle invasion by entomopathogenic fungi, when spores come in contact with the epicuticle and their germination begins, obtaining nutrients from the degradation of the insect protective layers (Jarrold et al. 2007; Schrank and Vainstein 2010). The pre-treatment with lipase inhibitors dramatically affects the pathogenicity of some parasitic fungi (Commenil et al. 1998; Berto et al. 1999; Silva et al. 2010), demonstrating the important role of lipases in host invasion.

Metarhizium anisopliae was characterized as an efficient producer of lipases, which seem to be specific for short acyl chain substrates (Santi et al. 2010), a feature pointed out as promising for application in industry (Silva et al. 2005; Silva et al. 2010). A comparative transcriptomic study of the genus *Metarhizium* showed the presence of a high number of secreted lipase genes (5 to 12, which is above the mean of other fungi), that may suggest a correlation with pathogenicity and host recognition (Gao et al. 2011). Also, the secretome of *M. anisopliae* revealed, upon

contact with the cuticle of *Dysdercus peruvianus*, the presence of specific types of lipolytic enzymes (not restricted to the EC 3.1.1.3 class) in the earliest stages of infection (Beys-Da-Silva et al. 2014).

Similar biochemical characterization of extracellular lipase activity was already achieved for a number of entomopathogens (Ali et al. 2010, 2014; Supakdamrongkul et al. 2010; Hussein et al. 2012; Pelizza et al. 2012; Hasan et al. 2013). Their relevance in the life cycle of fungi is well recognized, even in cases where the enzyme activity is not quite potent (Boguś et al. 2017). However, there seems to be a lack of studies that explore specifically the modulation of lipases through genetic engineering, in order to accomplish a better biocontrol performance. Still, a particular study used more elaborate techniques in order to assess the pathogenic potential of a *M. acridum* mutant, which was able to successfully infect a new type of host, upon the expression of an esterase gene from another *Metarhizium* species, *M. robertsii* (Wang et al. 2011). More initiatives like that could provide insights about the factors that determine host specificity and help to improve the usage of entomopathogenic fungi in biological control.

1.3.1.2 Bacterial Lipases

The infection of insect hosts by bacteria does not quite resemble the mechanisms used by entomopathogenic fungi. Usually, toxicity is achieved after ingestion of spores and parasporal bodies by the insect, resulting in an infective process that begins in the midgut (Ruiu 2015). Hence, active use of lipases by bacterial pathogens for invasion of the epicuticle is poorly described. Nevertheless, lipases are encountered in some entomopathogenic bacteria, as exemplified by the genomic analysis of *Pseudomonas entomophila*, which revealed the presence of four lipase encoding genes that are believed to contribute in hemolytic activity alongside other bacterial toxins (Vodovar et al. 2006). *Serratia entomophila* and *S. marescens* also had their lipolytic activity assayed (Grkovic and Mahanty 1996; Aucken and Pitt 1998; Tao et al. 2006; Salunkhe et al. 2013).

Interestingly, symbiotic bacteria of entomopathogenic nematodes exhibit a variety of secreted enzymes that contribute in insect death (septicemia) and posterior bioconversion of the cadaver (Nielsen-LeRoux et al. 2012). The genomic analysis of *Photorhabdus luminescens*, which is encountered in the gut of nematodes from the family Heterorhabditidae, found ten genes encoding lipase and phospholipase-like proteins (Duchaud et al. 2003). The Steinernematidae nematode *Xenorhabdus nematophilus* has similar characteristics, secreting lipases when in a physiological state called “phase I variant” (Thaler et al. 1998; Richards et al. 2008).

1.3.2 Proteases

Proteases are logical candidates for insect control, because proteolytic enzymatic activity can target and destroy essential proteins and tissues of pests. Indeed, proteases have evolved in plants for defense against herbivorous insects. In microbial pathogens of insects, proteases often play a role in host pathogenicity (Harrison and Bonning 2010).

1.3.2.1 Fungal Proteases

Proteases such as subtilisin-like enzymes (Pr1) and trypsin-like enzymes (Pr2) are considered important factors for insect cuticle degradation (Rosas-Garcia et al. 2014). The entomopathogenic fungus *Beauveria bassiana* has shown potential as a biological control agent of insects (Valero-Jiménez et al. 2016). A Brazilian isolate of *B. bassiana* (CG425), shows high virulence against the coffee berry borer (CBB), *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae). This insect is the most important coffee pest throughout the world. The data obtained in this study suggested that two proteases produced by *B. bassiana*, Pr1 and Pr2, were induced by *H. hampei* cuticle components. The secretion of these proteases could be fundamental for the pathogenesis of this fungus (Dias et al. 2008). Fang and co-workers (2009) expressed a fusion protein (CDEP1:Bbchit1) containing both protease and chitinase activities in *B. bassiana*. The transformants penetrated the cuticle of *Galleria mellonella* significantly faster than the wild type or the transformants expressing the single proteins. According to the authors, accelerating cuticle penetration by the fungus will potentially improve its utility as a BCA, by reducing the time of exposure to adverse environmental conditions such as Ultraviolet (UV) light, and to constitutive and inducible insect defenses such as melanization (Fang et al. 2009).

Metarhizium anisopliae has been widely studied as a model to understand virulence and pathogenicity processes against insect pests (Rosas-Garcia et al. 2014). The fungus produces, besides Pr1 and Pr2, several cysteine proteases (Pr4) and metalloproteases. It has been reported that at least 14 protease isoforms could be detected during growth in insects (Leger et al. 1987; Qazi and Khachatourians 2007). There are 11 Pr1 subtilisin genes in the *M. anisopliae* genome (Pr1A-Pr1K) (Bagga et al. 2004). However, genome sequencing has shown that there are 55 subtilisin genes in *M. anisopliae* (Gao et al. 2011). Some studies showed that *M. anisopliae* protease Pr1A digests cuticle proteins and is essential for virulence and cuticle penetration (St. Leger et al. 1987, 1988, 1992). The other subtilisins showed differences in regulation that could probably allow these virulence determinants to target different hosts and stages (Freimoser et al. 2005). Using liquid chromatography/tandem mass spectrometry (LC-MS/MS), Santi and co-workers detected carboxypeptidase and Pr1A protease produced by *M. anisopliae* and induced by the *Rhipicephalus microplus* cuticle. They also detected chitinase produced by the fungus when interacting with this tick (Santi et al. 2009).

Metarhizium robertsii can sense the presence of proteinase inhibitors and antifungal peptides produced by *Galleria mellonella*, and can counterattack by selectively expressing chymotrypsin-like proteases and metalloproteases that target the insect defense molecules for degradation (Mukherjee and Vilcinskas 2018). However, high protease activity can activate the prophenoloxidase cascade, a defense mechanism of the insect, resulting in high melanin production, which may result toxic to fungi, as mentioned above, but also to the host (Butt et al. 2016; Wang and Wang 2017).

The entomopathogenic fungus *Cordyceps sinensis* produced csp1 and csp2 proteases. These enzymes are novel members of the S8A subfamily of proteases. Both were cloned and expressed in *Pichia pastoris*. Bioassays using these proteins revealed the degradation *in vitro* of cuticle proteins of larval *Hepialus* sp. (Zhang et al. 2008). The fungus *Conidiobolus coronatus* produces enzymes such as proteases, chitinases and lipases, that may degrade *G. mellonella* cuticle. The composition of the cuticle, mainly the fat acids composition, are important for the insect susceptibility to the fungal enzymes (Wrońska et al. 2018).

1.3.2.2 Bacterial Proteases

Bacteria are widespread in the environment, interacting with organisms and some of them act as pathogens of insects. These interactions are due to co-evolution and development of strategies by some bacteria to invade and kill their hosts (Vilcinskas 2010; Ruiu 2015). Some pathogenic factors are linked to a secretion of hydrolases such as chitinase, lipase and protease, although it is postulated that other factors could also be involved (Patil et al. 2012). Interestingly, a metalloprotease from *Pseudomonas entomophila* AprA was purified and its effect was tested against the bean bug *Riptortus pedestris*. The study showed that AprA displays insectidal activity against bean bugs and this effect could be due the secretion of unidentified virulence factors during infection, modulating the host innate immunity (Lee et al. 2018). Proteases could provide a way for the pathogen to escape the host immune attack, as demonstrated in a study using *Pseudomonas aeruginosa* and *Tenebrio molitor* as a host model. It was shown that Proteases IV are involved in virulence factors causing the melanization and death of *T. molitor* larvae without the activation of antimicrobial peptides (AMP) (Park et al. 2014).

The bacterium genus *Serratia* could also be a reservoir of new genes and toxins to be used as pest control agents (Nuñez-Valdez et al. 2008). It was found that *S. marcescens* suppressed the immune cells in silkworm larvae, lowering the cellular immunity. A serralysin metalloprotease was suggested as responsible for this toxicity (Ishii et al. 2014). Another strain of *S. marcescens* (Sm81), showed mortality against *Phyllophaga blanchardi* larvae by oral and injection bioassays (Pineda-Castellanos et al. 2015). A novel serralysin family protease was produced by *S. marcescens* FS14, with thermostable properties and insecticidal activity against larvae of *Helicoverpa armigera* (Wu et al. 2016).

Photorhabdus luminescens is a Gram-negative bacterium that lives as a symbiont in the intestine of entomopathogenic nematodes (Forst et al. 1997). As described for

other pathogens, *Photorhabdus* also avoids the immune response of the infected host (Held et al. 2007). The proteolytic activation of toxins, like *Bacillus thuringiensis* (Bt) crystals by the insect midgut proteases (Xu et al. 2016), represents an additional approach in this same strategy.

1.3.3 Chitinases

As described above, insects offer two potential targets for chitinase action: the cuticle, that consists of a matrix of chitin and proteins, and the PM, that acts as a selective, permeable molecular filter (Lehane 1997; Fiandra et al. 2009). Consequently, any defect in PM reduces feeding and protection against microbial attacks. Due to the importance of chitinases, they have potential for use as eco-friendly agents in insect pest management (Patil and Jadhav 2015).

1.3.3.1 Fungal Chitinases

Chitinases have been characterized as fundamental in improving entomopathogenic fungi against insect pests. Entomopathogenic fungi, represented by the genera *Metarhizium*, *Beauveria*, *Isaria*, *Trichoderma* and others, could attack insects directly by penetrating their exoskeleton or cuticle (Charnley 2003). As these enzymes degrade chitin, it was speculated if they can damage the PM structure sufficiently to result in the larvae being unable to feed, consequently leading to their death (Binod et al. 2007). Interestingly, Binod and co-workers (2007), cultured *T. harzianum* by submerged fermentation using colloidal chitin as carbon source. The culture filtrate containing chitinase showed a potent antifeedant effect, reducing feeding rate and body weight of the *H. armigera* larvae. When applied topically, it reduced the successful pupation and increased larval and pupal mortality in a dosage-dependent manner. A similar effect was observed by the chitinase of *Penicillium ochrochloron*, applied topically on the back thorax of the fifth instar larvae of *H. armigera*, that infects cotton, tomato, corn and others. This chitinase reduced pupation and increased larval and pupal mortality (Patil and Jadhav 2015). The mycoparasites *T. harzianum* and *T. viride* are used to produce commercially-available chitinase cocktails. Berini and co-workers (2016) investigated the effect of one of such cocktails, containing a mixture of endo and exo-chitinases secreted by *T. viride*, in the PM of the lepidopteran *Bombyx mori*. They observed, *in vitro* and *in vivo*, a significant effect on the PM structure and permeability, leading to a delay of larval development, also inducing mortality (Berini et al. 2016). In another study, two fungal isolates JAB 68 and IBCB 35, identified by ITS (Internal Transcribed Spacer) region as *M. anisopliae* and *B. bassiana*, respectively, were analyzed for chitinase expression. Both fungi were capable to infect and kill the ootechae of crockroaches and reduced the amount of hatched nymphs (Baggio-Deibler et al. 2018).

Usually, the wild-type strain of fungi infects and kills the insect slowly, so when possible, the use of genetically modified strains overexpressing cuticle-hydrolyzing enzymes is desired (Berini et al. 2018). In *B. bassiana*, a hypervirulent strain overexpressing a chitinase gene (Bbchit1) fused with a protease gene (Bbcdep1) was produced. This construction increased the strain virulence against the aphid *Myzus persicae* (Fang et al. 2009). In *M. anisopliae*, overexpression of CHI2, involved in the pathogenicity of this fungus, was performed. The authors constructed strains overexpressing or lacking the CHI2 chitinase and tested their virulence against the cotton stainer bug, *Dysdercus peruvianus*. CHI2 overexpression constructs showed higher efficiency in host killing, reducing the time necessary to kill the insect. The knockout constructs showed decreased virulence to the insects as compared to the wild type strain (Boldo et al. 2009). The contribution of the gene CHI30 in the virulence of *M. anisopliae* against *D. peruvianus* was also studied (Staats et al. 2013). Interestingly, the chitinase gene of the fungus *Isaria fumosorosea (Ifchit1)* was cloned, characterized and a knockout mutant was constructed. This mutant and the wild type fungus were assayed against diamondback moth *Plutella xylostella* larvae, revealing a decreased infectivity of the *ΔIfchit1* strain compared to the wild type and complemented strain suggesting that the gene Ifchit 1 acts as a critical virulence factor in *I. fomosorosea* (Huang et al. 2016).

1.3.3.2 Bacterial Chitinases

The ability of chitinolytic bacteria to degrade vital chitinous structures in insects suggests their potential application in the field (Singh et al. 2016). Some nematode-associated bacteria, such as *Photorhabdus luminescens,* produce protein complexes (called ABC toxin), that display activity against insects (Bowen et al. 1998). The bacterium *Yersinia entomophaga* MH96, isolated from the coleopteran scarab *Costelytra zealandica,* was found to secrete this kind of toxin complex, called Yen-Tc (Hurst et al. 2011). This Yen-Tc complex as well as Yen-Tc K::9, a mutant lacking the B and C subunits, was studied and its 3D structure determined (Landsberg et al. 2011). Interestingly, one year later Busby and co-workers (2012) performed the structural analysis of chitinases Chi1 and Chi2 from *Y. entomophaga* that belong to the Yen-Tc complex and showed that both of them have endochitinase activity. Despite this, these chitinase isolates were not able to cause a lethal effect on insects, so it was suggested that they could be involved in the access of the toxin complex to the PM of larvae or involved in keeping the Tc complex structure (Hurst et al. 2011; Busby et al. 2012).

A purified extracellular chitinase from *Bacillus subtilis* showed a potent insecticidal activity against first, second, and third instars of *Spodoptera litura* Fab. (Chandrasekaran et al. 2012). A histological study showed that this chitinase affected the gut, PM and epithelial cells of *S. litura* larvae (Chandrasekaran et al. 2014).

Some studies reported the heterologous expression of bacterium chitinase genes and their characterization as insecticidal agents. Martinez and co-workers (2012)

for example studied two chitinolytic proteins from *Streptomyces albidoflavus*, expressed in *E. coli*, with significant biological activity against the coffee berry borer and the coffee leaf rust (Martínez et al. 2012). Three chitinase genes, (chiA, chiB, and chiC) from *Serratia marcescens* WW4 were also overexpressed in *E. coli* and their insecticidal toxicity against larvae of *Malacosoma neustria* and *H. armigera* were demonstrated (Danişmazoğlu et al. 2015).

The bacterium *Brevibacillus laterosporus*, isolated from soil in India, exhibited insect toxicity against larvae of diamondback moths, *P. xylostella*, reducing the time to reach 50% mortality upon infection with non-induced *B. laterosporus* from 3.3 to 2.1 days. This study provided evidence for the presence of inducible extracellular chitinolytic enzymes contributing to the insecticidal activity (Prasanna et al. 2013). Finally, a chitinase from *Pseudomonas fluorescens* strain MP-13 revealed 100% mortality against the tea mosquito bug, under *in vitro* conditions (Suganthi et al. 2017).

1.3.3.3 Viral Chitinases

A Chitinase A (ChiA) gene from *Autographa californica nuclear polyhedrosis virus* (AcMNPV, Baculoviridae), was expressed in tobacco plants. It was observed that the transgenic plants expressing an active ChiA were less damaged by fungal pathogens and lepidopteran larvae, while not having an inespecific effect on aphid populations (Corrado et al. 2008). The recombinant ChiaA, purified from tobacco leaves enhanced the permeability of the peritrophic membrane of larvae of Lepidoptera and inhibited spore germination and growth of the phytopatogenic fungus *Alternaria alternate* (Di Amaro et al. 2010). Furthermore, the AcMNPV ChiA was expressed in tobacco combined with *Aedes aegypti*-Trypsin Modulating Oostatic Factor, a peptide that inhibits synthesis of trypsin by the gut. Feeding experimental larvae showed a significantly inhibition on growth rate and development. The rate of mortality was also increased, when compared with plants expressing only one of the molecules (Fiandra et al. 2010).

Interestingly, another chitinase of viral origin was described. A chitinase of *Dendrolimus kikuchii* Matsumura nucleopolyhedrovirus (DkNPV), produced by the *DkChi* gene, was cloned, expressed in *E. coli* and purified. DKChi displayed an insecticidal activity against *S. exigua*, *Hyphantria cunea*, *Helicoverpa armigera* and *Lymantria dispar* (Wang et al. 2013).

1.4 Fungal Control

Fungi are considered as the most detrimental phytopathogens causing significant yield losses in most agriculturally important crops across the globe. Dean and co-workers (2012) classified the ten most scientifically/economically important fungi in this field: (1) *Magnaporthe oryzae*; (2) *Botrytis cinerea*; (3) *Puccinia* spp.; (4)

Fusarium graminearum; (5) *F. oxysporum*; (6) *Blumeria graminis*; (7) *Mycosphaerella graminicola*; (8) *Colletotrichum* spp.; (9) *Ustilago maydis* and (10) *Melampsora lini.* The authors also highlighted the importance of *Phakopsora pachyrhizi* and *Rhizoctonia solani* (Dean et al. 2012). Some of these species will be mentioned below in some examples of the literature on use of hydrolytic enzymes to control fungal growth.

One of the targets to control infection in plants is the fungal cell wall, as most of the components found in this structure are not present in plants (Fig. 1.2). The fungal cell wall provides both protective and aggressive functions and is also highly dynamic, changing during fungal cell division, growth and morphogenesis. The cell wall has protective action, because it acts as an initial barrier, and is in contact with hostile environments encountered by the fungus. If it is removed or weakened, the fungus dies, unless it is osmotically protected. It also provides an aggressive

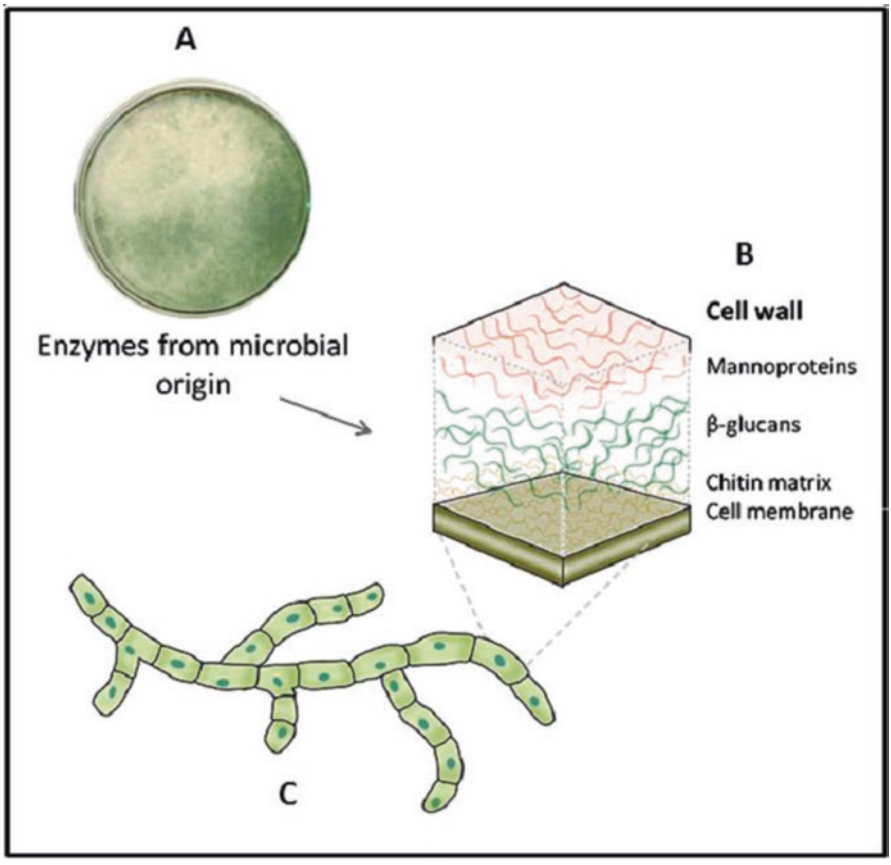

Fig. 1.2 Microbial hydrolytic enzymes such as chitinase, protease, glucanase and lipase (**a**) interact and degrade the fungal cell wall of the host (**b**) formed by layers of cell membrane, chitin, β-glucans and mannoproteins, present in the fungal hyphae (**c**)

function, as it harbors many hydrolytic enzymes and toxic molecules, most of them being in transit in the cell wall and required to invade its ecological niche (Adams 2004; Latgé 2007).

The fungal cell wall is a complex structure composed of chitin, glucans and other polymers. Polysaccharides account for more than 90% of the cell wall. The inner cell wall consists of a core of covalently attached branched β-(1,3) glucans with 3 to 4% interchain and chitin. These polysaccharides form intrachain hydrogen bonds and can assemble into fibrils and microfibrils that form a basket-like scaffold around the cell. This branched β-(1,3):β-(1,6) glucan is bound to proteins and/or other polysaccharides, whose composition may vary with the fungal species. Cell wall polymer branching and cross-linking, and the maintenance of wall plasticity during morphogenesis, may depend on the activities of a range of hydrolytic enzymes (glucanolytic and chitinolytic activities) found intimately associated with the fungal cell wall (Adams 2004; Latgé 2007, Gow et al. 2017). For a detailed review of the fungal cell wall and a comparison of the structures from different fungi, see Gow et al. (2017).

Glucanases and chitinases, when produced by plants, act as pathogenesis-related (PR) proteins, together with thaumatin, defensin and thionin. These enzymes can degrade fungal cell walls and have been widely used in plant protection as antifungal agents. The overexpression of PR genes solely or in combination has greatly increased the level of defense response in plants against a wide range of pathogens (Ali et al. 2018). Therefore, the production of transgenic plants expressing hydrolytic enzymes from microorganisms is also an interesting approach to combat fungal infection.

Lipolytic activity is reported in many microorganisms applied for biological control of phytopathogenic fungi (Chet and Inbar 1994; Cazorla et al. 2007; Magalhães et al. 2017; Mota et al. 2017; Durairaj et al. 2018), possibly having a synergistic effect alongside other hydrolytic enzymes (Calistru et al. 1997; Diby et al. 2005; Bach et al. 2016). However, the detailed function of these lipases during the infection remains elusive.

1.4.1 Glucanases

1.4.1.1 Fungal Glucanases

Many filamentous fungi and yeasts produce β-1,3-glucanases, constitutively or by induction. These enzymes are associated to the cell wall or only the interior of the cell (Rapp 1989; Pitson et al. 1993). Fungal extracellular β-1,3-glucanases typically act in fungal cell wall development, differentiation and also mycoparasitism (Pereira et al. 2007), with the latter being reported, for example, in *Trichoderma* spp. (Harman et al. 2004). According to these authors, some species of this genus are considered opportunistic, avirulent plant symbionts, as well as parasites of other fungi. *Trichoderma* species are prominent biocontrol agents used to control

Rhizoctonia solani, *Fusarium oxysporum*, *Sclerotium rolfsii*, *Alternaria alternata* and an array of foliar pathogens (*Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Sphaerotheca fusca*, *Pseudoperonospora cubensis*) (Ting and Chai 2015).

The antifungal activity of *Trichoderma* involves fungal cell-wall degrading enzymes, as well as antibiotics production and competition for key nutrients (Hjeljord and Tronsmo 1998). However, the production of cell wall-degrading enzymes has been proposed as the major mechanism of *Trichoderma* antagonistic activity against fungal plant pathogens (Chet et al. 1998). In particular, a *T. harzanium* commercial β-1,3-glucanase (Advanced Enzyme Ltd. Mumbai, India) was used to inhibit the growth of *B. cinerea*, a pathogen of grapes (Jadhav and Gupta 2016). The majority of *Trichoderma* preparations used commercially for biological control are *T. atroviride* or *T. harzianum*, as mentioned before (Marcello et al. 2010). *Trichoderma asperellum* produces at least two extracellular β-1,3-glucanases upon induction with cell walls from *Rhizoctonia solani* (Bara et al. 2003). Due to their potential as biocontrol agents, some researchers have investigated the use of *Thichoderma*-based consortia. However, the results were not encouraging, probably because *Trichoderma* isolates potentially parasitized other biocontrol agents in the consortia. This phenomenon is inevitable as *Trichoderma* species do not distinguish between pathogenic and non-pathogenic hosts (Kubicek et al. 2001). Ting and Chai observed the decrease of β-1,3 glucanase and chitinase production by *T. harzianum* when inoculated along with *T. viridescens*. Interestingly, the inoculation with the bacterium *Serratia marcescens* showed high production levels of the enzymes (Ting and Chai 2015).

Oomycetes are a unique group of diploid fungal-like organisms, related to chromophyte algae and other heterokont protists (Baldauf et al. 2000; Cooke et al. 2000). The Oomycete taxon is important since it contains numerous devastating plant pathogens including species of *Phytophtora*, *Pythium* and *Peronospora* (Alexopoulos et al. 1996). Oomycetes are also susceptible to the enzymes discussed here. Transgenic plants of pearl millet were produced with the gene *gluc78* from *T. atroviride*, that encodes a β-1,3 glucanase. One transgenic event reduced the incidence of the oomycete *Sclerospora graminicola* infection (O'Kennedy et al. 2011). Glucanases produced by yeasts are also important in biological control of fungi. The yeast *Pichia guillermondii* showed antagonistic activity against *Rhizopus nigricans* found in tomatoes during storage, due to the production of β-glucanases (Zhao et al. 2008). The commercial product "Aspire", recommended for the biological control of post-harvest rot in fruits such as apple and pear, includes in its formulation the yeast *Candida oleophila*, due to the production of β-glucanases (Bauermeister et al. 2010). The yeast *Debaryomyces nepalensis* produced β-1,3-glucanases in mangoes infected by *Colletotrichum gloeosporioides*. The production of this hydrolytic enzyme and other bioactive compounds, such as volatile compounds, helped to control the fungal infection (Zhou et al. 2018).

1.4.1.2 Bacterial Glucanases

Bacteria that colonize plant roots and promote plant growth are referred to as plant growth-promoting rhizobacteria (PGPR). Besides promoting plant growth, they can suppress different plant diseases. Several PGPRs produce hydrolytic enzymes, including glucanases, which can lyse a portion of the cell wall of many pathogenic fungi (Beneduzi et al. 2012). *Paenibacillus terrae* NK3-4 is an interesting BCA against rice blast, *Magnaporthe oryzae*. A 1,3-β-glucanase produced by this bacterium was identified and it was suggested it could be partially responsible for its antagonist activity (Yu et al. 2018). An endo-β-1,3-glucanase from *Paenibacillus* sp. was cloned and expressed in *E. coli*, the purified protein being active against *Candida albicans* and *Rhizoctonia solani* (Cheng et al. 2009).

Kweon et al. (2012) purified a 1,3-β-D-glucanase from *Streptomyces torulosus* PCPOK-0324. The purified glucanase inhibited the growth of *R. solani* and *Phytophthora capsici* (Kweon et al. 2012). An endo-β-1,3-glucanase from *Streptomyces matensis* ATCC 23935 was expressed in *E. coli.* The purified enzyme inhibited the growth of *C. albicans* (Woo et al. 2014). Another endo- β-1,3-glucanase was expressed heterologously in *E. coli*, a glucanases from *Streptomyces* sp. S27. The authors purified the enzyme and showed the activity of this protein against *R. solani* and *Fusarium oxysporum*, besides *Fusarium crookwellense* and *Paecilomyces variotii*, the latter two being mycotoxin producers (Shi et al. 2010).

Bacillus sp. strain 44, isolated from tomato rhizosphere, showed antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici*. The authors identified the production of β-1,3-glucanase, protease and chitinase, in addition to volatile and non-volatile metabolites. This isolate reduced by 36% the fungal infection in tomato plants grown under greenhouse conditions (Jangir et al. 2018). Two strains of *Bacillus velezensis* 5YN8 and DSN012 are potential BCAs for pepper gray mold, caused by *Botrytis cinerea*. These strains produced high activities of hydrolytic enzymes, including glucanases (Jiang et al. 2018).

Interestingly, the volatile compounds produced by bacteria can activate β-1,3 glucanases in plants. Volatiles of *Bacillus* sp. JS caused the up-regulation of PR-2 encoding β-1,3-glucanase in tobacco leaves damaged by *R. solani* and the oomycete *Phytophthora nicotianae* (Kim et al. 2015). Despite not being the focus of this chapter, it is important to highlight such plant-bacteria interactions, that caused the activation of the glucanases to combat the fungal infection.

1.4.2 Chitinase

1.4.2.1 Fungal Chitinase

The major fungal chitinases belong to the GH18 family (Hartl et al. 2012). In 2016, for the first time, a fungal chitinase from *Nosema bombycis* (NbchiA) was characterized as a glycoside hydrolase from family 19 (Han et al. 2016). Fungal

chitinases involved in fungicidal activity have been less investigated than the bacterial enzymes.

The chitinolytic enzyme purified by *Aspergillus terreus* showed ability to inhibit growth of *A. niger*, *A. oryzae*, *Penicillum oxysporium*, *R. solani*, *Candida albicans* and *F. solani* (Farag et al. 2016). Moreover, a chitinase isolated and purified from *Gliocladium catenulatum* inhibited the hyphal growth and conidial germination of various phytopathogenic fungi (Ma et al. 2012).

An interesting approach is the use of recombinant DNA technology to overexpress and increase the efficiency of the target enzymes. A study performed the overexpression of *chit*42 gene in *T. harzianum*, increasing by 4.9 fold its biocontrol effect against *S. sclerotiorum*, the causal agent of stem rot disease in canola (Kowsari et al. 2014). Chitinase genes (*chit2*, *chit3* and *chit4*) were cloned from *T. lanuginosus* SSBP and expressed in *Pichia pastoris*. Chit2 displayed antifungal activity against *Penicillium verrucosum* and *A. niger* (Zhang et al. 2015). Chitinase Chit42 from *T. atroviride* PTCC5220 was used to produce a chimeric chitinase fused to Chit42 a ChBD from *S. marcescens*. The chimeric chitinase showed higher antifungal activity toward phytopathogenic fungi (Matroodi et al. 2013). Another chimeric chitinase was also constructed by adding a chitin-binding domain to the N-terminal of Chit42 (that lacks it) from *T. atroviride*. The Chit42-ChBD transformants showed higher antifungal activity towards seven phytopathogenic fungal species (Kowsari et al. 2014).

1.4.2.2 Bacterial Chitinase

These enzymes occur in families GH18, GH19, their majority belonging to GH18 (Dahiya et al. 2006; Larsen et al. 2010). Chitinase-producing bacteria are usually present in the genera *Bacillus*, *Serratia, Vibrio*. They often produce different chitinases (Yu et al. 1991; Mehmood et al. 2010; Wang et al. 2014) and could be used to control phytopathogenic fungi. For example, a study demonstrated that a chitinase isolated from *B. subtilis* TV-125A was effective against *F. culmorum*, *Phythium ultimun* and other fungi (Chang et al. 2010). Chitinases purified from *Streptomyces* sp. DA11 and by *S. marcescens* B4A, also were reported to have antifungal activity (Han et al. 2009), the latter against *R. solani, Bipolaris* sp., *Alternaria raphani and A. brassicicola*. Interestingly, in another study, a chitinase from endophytic actinomycetes was purified and their antifungal activity was evaluated against the phytopathogens *R. solani, F. oxysporum, Alternaria alternata, Aspergilus niger, A. flavus, Sclerotinia scleoriorium, P. parasitica* and *B. cinerea*. This chitinase was toxic against all these microorganisms, suggesting its use for control (Haggag and Abdallh 2012).

Recombinant DNA and protein engineering technology remain relevant in improving the efficiency of bacterial chitinases in fungal control. Huang and coworkers produced a chimeric chitinase containing an antifungal chitinase ChiCW, produced by *Bacillus cereus* and a chitin-binding domain of *B. circulans*, increasing their antifungal activity (Huang et al. 2009). In another study, the gene sequence of

ChiS from *B. pumilus* was heterologously expressed in *E. coli* fused to *B. subtilis* spore coat protein (CotG), inhibiting the fungal growth of *R. solani* and *T. harzianum* (Rostami et al. 2017). Similarly, other studies performed the heterologous expression of chitinases in *E. coli* to investigate their effect in fungal control, i.e. chitinases PeChi68 of *Paenibacillus elgii* HOA73 (Kim et al. 2017), chi1 of *Serratia proteamaculans* (Wang et al. 2014), StmChiA and StmChiB from *Stenotrophomonas maltophilia* (Suma and Podile 2013), and a novel Chi18H8 isolated by suppressive-soil metagenome (Berini et al. 2017). These results are important to increase the spectrum of pest-resistance in the crop plants via co-expression of chitinases (Mehmood et al. 2010).

1.5 Conclusion

Biological control using microorganisms and the development of transgenic plants using genes of hydrolytic enzymes are promising approaches against pests. There is a great diversity of microorganisms that produce these enzymes and we have biotechnological tools to improve the activities of these proteins and obtain them in high amounts. Besides, we know only a little about their diversity. The possibility of high-scale genome sequencing increases significantly the possibility of finding new enzymes with interesting features to be used as biochemical pesticides. All this knowledge will allow us to build more specific and efficient enzymes. An eco-friendly approach to control diseases in plants may be developed from these enzymes, as these biopesticides are biodegradable, harmless to non-target organisms, and do not accumulate in the environment.

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Chapter 2
Role of Bacterial and Fungal Chitinases in Integrated Management of Pest and Diseases of Agro-Horticultural Crops

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Abstract Chitin is an important structural component of many plant pathogenic fungi. Similarly, it is also an important part of the insect cuticle and peritrophic matrices, which function as a permeability barrier, enhance digestive processes and protecting the brush border from mechanical disruption as well as from attacks by toxins and pathogens. Chitin degrading lytic enzymes (such as chitinases, and glucanase) produced by bacteria and other microorganisms can impede the growth of many insect pests and fungal phytopathogens that pose a severe risk to global crop production. Pathogenic microorganisms produce a variety of lytic enzymes such as proteases, chitinases, lipases etc. which play an important role in the virulence of entomopathogens. Many chitinolytic bacteria have the potential to control pests and fungal pathogens of crops owing to their ability to disintegrate chitin containing cellular structures. Currently, efforts are being made to discover producers of chitinolytic enzymes in nature. Production of lytic enzymes has been reported in a number of virulent pathogens such as *Serratia, Pseudomonas* or *Bacillus* spp. Bioprospecting and exploitation of chitinolytic bacteria will help in developing biocontrol agents, which have the potential to control fungal plant pathogens and insect pests. Thus, these bacteria-based biofungicides and biopesticides may replace or supplement the chemical fungicides and insecticides, reducing the negative impact of chemicals on the environment and supporting the sustainable development of agriculture-based ecosystem. This chapter focuses on the scope and potential of

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chitinolytic bacterial and fungal organisms in the management of insect pests and fungal pathogens of agricultural crops.

Keywords Chitinolytic organisms · Chitinase enzymes · Insect cuticle · Fungal cell wall · Chitin degradation mechanisms · Pests and pathogen biocontrol

2.1 Introduction

Pathogenic microorganisms and harmful insect pests affecting plant health are a major threat to food production and ecosystem stability, worldwide. Among the biotic factors, fungal phytopathogens and insects pests are responsible for considerable economic losses in a wide variety of cultivated crops. More than 65,000 diverse pest species are presently recognized, mainly fungi, weeds, and arthropods, causing up to 40% preharvest- and 10% post-production losses in agro-horticulture (Chandler et al. 2011). In India only, almost 30% of the crop yield, corresponding to 30 million tons of food, is wasted due to attacks by various insect pests, disease, and weeds (Koul 2011). Insects are one of the major natural hazards to any agro-horticultural products and encompass a remarkable group in the animal kingdom, accounting for 70% of all animals present in the world. According to an estimate, one third of the global agricultural production, amounting to about several billion dollars, is damaged annually by over 20,000 species of insects in field and storage conditions (Mariapackiam and Ignacimuthu 2008).

Similarly, pathogenic fungi are important causal agents of plant diseases of economic importance, and more than 60% of the literature in plant diseases is devoted to fungal infections (Hawksworth et al. 1995). In the past few decades, agricultural production in an intensified agricultural system was more and more dependent on agrochemicals as a relatively reliable method of crop protection. An excessive use of synthetic chemicals caused, however, several negative effects such as the development of fungicidal/insecticidal resistance in pathogens/pests, detrimental impacts on environment and human health, and harmful impact to their non-target insect/beneficial microflora, as well as possible bioaccumulation of toxic xenobiotics in the ecosystem and food supply chain (Hardy 2014; Czaja et al. 2015). Furthermore, higher cost of agrochemicals such as pesticides, mainly in poorer regions of the world, demanded for alternative and ecofriendly methods of crop protection, which have more and wider public acceptance. Among safer options, biological control using potential antagonistic microorganisms is being considered as an alternative or supplemental approach or method, reducing the chemicals use in sustainable agriculture (Gerhardson 2002; Dukare et al. 2011).

Microbe-mediated biological crop protection is an attractive and promising technology with no concern for negative impact on the environment and biodiversity

(Dukare and Paul 2018). The suppression of many crop pests and diseases using microbial biological agents holds great promise in facilitating and developing organic agriculture. Many biocontrol agents are safe to deliver, simpler in use, non-polluting, compatible with the conventional and low-input agricultural practices, induce host plant resistance and in many cases improve plant growth and yield by acting as bio fertilizers or phyto-stimulators.

Biological control of insect pest and fungal diseases using chitinase producing microorganisms has received a large amount of attention in recent times. Owing to the wider distribution of chitin in the ecosystem, microbial chitinases have lately achieved interest for their probable use as potential biopesticides in integrated pest management (IPM) strategies for controlling fungi, insects and nematodes (Hjort et al. 2014; Berini et al. 2016). Additionally, chitinase hydrolytic enzymes, produced by a diverse range of living organisms such as bacteria, fungi, nematodes, snails, insects, crustaceans and plants are receiving, for the purpose of pathogenesis, morphogenesis, parasitism, and defense, global attention with regard to their development as chemical defense proteins in transgenic plants and as microbial biocontrol agents. Widely occurring chitinolytic microbes have been preferred as a source of chitinase because of easy availability of raw materials for their cultivation and low production cost of chitinase enzymes.

Chitinases act as plant protection agents by hydrolyzing chitin. This polymer is an important constituent of the fungal cell wall, a structural component of insect exoskeleton and is present in the egg shells of nematodes. Chitin is degraded into a variety of products that include the deacylated oligomer chitosan, monomer N-acetylglucosamine and the disaccharide chitobiose. At the same time, chitinases are safe for plants and vertebrates as they are devoid of any chitin, therefore, holding a larger potential for IPM than other hydrolytic enzymes (Neeraja et al. 2010). The degradation of fungal cell wall leads to the loss of structural integrity, deformity, and eventual cell death (El-Tarabily 2006). Bacterial chitinases have been widely demonstrated as inhibiting fungal growth and can therefore be effective in controlling plant-pathogenic fungal diseases (Ordentlich et al. 1988). Similarly, extracellularly produced microbial chitinases hydrolyze chitin present in the exoskeleton and gut linings of insects, which leads to decline in the insect feeding rate and eventually yielding its death. Due to these properties, uses of chitinolytic microorganisms have stirred substantial interest in biological management of economically important insect pests of agricultural crops, during last years (Mubarik et al. 2010; Abdullah et al. 2014).

2.2 Occurrence of Chitin and Chitinolytic Organisms

Chitin is abundant in a broad range of earth environment and constitutes a structural component in many living organisms, e.g., fungi, nematodes, insects or crustaceans (Gooday 1990a, b). It is a characteristic component of the exoskeletons of arthropods and insects, of the fungal cell walls, as well as the radulae of mollusks and

internal shells of cephalopods. In accordance with the ubiquitous presence of the chitin polymer, chitinases, which degrade chitin, are found in numerous types of living organisms, such as bacteria, fungi (Gooday 1990a), archaea (Gao et al. 2003), phytoplancton (Štrojsová and Dyhrman 2008) and rotifers (Strojsova and Vrba 2005). The presence of chitinolytic microorganisms can be traced to a wide range of environments including all the regions of the earth's biosphere. Several microorganisms are able to produce chitinase and degrade chitin under both aerobic and anaerobic environments. It is believed that chitin decomposition in natural habitats is mostly and predominantly mediated by bacteria or groups of bacteria. Kielak et al. (2013) reported that hydrolysis of the chitin polymer in soil ecosystems is correlated with the abundance of bacteria. However, depending on the presence of various environmental conditions such as temperature and pH, fungi may constitute important agents of chitin hydrolysis in the later stages of its degradation process (Manucharova et al. 2011).

In general, fungal chitinases have been studied more extensively than the bacterial ones. However, there are several bacterial species known to produce extracellular chitinase enzymes in culture (Frandberg and Schnurer 1998; Viswanathan and Samiyappan 2001). Chitinase enzymes, which are involved in chitin degradation, are commonly distributed in several bacterial genera such as *Aeromonas*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Chromobacterium*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Klebsiella*, *Pseudomonas*, *Streptomyces*, *Serratia* and *Vibrio* etc. In natural ecosystems, chitin is degraded mainly by the above heterotrophic chitinolytic bacteria. Chitin-containing cell walls of plant pathogenic fungi are effectively hydrolyzed and ruptured down, individually or in combination, by the lytic enzymes such as chitinases, chitosanases and glucanases, of the chitinolytic bacteria. Bacterial chitinases have been extensively reported as fungal growth inhibitors, and can therefore be effective in controlling fungal plant diseases.

2.3 Chitinolytic Enzymes: Nomenclature and Classification

Chitinases (CHIs) are usually extracellular inducible enzymes secreted by a variety of microorganisms, insects, plants, and animals and even present in the blood serum of human (Gohel et al. 2006). CHIs break down the β-1,4-glycosidic bonds that are present between the N-acetyl-D-glucosamine residues of chitin polymer. The entire de-polymerization of chitin to free the N-acetylglucosamine (GlcNAc) residue is carried out by a diverse group of chitinolytic enzyme systems (Patil et al. 2000; Gohel et al. 2006).

The nomenclature of chitinolytic enzymes is ambiguous. Based on the location of the hydrolyzed bond, CHIs (EC 3.2.1.14) can be grouped into two classes. Endochitinases, the first class enzymes, slice chitin polymer randomly and produce low molecular weight oligomers such as diacetylochitobiose, chititriose and chitotetriose. The exochitinases generate chitobiose from either reducing or non-reducing sites of the polymer. In recent times, there were two other categories of

these enzymes which include chitobiases, accountable for the degradation of chitobiose, and β-N-acetylglucosaminidases that produce monomer unit of β-N-acetyl-D-glucosamine (Saks and Jankiewicz 2010). At present, chitobiase and β-N-acetylglucosaminidases are included in the enzyme family of β-N-acetylhexosaminidases (EC.3.2.1.52), (according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology) (Saks and Jankiewicz 2010). Based on the mechanism of hydrolysis, CHIs may also be classified into three types: a) β-1, 4-N-acetyl-glucosaminidases (EC 3.2.1.30), which catalyze the cleavage of the chitin polymer from the terminal end into individual units of GlcNAc; b) endochitinases (EC 3.2.1.14) randomly splitting at internal sites along the total extent of the chitin microfibril chain, and c) exochitinases (EC 3.2.1.14) that cause continuous release of diacetylchitobiose, in a stepwise manner, such that no monosaccharides or oligosaccharides are produced.

2.4 Family of Chitinase Enzymes

The diverse configurations of chitins present in nature are degraded by the chitinases produced by bacteria and related chitinase-producing organisms. Based on similarity in amino acid sequences, chitinolytic enzymes are grouped into the families 18, 19, and 20 of glycosyl hydrolase enzymes (Brzezinska et al. 2014). Family 18 includes CHIs enzymes obtained from bacteria, fungi, viruses, animals, and some plant chitinases, all of which have a diverse evolutionary background.

Plant chitinases (classes I, II, and IV) and some *Streptomyces* chitinases (Hart et al. 1995) are included in Family 19 of glycosyl hydrolases. Family 20 of glycosyl hydrolases includes N-acetylglucosaminidase from *Vibrio harveyi* and β-N-acetyl hexosaminidase enzymes from human and *Dictyostelium discoideum* (Patil et al. 2000; Duo-Chuan et al. 2005; Dahiya et al. 2006).

Based on the amino acid sequence of individual catalytic domains, bacterial chitinases are further grouped into three major subfamilies: A, B and C. *Bacillus licheniformis* produces five chitinases of 42, 49, 53, 62 and 66 kDa molecular weight, while *B. circulans* produces six distinct chitinases of which chitinase A1 is primarily responsible for chitin degradation. Many well known biocontrol bacteria including *Serratia marcescens*, *Pseudomonas aeruginosa* K-187, *Aeromonas* spp. and *Streptomyces griseus* are capable of synthesizing various CHIs (Brzezinska et al. 2014), with molecular weight ranging from 20 to 120 kDa (Joo 2005; Kavitha and Vijayalakshmi 2011). *Serratia marcescens* strain Nima produces an endochitinase (Chi60), an exochitinase (Chi50), and an N-acetyl glucosaminidase that showed a 43 fold higher chitinolytic activity than those of other strains (Ruiz-Sánchez et al. 2005). Mehmood et al. (2010) demonstrated that four chitinases secreted by *Aeromonas caviae* CB101 are encoded by a single chitinase gene *Chi1*. Furthermore, the well-known fungus *Trichoderma harzianum* secretes N-acetylglucosaminidases, endochitinases, and chitobiosidase (Haran et al. 1995). Aggarwal et al. (2015a) reported presence of two isoforms of chitinases in entomopathogenic *S. marcescens*

with molecular weight of ~51 kDa and 32 kDa. The optimum temperatures for CHIs functioning are around 40 °C with a broad pH range (5–8). Based on the source of CHIs, their activity can be stabilized, improved or inhibited in the presence of different metal ions.

CHIs are adaptive enzymes, produced only under definite conditions incited by specific factors. They are regulated by inducer/repressor compounds. Chitin is mostly an inducer molecule, while glucose or other easily storable carbon sources may act as a repressor molecule in the medium. An analysis using several carbon substrates have revealed the correlation between a metabolized carbon source and the extracellular production of chitinolytic enzymes. Inducing the production of chitinases via several chitin substances is a characteristic of a certain enzyme-producing species of bacteria or fungi. Many studies have used colloidal chitin to enhance production of CHIs. For examples, chitinolytic activity was found in bacteria grown on media constituting glycol or colloidal chitin, N-acetylglucosamine, chito-oligosaccarides, and/or the cell wall fractions of a few chitin containing molds. On the contrary, almost no or minimal activity was visible when same bacteria were cultured on media containing glucose or laminarin as the sole carbon source (Zhang et al. 2001; Miyamoto et al. 2007; Saks and Jankiewicz 2010). Molecular investigations have revealed the presence, in *Pseudoalteromonas piscicida, Streptomyces thermoviolaceus* and other bacteria, of a two-component signal transduction system consisting of two regulatory proteins (histidine kinase and a response regulator) that are implicated in the regulation of chitinase synthesis. In response to external stimuli/signals, an autophosphorylation occurs at the histidine residue of the bacterial kinase, followed by the transfer of a phosphate group to an asparagine residue in the response regulator. Eventually, the phosphorylated response regulator, in combination with a promoter sequence, causes the activation of and transcription of genes encoding for chitinase enzymes (Saito et al. 1998; Brzezinska et al. 2014).

2.5 Chitin as an Important Structural Component of Insect Pests and Fungal Pathogens

2.5.1 Structural and Functional Role of Chitin in Insects

The extracellular integument layer of insects, known as “cuticle”, is secreted by the insect epidermis and forms the outer covering of insects, as well as in other arthropods. Apart from the insect exterior, the cuticle covering can be observed on the interior parts including the foregut, hindgut, and trachea. The typical components of the cuticle layers, arranged in an exocuticle and endocuticle, include proteins, lipids, and chitin in cross-linking with each other, so as to provide varying degree of hardness and elasticity (Wigglesworth 1948). Chitin is also present in the peritrophic matrix (PM) of insect. This is a sleeve-like extracellular covering layer that encircles the food bolus present in the gut of most arthropods and insects (Hegedus

et al. 2009). In addition to chitin, the other important components of this matrix are proteins and proteo-glycans. This compound acts as a physical barricade towards invading pathogens, aids in the digestion process, protects gut epithelial cells from the detrimental impact of food particles and prevents the entrance of the swallowed insect pathogens and harmful toxins into the midgut epithelium (Tellam 1996). The PM is analogous to the protective mucosal layer that lines the digestive tracts of mammals. It also separates and organizes digestive processes within the midgut. The constituting components of PM such as membrane protein (peritrophins) and chitin may associate with other components such as enzymes and food molecules. The formation of chitin is usually found at the tips of midgut microvilli. The PM protein, peritrophins, has domains which are alike the gastrointestinal mucus proteins (mucins) and some other domains that are capable of binding to the chitin molecule. The PM formation can occur in the midgut or in the total organ (type I), or merely at the opening of the midgut (type II). Most insects contains type I PM, whereas type II PM is limited to larval and adult (except hematophagous) mosquitoes, flies (Diptera) and a few Lepidopteran adults. The PM is absent in Hemiptera and Thysanoptera, whose cells have perimicrovillar membranes.

Chitin chemical formula is $(C_8H_{13}O_5N)_n$. It is a long-chain polymer of N-acetylglucosamine, a derivative of glucose. It is widely distributed and the second most abundant polysaccharide in nature, after cellulose (Fig. 2.1). Numerous benefits are offered to animals possessing chitin as exoskeletons or as their structural component. To mention, chitin gives and defines the basic shape of the arthropods and provides protection from the probable desiccation and dehydration caused by adverse external factors (Anderson 1997). The chitin micro fibrils of PM form

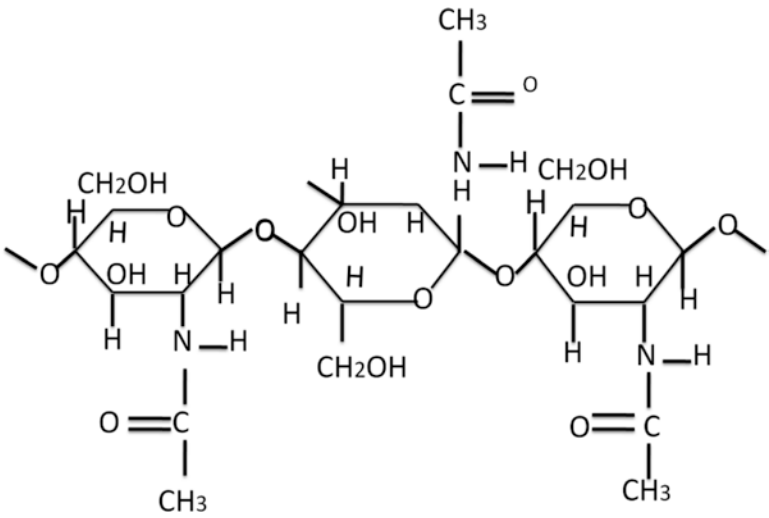

Fig. 2.1 Structure of chitin polymer found in cuticle and peritropic membrane of insects and cell wall of certain plant pathogenic fungi

association with the extremely hydrated proteoglycan matrix released by the gut cells of insects, contributing significantly in providing a tensile strength.

2.6 Mode of Action of Chitinolytic Bacteria in Biological Suppression

2.6.1 Mechanism of Action of Microbial Chitinolytic Enzymes in Insect Pests Biocontrol

Hydrolytic enzymes (including chitinsases, proteases and lipases) that can degrade and lyse the chitin-containing insect tissues can serve as an important virulence factor for the entomopathogenic microorganisms (Aggarwal et al. 2017). Chitinases have been isolated from numerous bacterial strains and reported to be effective against many pests and diseases (Gomaa 2012). A large number of bacteria that can cause pathogenesis/virulence in insect have a great potential for use in biological control of economically important pests of agricultural crops.

Chitin is insoluble polysaccharide made up of linear chains of GlcNAc residues, cross-linked by hydrogen bonds. It is the most important structural constituent of the external skeleton (50% of the cuticle is made up of chitin), hindgut, foregut, midgut lining of the PM. Therefore, it is also vital for ensuring the structural integrity of many insects and nematodes pests (Bhattacharya et al. 2007). The insect exoskeleton, cuticle and PM is broken down by individual or combined actions of a binary enzyme complex, involving chitinase and β-N-acetylglucosaminidases (NAG) enzymes (Filho et al. 2002).

Microbially produced chitinases complex de-polymerize chitin components into their monomeric or oligomeric units, thus degrading the foremost constituents of the insect exoskeleton. Sometimes, extra proteins that have one or more chitin-binding domains (which are however deprived of a chitinolytic activity) may augment the process of chitin degradation (Vaaje-Kolstad et al. 2005). This system, that likely operates in the gut during PM break down, increases the PM porosity, finally causing insects' death (Khajuria et al. 2010).

There is a correlation between virulence and chitinase production that can cause weakening and slimming of the insect cuticular structure. In addition, if these enzymes act on the insect intestine, a crucial damage to the PM may occur (Chandrasekaran et al. 2014). The hydrolytic action of chitinase and the subsequent damage caused to the gut of the larvae PM prevent the insect from feeding and, therefore, eventually leads to its death. In alternative it damages the cuticle resulting into abnormal molting (Suganthi et al. 2017). This reality has opened a new potentiality in relation to the exploitation of chitinolytic enzymes in the biocontrol of insect pests.

2.6.1.1 Effect of Chitinase on the Peritrophic Membrane

Chitin biosynthesis and its degradation are important metabolic pathways in several arthropods, and particularly most crucial in inhibition of the insect cuticle and PM (Husen et al. 2015). The capability of chitinases to degrade the PM of the insect gut has been explored for many years. In 1993, Shahabuddin et al. demonstrated that the addition of exogenous chitinase, isolated from *S. griseus* in a blood meal *in vivo* hampered the function of the PM in *Anopheles freeborni*. Likewise, feeding of the fifth instar larvae of *Spodoptera littoralis* on recombinant endochitinase ChiA caused holes and perforations in the PM (Regev et al. 1996). The excessive intake of chitinase has been found to have serious physiological consequences with death of exposed insects (Herrera-Estrella 1999). The midgut chitinases appear to be implicated in the development, puncture and degradation of the peritrophic matrix that protects the gut epithelium from destructive factors (Filho et al. 2002).

Histological study confirmed the significant negative effects of chitinases on the treated larval gut, epithelial cells and PM. SEM studies on PM from the midgut of *Spodoptera litura* larvae fed on a chitinolytic *S. marcescens* strain revealed considerable damage to the midgut (Aggarwal et al. 2017). Chitinase isolated from *B. subtilis* efficiently inhibited the gut enzyme activity, growth and development of *S. litura* larvae (Chandrasekaran et al. 2012). *Paenibacillus larvae,* a bacterial pathogen of honey bee larvae, expresses a chitin-binding and degrading protein which successfully degrades the chitin-containing gut line of PM (Garcia et al. 2013). This study demonstrated that the break down of the PM lining the midgut epithelium is a key step in the virulence of *P. larvae*. These observations confirmed that PM is the first barrier that bacteria have to overcome when trying to infringe the epithelium and penetrate the hemocoel. The mechanism of action of chitinase enzymes on insects are given in Fig. 2.2.

2.6.2 Mechanism of Action of Chitinolytic Enzymes on Fungal Pathogens

The extracellular hydrolytic enzymes of antagonistic microbes cause mycoparasitism of fungal pathogens, by attaching and degrading fungal hyphae. Direct parasitism or mycoparasitism is the ability of an antagonistic microorganism to attach hyphae to produce extracellular cell wall lytic enzymes (Dukare et al. 2018). Mycoparasitism of antagonists depends upon the sequential occurrence of the following events: (i) coming into close contact with the fungal pathogens, (ii) mutual recognition between antagonist and pathogen, (iii) lytic enzymes secretion and (iv) active growth of the antagonist inside the host (Talibi et al. 2014). The negative effects of myco-parasitism result in the killing of the pathogen propagules, lysis with destruction of its cellular structures and disintegration of the cellular integrity. The fungal cell wall, composed by chitin and glucan in combination with wall

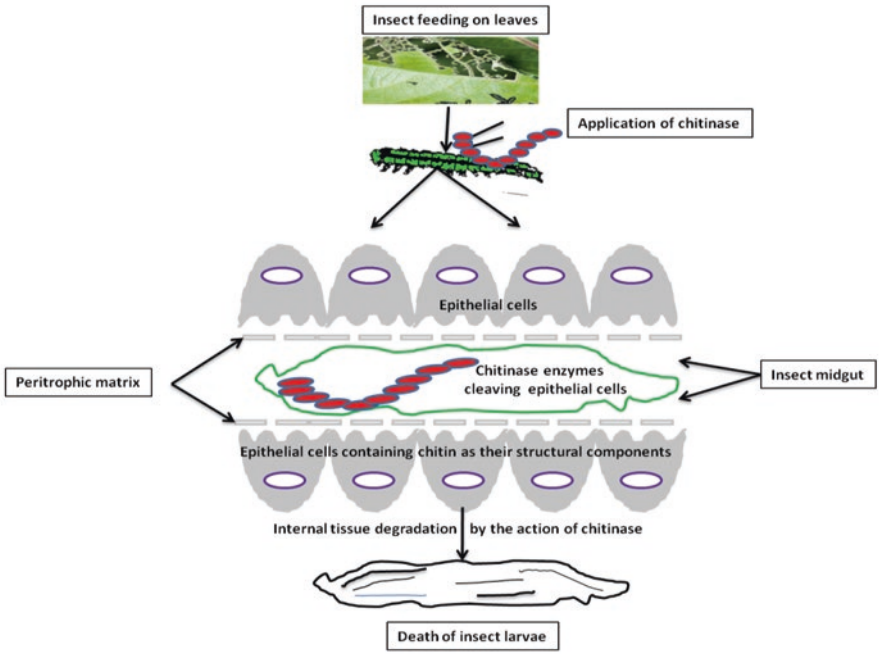

Fig. 2.2 Schematic representation of chitin degradation in insect by chitinase, and chitinase mode of action in insect. (Adapted in modified from Chandrasekaran et al. 2012)

proteins, is disintegrated by an individual or collective actions of lytic enzymes such as chitinases, glucanases, chitosanases, cellulase, and proteases produced by the antagonists, thus contributing to the biocontrol activity (Spadaro and Droby 2016). Figure 2.3 demonstrates the action of different chitinolytic enzymes in the complete dissolution of chitin-containing cell wall of pathogenic fungi. Moreover, these enzymes adversely affect pathogen's conidial germination, germ tube elongation, and may cause damages to the oospores (El-Tarabily 2006). Accordingly, several reported microbial biocontrol agents of different fungal pathogens produce extracellular hydrolytic enzymes. For example, the biocontrol action of the antagonist yeast *Candida oleophila* against *Penicillium expansum*, causing blue mold decay in apple, was mediated through an extracellularly produced β-1,3-glucanase enzyme (Urbina et al. 2016). In the process, the action of the purified glucanase enzyme reduced the *P. expansum* conidia germination and caused mycelium inhibition. Banani et al. (2015) reported chitinase activity of the antagonistic yeast *Metschnikowia fructicola* and demonstrated that chitinase gene *MfChi* was over induced in the presence of the yeast *Monilinia fructicola* cell wall. An overexpressed MfChi chitinase in *Pichia pastoris* controlled the growth of *M. fructicola* and *M. laxa in vitro*, and *in vivo* on peach fruits. The antimicrobial actions of many species of *Bacillus* and *Pseudomonas* are credited to the extracellular chitinolytic enzymes they release (Yu et al. 2008). Some other detrimental effects of enzymatic actions on pathogens are cellular deformities, protoplasmic damages, mycelial

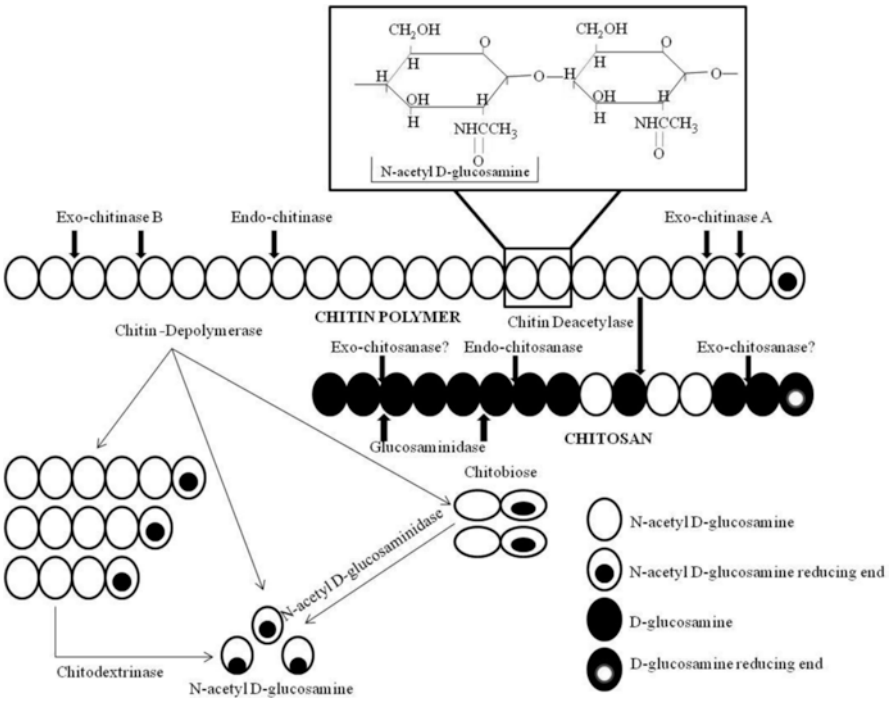

Fig. 2.3 Schematic diagram representing action of different chitinolytic enzymes involved in the complete dissolution of chitin-containing pathogenic fungal cell wall. (Adapted from Dukare et al. 2018)

distortion and lyses, leakage of cellular contents, and changes in membrane permeability (Di Francesco et al. 2016). Therefore, the loss of fungal cytoplasm due to the enzymatic cell wall disintegration is one of the key modes accountable for biocontrol actions of chitinase and other lytic enzymes secreted by microbial antagonists.

2.7 Chitinase Deployed as Successful Biocontrol Agents

2.7.1 Chitinases in Pest Management of Insects

Bacterial chitinases are active at an alkaline pH range. They can consequently degrade the chitin present in the insects gut lining, which has an alkaline pH (Bhattacharya et al. 2007; Aggarwal et al. 2015b). Chitin metabolism is an excellent target for selective pest control (Shternshis et al. 2005). Most chitinases deployed as insecticides are isolated and purified from the bacterial genera such as *Bacillus, Pseudomonas, Serratia, Streptomyces* spp. etc. In addition, Nawani and Kapadnis (2003) reported the presence of chitin degrading actinobacterial genera including *Saccharopolyspora, Kitasatospora, Nocardiopsis, Nocardioides, Herbidospora, Microbispora,*

Micromonospora, and *Actinoplanes*. Their investigation formed a comprehensive base to study the diversity of chitinolytic systems in actinobacteria.

In recent times, microbial chitinases have been utilized as insecticide and fungicide for pest and disease control (Berini et al. 2018). Chitinolytic enzymes derived from bacteria are being used to augment the efficiency of microbial insecticides such as *B. thuringiensis* and Baculoviruses. These enzymes, by disrupting the PM, enable microbes and entomopathogens to march into the hemocoel of insects (Hegedus et al. 2009).

The soil bacterium *S. marcescens* produces different extracellular enzymes including chitinases exhibiting insecticidal effects (Petersen and Tisa 2013). This bacterium is a well recognized soil microbe for the rapid degradation of chitin present in the ecosystem. In a range of experimental setups, chitinases, and chitinase genes of *S. marcescens* have demonstrated their potential as effective biocontrol agents against several pests (Brurberg et al. 2000; Aggarwal et al. 2015b). *Serratia marcescens* strain STS caused a 90% mortality in *S. litura* grown on a semi-synthetic diet (Aggarwal et al. 2014). Another *S. marcescens* strain SEN, isolated from diseased insects, showed predominant chitinase activities including exochitinase, endochitinase and chitobiosidase (Aggarwal et al. 2015b). This strain had strong insecticidal properties against all growth stages of *S. litura* larvae. The intake of sub lethal doses of this strain reduced larval and pupal weight, normal pupation, emergence of adult with a delay in the larval period. In addition, fecundity and hatchability was also significantly affected. Similarly, purified chitinase from *Psuedomonas fluorescens* exhibited potent insecticidal activity against the tea mosquito bug *Helopeltis theivora* (Suganthi et al. 2017). The implicated mechanism of insecticidal action was attributed to the hydrolysis of chitin.

Another bacterium, *Bacillus thuringiensis*, well known for its pesticidal activity, also produces many chitinases with varying molecular weights (66, 60, 47 and 32 kDa) (Thamthiankul et al. 2001). Larvae of the spruce budworm *Choristoneura fumiferana* died more quickly when exposed to a chitinase-*Bacillus* mixture (Thamthiankul et al. 2001). The biocontrol potential of the soil bacterium *Paenibacillus* spp. D1 against larvae of *Helicoverpa armigera*, commonly known as legume pod borer, was ascribed to its high rate of chitinase production (Singh et al. 2016). The exposure of insects to this bacterium noticeably reduced their feeding rate and larval body weight. This effect was attributed to the hydrolysis and degradation of the insects' chitinous structures. This was apparent from the decrease in the total chitin content and increased larval mortality when treated with *Paenibacillus* spp. D1 and chitinase, as compared to untreated control.

The application of specific metabolites such as chitinase is always beneficial in comparison to the use of living microbial cell (Shternshis et al. 2005). Chitinase functions as both a systemic toxic and contact component, causing insect death. Therefore, this strategy is opening a new avenue in the development of chitinase-based biopesticidal formulations. The production of low-cost chitinases has received interest as a potential biocontrol molecule for management of many insects. It is a rising field of research, and has been evaluated for numerous pests of agricultural crops. Table 2.1 shows the list of chitinolytic microorganisms (bacteria and fungi) and their produced chitinases implicated in the biological control of insect pests.

Table 2.1 Chitinolytic microorganisms (bacteria and fungi) and their chitinase enzymes implicated in the biological control of insect pests

Chitinolytic microorganism	Reported chitinase	Target insect pests	References
Bacillus circulans No. 4.1	Not available	*Lymantria dispara*	Lertcanawanichakul et al. (2004)
Bacillus subtilis	Not available	*Spodoptera litura*	Chandrasekaran et al. (2012)
Bacillus licheniformis	ChiBIA	*Spodoptera exigua*	Thamthiankul et al. (2001)
Bacillus thuringiensis subsp.*tenebrionis* YBT-9602	Chi 960235–459	*Helicoverpa armigera*	Ni et al. (2015)
Bacillus thuringiensis subsp. *colmeri*	Chi A	*Spodoptera exigua* and *Helicoverpa armigera*	Liu et al. (2010)
Brevibacillus laterosporus Lak1210	Mixture of six chitinases	*Plutella xylostella*	Prasanna et al. (2013)
Pseudomonas fluorescens MP-13	Not available	*Helopeltis theivora*	Suganthi et al. (2017)
Serratia marcescens	ChiAII	*Spodoptera littoralis*	Regev et al. (1996)
Serratia marcescens SEN	Mixture of two chitinases	*Spodoptera litura*	Aggarwal et al. (2015a, b)
Serratia marcescens WW4	Chi A, Chi B, and Chi C	*Malacosoma Neustria, Helicoverpa armigera*	Danişmazoğlu et al. (2015)
Serratia marcescens Xd1	Chi B, Chi C	*Galleria mellonella Drosophila melanogaster*	Ozgen et al. (2013)
Yersinia entomophaga MH96	Chi1 and Chi2	*Costelytra zealandica, Adoryphorus couloni, Acrossidius tasmaniae,* and *Plutella xylostella*	Busby et al. (2012)
Trichoderma harzianum	Not available	*Helicoverpa armigera*	Binod et al. (2007)
Trichoderma viride	Mixture of at least four chitinases	*Bombyx mori*	Berini et al. (2016)
Beauveria bassiana	BbChit1	*Myzus persicae*	Fang et al. (2009)
Beauveria bassiana	Chimeric BbChit1	*Myzus persicae*	Fang et al. (2009)
Metarhizium anisopliae	Chi2	*Dysdercus peruvianus*	Boldo et al. (2009)
Isaria fumosorosea	IfChit1	*Plutella xylostella*	Huang et al. (2016)

2.7.2 *Chitinolytic Microbes and Enzymes as Biocontrol Agents of Plant Fungal Diseases*

Chitinolytic enzymes have received attention for the biological control of soil borne fungal phytopathogens. Hydrolytic enzymes such as chitinases, chitosanases and glucanases, individually or in combination, effectively hydrolyze and break down the chitin containing cell walls of fungi. Those enzymes possessing activity of chitin degradation are generally referred to as chitinolytic enzymes. A positive correlation between biological control of some soil-borne fungal diseases of crop plants with chitinase enzyme production by chitinolytic bacteria has been demonstrated. Several studies have demonstrated *in vitro* lysis of fungal cell walls either by chitinase or β-1, 3-glucanase, alone or in combination. Two chitinase enzymes from *B. amyloliquefaciens* V656 exhibited inhibitory activities on the growth of *Fusarium oxysporum* (Wang et al. 2002). Antifungal chitinase of *B. subtilis* showed strong inhibitory activity against several phytopathogenic fungi (Chang et al. 2007; Yang et al. 2009). Biocontrol ability of *Stenotrophomonas maltophilia* and *S. marcescens* for controlling growth of *Rhizoctonia solani*, *F. oxysporum* and *Botrytis cinerea* is mainly attributed to their chitinase enzymes produced (Someya et al. 2000). Table 2.2 provides a partial list of chitinase-producing bacterial and fungal agents, deployed for phytopathogens control.

El-Mougy and Abdel-Kader (2009) reported that chitinolytic bacteria such as *B. subtilis* and *P. fluorescens*, and their chitinase enzymes (culture filtrates), exhibited antagonistic activity against selected soilborne root rot pathogens (*R. solani* and *F. solani*) *in vitro*. *In vitro* antagonistic assays were performed with dual culture and agar diffusion techniques. Anitha and Rebeeth (2009) reported that chitinase producing *S. griseus* was able to inhibit the growth of some soil borne plant pathogens of tomato such as *F. oxysporum*, *F. solani, R. solani* and *Alternaria alternata*.

Fungal spore germination and hyphal growth of pathogenic *F. oxysporum* and *Aspergillus niger* was inhibited significantly by chitinase from *Serratia proteamaculans* (Mehmood et al. 2009). Chitinase from *B. thuringiensis* contributed to the biocontrol of *Sclerotium rolfsii* and other phytopathogenic fungi in soybean seeds (Reyes-Ramírez et al. 2006). Spore germination of two fungal pathogens was inhibited by chitinase A produced by *B. thuringiensis* subsp. *colmeri* (Liu et al. 2010). Gupta et al. (2006) showed that chitinase producing *P. aeruginosa* GRC1 was strongly antagonistic against *Sclerotinia sclerotiorum*, the causal organism of stem end rot of peanut, *in vitro* and *in vivo*. They also observed increased seed germination and reduction in stem-rot infestation when peanut seeds were treated with *P. aeruginosa* GRC1 strain *in vivo*. High levels of chitinase production and *in vitro* antifungal ability against *Sclerotinia minor*, a pathogen causing basal drop disease of lettuce, was observed among 23 bacteria, 38 streptomycete and 15 non-streptomycete actinomycetes., *In vitro* assays were used to test the antifungal potential of *Serratia marcescens*, *Streptomyces viridodiasticus* and *Micromonospora carbonacea* under pot conditions, based on production of β-1,3-glucanase enzyme, antifungal activity, ability to colonize roots and rhizosphere of lettuce. Under

Table 2.2 Partial list of chitinase producing bacterial and fungal agents deployed for controlling main phytopathogens of agricultural crops

Biocontrol agent involved	Target pathogens/diseases	References
Species of *Acinetobacter, Bacterium, Burkholderia, Paenibacillus, Pseudomonas, Rhizobium, Shewanella, Sphingomonas* and *Stenotrophomonas*	*Acremonium, Alternaria, Fusarium, Penicillium*	Medina-de la Rosa et al. (2016)
Bacillus licheniformis	*Fusarium solani, Fusarium oxysporum, Rhizoctonia solani, Phytophthora cinnamomi*	Jankiewicz et al. (2016)
Bacillus subtilis	*F. oxysporum* f. sp. *ciceris, F. oxysporum* f. sp. *ricini, and R. solani*	Basha and Ulaganathan (2014)
Bacillus amyloliquefaciens and *Serratia marcescens*	*Ganoderma boninense*	Azizah et al. (2015)
B. thuringiensis subsp. *tenebrionis* DSM-2803	*Colletotrichium gloeosporioides*	Fuente-Sacido et al. (2016)
Bacillus licheniformis	*Phoma medicaginis*	Slimene et al. (2015)
Stenotrophomonas maltophilia	*Alternaria alternata, Rhizoctonia solani, Fusarium solani, F. oxysporum.*	Jankiewicz et al. (2016)
Streptomyces sp. ACT7	*F. oxysporum, Alternaria* sp.	Thirumurugan et al. (2015)
Bacillus subtilis, and *B. pumilus*	*Fusarium oxysporum* f. sp. *cubense*	Nawangsih, and Purba (2013)
Streptomyces hygroscopicus	*Colletotrichum gloeosporioides, Sclerotium rolfsii*	Prapagdee et al. (2008)
Serratia proteamaculans 3095	*Fusarium oxysporum*	Chung and Kim (2007)
Alcaligenes xylosoxydans	*Fusarium* spp., *Rhizoctonia bataticola*	Vaidya et al. (2003)
Lactobacillus plantarum	*Aspergillus* spp., *Fusarium culmorum, Penicillium* spp., *Cladosporium* spp.	Russo et al. (2017)
Leucosporidium scottii	*Penicillium expansum, Botrytis cinerea*	Vero et al. (2013)
Rhizobium spp.	*Aspergillus* spp., *Curvularia lunata, Fusarium* spp., *Sclerotinia sclerotiorum*	Sridevi and Mallaiah (2008)
Enterobacter NRG4	*Aspergillus niger, Fusarium moniliforme, Mucor rouxi, Rhizopus nigricans*	Dahiya et al. (2006)
Bacillus cereus QQ308	*Fusarium* spp., *Pythium ultimum*	Chang et al. (2007)
Bacillus circulans GRS 243	*Phaeoisariopsis personata*	Kishore et al. (2005)
Pseudomonas aeruginosa	*GRC1 Sclerotinia sclerotiorum*	Gupta et al. (2006)
Serratia proteamaculans 336x	*Gaeumannomyces graminis*	Wang et al. (2014)

(continued)

Table 2.2 (continued)

Biocontrol agent involved	Target pathogens/diseases	References
Paenibacillus elgii HOA73	*Cladosporium* spp., *Botrytis cinerea*	Kim et al. (2017)
Brevibacillus laterosporus Lak1210	*Fusarium equiseti*	Prasanna et al. (2013)
Trichoderma harzianum ABRIICC T8-7MK	*Rhizoctonia solani, Fusarium* spp., *Sclerotinia sclerotiorum, Verticillium dahliae, Alternaria brassicola*	Kowsari et al. (2014)
Trichoderma harzianum CECT 2413	*Botrytis cinerea*	Limón et al. (2001)
Trichoderma atroviride PTCC5220	*Rhizoctonia solani, Sclerotinia sclerotiorum, Alternaria alternata*	Matroodi et al. (2013)
Talaromyces flavus CGMCC 3.4301	*Verticillium dahliae, Sclerotinia sclerotiorum, Rhizoctonia solani, Alternaria alternata, Fusarium moniliforme, Magnaporthe grisea*	Duo-Chuan et al. (2005)
Clonostachys rosea	*Sclerotinia sclerotiorum*	Sun et al. (2017)
Aspergillus terreus	*Aspergillus* spp., *Penicillium oxysporium, Rhizoctonia solani, Candida albicans, Fusarium solani*	Farag et al. (2016)

controlled glasshouse conditions, all the three isolates, individually or in combination, were antagonistic to *S. minor* and significantly reduced incidence of basal drop disease (El-Tarabily et al. 2000).

Quecine et al. (2008) reported that twenty five chitinase-producing endophytic isolates of *Streptomyces* spp., proceeding from citrus and soybean, exhibited antifungal activity *in vitro* against *Guignardia citricarpa*, *Phytophthora parasitica*, *Colletotrichum sublineolum*, *R. solani*, *Pythium* sp. and *F. oxysporum*. These authors also found a positive genetic and phenotypic correlation between chitinolytic potential and antagonistic activity against *C. sublineolum*, *R. solani*, *G. citricarpa* and *F. oxysporum*. Yasir et al. (2009) enriched chitinolytic bacterial communities in vermicompost that reduced the spore germination of *F. moniliforme.* Two chitinolytic bacterial strains, *Paenibacillus* spp. 300 and *Streptomyces* spp. 385, suppressed fusarium wilt of cucumber (*Cucumis sativus*) caused by *F. oxysporum* f. sp. *cucumerinum* in non-sterile, soil-less potting medium. A mixture of the two strains in a ratio of 1:1 or 4:1 gave significantly better control of the disease then each of the strains used individually or in other ratios. Malathi and Viswanathan (2013) demonstrated the role of microbial chitinase (*Trichoderma* spp. and *Pseudomonas* spp.) against *Colletotrichum falcatum in vitro* and *in vivo* conditions. Under *in vivo* conditions, the native rhizospheric strains of fluorescent pseudomonads and *T. harzianum* from sugarcane were able to protect the crop from soil borne inocula of red rot, with an efficacy positively correlated with the soil chitinase activity.

There are many reports of chitinase activity of biocontrol agents, as further influenced by inoculations with one or more pathogens. Hjort et al. (2014) targeted chitin degrading enzymes of an uncultured bacterial community through a functional metagenomics approach. They identified a novel bacterial chitinase, Chi18H8 using

a fosmid library of a suppressive soil metagenome. This enzyme showed antifungal activity against several important phytopathogens such as *Colletotrichum gloeosporioides*, *F. oxysporum*, *Penicillium chrysogenum*, *A. alternata*, *Aspergillus niger*, and *Rhizopus stolonifer.* These authors also reported that this was the first chitinase isolated from a metagenome library with a potential for controlling fungal crop diseases. Muhammad et al. (2014) isolated a *B. thuringiensis* producing chitinase with a maximum activity at 35 °C and neutral pH. *In vitro* and detached leaf assays showed that chitinolytic bacteria have an antagonistic activity and fungistatic efficacy against *Colletotrichum gloeosporioides* and *Curvularia affinis.* Saleem and Kandasamy (2014) reported presence of chitinase Chi25 in *B. subtilis* strain BC121 and demonstrated its antagonistic activity vs *F. oxysporum* f. sp. *ciceri* through the inhibition of spore germination and hyphal extension. Glasshouse experiment also revealed presence of antifungal activity against pathogenic fungi whereby it reduced incidence of fusarium wilt by 80%. These authors also reported that the fungistatic activity of the strain against plant pathogenic fungi both *in vitro* and *in vivo* was mediated by a chitinase.

Siti et al. (2015) reported the antifungal activity of chitinolytic *B. amyloliquefaciens* and *S. marcescens* against *Ganoderma boninense*, the pathogen responsible for deadly Basal Stem Rot (BSR) disease of oil palm trees. Under *in vitro* conditions, both bacteria showed percentage inhibition of *G. boninense* by 68.19 and 40.29%, respectively, using a dual culture method. *In vitro* hyphal lysis demonstrated the role of chitinase in growth inhibition. Thirumurugan et al. (2015) isolated actinobacteria from twelve sediment samples and found them positive for chitinase activities using enrichment chitin agar media. The most potential chitinase producing isolate, *Streptomyces* spp. ACT7, showed antagonistic activity vs *F. oxysporum* and *Alternaria* sp. under *in vitro* conditions. Imen et al. (2015) isolated nine chitinase-producing cultures from Tunisian soil, among which culture S213, identified as *B. licheniformis*, exhibited strong chitinolytic activity. SDS-PAGE analysis of the secreted colloidal chitin-induced proteins showed presence of a 65 kDa chitinase. Bacterial culture supernatant containing extracellular chitinase inhibited growth of several phytopathogenic fungi including *Phoma medicaginis*. The strain was efficient in reducing the damping-off caused by *P. medicaginis* in *Medicago truncatula*.

Out of thirteen cultures of actinobacteria isolated from mangrove soils, eleven produced chitin hydrolysis zone on chitinase mineral agar medium. Seven isolates showed antifungal activity against *F. oxysporum* on potato dextrose agar, under *in vitro* conditions. Out of these seven isolates, three actinobacteria (VMK1, VMK4 and VMK9) were found as most potent and showed reduction in disease incidence up to 80%, under *in vivo* conditions. Selected cultures were identified as *Streptomyces* spp. based on morphological and microscopic observations (Vaijayanthi and Vijayakumar 2016).

Fuente-Sacido et al. (2016) cloned and expressed the endochitinase *chiABtt* gene of *B. thuringiensis* subsp. *tenebrionis* DSM-2803 in *Escherichia coli*. They also reported, for the first time, characterization of a chitinase synthesized by *B. thuringiensis* subsp. *tenebrionis* DSM-2803 and inhibition effects on radial growth and

hyphae of *C. gloeosporioides,* the etiological agent of anthracnose in crops. Jankiewicz et al. (2016) reported presence of at least two different isoforms (Chi1 and Chi2) of chitinase in *B. licheniformis* M3. They also reported that this bacterium was antagonistic towards pathogenic *F. solani,* responsible for wilting in solanaceae family crops. Use of formulations based on antifungal chitin-degrading enzymes directly for the suppression of plant fungal diseases has opened a new era in biological disease control.

2.8 Conclusions

Chitinolytic microorganisms may be produced for a potential biotechnological application in various natural environments. Their application is not limited to degradation of the waste containing chitin. Numerous studies indicated the possibility of their use for production of chitinolytic enzymes with fungicidal or insecticidal activity against some fungal phytopathogens and insect pests, respectively. In biological control of insect pests and crop pathogens, the application of various metabolites, including CHIs, appears to be more efficient, since they show stronger pesticidal and fungicidal activity than purified enzymes. The use of agents in a consortium of chitinolytic microorganisms seems to bring better results in controlling insect pests and fungal phytopathogens of crops. Chitinases are potent weapons in the armory of strategies developed, during time, by microorganisms for virulence. A benefit can be obtained by exploiting microbial chitinases as biotechnological tools for controlling different plant pests and pathogens. Increasingly stringent regulation on use of synthetic pesticides globally, and the urgent need for sustainable IPM strategies, may impel a more systematic analysis of the potential of chitinases as biopesticides in the coming era.

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Chapter 3
Hydrolytic Enzymes and Integrated Pest Management

Tariq Ahmad and Ajaz Rasool

Abstract Growing concern and general awareness of the increased use of pesticides and their overall adverse effects on ecosystem and human health, bioaccumulation in food chains, pest resistance and persistence of chemicals in the environment elicited the search for alternate pest controlling strategies. Hydrolytic enzymes including chitinases, proteases, lipases and glucanases, which are key biochemical components of insect metabolism and life cycle, have become new arsenal for controlling pests. Owing to the presence of cuticle covering, hydrolytic enzymes act on and degrade the insectbarriers, and start the body infection process. Various entomopathogenic fungi, bacteria and viruses have been reported to release hydrolytic enzymes against pests. This chapter provides an insight of hydrolytic enzymes and their role in Insect Pest Management (IPM). Potential use of these hydrolytic enzymes, their virulence and mechanism of action could be valuable for producing more potent and safer insecticides. Comprehensive understanding and knowledge-based chemistry and regulation of chitin metabolism, part of the insect pest which is more susceptible to hydrolytic enzymes, life cycle and stages of insects and thorough understanding of metabolic pathways will help in control of pests and achieving IPM goals.

Keywords Hydrolytic enzymes · Chitinase · Protease · Lipase · Entomopathogenic fungi · IPM

3.1 Introduction

Agriculture sector today is facing burgeoning pressure to support booming population around the globe while past 50 years have seen human population getting doubled. Food and Agriculture Organization (FAO) predicts it to reach 10 billion by

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2050 (Alexandratos and Bruinsma 2012). The main threat to the food security around the world is global warming and pests while over 65,000 pest species including weeds, fungi and arthropods are responsible for about 40% pre- harvest and 10% post harvest losses in different crop productions (Chandler et al. 2011). Therefore, efforts have been laid to increase yields with high quality varieties and increased resistance to pests and different diseases, which account for 20–40% crop losses annually (FAO 2012). To suppress the pest population below economic injury levels, multiple approaches and methods, both physical and technical have been applied from time to time. Use of pesticides to control different species has been the best practice of pest management in industrialized countries and over 2.3 billion kg of pesticides are used annually around the globe (Atwood and Paisley-Jones 2017). While concern and general awareness are growing about the increased use of pesticides and their overall adverse effects on ecosystem and human health, bioaccumulation in food chains, pest resistance and persistence of chemicals in environment came to the limelight in recent past (Chandler et al. 2011; Hardy 2014; Czaja et al. 2015). These circumstances led to the regulation of chemical pesticides for progressively lowering their usage for crop protection, while alternate pest controlling strategies were fostered for Integrated Pest Management (IPM).

IPM, a broad based approach, accounts for regulation of pest population by integrating various approaches. Main emphasis of IPM is healthy crop and least disturbance to the surrounding agro-ecosystem. Cultural and physical barriers, use of pesticides as chemical control and biological control, which includes adding natural enemies and predators for pest management, all together form the important network of IPM. Of late, use of biological control agents to regulate pest population has spiked up owing to its benign and benevolence towards surrounding environment. It reduces human exposure towards harmful chemicals besides being economically cheap, while employment of organisms and their products to fight pests form a fundamental pillar of IPM (Chandler et al. 2011).

Introducing living organisms for agricultural practices is the first frontier of biological control. It comprise of parasitoids, predators and pathogens while most of these bio-control agents are fungi-based insecticide products including Ascomycetes viz., *Beauveria bassiana*, *B. brogniarti*, *Isaria fumosoroseus*, *Metarhizium anisopliae*, and *Lecanicilium lecanii* (Mancini and Romanazzi 2014; Ahmad et al. 2019).

Other than entomopathogens, bacterial and viral hydrolytic enzymes have also been exploited for controlling pests (Subbanna et al. 2018). Biocontrol action is mostly due to multiple synergic mechanisms and generally includes production and secretion of antibiotics and secondary metabolites. In addition, secretions of lytic and defensive enzymes like chitinases, glucanases, peroxidases, lipases etc. form a novel approach in pest regulation (Leahy et al. 2014; Parnell et al. 2016). Owing to unpredictable efficacy under ever changing field conditions, long time for action against pests and difficulty to displace indigenous microbiota, the role of biocontrol organisms in IPM strategies have become limited (Neeraja et al. 2010; Parnell et al. 2016). Alternative approaches have been developed over the time and include generating and optimizing cocktail of microbial enzymes which mimic the multiplicity of biocontrol mechanisms without getting influenced by the inherent limitations in

using live organisms (Karasuda et al. 2003; Huang and Chen 2008). These mostly include hydrolytic enzymes like glucanases, proteases, lipases and chitinases which are key biochemical components of insect metabolism and life cycle. Main advantage of these enzymes and metabolites is that they can be exploited and improved at any time to make them best fit for a particular pest under varying environmental conditions (Berini et al. 2017).

Chitin is one of the most abundant substances on earth. Predominantly, it is found in exoskeleton of arthropods, their peritrophic membrane linings and cell walls of fungi. It acts as a significant barrier and helps insects in combating surrounding harsh conditions. For insect species which act as pests, breaching of this cuticle border with the help of hydrolytic enzymes is key to their control. Chitinases are the main enzymes involved in chitin metabolism. Hydrolytic enzymes with particular emphasis on chitinase enzymes from entomopathogens (*Beauveria bassiana*, *B. brogniarti*, *Isaria fumosoroseus*, *Metarhizium anisopliae* and *Lecanicilium lecanii*), bacteria (*Serratia marcescens*, *Aeromonas caviae*, *Pseudomonas aeruginosa*, *Streptomyces coelicolor*) and some viruses (*Autographa californica* multicapsid nucleopolyhedrovirus, *Dendrolimus kikuchii* Matsumara NPV) have been exploited for management of some insect pests.

Entomopathogenic fungi have become a driving force and key component of IPM techniques against insect pests in horticulture, forestry and agriculture. Virulence of different entomopathogens like *B. bassiana*, *B. brogniarti*, *I. fumosoroseus*, *M. anisoplia*, and *L lecanii* has been related with cuticle degrading enzymes which can be regulated on different nutrient conditions (Petrisor and Stoian 2017). Since chitin is widely distributed across animal kingdom, role of chitinases have recently gained utmost importance for their possible use as promising biopesticides in controlling insects, fungi and nematodes in various IPM programs (Neeraja et al. 2010; Hjort et al. 2014; Berini et al. 2016, 2017). Even though chitinases degrade different vital structures of pests like the peritophic matrix and cuticle in insects, cell wall in fungal phytopathogens and eggshells in nematodes, they are non detrimental for plants and animals which lack chitin. Therefore, chitinases hold great potential for various IPM programs than any other hydrolytic enzymes such as proteases, glucanases or lipases (Neeraja et al. 2010). Potential use of these hydrolytic enzymes, their virulence and mechanism of action could be valuable for producing more potent and safer insecticides. Some of the reported chitinases from bacteria, fungi and viruses with target pests are shown in Table 3.1.

This chapter, therefore, provides an insight of hydrolytic enzymes, their role in IPM and futuristic considerations. Current strategies in utilization of hydrolytic enzymes dependent on pest management studies with either direct application of organism as bioagents, or associated genes encoded in transgenic development, either separately or in combination with other genes.

Table 3.1 Fungal, bacterial and viral chitinases with insecticidal activity and target pests

Source	Name	Target insect/pest	Reference
Fungal chitinases			
Beauveria bassiana	BbChit1; chimeric BbChit1	*Galleria mellonella*, *Myzus persicae*	Fang et al. (2005, 2009)
Isaria fumosorosea	IfChit1	*Plutella xylostella*	Huang et al. (2016)
Metarhizium anisopliae	Chi2	*Dysdercus peruvianus*	Boldo et al. (2009)
Trichoderma harzianum	N/A[a]	*Helicoverpa armigera*	Binod et al. (2007)
Trichoderma viride	Mixture of at least four chitinases	*Bombyx mori*	Berini et al. (2016)
Bacterial chitinases			
Bacillus circulans	N/A	*Lymantria dispar*	Lertcanawanichakul et al. (2004)
Bacillus licheniformis	ChiBIA	*Spodoptera exigua*	Thamthiankul et al. (2004)
Bacillus subtilis	N/A	*Spodoptera litura*	Chandrasekar an et al. (2012)
Bacillus thuringiensis subsp. *colmeri*; *Bacillus thuringiensis* subsp. *tenebrionis* YBT 9602	ChiA, Chi96023 5–459 (truncated and mutageni zed form)	*Helicoverpa armigera*; *Plutella xylostella*	Ni et al. (2015) and Liu et al. (2010)
Pseudomonas fluorescens MP-13	N/A	*Helopeltis theivora*	Suganthi et al. (2017)
Serratia marcescens; S. marcescens SEN; *S. marcescens* Xd1; *S. marcescens* WW4	ChiAII; Mixture of two chitinases; ChiB and ChiC; ChiA, ChiB, and ChiC	*Spodoptera littoralis*; *Spodoptera litura*, *Galleria mellonella*, *Malacosoma neustria*, *Helicopverma armigera*	Regev et al. (1996), Aggarwal et al. (2015), Ozgen et al. (2013) and Danismazoglu et al. (2015)
Viral chitinases			
Autographa californica Multicapsid nucleopolyhedrovirus	ChiA	*Bombyx mori* and *Heliothis virescens*	Corrado et al. (2008), Di Maro et al. (2010), Fiandra et al. (2010) and Rao et al. (2004)
Dendrolimus kikuchii Matsumara nucleopolyhedrovirus	DkChi	*Spodoptera exigua*; *Hyphantria cunea*; *Helicoverpa armigera*; *Lymantria dispar*	Wang et al. (2013)

[a]*N/A* not available

3.2 Fungal Hydrolytic Enzymes

3.2.1 Role of Chitinases

Production of cuticle degrading enzymes by entomopathogens has been proposed as an important attribute towards their hosts. Pathogenesis during insect infection by fungi represents a complex of physiological, biochemical and genetic processes. Insect cuticle is highly heterogenous varying greatly in composition. It is composed of wax, protein, lipids and phenolic compounds, representing a tough barrier to the entomopathogen infection (Ortiz-Urquiza and Keyhani 2013). In response to these barriers, entomopathogens secrete diverse enzymes as virulence factors. With their unique mode of action, entomopathogens on contact with host cuticle, breach it with the help of enzymes and physical mechanisms (Charnley and Collins 2007; Mondal et al. 2016). Later on, a germ peg which is formed in entomopathogen infection, invades tissues producing more and more mycelia and spores, killing its host by starvation or production of toxins.

Chitinases are among diverse enzymes involved in chitin metabolism. Although a wide range of cuticle degrading enzymes are produced by entomopathogenic fungi, initial step of degradation is performed by proteases working synergistically with chitinases (St. Leger and Cooper 1986). Peanut rust, *Puccinia arachidis* produces an endo-chitinase inhibiting germination of uredospores of the rust fungus, *Fusarium chlamydosporum* (Mathivanan et al. 1998) while chitinolytic enzymes isolated from *Trichoderma harzianum* were found to be potent against a wide range of harmful fungi (Lorito et al. 1993). The high levels of extracellular enzymes like chitinase, proteinase and b-1,3-glucanase produced by *M. verrucaria* are effective against *Sclerotium rolfsii*, a soil born fungal pathogen which causes disease on a wide variety of plants, including vegetables, fruits, and ornamental crops (Vyas and Deshpande 1989; Deshpande 1999).

For longhorn beetles and aphids, enzymatic treatment along with entomopathogenic fungus was successfully tried (Higuchi et al. 1998) while *M. anisopliae* and *B. bassiana* along with mycolytic and cuticle degrading enzymes were applied for control of pathogens and different pests in agriculture (Kulkarni et al. 2008; Nahar et al. 2008; Chavan 2009; Chavan and Deshpande 2013). Experimental studies of Chui-Chai et al. (2012) revealed higher hydrolytic enzyme activities and insecticidal efficacy of *Metarhizium* isolates in comparison to *Beauveria* isolates. Boldo et al. (2009) demonstrated that over expression of endochitinase Chi2 was marked with high virulence against cotton stainer bug, *Dysdercus peruvianus*. Similarily over expresssion of chitinase gene Bbchit1 enhanced efficacy of *B. bassiana* against the aphid, *Myzus persicae* (Fang et al. 2005). Huang et al. (2016) demonstrated the role of chitinase gene (Ifchit1) in *Isaria fumosorosea* in cuticle penetration. A split marker transformation system was used for knocking- out Ifchit1 gene. Bioassays using larvae of diamondback moth, *Plutella xylostella* showed that the tailored strain (ΔIfchit1) possessed a decreased infectivity i.e. increased LT_{50} and LC_{50} as compared to complemented and wild type strains.

Binod et al. (2007) stimulated chitinase production in *T. harzianum* grown in submerged fermentation by means of colloidal chitin as carbon source while cotton bollworm, *Helicoverpa armigera* larvae treated with colloidal chitin filtrate were grown on cotton leaf disks. The results showed a drastically lower growth rate than the controls. Topical application of the filtrate on larval thorax decreased the pupation percentage (from ca. 50% in control to 0% with highest chitinase concentration) and increased larval and pupal mortality in dose-dependent manner (from 10% in the control to 70% at the highest chitinase dosage). Likewise, Berini et al. (2016) investigated *in vitro* and *in vivo* effects of a chitinases derived from *T. viride*, on peritrophic matrix of the silkworm, *Bombyx mori*. Significant *in vitro* effects were seen on the structure and permeability of the peritrophic matrix, leading to adverse consequences in overall larval development.

Conidia of *M. anisopliae* and cuticle degrading enzymes of *M. verrucaria* were successfully used for the control of woolly aphid, *Ceratovacuna lanigera* on sugarcane (Chavan 2009) while mycolytic enzymes of *M. verrucaria* have also been reported to control root infecting fungal pathogen, *Sclerotium rolfsii* (Vidhate and Deshpande 2013). Similar studies on *M. verrucaria* by Vidhate et al. (2015) showed that its hydrolytic enzymes were effective against mealy bug, and retained activity (66–70%) even after 5 hrs exposure to chemicals. Sequential application of hydrolytic enzymes followed by *M. anisopliae* conidia showed noteworthy control of mealy bugs.

Beauveria brongniartii and *M. anisopliae* mutants deficient in chitinase production were shown to have reduced virulence towards *Melolontha melolontha* and *Dysdercus peruvianus*, thus highlighting importance of chitinases (Boldo et al. 2009; Montesinos-Matías et al. 2011). Studies of Montesinos-Matías et al. (2011) also indicated higher virulence of the *B. bassiana* mutant was linked to increased production of chitinases compared to wild type. Greater virulence of *Noumuraea rileyi* strains on *Trichoplusia ni* larvae were reported to be related with high levels of exochitinase and total chitinase (El-Sayed et al. 1989). High levels of exochitinase in *B. bassiana* SFB-205 were associated with increased potency towards *Aphis gossypii* (Kim et al. 2010). *Beauveria bassiana* and *M. anisopliae* comprehensively produce N-acetyl-D-glucosaminidase, exochitinase and endochitinase, with increased virulence against various insect pests (Bidochka et al. 1993; Pinto et al. 1997). Over expression of a chitinase gene (Bbchit 1) was reported to enhance the virulence of *B. bassiana* towards the aphid *Myzus persicae* (Fang et al. 2005). In another study, Dhawan and Joshi (2017) reported isolates of *B. bassiana* with highest mean chitinase activity correlated with mortality rate towards third instar larva of *Pieris brassicae*. In addition, *B. bassiana* isolates with highest levels of chitinase activity were observed to be more virulent against *Tropida criscollaris* (Pelizza et al. 2012).

Two well know fungi, *Isaria farinose* and *I. fumosorosea* have successfully been used for biocontrol of white flies. They produce cuticle destroying enzymes like proteases (Pr1 and Pr2), chitosanases, chitinases and lipases while *I. fumosorosea* chitinase gene, (Ifchit1) encodes a chitinase which proved to be a most virulent factor to its host (Zhen et al. 2016).

The entomopathogen *Trichoderma* sp. secretes, while infecting the host, hydrolytic enzymes like chitinases, β-(1,3)-glucanases, proteases and lipase with a potential to act as biocontrol agent against insect pests and plant pathogenic fungi (Haran et al. 1995; Harman et al. 2004). There are four endochitinases and two β-(1,4)-N-acetylglucosaminidases present in *Trichoderma* and this number varies from species to species. In *T. harzianum* its chitinolytic system comprises of five to seven distinct enzymes which work with a complementary mode of action (Haran et al. 1995). In addition, two extracellular enzymes namely β-1,6-glucanase and hydrolases are also produced while growing on chitin. There are certain soluble sugars released by the enzymes which result in the formation of hydrolytic thalli on the host. On the whole, all cell degrading enzymes such as β-1,6-glucanases and chitinases dissolve fungal cell wall resulting in inhibition of the host growth (Kubicek et al. 2001).

3.2.2 Role of Proteases

Proteins constitute the majority of the insect's cuticle (roughly 70%). Proteases, including aminopeptidases, endopeptidases and carboxypeptidases, attack the cuticle before chitinases. On the other hand enzymes like chitinases, esterases, proteases, endopeptidases, carboxypeptidase A and chymoelastase serine protease act synergistically, breaching the cuticle to start the infection process (St. Leger and Cooper 1986, 1987). Studies by Bidochka and Khachatourians (1990) confirmed virulence of *B. bassiana* on migratory grasshopper, *Melanoplus sanguinipes* under controlled laboratory conditions. They reported extracellular protease as a virulence factor in pathogenesis. Early onset of mortality in larvae of greater wax moth, *Galleria mellonella*, was also reported with high levels of proteases produced by *B. bassiana* (Gupta et al. 1994). Considerable differences in proteolytic activities of *B. bassiana* isolates was correlated with their virulence activity (Zare et al. 2014). Higher proteolytic activity was in consonance with higher virulence. However, studies of Dias et al. (2008) suggest no relation between virulence and protease activity, that were expressed differently according to differences in cuticle.

A subtilisin-like serine protease (Pr1) produced by *M. anisopliae* has been shown to degrade insect cuticle proteins (St. Leger et al. 1987). Its role in host invasion has also been clearly demonstrated (St. Leger et al. 1988). *Lacanicillium* spp. also have been reported to have high potential against insect pests, while using both mechanical pressure and hydrolytic enzymes to pierce the integument for pathogenesis in aphids, thrips, mealy bugs, scale insects and white flies (Ekbom 1979; Kanagaratnam et al. 1982; Goettel et al. 2008). It also secretes a specific insecticidal toxin which is effective against sap sucking insects and noctuid pests (Ye et al. 1993; Anand et al. 2009). The studies carried by Donatti et al. (2008) on the production of subtilisin-like activity (Pr1) and trypsin-like activity (Pr2) proteases by *B. bassiana* in presence of *Rhammatocerus scistocercoides* cuticle, revealed that the highest levels of Pr1 and ZPr2 proteases were found in culture supernatants of grasshopper cuticle.

3.2.3 Role of Lipases

In addition to the proteins and chitin, insect tegument comprises of associated lipids and phenolic compounds serving as a barrier against invading pathogens (St. Leger 1991). Hydrolysis of ester bonds of fats, lipoproteins and waxes, found in the interior of the insect tegument, is acted upon by lipases (Ali et al. 2009; Mondal et al. 2016). Mutant of *M. anisopliae* with high amylase and lipase activity shows increased virulence against kissing bug, *Triatoma infestans* (Silva et al. 1989). The significance of lipases involved in the host infection process viz., integument penetration and breaking down, has already been demonstrated (Silva et al. 2010) while its production by microorganisms varies not only by lipid source but also with concentration.

Lipase production in *M. anisopliae*, induced by soybean oil, olive oil, sesame oil, rice oil and hydrogenated soybean fat, has been observed to potentially act against Asian blue tick, *Rhipicephalus* (*Boophilus*) *microplus* (Silva et al. 2005). Addition of fatty acids like myristic acid, stearic acid, linoleic acid, arachidic acid to *B. bassiana* culture inhibited both growth and lipase secretion, notwithstanding the addition of olive oil reverses this inhibition (Hegedus and Khachatourians 1988). According to Dhawan and Joshi (2017) MTCC 4495 strain of *B. bassiana* exhibits highest levels of lipolytic activity and virulence against third instar larvae of cabbage butterfly, *Pieris brassicae*. This lipase secreted by entomopathogens is drawn in early stages of the adhesion and penetration of insect hosts (Silva et al. 2010). Nevertheless, the activity of extracellular lipase has been reported to be higher than that of protease and chitinase. It is, therefore, concluded that lipase acts as an essential enzyme in the metabolic activities of *B. bassiana*.

3.3 Bacterial Hydrolytic Enzymes

A number of bacteria are reported to degrade native chitin of pest organisms thereby exerting pathogenicity. Owing to their efficacy and synergistic potential, chitinolytic bacteria are being formulated as biocontrol agents or used for development of transgenic plants (Subbanna et al. 2018). Accretion of chitinases in plants as a response against infestation by bacteria (Robert et al. 2002), viruses (Bol et al. 1990), fungi (Krishnaveni et al. 1999; Robert et al. 2002) or insects (Krishnaveni et al. 1999) is well documented. Moreover, huge diversity in molecular structure and catalytic mechanisms of various bacterial chitinases make them potential candidates in pest control programs (Frederiksen et al. 2013). Apart of degrading peritrophic memebranes, chitinases cause structural deformities in epithelial cells of midgut (Wiwat et al. 2000), suggesting their key role in IPM (Singh et al. 2016). A significant reduction has been reported in the larval development of *Trichoplusia ni* (Broadway et al. 1998), *Malacosoma neustria* (Danismazoglu et al. 2015),

Helicoverpa armigera (Chandrasekaran et al. 2012; Singh et al. 2016) and sap sucking pests like *Myzus persicae* (Rahbe and Febvay 1993; Broadway et al. 1998).

Some bacterial chitinase genes have been cloned in transgenic plants to confer resistance against pathogenic fungi. Transgenic tomato expressing a chitinase gene of *Streptomyces albidoflavus* showed enhanced resistance against cabbage looper (Gongora et al. 2001). Chitinases from bacteria have also been proposed as biopesticides against pests (Herrera-Estrella and Chet 1999; Bahar et al. 2012). Two endochitinases viz., Chi1 and Chi2, reported to be associated with toxin complex (Yen-Tc) of *Yersinia entomophaga* MH96 are responsible for the insecticidal activity on the larvae of *Adoryphorus couloni*, *Acrossidius tasmaniae*, *Costelytra zealandica* and *Plutella xylostella* (Landsberg et al. 2011; Busby et al. 2012). Even though direct effect on insect larvae was not notable (Hurst et al. 2011), degradation of the chitin component of peritrophic matrix was reported (Landsberg et al. 2011). Infact Yen-Tc is the only reported case of bacterial toxin complex including a chitinase activity (Berini et al. 2018).

The culture broth of *S. marcescens* strain SEN showed occurrence of two chitinases that caused high mortality rates in *Spodoptera litura* (Aggarwal et al. 2015). An insecticidal activity of three chitinases (ChiA, ChiB, and ChiC) from *S. marcescens* WW4 has been observed on the larvae of *Malacosoma neustria* and *Helicoverpa armigera* (Danismazoglu et al. 2015). Similarly, chitinases from *Brevibacillus laterosporus* Lak1210 also contribute to the management of diamond back moth, *P. xylostella* (Prasanna et al. 2013). Insecticidal activity of *B. thuringiensis* strains engineered with chiB and chiC genes of *S. marcescens* Xd1, tested on *Galleria mellonella* larvae, showed mortality rates higher by at least 50%, when compared to parental strains (Ozgen et al. 2013). Similarly, a chitinase gene from *B. circulans* 4.1 cloned into *B. thuringiensis* subsp. *Aizawai* caused a 27% higher insecticidal activity against gypsy moth, *Lymantria dispar* when compared to the parental type (Lertcanawanichakul et al. 2004). Finally, exposure of *S. exigua* to *B. thuringiensis* subsp. *aizawai* (comprising the chitinase gene chiBIA from *B. licheniformis* engineered with a sporulation-related gene from *B. thuringiensis* subsp. *israelensis*), caused perforations in the host peritrophic matrix (Thamthiankul et al. 2004).

3.4 Viral Hydrolytic Enzymes

. The hydrolytic enzymes secreted by viruses from the family Baculoviridae have been extensively studied in IPM. However, owing to prolonged killing and high cost of commercial production, viruses had a limited usage in IPM. Enzymes like AcMNPV ChiA, which induces a liquefaction of infected insects, showed both endo and exo-chitionolytic activities (Hawtin et al. 1997). It alters the structural organization of the peritrophic matrix and increases its permeability in a dose dependent manner (Rao et al. 2004). Therefore, ChiA could represent a potential resource in IPM strategies as a gut permeation enhancer to increase toxicity of bioinsecticides. Another viral chitinase with insecticidal activity, DkChi from the

nucleopolyhedrovirus *D. kikuchii* Matsumara has been reported to increase mortality in larvae of *H. armigera*, *L. dispar*, *S. exigua* and *Hyphantria cune* (Wang et al. 2013).

The juvenile hormone has an important role in insect development and reproductive function. It is primarily metabolized by hydrolytic enzymes viz. JH esterase and JH epoxide hydrolase. For effective and potent effect on insect pests, these two genes were inserted in the baculovirus genome via a recombinant technology (El-Sheikh and Mamatha 2012). It resulted in a long term stability and efficiency in pest control promoting the use of these hydrolytic enzymes as potent biopesticides (El-Sheikh and Mamatha 2012).

3.5 Future Prospects

Management of pests is the ultimate goal of IPM programs. Due to pesticide resistance, high cost, difficulty in keeping alive natural predators and precarious weather conditions, some pest populations showed a recurrent capability to destroy crops. Futuristic considerations for pest management should include more efficient and robust DNA based methods for surveying and identification of candidate agents, monitoring of biocontrol agents in field conditions and elucidation of the mode of action of various pest killing agents. Cloning of chitinolytic enzyme genes from various insects has opened the possibilities and new vistas for transgenic crop plants with improved resistance. Insect chitinase, which has a short half life in insects, might be augmented in transgenic plants by deleting regions in the chitinase gene that make it susceptible to proteolysis. Modifications, by site directed mutagenesis or deletions, or shifting the frame of specific functional domains may also help in increasing virulence, potency and stability of chitinases. To improve the expression levels of enzymes using chimeric gene combinations of chitinase and other insecticidal genes, additional studies need to be conducted to for the range of applications of transgenic plants and microbial biocontrol agents. Comprehensive understanding and knowledge on chemistry and regulation of chitin metabolism, on the susceptibility of insect pests to hydrolytic enzymes, their life cycle with thorough understanding of metabolic pathways could help to smoothen the progress towards novel methods and ways to control pests.

3.6 Conclusion

Insect pests are nuisance to crops and human beings. Their management is a prime requisite to feeding global human population. Although IPM programmes managed to keep a number of pests under control by means of predators, parasitoids, entomopathogens, bacteria and viruses, hydrolytic enzymes which usually comprise of chitinases, proteinase and lipase have become the latest arsenal in pest management

strategies. Since insect cuticle is made up of chitin, use of hydrolytic enzymes in perforating and degrading this outer covering have severe consequences on particular insect pests. Chitinases are the most common hydrolytic enzymes used against pests. Chitinases, proteases and lipases derived from entomopathogens, bacteria and viruses have been either used directly or manipulated by genetic engineering to become more virulent and potent against some pest species. Diverse extracellular enzymes are produced by pathogens in different hosts and habitats. Proper use of recent molecular techniques, identification of virulent genes, increased knowledge about transgenic plants and crops possessing insecticidal genes will help in achieving most IPM goals.

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Chapter 4
Microbial Metabolites as Pesticides

Surendra K. Dara

Abstract Beneficial microbes used for the control of crop pests have one or more modes of action against their target arthropods, nematodes, pathogens, or weeds. Metabolites, enzymes, volatile compounds, and other bioactive materials help them to antagonize and suppress target pests. The pesticidal activity of several microbial metabolites has been extensively studied, but only a few have commercial potential. While microbes themselves, rather than their metabolites, are primarily used for plant disease control, metabolites such as anisomycin, avermectins, bialophos, and spinosad are some examples of successful commercialization for insect, mite, nematode, and weed control. Biopesticides based on fermentation solids and solubles, without live microbes, are also available in the market. Pesticides based on *Burkholderia rinojensis* and *Chromobacterium subtsugae* for insect and mite control and *Myrothecium verrucaria* for nematode control are some of such examples that do not have viable microbes. Microbial biopesticides play a critical role in integrated pest management and maintaining crop productivity. This chapter focuses on microbial metabolites and metabolite-producing microbes that are commercialized and briefly discusses the potential of others.

Keywords Microbial metabolites · Pesticides · Microbial control

4.1 Introduction

Plants have a close association with microbes that are both beneficial and harmful. While several microbes cause plant diseases, those that have mutualistic or symbiotic relationships outnumber the disease-causing microbes and play a critical role in maintaining crop health (Kalita et al. 1996; Butt et al. 2001a; Sturz and Christie 2003; Ackert Jr 2006; Suman et al. 2016). Some are in the rhizosphere, some are on the phylloplane, and whether they are endophytes or ectophytes, all of them have coevolved and coexist with plants. Some of the metabolites that these

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microorganisms produce for their survival or defense can be explored and exploited for plant protection. Among these microflora, entomopathogens are the natural enemies of several arthropod pests and contribute to crop productivity and health through microbial control, which is an important part of integrated pest management (IPM) (Lacey 2016; Dara 2019). Similarly, bacteria, fungi, and yeasts have been used for controlling several plant pathogens and plant-parasitic nematodes (Thomashow and Weller 1996; Benbow and Sugar 1999; Butt et al. 2001b; Punja and Utkhede 2003; Dong and Zhang 2006). While naturally occurring and commercially formulated and released microbes cause infections in arthropods through various mechanisms or antagonize plant pathogens to suppress their pressure, metabolites derived from certain microbes cause mortality or suppression of pest (arthropod, nematode, plant pathogen, or weed) populations through toxicity in one or more pathways (Lamberth 2016; Subbanna et al. 2019). There is an extensive list of microbial metabolites and their potential in agriculture from earlier studies and review articles (Mishra et al. 1987; Chattopadhyay et al. 2004; Berdy 2005; Saxena 2013; Lamberth 2016), but only a few of these metabolites have been developed into pesticides. This chapter will provide a brief introduction of various microbial metabolites against different pests.

4.2 Metabolites for Arthropod Control

Avermectins produced by the bacterium *Streptomyces avermitilis* are an earlier example of successful isolation and commercialization of microbial metabolites for pest management (Campbell 2012). After avermectins were first derived in the mid-1970s, a commercial product was developed through bacterial fermentation by the early 1980s. Currently, abamectin (avermectin B1) is a popular active ingredient in several acaricide/insecticide/ nematicide formulations against a number of mite, insect, and plant-parasitic nematode species. Emamectin, derived from abamectin and formulated as emamectin benzoate, is also a popular active ingredient in multiple acaricide/insecticide/nematicide formulations. Several derivatives of avermectins showed improved pesticidal properties in studies (Pitterna et al. 2009), but the earlier active ingredients continue to have widespread use.

Spinosad, derived from *Saccharopolyspora spinosa*, is another example of a bacterial metabolite that has been developed into a pesticide and successfully used for insect control (Thompson et al. 1997; Sparks et al. 1999). After the isolation of the bacterium in the 1980s from a soil sample from an abandoned sugar mill rum still from the Virgin Islands, spinosad, a mixture of metabolites spinosyn A and spinosyn D, was extracted and studied in the following years (Mertz and Yao 1990; Kirst 2010). Spinosad is now available in different formulations for use in both organic and conventional agriculture. In 2007, a semi-synthetic derivative of spinosyn with improved efficacy, residual activity, and an expanded spectrum was released as spinetoram (Galm and Sparks 2016). Isolated from *Streptomyces hygroscopicus*

subsp. *aureolacrimosus*, milbemycin is an acaricidal and insecticidal compound (Mishima et al. 1983).

Martin et al. (2007) isolated a new bacterial species, *Chromobacterium subtsugae*, with insecticidal activity in 2000 and later discovered the insecticidal activity of its metabolites. A biopesticide was developed by extracting secondary metabolites from the fermentation of the *C. subtsugae* strain and is now used in many cropping systems for its insecticidal, repellent, and antifeedant activities (Koivunen et al. 2009). A new species of another bacterium, *Burkholderia rinojensis* was isolated from soil in Japan in 2008 with insecticidal and acaricidal properties (Cordova-Kreylos et al. 2013). While *Burkholderia cepacia* is a plant pathogen and other *Burkholderia* spp. are used for nitrogen fixation, biological control of plant pathogens, or bioremediation of soil or water, *B. rinojensis* possesses insecticidal and acaricidal characters. Cell-free extracts from the bacterial fermentation of *B. rinojensis* containing the toxic metabolites are now commercially available for insect and mite control.

Another bacterium, *Bacillus thuringiensis,* and its subspecies are active against several lepidopteran, coleopteran, and dipteran pests, and several commercial formulations have been effectively used for controlling agricultural pests and mosquitoes (Sanchis 2011; Lacey 2016). Several crystal proteins (known as *cry* toxins) produced by *B. thuriengiensis* are responsible for its insecticidal properties (Höfte and Whiteley 1989). Although the genes responsible for producing these toxins are inserted into cotton, corn, soybean, and others to develop genetically modified crops, the toxins are not commercially formulated (Huesing and English 2004; Sanchis 2011).

Insecticidal proteins with a high molecular weight that were active against four insect orders were isolated and characterized from *Photorhabdus luminescens*, a bacterium present in the intestinal tract of entomopathogenic nematodes (Bowen and Ensign 1998; Bowen et al. 1998). Unlike the toxins of *B. thuringiensis*, which are activated in the insect gut, the *P. luminescens* toxin complex acts in the hemocoel of the insect (Blackburn et al. 1998). Similarly, the toxin complex from another such enterobacterium, *Xenorhabdus nematophilus*, is also insecticidal and helps the entomopathogenic nematodes it is associated with (Sheets et al. 2011). However, no insecticides or transgenic crops were developed based on *P. luminescens* or *X. nematophilus* toxins (Chattopadhyay et al. 2004), and their potential is limited by the use of entomopathogenic nematodes.

Entomopathogenic fungi, *Beauveria* spp., *Hirsutella thompsonii*, *Cordyceps fumosorosea*, *Lecanicillium lecanii*, *Metarhizium* spp., and *Tolypocladium* spp., produce several toxic compounds such as beauvericin, destruxins, dipcolonic acid, efrapeptins, hirsutellins, and oosporein that are active against insects (Vey et al. 2001). However, they have not been explored as biopesticides. Pyripyropenes, produced by *Aspergillus fumigatus*, have been extracted and studied for their insecticidal activity, but have not been developed as biopesticides although pyripyropene A appeared to be a potent aphicide (Tomoda et al. 1994; Horikoshi et al. 2017).

4.3 Metabolites and Microbes for Disease Control

Unlike the infections caused by entomopathogens for arthropod control, the primary mode of action of beneficial microbes for plant disease control is through the antibiotic or antimycotic (antifungal) compounds they produce. This section covers some of the microbes that are currently available for commercial use and their bioactive compounds.

4.3.1 Bacteria

Bacillus spp. produce antifungal lipopeptide compounds such as surfactins, iturins, and fengycins, as well as antibiotics, antifungal volatiles, and other bioactive materials which make them good biocontrol agents for plant diseases (El-hamshary and Khattab 2008; Ongena and Jacques 2008; Elkahoui et al. 2014; Pretorius et al. 2015; González-Jaramillo et al. 2017). In addition to having an antagonistic effect against bacteria, fungi, and oomycetes, *Bacillus* spp. also activate plant immune responses (Ongena and Jacques 2008). The antagonistic effect of *Bacillus* lipopeptides has been reported against *Rhizoctonia solani* (Asaka and Shoda 1996) and *Pythium aphanidermatum* in tomato (Leclère et al. 2005); *Podosphaera fusca* in melons (Romero et al. 2007); *Botrytis cinerea* in apple (Toure et al. 2004); and *B. cinerea* (Pretorius et al. 2015), *R. solani*, and *Sclerotinia sclerotiorum in vitro* (Elkahoui et al. 2014). Phenaminomethylacetic acid produced by a strain of *Bacillus methylotrophicus* antagonized *Magnaporthe oryzae*, the causal agent of rice blast (Shan et al. 2013). Yu et al. (2011) found out that a Chinese isolate of *B. subtilis* not only antagonized *Fusarium oxysporum* Schl. f. sp. *capsici*, but also produced an iron-chelating siderophore and promoted the growth of pepper plants. Similarly, *Bacillus mojavensis* antagonizes *Fusarium verticillioides* and promotes plant growth (Rath et al. 2018). *Bacillus amyloliquefaciens* also stimulates plant growth due to the extracellular phytase activity (Idriss et al. 2002). Such plant-growth-promoting properties further help these microbes' role in disease management. Some strategies, such as genetic modification (Leclère et al. 2005) or manipulation of the production process (Pretorius et al. 2015; Rath et al. 2018), can increase the overproduction of certain lipopeptides in *Bacillus* spp. Commercial formulations of these microbes with improved metabolite activity can be effective non-chemical alternatives for disease management.

A review by Dowling and O'Gara (1994) described several metabolites of *Pseudomonas* spp. that suppress *Agrobacterium tumefaciens*, *Fusarium oxysporum*, *Gaeumannomyces graminis* var. *tritici*, *Pyrenophora tritici-repentis*, and *Pythium* spp. in grains, fruits, vegetables, and other crops. Iron-chelating siderophores such as salicylic acid, pyochelin, and fluorescent pseudobactins produced by pseudomonads also promote plant growth. Another recent review by Anderson and Kim (2018) discussed various isolates of *Pseudomonas chlororaphis* with activity against

insects, nematodes, and bacterial, fungal, and viral diseases. Insecticidal proteins, iron-chelating fluorescent pyoverdine-like siderophore, hydrogen cyanide, phenazines, pyrrolnitrin, and dialkylresorcinols impart these properties.

4.3.2 Fungi

Species of *Clonostachys*, *Gliocladium*, *Glomus*, and *Trichoderma* are effectively used for controlling plant diseases in multiple cropping systems. These fungi are present in soil or have a mycorrhizal relationship with plants. They suppress pathogens through antibiotics, competition for nutrients, mycoparasitism, enhancing plant immune response, and promoting nutrient uptake and plant growth (Shoresh et al. 2010; Naher et al. 2014; Zhai et al. 2016). Toxins or secondary metabolites, such as gliotoxin and bisorbicillinoids, in some of these fungi (Johnson et al. 1943; Zhai et al. 2016), or lytic enzymes in others (Braun et al. 2018), help them suppress pathogens. An extracellular chitinase from *Myrothecium verrucaria* was antagonistic to the peanut rust fungus *Puccinia arachidis* (Govindsamy et al. 1998). Chitinase production and activity can be increased by adding urea or oxgall to the culture medium (Vyas and Deshpande 1989). *Muscodor albus* is an endophyte that produces a mixture of volatile compounds (acids, alcohols, esters, ketones, and lipids) with antimicrobial activity against *Aspergillus fumigatus*, *Pythium ultimum*, *Phytophthora cinnamomi*, *Rhizoctonia solani*, *Verticillium dahlia* and others (Strobel et al. 2001). Several endophytic fungi also produce a variety of antimicrobials including alkaloids, flavonoids, phenols, quinones, steroids, and terpenoids that antagonize bacteria, fungi, and yeasts (Pavithra et al. 2020).

Aureobasidium pullulans, a yeast-like fungus that is found in a variety of environments and has a close association with plants, produces several bioactive compounds including an iron-chelating siderophore, extracellular enzymes, and pullulan (a polysaccharide) and has a biocontrol potential (Schena et al. 2003; Chi et al. 2009). Lytic enzymes produced by a strain of *A. pullulans* inhibited post harvest pathogens – *Botrytis cinerea*, *Monilinia laxa*, and *Penicillium expansum* – on apple, peach, and plum (Zhang et al. 2010). Other fungi inhibited by *A. pullulans* strains or their derivatives include *Alternaria alternaria*, *Aspergillus niger*, *Monilinia fructicola*, *Phytophthora infestans*, *Rhizopus stolonifer*, on multiple hosts through antagonism by metabolites, enzymes, or by promoting plant immune responses (Ippolito et al. 2000; Castoria et al. 2001; Di Francesco et al. 2017).

4.3.3 Yeasts

Yeasts can also be used for controlling plant pathogens, especially those causing postharvest diseases, through multiple mechanisms including the production of antibiotics and enzymes, competition, or inducing host plant resistance (Benbow

and Sugar 1999; Urquhart and Punja 2002; Punja and Utkhede 2003; Zhang et al. 2018). Anthracnose caused by *Colletotrichum gloeosporioides* on papaya fruit was controlled by the yeast *Debaryomyces hansenii* through volatile organic compounds, hydrolytic enzymes, and competition for nutrients (Hernandez-Montiél et al. 2018). Nally et al. (2015) investigated the modes of action of *Saccharomyces* and non-*Saccharomyces* yeasts isolated from sour and grey rot in grapes and found out that metabolites, enzymes, antifungal volatiles, and siderophores were responsible for the inhibition of mycelial growth. Although the mechanism of action was not investigated, an isolate of the yeast *Metschnikowia andauensis* effectively reduced the postharvest pathogens *B. cinerea* and *P. expansum* on apples and pears, and *Penicillium digitatum* and *P. italicum* on oranges (Manso and Nunes 2011).

4.4 Metabolites for Nematode Control

Avermectins, isolated from *S. avermitilis* from a soil sample in Kitasato, Japan, were initially studied for their nematicidal activity against the gastrointestinal nematode *Nematospiroides dubius* but were found to have efficacy against a variety of nematode species (Burg et al. 1979). An earlier study by Mishra et al. (1987) evaluated metabolites from 942 bacterial and fungal isolates for their insecticidal (against *Aedes aegypti* larvae) and nematicidal activity (against *Panagrellus redivivus*). Among those, 12 *Streptomyces* isolates showed nematicidal activity. While milbemycin from *S. hygroscopicus* subsp. *aureolacrimosus* is insecticidal and acaricidal, its synthetic derivative, milbemycin oxime, is nematicidal and acaricidal and is used for preventing or treating parasitic worms (Tsukamoto et al. 1991; Grieve et al. 1991) and mites in dogs (Garfield and Reedy 1992). Although not directly from their metabolites, *B. cereus* and *Pseudomonas putida* promoted the growth of patchouli (*Pogostemon cablin*), an aromatic and medicinal herb, and imparted resistance against the root-knot nematode, *Meloidogyne incognita*, by boosting the plant immune system and stimulating the production of nematicidal flavonoids (Borah et al. 2018).

Nematicidal compounds of fungal origin include omphalotin A (from *Omphalotus olearicus*), caryospomycin (from *Caryospora callicarpa*), dicarboxylic acid (from a *Paecilomyces* sp.), and gliocladin C and 5-n-heneicosylresorcinol (from *Clonostachys rosea*), but none of them have been developed into commercial products (Mayer et al. 1999; Dong et al. 2007; Liu et al. 2009; Song et al. 2016). However, *M. verrucaria* isolated from the cysts of the soybean cyst nematode, *Heterodera glycines*, in the early 1980s, demonstrated nematicidal properties and was developed into a nematicide (without viable fungal cells) in the late 1990s and is still in use (Gintis et al. 1983; Warrior et al. 1999). Nguyen et al. (2018) reported that the nematicidal metabolites verrucarin A and roridin A from an isolate of *M. verrucaria* effectively controlled *M. incognita* and reduced gall formation in tomato and melon.

4.5 Metabolites for Weed Control

There are several phytotoxic compounds produced by bacteria and fungi (Duke and Lydon 1987; Tanaka and Ōmura 1993). Bialophos, hydantocidin, vulgamycin from *S. hygroscopicus*, herboxidiene from *Streptomyces chromofuscus*, and cornexistin from *Paecilomyces variotii* are among several herbicidal microbial metabolites. *Alternaria*, *Aspergillus*, *Fusarium*, and *Streptomyces* are important genera among many that produce numerous metabolites of herbicidal nature (Singh et al. 2003; Batish et al. 2006). Anisomycin from *Streptomyces toyocaensis* (Singh et al. 2003) and bialophos from *S. hygroscopicus* (Tanaka and Ōmura 1993) were among the first to be used as herbicides. In their 1993 annual review, Tanaka and Ōmura stated that most of the new insecticides and herbicides developed in the late 1980s to the early 1990s were of *Streptomyces* origin. Yamada et al. (1972) isolated the antibiotics – anisomycin and toyocamycin – from *S. toyocaensis* and tested for phytotoxicity against barnyard grass and crabgrass. A synthetic analog of anisomycin was later developed as a commercial herbicide (Yamada et al. 1974). The amino acid L-phosphinothricin, is the actual herbicidal molecule in bialophos, which was later commercialized as glufosinate (ammonium salt of DL-phosphinothricin) (Singh et al. 2003). Another microbial metabolite, rhizobitoxine, derived from *Rhizobium japonicum*, also has herbicidal properties. Owens (1973) showed that it was as phytotoxic as amitrole towards multiple plant species. Tentoxin is a cyclic tetrapeptide compound from *Alternaria alternata* causing chlorosis in several monocot and dicot species (Duke 1986). Duke and Dayan (2012) discussed the mode of action of several phytotoxins produced from pathogenic and non-pathogenic microbes. Among AAL-toxin, auscaulitoxin aglycone, hydantocidin, tabtoxin, tentoxin, and thaxtomin, some have novel modes of action while others have modes that are similar to commercial herbicides.

4.6 Strategies for Enhanced Efficacy of Beneficial Microbes and Microbial Metabolites

While many beneficial microbes and their metabolites can play a significant role in disease suppression, improving their efficacy through one or more strategies will promote their practical use. Abeysinghe (2009) showed that treating the seeds of eggplant and pepper with *B. subtilis* followed by soil application of *Trichoderma harzianum* significantly improved control efficacy against *R. solani*, compared to individual treatments. The combined application of *Pseudomonas nonteilii* and the mycorrhizal fungus *Glomus fasciculatum* worked synergistically in reducing *Ralstonia solanacearum* in *Plectranthus barbatus* (=*Coleus forskhohlii*), a medicinal herb (Singh et al. 2012).

Glomus mosseae improved the biomass of tomato and leek, and increased *Pseudomonas fluorescens* populations in the rhizospheres when both beneficial

microbes were applied together (Edwards et al. 1998). Improved nutrient absorption, plant growth, and tuber yields were also seen in addition to the disease suppression from the combined application of the bacterium and the fungus. Lower rates of avermectins and the entomopathogenic fungus *Metarhizium robertsii* had a synergistic effect on controlling the larvae of *Leptinotarsa decemlineata* (Tomilova et al. 2016). Avermectins suppressed insect immune responses and enhanced fungal infections. Zhang et al. (2018) discussed improving the efficacy of yeasts and other beneficial microbes with unconventional chemical compounds, including biological materials, against postharvest diseases. Wang et al. (2018) reported that β-glucan, a polysaccharide found in yeasts, fungi, and plants, enhanced the growth of the yeast *Cryptococcus podzolicus,* its efficacy against postharvest decay of apples by *P. expansum*, and the production of resistance-related enzymes in apple. In another study, Gramisci et al. (2018) found that calcium chloride and certain amino acid additives improved the growth and efficacy of the yeasts, *Vishniacozyma victoriae* and *Pichia membranifaciens* against *B. cinerea* and *P. expansum* on pears. While effective disease suppression was seen in tomato with the combined use of *T. harzianum* and *Glomus intraradices* against *Fusarium oxysporum* f.sp. *radices-lycopersici* (Datnoff et al. 1995), *T. harzianum* was negatively impacted by *G. intraradices* in root-free soil (Green et al. 1999). Similarly, *Clonostachys rosea* and *G. intraradices* demonstrated mutual inhibition but still promoted tomato plant growth in another study (Ravnskov et al. 2006). So, it is important to understand these interactions to ensure the proper use of one or more beneficial microbes and other materials for disease management. In a recent article, Dikbaş and Cinisli (2019) entertained the idea of using microbial metabolites (e.g., chitinase) with nanoparticles (e.g., zinc oxide) to improve the control efficacy of arthropods. As scientific research in sustainable crop protection advances, one or more of these tools can be put to practical use.

4.7 Conclusion

While several studies showed the pesticidal properties of microbial metabolites and discussed their potential for commercialization, only a few have been developed as commercial formulations for insect, disease, or weed control. Applictions may consider using the microbes that produce toxic metabolites and/or the metabolites extracted from them. Both approaches have their advantages and disadvantages in relation to scaling up to a commercial level, formulating them into a product, cost of production, storage and handling, and applying for the intended purpose. Although microbial metabolite-based pesticides are fewer in the market, there are numerous pesticides that include live organisms. Efficacy and cost-effectiveness of producing and using a product are always major factors in the commercial potential of microbial metabolites. There is already sufficient knowledge about their potential, and it is only a matter of commercial-scale production technology that can put them into practical use. In the meantime, the market for biopesticides,

biostimulants, and soil amendments based on beneficial microbes is continuously increasing. Microbial pesticides play an important role in IPM and contribute to increased pest control efficacy, alone or in combination with other control options. Alternating chemical pesticides with microbial pesticides is also critical to reduce the risk of resistance development. However, excessive use of certain microbial metabolite-based pesticides can also lead to pest resistance, so it is always ideal to use pesticides of any nature judiciously.

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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.

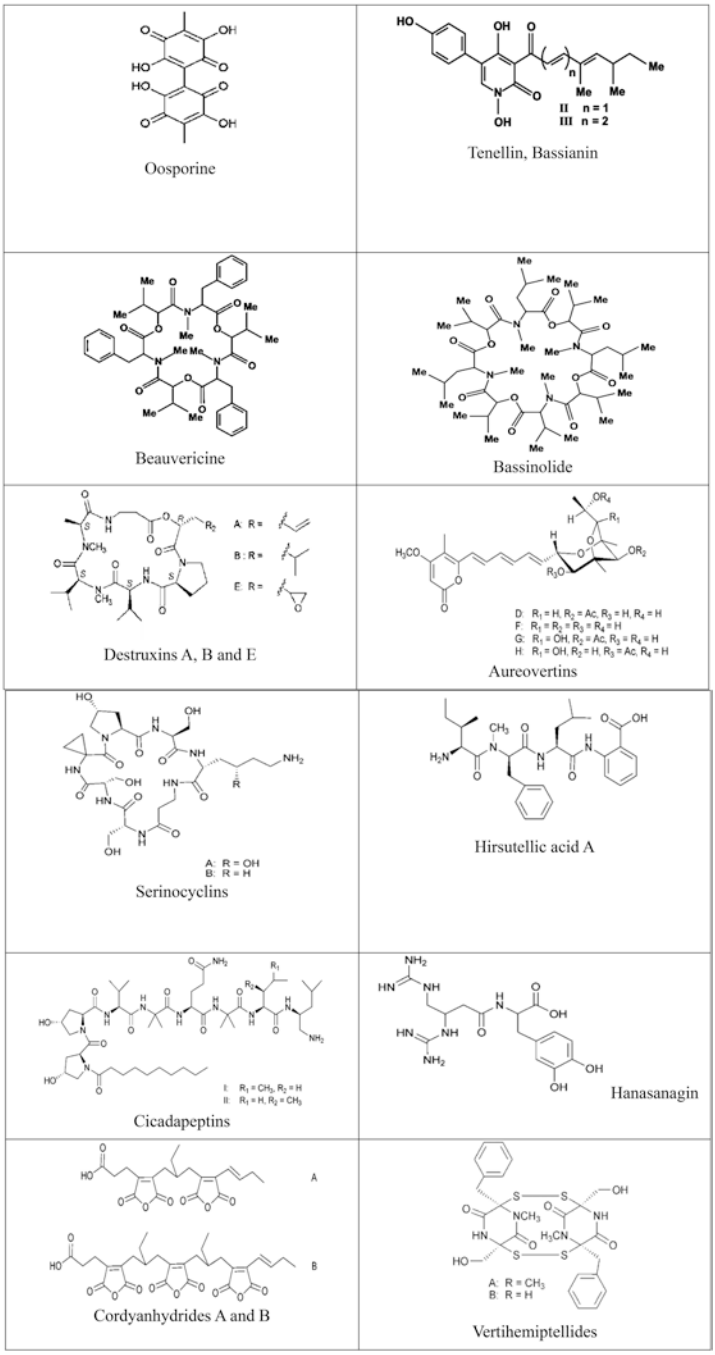

Fig. 5.1 Chemical structure of some important secondary metabolites (Molnar et al. 2010; Patocka 2016)

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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Chapter 6
Toxic Secondary Metabolites and Virulence Factors Expression by Entomopathogenic Fungi during Insect Infection and Potential Impact as a Tool for Pest Management

M. Constanza Mannino, Belén Davyt-Colo, and Nicolás Pedrini

Abstract Entomopathogenic fungi interact with their insect hosts by infecting and colonizing their bodies as part of their life cycle. After breaching the host cuticle, a variety of toxic secondary metabolites is secreted into the hemocoel facilitating a successful invasion and colonization. The production of fungal toxins, e.g. beauvericin and destruxin in some model fungi such as *Beauveria bassiana* and *Metarhizium anisopliae*, represents a powerful defense tool system for the fungal species but also an opportunity to exploit its efficacy against prejudicial insects. Most of these compounds, such as non-ribosomal peptides, alkaloids, terpenes, and polyketides, are referred to as virulence factors and their synthesis and secretion regulation is tightly controlled. In the last decade few informations were available on how these metabolites work when secreted, and how to harness their potential regarding biological control applications. In recent years, with the advent of next-generation sequencing techniques and the advances in genetic manipulation of fungal species, vast information became available on the genes involved in the interaction between host and entomopathogenic fungi, including those involved in the synthesis and regulation of toxic secondary metabolite production. The design and application of transgenic entomopathogens with enhanced virulence factors are currently being addressed as a more effective alternative in traditional biological control strategies. The ecological importance of fungal secondary metabolites and virulence factors, and their role in the effectiveness of different species relying on toxins production, are key to enhance control of detrimental insect population, in an environmentally friendly and sustainable manner.

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6.1 Introduction

Some insects represent a major problem for agriculture and food industry as well as for human and animal health, due to their role as pests of crops and stored products, and disease vectors, respectively. Around one third of the total food production in the world is annually lost due to insect pests, representing economic losses estimated in $100 million (Tobergte and Curtis 2013). Increasing productions and reduction of losses are needed to improve food accessibility and availability. These will become a necessity according to FAO estimated population growth rates (Tobergte and Curtis 2013). Insect vectors of diseases also represent a severe health risk. Vector-borne diseases such as malaria, dengue, trypanosomiasis, leishmaniasis and schistosomiasis account for more than 17% of all infectious diseases and cause more than 700,000 deaths, annually (WHO 2017).

Entomopathogenic fungi (EF) have developed a complex relationship with their arthropod hosts, through an evolutive arms race for survival still ongoing in these interacting systems. Through the infection and colonization processes, fungi display a series of resources to overcome the insect immune system. They will allow the pathogen to survive, depending on its capability to produce virulence factors in each step of the parasitic mycosis (Mannino et al. 2018; Pedrini 2018). A virulence factor is considered to be a trait contributing to fungal pathogenicity, that helps the pathogen to invade and circumvent the insect defenses, and to use its nutrients to germinate and proliferate into the host (Cross 2008). To understand what constitutes a fungal virulence factor, three considerations have to be taken into account: (1) the strategy employed in infecting hosts; (2) the existence of shared pathways with other functions, and (3) the existence of gene families with potential redundancy (Ortiz-Urquiza and Keyhani 2015). The development of insect mycosis suggests that the fungus overwhelms insect defenses using a battery of degradative enzymes and toxic molecules, with a rapid assimilation of nutrients, an inhibition of defense responses, or resistance against them (stress), and a rapid growth (Ortiz-Urquiza and Keyhani 2015; Pedrini 2018).

Among the plethora of virulence factors that fungi display, several degradative enzymes have been already characterized and even used as enhancers of virulence (Lovett and St. Leger 2018). Secondary metabolites, on the other hand, represent a different challenge since several gene clusters, which would be linked to fungal secondary metabolites, do not have an associated product and *vice versa* (Gibson et al. 2014). This group of molecules with chemically diverse origins have antimicrobial activities and functions, acting as immunosuppressants during fungal infection with a varying degree of virulence, although in many cases their role remains

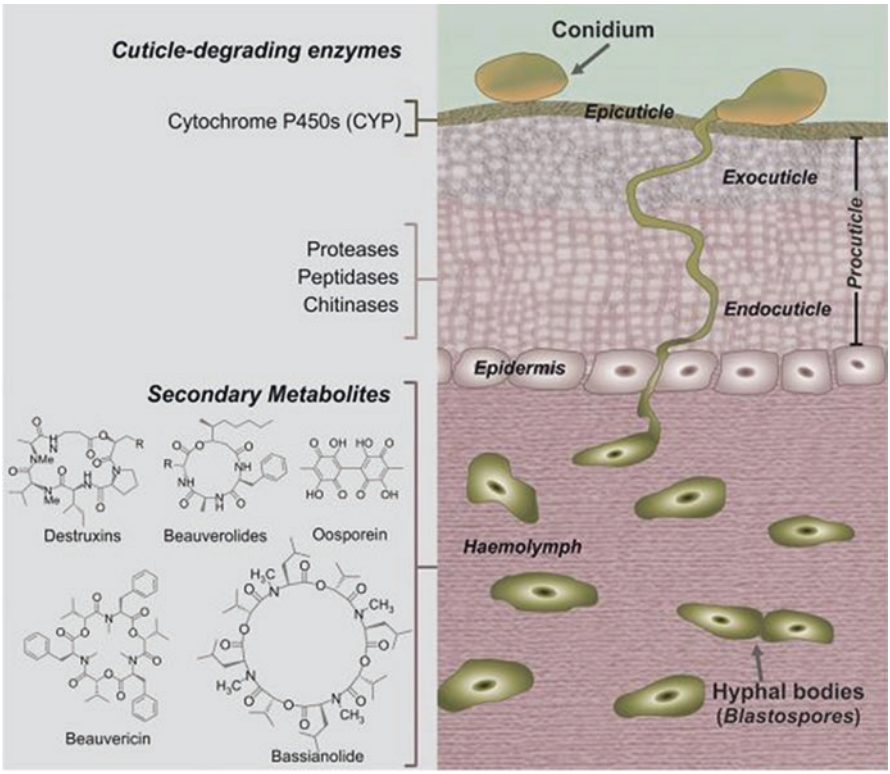

Fig. 6.1 Scheme of the infection process of an entomopathogenic fungus in an insect host. Some examples of fungal cuticle-degrading enzymes and fungal toxic secondary metabolites secreted in haemolymph are shown. (Modified from Pedrini 2018)

poorly understood or even unknown (Rohlfs and Churchill 2011; Trienens and Rohlfs 2012; de Bekker et al. 2013) (Fig. 6.1).

Commercially available mycoinsecticides have similar cost as chemical pesticides since they are successfully mass-produced, but they often have an inconsistent field performance and/or low virulence. For that reason, research plans have been conducted to enhance the production of one or more enzymes and/or toxins, in order to improve the fungal virulence (Lovett and St. Leger 2018), some of which will be addressed below.

6.2 Fungal Infection Processes

As a part of many fungal life cycles, host infection occurs mainly through cuticle contact , although fungi can also enter through the oral and/or other cavities. Infection of the insect host by the pathogen usually starts with the attachment of

conidia to the surface by nonspecific hydrophobic and electrostatic mechanisms. Furthermore, some fungal strains also secrete mucous substances that help conidia adsorption (Boucias and Pendland 1991). If the spores find an appropriate cuticular microclimate, they germinate. The host surface is determinant for these processes since it produces germination stimulators and inhibitors on its cuticle (Pedrini and Juárez 2008). Subsequently, a specialized structure named *appressorium* is formed by the EF to penetrate the cuticle. For breaching, these swollen cells form in turn a penetration hypha (or peg) that utilizes a combination of enzymatic and mechanical (pressing) mechanisms to pass through the different cuticular layers and reach the hemolymph (Ferron 1985).

EF have the ability to degrade the insect cuticle with a battery of hydroxylating enzymes that degrade and assimilate the hydrocarbon blend. These are the main compounds from the first cuticle layer (epicuticle) that the fungus finds (Pedrini et al. 2007). Hydrocarbon-grown fungi appeared more virulent than glucose-grown ones, increasing insect mortality or lowering the average lethal time, when tested against different insect hosts (Crespo et al. 2002; Pedrini et al. 2009). The procuticle is the next layer that the fungus must penetrate, composed mainly by proteins and chitin. Around 40 years ago a variety of hydrolytic enzymes implicated in procuticle degradation was identified, amongst which there were proteases, peptidases, and chitinases (Lovett and St. Leger 2017).

After the fungus reaches the insect hemolymph, its morphology switches to a different, yeast-like type cells, called hyphal bodies. These cells can invade the entire host by a tissue-specific sequential process and finally produce insect mummification. They can also secrete toxic compounds which are known as secondary metabolites (Boucias and Pendland 1998). These substances can either facilitate the fungal invasion (Ferron 1985) or act as immunosuppressive compounds conferring resistance against host defenses (Trienens and Rohlfs 2012). These secondary metabolites have different chemical natures such as non-ribosomal peptides, alkaloids, terpenes, and polyketides, and act as fungal virulence factors. Even though the precise role of secondary metabolites is poorly understood, they are usually linked to strains virulence levels (Pedrini 2018).

Another infection route that fungal pathogens might exploit is through the host oral cavity (Mannino et al. 2019). Some reports suggest that this is in fact used by *B. bassiana* which shares common virulence factors with entomopathogenic bacteria and may use them to infect, colonize, and kill their hosts (Xiao et al. 2012). Very little information is available regarding this route of infection for EF, but massive sequencing and the availability of several genomes of fungi evidenced the presence of the mentioned virulence factors. This raises the question if fungi are able to use them and therefore infect and colonize effectively through the digestive tube. This group of virulence factors will be further discussed in the following sections.

6.3 Virulence Factors and Secondary Metabolites: From the Insect Cuticle to the Hemolymph

EF find their hosts among the vast arthropod world, and in this wide and varied menu they come across susceptible and resistant hosts. It was found that out of the 30 insect orders, 20 are infected at least by one type of fungus (Araujo and Hughes 2016). Once contact and cuticle adhesion are achieved, fungal pathogens produce a series of enzymes that help degrade the cuticle layers. Among the hydroxylating enzymes that EF produce to penetrate the epicuticle, *B. bassiana* synthesizes a variety of cytochrome P450s (CYP), catalases, lipase/esterases, long chain alcohol and aldehyde dehydrogenases, which are likely implicated in cuticular lipid degradation by *B. bassiana* (Pedrini et al. 2010, 2013; Zhang et al. 2012; Huarte-Bonnet et al. 2018a; Keyhani 2018). A functional study of 8 P450 (CYP) genes (out of the 77 known), carried out through mutant strains experiments, showed that the battery of P450 variants of *B. bassiana* not only have overlapping substrates – the cuticular hydrocarbons – but also are involved in the production of secondary metabolites, once inside the insects cavity (Zhang et al. 2012; Ortiz-Urquiza and Keyhani 2013; Pedrini et al. 2010, 2013). Although lipases/esterases are recognized as a part of the enzyme repertoire that helps breaching the cuticle, functional characterization has not been reported to date due to difficulties in obtaining single mutant phenotypes (Gao et al. 2011; Xiao et al. 2012). These enzymes may not affect virulence directly but help to improve the nutrient uptake, leading to a better fungal fitness by the recognition of cuticular lipids. Their assimilation represents an important metabolic adaptation with activation of signaling cues that contribute to entomopathogenesis (Keyhani 2018). *Beauveria bassiana* cultivated in insect-like hydrocarbons showed increased catalase activity in the peroxisomes, where lipid degradation (β-oxidation) takes place, and exhibited oxidative stress and peroxisome proliferation (Pedrini et al. 2006; Huarte-Bonnet et al. 2015, 2018b).

The direct penetration of the proteinaceous cuticle (procuticle) is achieved using a series of proteases, peptidases and chitinases, already recognized as virulence factors (Ortiz-Urquiza and Keyhani 2013; Joop and Vilcinskas 2016). In some cases, insects can partially inactivate these proteases by synthesizing inhibitors. When they cannot be completely inactivated, they act as virulence factors (Vilcinskas and Götz 1999; Vilcinskas 2010). An example of these mechanisms is given by *M. anisopliae*, which produces, during the growth on the cuticle, at least three different types of proteases. These include the subtilisin-like serine protease Pr1, the trypsin-like serine protease Pr2 and a metalloprotease (Vilcinskas 2010). This group of proteases not only sustains the cuticle penetration process, but also allows the fungus to degrade and use hosts proteins as nutrients suppressing degrading hosts defenses (Vilcinskas 2010). In case of failure, *M. anisopliae* has also an extra enzyme, a thermolysin-like metalloprotease, which works as a back-up system (St. Leger et al. 1994). A peculiar case is the interaction of *B. bassiana* – *Tribolium castaneum*, where *B. bassiana* produces an enzyme, NAD(P)H:1,4-benzoquinone oxidoreductase (*BbbqrA*), which reduces a group of benzoquinones, produced by the insect as

defense pheromone, to harmless metabolites and partially allow the fungus to enter the insect body (Pedrini et al. 2015). *BbbqrA* is part of the fungal detoxification system more than a virulence factor *per se*, but it aids the pathogen to successfully enter the host. In this particular case, fungal infection is mostly avoided by the insect.

Secondary metabolites have a leading role when the EF breach the cuticle layers and gain access to the insect hemolymph. This group of compounds is crucial for EF survival and for their interaction with other organisms (Pichersky and Gang 2000). The link of secondary metabolites with virulence is already ascertained, but their roles during infection and genetic origin are not fully understood. Compounds that act as immunosuppressants and facilitate infection such as beauvericin, bassianolide, beauverolides, oosporein, bassiantin and tenellin are produced by *B. bassiana*, whereas *Metarhizium* species produce mainly destruxin (Pedrini 2018). Differences in secondary metabolites production on either live tissue, i.e. *B. bassiana* beauverolides, or dead tissues such as the *M. brunneun* destruxins, attribute different purposes to different compounds (de Bekker et al. 2013). While beauverolides may be used to help killing the host, *M. brunneun* destruxins could be mainly used for antimicrobial purposes (de Bekker et al. 2013).

It is known that secondary metabolites are synthesized from gene clusters, including non-ribosomal peptides synthetases (NRPS), polyketides synthetases (PKS), and hybrid NRPS-PKS genes (Süssmuth et al. 2011). Induction of these genes is achieved when EF are confronted with insects or insect tissues, but several of the known clustered genes have no secondary metabolite assigned and *vice versa* (Gibson et al. 2014). Whereas bassiacridin and beauverolides are known secondary metabolites with unknow origin, there are many characterized metabolites with known biosynthetic pathways, e.g., for destruxins, tenellin, beauvericin, and bassianolide. Functional studies targeting NRPS and PKS assigned a very important role in virulence against insect hosts to bassianolide and an important but dispensable role to beauvericin (Xu et al. 2008, 2009). While tenellin does not seem to contribute to *B. bassiana* virulence (Eley et al. 2007), oosperein directly evades insect immunity and facilitates fungal growth (Feng et al. 2015). Broad insecticidal effects are assigned to destruxins. However, it seems to be strongly related to the host that is being targeted (Pedras et al. 2002; Wang et al. 2012). Measurements of gene expression in biosynthetic pathways of secondary metabolites after insect invasion were developed by Lobo et al. (2015). Absolute quantification by qPCR showed that *B. bassiana* induces toxin genes during the first days of infection, likely used as virulence factors, and then in moribund insects and/or cadavers, to protect them from competitive microorganisms (Lobo et al. 2015).

The complex combination of enzymes and secondary metabolites described shows how (in most cases) EF are winning the arms race for survival in the host-pathogen relationship (Pedrini 2018). More importantly, this pool of resources can be, and in some cases already are, manipulated to favor infection, thus allowing their use as part of pest and vector management programs.

6.4 Genes and Transgenic Enhancement of Virulence Factors

Genetic engineering of fungal pathogens to improve their virulence has been a useful tool since many genomes and recombinant DNA technologies became available, facilitating the design of multiple fungal pathogens with enhanced virulence and stress resistance (Lovett and St. Leger 2018). Environmental influence has a major impact on fungi and makes it difficult to obtain consistent field results, representing a hurdle to their application as biological control agents (Lovett and St. Leger 2018). To date, many natural and synthetic genes have been inserted into EF genomes. The most important genes exploited to gain virulence are those encoding neurotoxic peptides, that manipulate host physiology, and proteases or chitinases, that degrade the insect cuticle (Lovett and St. Leger 2018). Different approaches can be taken when designing an enhanced biopesticide. They include the use of its own virulence factor as targets for a transformation, or the use of virulence factors that belong to other organisms that could help fungal pathogens to perform better. Synthetic genes can also be used for this purpose.

The variety of EF endogenous genes suitable for genetic engineering is enormous. The adhesins, species-specific toxin-encoding genes and the systems allowing evasion of the host immunity have evolved independently in many insect pathogens (Zhao et al. 2016). The first recombinant pathogen with enhanced virulence was a strain of *M. robertsii* which constitutively overexpressed the cuticle-degrading protease Pr1, that triggered a host protease. This in turn triggered a massive melanization in the body cavity and resulted in a reduction of the survival time. The melanized insects did not favor fungal growth and sporulation, reducing transmission of the recombinant fungus (St Leger et al. 1996). A similar experiment was conducted with chitinase CHIT1 in *B. bassiana*, with similar results (Fang et al. 2005). Another case of fungal virulence factor used to tackle resistance in insects is that of *B. bassiana* and *T. castaneum*. It was identified that the source of resistance was given by the volatile compounds secreted by the beetle as a first line of defense. This group of quinones inhibits fungal growth and avoids the adhesion to the insects cuticle (Pedrini et al. 2015). A transformant fungal strain was obtained, and it was proven that it displayed a higher virulence against *T. castaneum* adults, showing at least twice the cumulative mortality rates than those achieved with the wild type strains (Pedrini et al. 2015).

The use of pathogenicity-related genes from one EF can be used to improve virulence of other fungi, and combinations of cuticle-degrading enzymes have also been tested and found to be synergistic providing, in some cases, improved virulence (Lovett and St. Leger 2018). When fungal pathogens reach the insect's body cavity, the yeast-like cells use trehalose (the main carbohydrate present in the insect hemolymph) to proliferate. Specifically, *M. acridum* secretes an acid trehalase (ATM1) which has been overexpressed and shown to cause an eightfold increase in locusts mortality compared to the wild type strain (Zhao et al. 2016). Over 80% of the putative secondary metabolites-associated genes identified in *Metarhizium* spp. and *B. bassiana* have no identified specific products and their sequences are unique to

EF (Gibson et al. 2014). It is also important to note that upon direct injection into the heamolymph, individual secondary metabolites did not cause significant mortality or macroscopical alterations, with the exception of beauverolides and destruxins. However, this does not mean that they are not important as they may act concertedly and/or their exact roles are yet to be understood (Molnár et al. 2010).

The key to unlock secondary metabolite potential is directly related to the ability to understand and manipulate the complex regulatory networks controlling gene expression in fungi (Molnár et al. 2010). Even though some secondary metabolites biosynthetic pathways have been studied, most of the secondary metabolites gene clusters, their biological role(s), and the resulting compounds remain to be uncovered (Gibson et al. 2014). Biosynthesis of secondary metabolites is an energy-consuming process and it would be expected to occurr only under the right ecological conditions, e.g., when an insect immune system is attacking the pathogen (Rohlfs and Churchill 2011). Little is known about environmental or host signals responsible for induction of secondary metabolites synthesis genes during the host infection course. There is indeed still a lack of conclusive knowledge about the ecological function of fungal secondary metabolites. This, together with the lack of information about the biosynthetic pathways and products at the molecular level, might be the main reason why there is no extensive use of EF secondary metabolites as a tool for recombinant strains (Zhao et al. 2016).

A foreign protein approach was tested with the bacterial protein Vip3Aa1, originally found in *Bacillus thuringiensis*. It is secreted at the vegetative and stationary growth stages and shows insecticidal activities only in the insect intestine, with a broad host spectrum (Qin et al. 2010). This protein was transformed into *B. bassiana* and used to compare virulence against mosquitos. The assays showed that the larvae, in presence of the protein, were killed mainly by the Vip3Aa1 released from the ingested conidia, at least in the first 3 days (Qin et al. 2010). An additional possibility to enhance virulence is the use of insect proteins to genetically engineer EF (Zhao et al. 2016). An example could be a sterol transporter (Mr-NPC2a) acquired by the fungus from an insect by horizontal gene transfer and reproduced in a *B. bassiana* lacking the gene, showing an increased virulence (Zhao et al. 2016). Recently, spider-derived toxins with combined functions that voltage-gated sodium (Na_V), potassium (K_V), and calcium (Ca_V) channels were cloned into *M. anisopliae* under the Mcl1 promoter that targets expression to the hemocoel of infected insects. Each toxin improved the median lethal time (LT_{50}) compared to WT, representing a potential arsenal that could be rotated and/or used combined in a mosquito control program aimed at mitigating resistance (Bilgo et al. 2017).

The option of using synthetic multifunctional genes and protein engineering is also available to create a more virulent fungal strainwith cuticle as first target. Expression of the fusion protein CDEP1:Bbchit1, containing the Pr1A-like protease CDEP1 and the chitinase Bbchit1, accelerated cuticular penetration by *B. bassiana* when compared to the wild type strain or transformants overexpressing each gene alone (Fang et al. 2009).

As discussed, there are many proven and potential candidates to enhance virulence in EF strains. A series of experiments needs to be conducted in order to test

effectiveness and assess possible risks for a further application of the recombinants in the field.

6.5 Virulence Factors Applications: Biological Control Strategies and Possibilities

Biological control is a useful tool in integrated pest management (IPM) and vector control programs that helps circumvent the development of chemical insecticides resistance in many insects of economical and health importance. EF represent well-suited candidates for this task, because their life cycle and broad host spectrum allow them to infect and colonize a wide range of insects and arthropods. They represent an ecologically friendly alternative to chemical pesticides and there are no reports on the generation of insect populations resistant to EF. In many cases, these agents are also compatible with other control strategies, including traditional pesticides, BT transgenic plants, or other biological control agents such as predators and parasitoids. They thus offer an alternative and complementary tool for use in IPM programs (Gonzalez et al. 2016).

The enhancement of EF performance using recombinant DNA technology together with a better understanding of fungal pathogenesis and ecology, to aid insect colonization and population reduction, offer numerous opportunities to optimize cost-effective mycoinsecticides (Lovett and St. Leger 2018). Even though, as discussed in the previous section, many genes involved in different fungal infection steps were used to enhance virulence through transformation protocols, there are still many questions to be answered before a recombinant product for can be obtained field application . Nevertheless, there were a few experiences that reached the stage of field trial and even got US EPA approval. Next, some of these cases related to agricultural pests and vectors control will be discussed.

6.5.1 Agricultural Pests

Biological control in IPM programs have been implemented worldwide to control several crop pests. Although commercial products consisting in fungal formulations have been around for quite some time, the insertion of genetically modified EF products in the market will depend on many variables, ranging from safety of use to product cost-effectiveness. The first EF transgenic strain, approved by the US EPA for a field trial, was a Pr1A overexpressing strain of *M. anisopliae*, providing a precedent that paves the way for future trials (Zhao et al. 2016). This strain showed an increased virulence against *Manduca sexta*, reducing by 25% its survival time (Hu and St. Leger 2002). Few data are available on field trials including transgenic EF, as stability of constructions, transformants effect on native fungal populations

and impact of many environmental variables must be addressed before any further field experiments are carried out.

6.5.2 Vector Management

From malaria to Chagas disease or dengue fever, a wide spectrum of vector-borne diseases is transmitted through insects and arthropods. The majority of them are susceptible to EF attacks but better, faster, more efficient, and cost-effective agents are needed to compete with chemical pesticides. Trials have been carried out with recombinant EF in the fight against malaria vector. Recombinant strains of *M. pingshaense* expressing a toxin that targets Ca_V and K_{Ca} channels (Met-Hybrid), also known as Versitude™, were chosen for a study approved by the US EPA for use as a stand-alone insect control agent (Bilgo et al. 2017). These strains improved the median lethal time, compared to wild type. The insecticide resistance did not alter the susceptibility of the mosquito populations, and it was species-specific since it did not affect *Apis mellifera* (Bilgo et al. 2017). Additionally, this Met-Hybrid passed the metrics control threshold suggested by the World Health Organization for a successful vector control agent. Another US EPA approved toxin, AaIT, which targets Na_V channels, was cloned and combined with Hybrid in a single fungal strain (Met-Hybrid/AaIT). The combination displayed synergistic interactions making this strain kill faster than the single recombinants alone (Bilgo et al. 2017, 2018).

6.6 Conclusion

Pest control strategies that are environment friendly and safer than chemical pesticides are needed. This request arose as a deeper knowledge as been achieved on the impact that pesticides have on the ecosystem and because of resistant insect populations found around the world. EF have been used for years as part of many IPM and vector management programs. Many commercial products are on the market today, but they are yet considered to have poor efficacy compared to chemicals, mainly due to environmental abiotic stress factors. To overcome these obstacles, the development of transgenics displaying an increased virulence and tolerance to abiotic factors has been proposed as a suitable option. Their use together with chemical pesticides – even though resistance is an increasing phenomenon – is a path to be further explored in the future (St. Leger and Wang 2010).

The main issue with genetically modified organisms concerns the assessment of their real impact on native species such as pollinators and other beneficial insects, their ecological niche and the safety of their use. Even though some transgenic fungal strains have been developed and even had field trials, there is still a long way to go before their full understanding. Issues concern aspects such as the metabolic costs and the pathways involved in the overproduction of fungal and external

virulence factors . Furthermore, when it comes to the very promising group of virulence factors that secondary metabolites represent for this objective, there is a lot to be known. Data are needed on the active biosynthetic pathways, linking genes to products. We also need to know the concrete function and mode of action of this very diverse group of metabolites. Advancements have been achieved in the exploration and exploitation of the EF secondary metabolites potential. New approaches are necessary to open a wide range of possibilities. Dual RNA sequencing allows exploring the mechanisms involved during the infection process (Pedrini 2018), making it possible the search for candidate genes in host-pathogen interactions and even identify key virulence factors acting in the different infection stages.

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Chapter 7
Microbial-Based Nanoparticles as Potential Approach of Insect Pest Management

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Abstract Over the past decades, chemical pesticides have been successfully used to control insect pests. However, excessive use of insecticides has led to the development of pesticide resistance in the targeted insects, as well as caused several environmental and human health hazards. Nanotechnology has emerged as one of the highly attractive alternative approaches to chemical pesticides. Various chemical, physical and biological methods are used to generate a variety of organic and inorganic nanoparticles (NPs). However, NPs generated by non-biological methods are unstable, expensive and environmentally hazardous due to the use of toxic chemicals and energy expensive methods. In the recent years, microbial synthesis of NPs has become popular and microorganisms are considered as potential sources of bioactive NPs. Bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Pseudomonas aeruginosa*, *Serratia*, *Escerichia coli*, cyanobacteria such as *Plectonema boryanum,* actinobacteria such as *Thermomonospora*, *Actinobacter*, yeasts such as *Candida glabrata*, *Schizosaccharomyces pombe* and fungi (*Verticillium, Fusarium*) are widely used for the synthesis of nanomaterials. Toxic effects of metal NPs such as Ag, Au, Al, Si, Zn, and ZnO have been proven successfully against a wide range of insects. NPs have significant impact on the insect's antioxidant and detoxifying enzymes, protein synthesis, gene regulation thus leading to oxidative stress, disrupting development and reproduction, enzymes

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denaturation and cell death. NPs have been mainly tested against a wide number of arthropod pests and vectors and their usage in crop pest management is under progress. Currently, studies are being carried out to improve the quality and synthesis efficiency of microbial-based NPs and nanopesticides.

Keywords Nanotechnology · Biosynthesis · Microorganisms · Nanopesticides · Nanoencapsulations · Nanocarriers

7.1 Introduction

Nanotechnology is a novel scientific approach that involves the use of materials and equipment capable of manipulating physical as well as chemical properties of a substance at nanometer levels. It deals with the physical, chemical and biological properties of matter considered at the nanoscale (1–100 nm) (Holdren 2011). Nanoparticle behaves differently than larger particle with same atoms composition because of differences in optical and electronic properties. Nanotechnology is an expanding field of science with applications in almost every sphere of human life including medicine, agriculture, plant protection, sanitation and environmental protection. Nanotechnology has the potential to revolutionize the agricultural and food industry with novel tools for the molecular management of diseases, rapid disease detection, enhancing the ability of plants to absorb nutrients, pathogens detection and protection of the environment (Welch and Graham 1999).

Crop and storage losses due to insect pest infestation are a major threat to agricultural production and food security. Insects have the potential to reduce crop production substantially. In the developing world, loss in crop yields caused by pests, crop diseases or post-harvest losses are estimated as nearly 40–50%. Dhaliwal et al. (2007) mentioned that food plants are damaged by more than 10,000 species of insects, 30,000 species of weeds, 100,000 species causing diseases and 1000 species of nematodes. Pest control in agriculture is largely based on the use of chemical pesticides which results in many environmental and health hazards. Worldwide, around 318.4 thousand metric tonnes of insecticides are used annually to control insects (Heisey and Norton 2007). It was estimated that as many as 25 million agricultural workers worldwide experience unintentional pesticide poisonings each year. Reports show that approx. 70% of the applied pesticides are not absorbed by plants and that a large portion seeps into soil and groundwater. As most of the registered pesticides are neurotoxic, their use has been related to mammalian toxicity, bioaccumulation and environmental contamination. Most of the currently used synthetic pesticides are made of molecules with a reduced water solubility, which are expensive, flammable and toxic. They also require high-energy inputs during their manufacturing. Overuse of pesticides also leads to the development of pesticide-resistant pathogens and pests. Therefore, developing alternative methods for pest

control became imperative. Recent studies focusing on the use of nanotechnology in insect control indicated a potential of nanopesticides as a possible alternative for chemical pesticides.

Various physical and chemical strategies are being explored for the production of NPs (Tarafdar and Raliya 2011). Nonetheless, NPs synthesized using these strategies are unstable, cost intensive and furthermore, include the utilization of toxic chemicals in their synthesis. Therefore, there was a serious need to develop reliable, economical, biocompatible and naturally safe processes of NPs synthesis. One conceivable way to fulfill this objective was the use of microorganisms to produce NPs. The biological synthesis of NPs by using microorganisms like bacteria, fungi, actinomycetes, yeasts and algae has been a topic of interest for the past decades. Biosynthesized nanonutrients gives three-fold increment in nutrient use efficiency, 80–100 times less requirement of chemical fertilizers, 10 times higher stress tolerance by plants, 30% more nutrient mobilization in the rhizosphere, 17–54% improvements in crop yields and in soil aggregation, moisture retention and carbon build up in soil. Data showed a high potential of biosynthesized nanoparticles in agricultural ecosystems (Tarafdar and Rathore 2016). Hence, in this chapter, we have discussed in detail about the role of several microorganisms in the synthesis of NPs and possible utilization of nanotechnology in insect pest control.

7.2 Applications of Nanotechnology in Agriculture

The application of nanotechnology in agriculture mainly aims towards minimizing nutrient losses in fertilizer application, increasing the yield through optimized nutrient management and reducing the application of plant protection products.

Nano-Fertilizers To enhance the nutrient use efficiency and overcome chronic problem such as eutrophication, nano-fertilizers might represent the best alternative way. Nano-fertilizers are synthesized in order to regulate the release of nutrients depending on the requirements of the crops, resulting in more efficient than conventional fertilizers (Dimkpa et al. 2012). They could be used to reduce nutrient losses, especially nitrogen due to leaching, emissions and long-term incorporation by soil. Slow and controlled release of nano-fertilizers may also improve soil health by decreasing the toxic effects associated with fertilizers over-application (Suman et al. 2010). Nano capsules and nano-particles as fertilizers are useful for the enhancement of nutrients absorption by the plants and the targeted delivery of nutrients to a specific site (Dimkpa et al. 2012).

Nano-Herbicides Improvements in the efficacy of herbicides through the use of nanotechnology could result in higher crop productivity, without causing any harmful effects to agricultural workers who are supposed to physically remove weeds if no application of herbicides is practiced. Properly functionalized nano-capsules

provide better penetration through the cuticle and allow slow and controlled release of active ingredients on reaching the target weed. The easiest way to eliminate weeds is to destroy their seed banks in the soil and prevent them from germinating, when weather and soil conditions become favourable for growth. Being very small, nano-herbicides will be able to blend with the soil, eradicate weeds in an eco-friendly way without leaving any toxic residues, and prevent the growth of weed species that have become resistant to conventional herbicides. Whether the nano application is due to a nano-sized active ingredient or the creation of a nano-sized formulation through the use of an adjuvant, the use of nano application is same. If the active ingredient is combined with a smart delivery system, the herbicide will be applied only when necessary, according to the conditions of the agricultural field. The soil with weed contamination has gradually shown lower yields in agricultural field. Nanosilver is the most studied and utilized nano particle for these bio-system.

Nanotechnology in Plant Disease Control NPs can be used as effective pesticides for controlling plant diseases. Reports showed that biosynthesized silver NPs act as strong fungicides against various phytopathogens and have successfully controlled the plant diseases they cause (Jaidev and Narasimha 2010; Mala et al. 2012; Gopinath and Velusamy 2013; Mishra and Singh 2015). NPs suppress the pathogenic organisms by affecting cell permeability, formation of free radicals, inactivation of vital enzymes, affecting DNA replication and by inhibiting signal transduction (Mishra and Singh 2015). Additionally, reports are also available on the applications of nanotechnology for the management of postharvest diseases, preservation techniques, nano-sensors for monitoring agroecosystems and improvement in agricultural machineries. The use of nano-biopesticide is more acceptable since they are safe for the plants and cause less environmental pollution in comparison to conventional chemical pesticides (Barik et al. 2008).

7.3 Synthesis of NPs

Nano particles are categorized as organic, such as carbon NPs, and inorganic, like metals (eg. gold and silver) and semi-conductor NPs (i.e. titanium oxide and zinc oxide). Two basic approaches are followed for nanoparticle synthesis. In the bottom-up approach, smaller components are arranged into more complex structures with their respective basic units, using chemical or physical forces. In the top-down approach, the NPs are synthesized by disintegrating larger molecules. To attain nano sized particles with the desired geometry, various chemical and physical methods are applied, including photolithography, electron, ion beam lithography, dip pen lithography, micro contact printing, electrochemical synthesis and nano imprint lithography. The geometries can be achieved by employing physical approaches. The chemical processes begin with decreasing the metal to atoms which is pursued by controlled amounts of atoms. Majority of the chemical and physical techniques employed for the synthesis of NPs are very costly and use harmful chemicals,

requiring the development of eco-friendly and economic technologies for nanoparticle synthesis (Moghaddam et al. 2010).

Biosynthesis of NPs is a relatively recent addition to the synthesis methods, which employs microbial cells or plant extracts for production. It is a cost effective, rapid, stable and green approach (Elemike et al. 2017). A vast array of biological resources available in nature, including plants and plant products, algae, fungi, yeast, bacteria and viruses, could all be employed for the synthesis of NPs.

Numerous microorganisms have the ability to synthesize NPs from heavy metals through a bioreduction process, which acts as a detoxification mechanism. The role of microorganisms in the remediation of toxic metals is well documented, but their use in the synthesis of NPs has also been defined. Several microorganisms are capable of producing nanomaterials naturally through biomineralization. This process exploits biomolecular templates that interact with the inorganic material at the nanoscale, resulting in an extremely efficient and highly controlled synthesis. Microorganisms are provided with the asset of natural occurrence of inorganic nanomaterials, either intra or extracellularly (Mann 1996).

Best known examples of microorganisms naturally producing NPs are magnetotactic bacteria which produce magnetite NPs (Lang et al. 2007; Faivre and Schuler 2008), S-layer bacteria which produce gypsum and calcium carbonate layers (Pum and Sleytr 1999; Sleytr et al. 1999), and diatoms which produce siliceous materials (Shankar et al. 2004). Apart from these, simpler organisms such as bacteria, yeast and fungi have also developed highly specialized strategies for biomineral synthesis over the course of their evolution. These features of microorganisms encourage their use in nanoparticle synthesis as a potential alternative.

7.4 Nanoparticle Synthesis by Microorganisms

The use of microorganisms in the synthesis of NPs is considered as a green and environment friendly approach, which interconnects nanotechnology and microbial biotechnology. Microorganisms are capable of synthesizing NPs both intra and extracellularly for their chemolithotrophic growth. They can use metal ions for specific functions, e.g. synthesis of magnetosomes or terminal electron acceptors and detoxification mechanisms (Krumov et al. 2007). Many microorganisms can survive in toxic environments and are capable of removing toxic metals through multiple mechanisms involving bioreduction, biosorption, bioaccumulation and efflux of metal ions. Thus, the metal ions undergo reduction and subsequent aggregation in NPs (Moghaddam 2010). Several microorganisms such as *Bacillus subtilis, Fusarium oxysporum, Shewanella algae,* magnetotactic bacteria*, Saccharomyces cerevisiae,* etc. have been explored for the synthesis of different types of metallic NPs *viz.* gold, silver, platinum, zirconium, palladium, iron, cadmium and metal oxides such as titanium oxide, zinc oxide, etc. (Salunke et al. 2016).

The biosynthesis of gold NPs was first reported by Beveridge and Murray (1980) using *B. subtilis.* Mukherjee et al. (2001) demonstrated that exposure of the fungus,

Verticillium sp. to aqueous $HAuCl_4$ solution led to the accumulation of gold NPs intracellularly. Singh et al. (2014) have reported the extracellular production of gold NPs by bacterium *Bacillus licheniformis.* Biosynthesis of silver NPs using microorganisms has also gained attention due to their inhibitory and bactericidal properties. The bacterium *Pseudomonas stutzeri* AG259 isolated from a silver mine has been known to produce silver NPs in the cell periplasm. Synthesis of silver nano particle using the fungus *Pleurotus ostreatus* was reported by Devika et al. (2012). The aqueous silver ions (Ag^+) were reduced to silver metal NPs (Ag *m*-NPs), when treated with the fungal supernatant for 72 hours. Extra and intracellular formation of silver NPs (AgNPs) by bacteria (*P. stulzeri*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Vibrio cholerae*, *Salmonella* sp. and *Staphylococcus aureus* has been investigated by Lengke et al. (2007).

Basavaraja et al. (2008) have demonstrated the formation of AgNPs when the culture filtrate of *Fusarium semitectum* was incubated with silver ions. *Neurospora crassa*, a non-pathogenic filamentous fungus was reported to produce AgNPs both intracellularly and extracellularly (Castro-Longoria et al. 2011). Studies have found that AgNPs were produced in the form of a film or in the solution or accumulated on the cell surface when fungi, *Verticillium* sp., *F. oxysporum* or *Aspergillus flavus*, were used (Senapati et al. 2004; Jain et al. 2011). *Rhodococcus* sp. and *Thermospora* sp. have been used to produce AuNPs. Shaligram et al. (2009) have used the fungus *Penicillium brevicompactum* WA 2315 to obtain silver NPs. In addition to Au and AgNPs, many workers have studied other metals and oxides for NPs formation by microorganisms. In this context, many magnetotactic bacteria (*Magnetospirillum magnetotacticum*, *Magnetobacterium bavaricum*, *Magnetospirillum gryphiswaldense*) are well known for the accumulation of magnetite (Fe_3O_4) or greigite (Fe_3S_4) (Lang et al. 2007). Moreover, other oxides including SiO_2, Sb_2O_3, TiO_2, $BaTiO_3$, and ZrO_2 NPs have also been synthesized using microorganisms (Bansal et al. 2005; Jha and Prasad 2009; Jha et al. 2009).

Metal sulphide NPs have also received considerable attention due to their optical, magnetic and electronic properties. Dameron et al. (1989) showed the intracellular production of CdS NPs (CdSNPs) using *Schizosaccharomyces pombe* and *Candida glabrata* when exposed to cadmium salt solution. Extracellular production of CdSNPs was observed when the cell free extracts of *F. oxysporium* were exposed to $CdSO_4$ solution (Ahmad et al. 2002). Apart from CdS NPs, ZnS and PbS NPs have also been shown to be produced by *Rhodobacter sphaeroides* (Bai et al. 2006; Bai and Zhang 2009). Khan and Ahmad (2013) have shown the production of cerium oxide NPs when the thermophillic fungus *Humicola* sp. was incubated in aqueous solution of cerium (III) nitrate hydrate.

Recently, virus mediated assembly of NPs has also been explored. In a study by Lee et al. (2002), viral assembly of ZnS NPs have been demonstrated using genetically engineered M13 bacteriophage. Besides the above mentioned examples, other NPs synthesized by microorganism are summarized in Table 7.1.

Table 7.1 Microorganisms in nanoparticle synthesis

Nanoparticles	Microorganisms	References
Au	*Bacillus subtilis*	Beveridge and Murray (1980)
	Rhodopseudomonas capsulata	He et al. (2007)
	Bacillus licheniformis	Singh et al. (2014)
	Verticillium sp.	Mukherjee et al. (2001)
	Trichothecium	Ahmad et al. (2005)
	Colletotrichum sp.	Shankar et al. (2003)
	Rhodococcus sp.	Ahmad et al. (2003a)
	Thermomonospora sp.	Ahmad et al. (2003b)
	Plectonema boryanum UTEX 485 (Cyanobacterium)	Lengke et al. (2006a, b)
Ag	*Pseudomonas stutzeri* AG259	Klaus et al. (1999)
	Salmonella typhimurium	Ghorbani (2013)
	Escherichia coli	Kushwaha et al. (2015)
	Morganella sp.	Parikh et al. (2008)
	Fusarium oxysporum	Duran et al. (2005)
	Aspergillus flavus NJP08	Jain et al. (2011)
	Neurospora crassa	Castro-Longoria et al. (2011)
	Fusarium semitectum	Basavaraja et al. (2008)
	Trichoderma asperellum	Mukherjee et al. (2008)
	Chlorella pyrenoidosa	Aziz et al. (2015)
CdS	*Rhodopseudomonas palustris*	Bai et al. (2009)
	Klebsiella aerogenes	Holmes et al. (1995)
	Fusarium oxysporum	Ahmad et al. (2002)
	Candida glabrata	Dameron (1989)
	Schizosaccaromyces pombe	Kowshik et al. (2002)
ZnS	*Rhodobacter sphaeroides*	Bai et al. (2006)
	M13 bacteriophage	Lee et al. (2002)
	M13 viral capsid	Mao et al. (2003)
CoPt	M13 bacteriophage	Mao et al. (2004)
PbS	*Rhodobacter sphaeroides*	Bai and Zhang (2009)
Sb_2S_3	*Serratia marcescens*	Bahrami et al. (2012)
Palladium	*Desulfovibrio desulfuricans*	Yong et al. (2002)
	Plectonema boryanum (Cyanobacterium)	Lengke et al. (2007)
CeO	*Humicola* sp.	Khan and Ahmad (2013)

(continued)

Table 7.1 (continued)

Nanoparticles	Microorganisms	References
Magnetic Fe_3O_4 and greigite (Fe_3S_4)	Magnetotactic bacteria	Roh et al. (2001)
Magnetite	*Fusarium oxysporum*	Bharde et al. (2006)
Iron sulfide (FeS) and siderite ($FeCO_3$)	*Geobacter* sp.	Kim et al. (2015)
Pt	*Shewanella algae*	Konishi et al. (2007)
	Fusarium oxysporum	Syed and Ahmad (2012)
Se	*Bacillus subtilis*	Wang et al. (2010)
Co	*Bacillus thurigiensis*	Marimuthu et al. (2013)
ZnO	*Aspergillus aeneus* NJP 12	Jain et al. (2013)
	Saccharomyces cerevisiae	Pavani et al. (2015)
Zr	*Fusarium oxysporum*	Bansal et al. (2004)
Pd	*Desulfovibrio desulfuricans*	Yong et al. (2002)
	Shewanella oneidensis	Windt et al. (2005)
	Aspergillus sp.	Pavani et al. (2012)
Barium titanate	*Fusarium oxysporum*	Bansal et al. (2006)
MnO_2	*Saccharomyces cerevisiae*	Salunke et al. (2015)
Copper	*Rhodotorula mucilaginosa*	Salvadori et al. (2014)
Silica and titanium Particles	*Fusarium oxysporum*	Bansal et al. (2005)
TiO_2	*Lactobacillus* sp.	Jha et al. (2009)

7.5 Mechanisms of NPs Synthesis by Microorganisms

The exact mechanisms for the biosynthesis of NPs is not fully understood yet, as the process involves different biomolecules like cell wall components, enzymes and proteins. Different biological agents have distinct mechanisms of producing NPs. The cell wall components and proteins of *Saccharomyces cerevisiae* play an important role in MnO_2 nanoparticle formation and stabilization (Salunke et al. 2015). In a study by Mourato et al. (2011), it was demonstrated that the cell wall and proteins released by the yeast cells played a significant role in the formation of gold and silver NPs, respectively. Different enzymes are also known to be involved in the biosynthesis of NPs. For example, NADH-dependent reductase enzyme is considered as key enzyme responsible for nanoparticle formation.

Biogenesis of NPs can be grouped into intra- and extracellular synthesis, according to the site of nanoparticle formation. The intracellular route of synthesis is essentially a bioaccumulation process, which involves the transport of metal ions into the microbial cells and subsequent reduction to the nanoparticle, by the action of intracellular enzymes. On the other hand, extracellular synthesis involves the reduction of metal ions present in the environment to their elemental form. The process is mediated by extracellular enzymes residing on the cell membrane or

present in the cell-free suspension. The mechanism of biosynthesis involves trapping of metal ions, bioreduction of the metal ions and stabilization of the NPs.

Trapping of Metal Ions In the process of nanoparticle formation, microorganisms grab metal ions from the environment essentially through electrostatic interactions and/or secretion of adhesive materials (Manti et al. 2008).

Bioreduction of Metal Ions to NPs The process of bioreduction of metal ions to the elemental form is catalyzed by enzymes generated by microorganisms. Different enzymes are found to be involved in the biosynthesis of different NPs. Some of the reported enzymes include NADH/NADPH-dependent reductase, nitrate reductase, sulfate and sulfite reductase, cysteine desulfhydrase, oxidoreductase, and hydrogenases (Duran et al. 2005; Bai et al. 2006, 2009; He et al. 2007; Jha and Prasad 2009; Riddin et al. 2009). Besides enzymes, other components, such as amino acids (Selvakannan et al. 2004) and lipids (Kumar et al. 2010), are also responsible for the bioreduction of Au and Ag ions.

Stabilization of NPs Several biomolecules such as polysaccharides, proteins and lipids can act as capping agents to enhance the stability of NPs. The Ag NPs produced by *Aspergillus niger* were stabilized with the use of proteins released by the fungus (Gade et al. 2008).

Synthesis of NPs using microorganisms has been emerging as an important research area in nanotechnology. Different biological entities are used in the production of NPs, forming a better alternative to the conventional methods. The mechanisms of synthesis of NPs by microorganisms are given in Fig. 7.1.

7.6 NPs as Novel Insecticides

Due to the high cost and risks posed by the synthetic pesticides, there has been an increasing demand for alternative pest control strategies and pesticides with more effective, non-persistent nature. Nanotechnology has been exploited to develop pesticides, and has a potential to revolutionize modern day pest control in agriculture (Harper 2010). Pest control using NPs synthesized by microorganisms has been emerging as a cost effective and eco-friendly approach. The use of NPs is gaining importance as they possess distinct physical, chemical and biological properties associated with their atomic strength. The key parameters which define many outstanding properties, relevant for their use in pesticide application and toxicity, are the distinct size, shape, surface to volume ratio, crystal phase and chemical composition. Different types of NPs, i.e. silver, aluminum oxide, zinc, titanium NPs etc., were experimented for control of insect pests (Routray et al. 2016). For more effective pest control, new substances are being formulated including smart pesticides and targeted pesticide delivery systems (Rai and Ingle 2012).

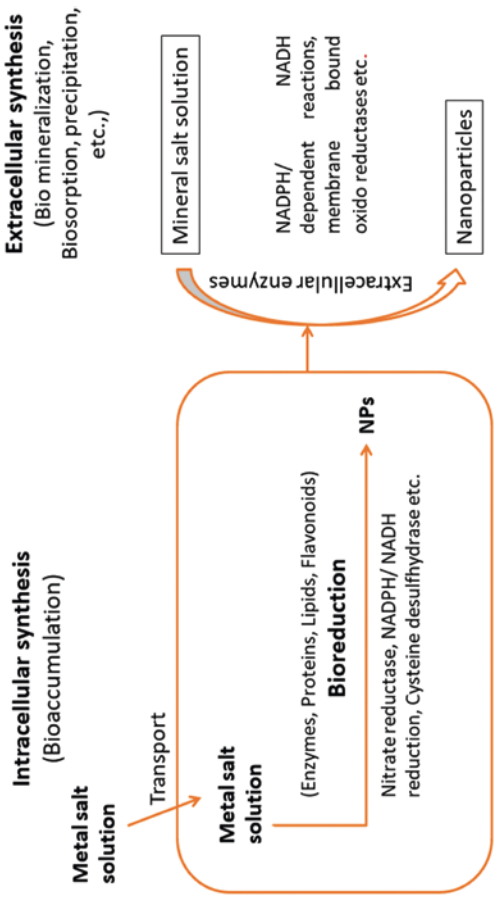

Fig. 7.1 Schematic representation of microbial synthesis of nanoparticles

Nanopesticides are developed based on the following two major concepts: improving already available pesticide formulations and usage as nanocarriers for effective delivery and increasing pesticide efficiency, and usage as active pesticide agents. Some of the common benefits of nanopesticides over conventional methods are: (i) increased solubility of the water-insoluble active ingredients and formulations, (ii) elimination of toxic organic solvents, (iii) slow release and improved stability of the active ingredients, for higher use efficiency, (iv) improved mobility (v) higher insecticidal activity due to smaller particle size and (vi) larger surface area, extending longevity and reactivity (Sasson et al. 2007; Athanassiou et al. 2018). Figure 7.2 describes the various uses of NPs in insect control.

7.6.1 Improving Pesticide Formulations and Usage as Carrier for Improved pesticide Delivery System

Traditional pesticide formulations available in the market have several disadvantages such as high organic solvent content, dust drift, poor dispersibility and low effectiveness. Most of the applied pesticide is lost to the environment and less than 1% remains on the target, which contributes to serious environmental pollution. Controlled pesticide delivery systems mainly focus on suitable active ingredients at specific concentrations and timely administration routes, to precisely regulate the target pest by maintaining full biological efficiency of the pesticides. It also aims at reducing waste, production costs and environmental pollution associated with the pesticide, while also extending the duration of the pesticide activity on crops. Properties such as high surface area, easy attachment to single and several different pesticide molecules, and reasonably fast mass transfer to the target make NPs effective carriers for delivery systems. NPs delay the degradation of pesticides, provide more controlled and gradual release over time, higher loading and simpler production, resulting in lower costs (Nuruzzaman et al. 2016).

Polymer NPs, synthetic silica, titania, alumina, Ag, Cu and natural minerals with nanoscale dimensions are mostly used as nanocarriers. Active pesticide molecules are loaded on NPs by absorption, covalent attachment mediated by different ligands, encapsulation and entrapment inside NPs (Athanassiou et al. 2018). Nanoencapsulation is a process through which chemicals like insecticides are slowly but efficiently released on a particular host plant for insect pest control. Nanoencapsulation with NPs in the form of pesticides allows for proper absorption of the chemicals into the plants (Scrinis and Lyons 2007). Release mechanisms of nanoencapsulation include diffusion, dissolution, biodegradation and osmotic pressure with a specific pH. Nanoencapsulation is the most promising technology for protection of host plants against insect pests. Most leading chemical companies focus on formulation of nanoscale pesticides for delivery into the target host tissue, through nanoencapsulation (Kumar and Yadav 2009).

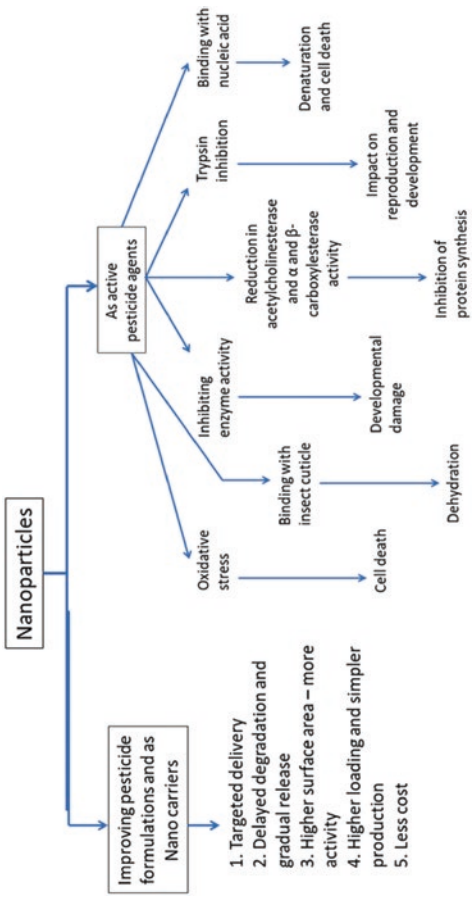

Fig. 7.2 Uses of nanoparticles in insect pest control

Hollow silica NPs were used as carriers for the controlled release and UV shielding of avermectin and validamycins (Li et al. 2006). Insecticidal activity of garlic essential oil against *Tribolium castaneum* was observed to increase with PEG-coated NPs (Yang et al. 2009). Bioavailability of plant protection molecules from PEG polymer nanoformulations such as imidacloprid (Adak et al. 2012), thiamethoxam (Sarkar et al. 2012), carbofuran (Pankaj et al. 2012) and β-cyfluthrin (Loha et al. 2011) was significantly increased by slower release as compared to commercial formulations. Some examples of NPs as carriers for improving pesticide efficiency are given in Table 7.2.

Nanoemulsions are formulations introduced to provide active ingredients as NPs ranging in sizes from 20 to 100 nm (Muller and Junghanns 2006). Elek et al. (2010) prepared NPs of navaluran, a water insoluble insecticide. Size of particles in the formulation was 200 ± 50 nm. It was tested against cotton leafworm, *Spodoptera litura*. Bioavailability of pesticide was increased and toxicity resembled that of conventional commercial formulation. Zhang et al. (2008) produced nanodispersion of triclosan, which showed greater activity than the organic/aqueous solutions, suggesting a potential to decrease application doses and lower rates of resistance development. Table 7.3 summarizes some examples of nanoformulations.

7.6.2 NPs as Active Pesticide Agents

NPs with insect toxicity can be used not only as nanocarriers, but also as an active pesticide agent itself (Barik et al. 2008; Elango et al. 2016). Insecticidal properties of various metal NPs like silica, aluminium oxide or nanostructured alumina, zinc or titanium oxide, silver and gold have been reported by many authors (Table 7.4).

Rouhani et al. (2013) tested the effects of silica and silver NPs on larval and adult stages of cowpea seed beetle, *Callosobruchus maculatus*. In the experiments, the LC_{50} value for SiO_2 and Ag NPs were calculated as 0.68 and 2.06 g kg^{-1} cowpea seeds on adults and 1.03 and 1.00 g kg^{-1} on larvae, respectively. Results showed that silica and silver NPs were highly effective on adults and larva with 100% and 83% mortality, respectively. Debnath et al. (2011) reported that silica NPs could cause

Table 7.2 Nanoparticles as carriers for improving pesticide efficiency

Nanoparticle	Pesticide	References
Meso porous silica	Imidacloprid	Popat et al. (2011)
Porous hollow silica	Validamycin	Liu et al. (2006)
Porous hollow silica	Avermectin	Liu et al. (2006)
PEG-coated	Garlic essential oil	Yang et al. (2009)
PEG-coated	Imidachloprid	Adak et al. (2012)
PEG-coated	Thiamethoxam	Sarkar et al. (2012)
PEG-coated	Carbofuran	Pankaj et al. (2012)
PEG-coated	β-Cyfluthrin	Loha et al. (2011)

Table 7.3 Nano formulations for effective insect control

Nanoformulation	Type of formulation	References
Bifenthrin	Insecticide, slow release	Liu et al. (2008)
Navaluran	Against *Spodoptera litura*	Elek et al. (2010)
Triclosan	Insecticide, slow release	Zhang et al. (2008)

Table 7.4 Nanoparticles as active pesticide agents

Nanoparticle	Insect	References
Silica	*Callosobruchus maculatus* (cowpea seed beetle)	Rouhani et al. (2013)
Silica	*Sitophilus oryzae* (rice weevil)	Debnath et al. (2011)
Silica	*Corcyra cephalonica* (stored grain pest)	Vani and Brindhaa (2013)
Cadmium	*Spodoptera litura*	Chakravarthy et al. (2012)
Nano-TiO_2	*Spodoptera litura*	Chakravarthy et al. (2012)
Nano-Ag	*Spodoptera litura*	Chakravarthy et al. (2012)
Amorphous lipophilic silica	*Tribolium castaneum*	Debnath et al. (2012)
Silica	*Sitophilus oryzae*	Zahir et al. (2012)
Nanoalumina	*Rhyzopertha dominica*	Stadler et al. (2010)
Nanoalumina	*Sitophilus oryzae*	Stadler et al. (2010)
Nano-silica	*Spodoptera litoralis*	El-bendary and El-Helaly (2013)
Silver	*Aphis nerii*	Rouhani et al. (2012)
Zinc	*Aphis nerii*	Rouhani et al. (2012)

100% mortality in adults of rice weevil, *Sitophilus oryzae*. Entomotoxic effects of silica NPs against the stored grain pest, *Corcyra cephalonica* has been reported by Vani and Brindhaa (2013). Silica NPs of size range from 70–80 nm were found to be highly effective against this insect, causing 100% mortality, indicating effectiveness for pests control.

Chakravarthy et al. (2012) studied the insecticidal potential of CdS, Nano-Ag and Nano-TiO_2 NPs against *S. litura*. Higher larval mortality of 93.79%, 73.79% and 56.89% were caused by CdS, Nano-TiO_2 and Nano-Ag, respectively, at 2400 ppm. The three NPs tested proved effective against *S. litura* larvae and hence could be selectively used for pest suppression. Debnath et al. (2012) reported the insecticidal efficacy of amorphous lipophilic silica on red flour beetle (*T. castaneum*), a stored grain insect pest. Zahir et al. (2012) assessed the pesticidal activity of silica NPs of 25–80 nm size against the adults of *Sitophilus oryzae* and reported 90% mortality at 168.28 mg kg^{-1} concentration.

Stadler et al. (2010) successfully used nanoalumina for controlling stored grain pests like *S. oryzae* and *Rhyzopertha dominica*. Significant mortality was observed after 3 days of continuous exposure to nanostructured alumina treated wheat. Nanosilica application could minimize *Spodoptera littoralis* infestation in tomato plants by affecting the feeding preference and reproductive potential of the pest

(El-bendary and El-Helaly 2013). Rouhani et al. (2012) reported that silver and Zn NPs could be used as a valuable tool in the pest management programs of *Aphis nerii*.

7.7 Mechanisms of Action of NPs Against Insects

The NPs pesticidal actions against various insects were reported by many authors (Rai et al. 2014; Athanassiou et al. 2018). NPs have a major impact on the induction of insects antioxidant and detoxifying enzymes. Nanosilver induced oxidative stress in the insects guts and cell death was observed in the Asian armyworm, *S. litura* and castor semilooper, *Achaea janata* (Yasur and Usha Rani 2015). SiO_2 and Al_2O_3 NPs kill the insects by binding with its cuticle. Stadler et al. (2010) reported the action of nanoalumina against *S. oryzae*. Nanostructured alumina binds to the beetle's cuticle due to triboelectric forces, sorbing its wax layer by surface area phenomena, resulting in the insect dehydration.

Polystyrene NPs inhibited the activity of the key drug metabolizing cytochrome P450 isoenzyme, thus leading to a developmental damage (Frohlich et al. 2010). Metal NPs can bind to sulfur in the proteins and phosphorous in the nucleic acids, leading to a decrease in the membrane permeability and disruption in proton motive force, with organelle and enzyme denaturations, followed by cell death (Jiang et al. 2015; Benelli 2016). NPs have also a major impact on protein synthesis, reducing acetylcholinesterases and α- and β- carboxylesterase activity (Fouad et al. 2018).

Nair and Choi (2011) observed the down regulation of the gene *CrL15,* involved in the regulation of ribosomal assembly and protein synthesis by Ag NPs. Upregulation of genes *CrGnRH1* and *CrBR2.2* was also observed, which indicated the activation of gonadotrophin-releasing, hormone-mediated signal transduction pathways, related to developmental damage and reproductive failure. Patil et al. (2016) reported trypsin inhibition in beetles and mealy bugs upon exposure to Au NPs, leading to a disruption in development and reproduction. Examples of bio-synthesized NPs in pest management are given in Table 7.5.

Table 7.5 Biosynthesized nanoparticles used in insect pest management

NPs	Microorganism used	Insect	References
AgNPs	*Cochliobolus lunatus*	*Aedes aegypti*, *Anopheles stephensi*	Salunkhe et al. (2011)
Ag and AuNPs	*Chrysosporium tropicum*	*Aedes aegypti*	Soni and Prakash (2012)
AgNPs	*Trichoderma harzianum rifai*	*A. aegypti*	Sundaravadivelan and Padmanabhan (2014)
AgNPs	*Bacillus thuringiensis kurstaki*	*Trichoplusia ni*, *Agrotis ipsilon*	Sayed et al. (2017)

7.8 Current Knowledge on Insect Control Using Biosynthesized NPs

Biosynthesized metal NPs have revealed interesting prospects in the management of insect pests. Salunkhe et al. (2011) used Ag NPs synthesized by the fungus *Cochliobolus lunatus* for control of *Aedes aegypti* and *Anopheles stephensi* larvae. The AgNPS synthesized by the filamentous fungus *C. lunatus* measured 3–21 nm in size and showed complete mortality among 2nd to 4th instar larvae, at 5 to 10 ppm concentrations after 24 hours of exposure. The potential larvicidal activity may be due to penetration of AgNPs through the larvae membranes.

Soni and Prakash (2012) studied the larvicidal activity of myco-synthesized gold and silver NPs against *Ae. aegypti*. *Chrysosporium tropicum* was used for the synthesis of gold and silver NPs of 2–15 and 20–50 nm sizes, which were highly toxic, causing 100% mortality, to the 2nd instar after 1 hour of exposure and to the 1st instars, after 24 hour of exposure, respectively. Ag NPs synthesized by the extracellular filtrate of the entomopathogenic fungus *Trichoderma harzianum rifai* resulted in 92, 96 and 100% mortality of 1st, 2nd and 3rd–4th instar larvae or pupae of *Ae. aegypti*, respectively, at 0.25% concentration after 24 hours of exposure (Sundaravadivelan and Padmanabhan 2014).

Sayed et al. (2017) synthesized biocompatible silver NPs using the entomopathogenic bacterium *Bacillus thuringiensis kurstaki* as a low-cost and eco-friendly production system. The insecticidal efficacy of bacterial synthesised AgNPs against larvae of the cabbage looper, *Trichoplusia ni* and the black cutworm, *Agrotis ipsilon* was tested. Results demonstrated that the treatments of either bacterial synthesised AgNP(s) made with *B. thuringiensis* supernatant or pellet were significantly more virulent toward larvae of *T. ni*.

The use of biosynthesized NPs in insect pest control is still in infancy, but these novel products are more effective at managing pests with lowered quantities of pesticides, and could contribute to enhancement of agricultural productivity involving integrated pest management. There is need for research on the utilization of biosynthesized NPs in pest management, implementation of these approaches on a large scale and their commercial application.

7.9 Biosafety

The nano-toxicity studies related to the potential of biosynthesized NPs in agriculture are still very limited. The toxicity test for NPs, parameters and methodology for monitoring and evaluation should be formulated based on the usage dose, crop and economic products. The potential toxicity and environmental implications of nanomaterials to aquatic organisms need to be evaluated. Risk assessment studies related to the toxicity of these compounds to plants and insects have to be strengthened.

7.10 Conclusions

Chemical pesticides used for pest control have an adverse impact on human health and environment. By increasing the efficiency and targeted release, and by reducing the application dose in the form of nanoemulsions, nanocarriers, nanoencapsulation and using NPs as active pesticide agents, nanotechnology opened a way towards a better alternative for chemical pesticides. Green synthesis of NPs using microorganisms is an environment friendly and cost effective approach which avoids the harmful effects of chemicals and physical methods of synthesis. Use of biosynthesized NPs in insect control is a promising and eco-friendly tool for agriculture, but more research is needed on large scale production, toxicity studies and commercialization, before it is adopted on a large scale by farmers.

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Chapter 8
Role of Recombinant DNA Technology to Improve the Efficacy of Microbial Insecticides

Ugur Azizoglu and Salih Karabörklü

Abstract Recombinant technology has great potential for use in the development of new microbial insecticides to control insect pests. Recombinant DNA technology is a biotechnological process that allows the manipulation of DNA to achieve practical benefits. In fact, recombinant DNA technology is synonymous with genetic modification (GM) because the genes of an organism are changed during the process and its DNA is recombined. Genetically engineered microbial insecticides have many advantages compared with natural microbial insecticides, such as their higher efficacy, lower insect resistance and lower spraying requirements. In addition, they can decrease the amount of chemicals used by farmers for pest control. In this chapter, we address some concepts that demonstrate the important roles of recombinant DNA technology in enhancing the efficacy of microbial insecticides.

Keywords Microbial biotechnology · Genetically engineered entomopathogenic agents · Insect pest management

8.1 Introduction

The use of chemical insecticides in the fight against insect pests was accelerated by the discovery of the Nobel Prize-winning compound DDT in the 1940s. However, due to the negative effects of chemicals such as DTT on the environment, human

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health and nontarget organisms, scientists have been forced to discover and implement more environment friendly techniques (Karabörklü et al. 2018).

Eco-friendly microbial insecticides are composed of microorganisms (viruses, bacteria, fungi, microsporidia and nematodes) or their toxin products (Azizoglu et al. 2012). In many cases, these toxins do not exert toxic effects on non-target organisms and can be used together with conventional pesticides. Microbial insecticides can be applied even during the harvest period, which is a great advantage over traditional insecticides. However, some disadvantages are their sensitivity to adverse weather conditions and UV light during application, the development of pest resistance, and the short-term insecticides persistence in the field. As a result, scientists have used recombinant DNA technology and biotechnology to overcome these disadvantages.

Recombinant DNA technology refers to molecular applications such as gene isolation, genetic manipulation, and gene cloning and expression (Mazin 1976). Advances in recombinant DNA technology have allowed the emergence of new strategies to manage insect pests. One of these new strategies is the use of genetically engineered microbial insecticides, which have many advantages over natural microbial insecticides, such as low spraying requirements, long-term persistence, lower insect resistance and higher efficacy (Castagnola and Jurat-Fuentes 2012; Karabörklü et al. 2018). In this chapter, we emphasize the role of recombinant DNA technology in improving microbial insecticides and the superior aspects of genetically engineered microbial insecticides over wild types.

8.2 A Brief History of Recombinant DNA Technology

Humans have altered the genetic structure of living organisms through the selective breeding of plants and animals, for many years. The deliberate modification of the genetic material by directly altering its nucleic acids is termed genetic engineering or gene manipulation. These modifications are achieved by various methods that are collectively described as recombinant DNA technology. The key breakthrough in recombinant DNA technology began with the discovery of restriction endonucleases in microorganisms by Werner Arber, Daniel Nathans and Hamilton O. Smith in the late 1960s. In 1969, Herbert Boyer showed that the restriction enzyme *Eco*RI, which was isolated from *Escherichia coli*, cut DNA between the G and A nucleotides in the sequence GAATTC (Hedgpeth et al. 1972).

After this discovery, rapid progress in recombinant DNA technology continued to be achieved. Some milestones of recombinant DNA technology include the discovery of reverse transcriptase from retroviruses (by H. Temin and D. Baltimore), the first recombinant DNA molecules (by D.Jackson, R.Symons and P. Berg), the development of a recombinant plasmid (a high-copy-number vector within a bacterial host, by S.N. Cohen and H. Boyer) and the detection of specific DNA fragments (isolation of a gene from a complex mixture of DNA, by E.M. Southern). Further achievements are: the DNA sequencing method (by F. Sanger, G. Brownlee and

B. Barrell); the first gene cloning (for human growth hormone) and the construction of recombinant DNA (to produce insulin, by J. Baxter); the production of recombinant human insulin protein in bacteria; the first genetically modified crop (an antibiotic-resistant tobacco plant); the first recombinant vaccine (Hepatitis B); the first field test of genetically engineered baculoviruses to kill cabbage caterpillars; the first mammalian clone, obtained via nuclear transplantation from a non-reproductive cell of an adult animal (Dolly).

8.3 Commonly Used Microbial Insecticides in Insect Pest Management

Commercial microbial insecticides are available for controlling insect pests in the agricultural and horticultural sectors. These insecticides include insect pathogens (entomopathogenic bacteria, particularly *Bacillus thuringiensis*, entomopathogenic fungi, baculoviruses and microsporidia) and entomopathogenic nematodes (Wakefield 2018). They can be formulated in the form of sprays, powders, liquid concentrates, wettable powders and granules. The unique characteristics of each product determine the most effective approaches for their use (Weinzierl et al. 1995).

Although approx.100 bacterial groups have been identified as exo- and endopathogens of arthropods, only a few are commercially used in insect pest control (Thacker 2002; Chattopadhyay et al. 2017). Currently, *Bacillus thuringiensis* (*Bt*), *Paenibacillus popilliae*, *Lysinibacillus sphaericus*, *Clostridium bifermentans*, *Pseudomonas alcaligenes*, *P. aureofaciens*, *Saccharopolyspora spinosa*, *Serratia entomophila* and *Streptomyces avermitilis* are among the commercially available bacteria (Chattopadhyay et al. 2017). Two bacterial insecticides, the spore-forming soil bacterium *Bt* and the non-spore-forming bacterium *S. entomophila,* have become popular biological control agents (Inglis and Lawrence 2001; O'Callaghan and Gerard 2005; Bizzarri et al. 2008; Porcar et al. 2008; Azizoglu et al. 2011, 2012, 2016, 2017; Chattopadhyay et al. 2017; Karabörklü et al. 2018; Wakefield 2018). *Bt* is a gram-positive soil bacterium of economic importance that exerts a toxic effect on many agricultural and forest pest larvae. It can be isolated from soil, rhizospheres, leaves, clean water, grain dust, insects, crustaceans, ringworms and insectivores (Hendriksen and Hansen 2002; Broderick et al. 2006; Bizzarri and Bishop 2008; Raymond et al. 2008, 2009, 2010; Broderick et al. 2009; Johnston and Crickmore 2009; van Frankenhuyzen et al. 2010; Azizoglu et al. 2011; Yilmaz et al. 2017). *Bt* controls insect pests by causing damage to the midgut of larvae or by causing septicemia during the larval periods, and these effects are exerted on species belonging to different insect orders (Raymond et al. 2010). Commercial formulations of parasporal crystal proteins (Cry toxin) formed by *Bt* during its sporulation have been used for biological control (Table 8.1) (Tamez-Guerra et al. 2004). Cry proteins have toxic effects on specific insect species belonging to the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera and Hemiptera (Karabörklü et al. 2018). These proteins have also showed toxic effects against other invertebrates such as nematodes and mites (Schnepf et al. 1998; van Frankenhuyzen 2009; Crickmore et al. 2016).

Table 8.1 Some commercially available entomopathogenic bacteria and their products[a]

Product name	Microorganism	Target pest insect order	Producer
Doom	*Bacillus popilliae*	Coleoptera	Fairfax Biological Laboratory
Japademic			Fairfax Biological Laboratory
VectoLex GC	*Lysinibacillus sphaericus*	Diptera	Valent BioSciences
Agree 50WP	*Bt* subsp. *aizawai*[b]	Lepidoptera	Certis USA
Agree® WG			Certis USA
Able® 50 WDG			Certis USA
Florbac			Valent BioSciences
Solbit			Green Biotech
Turex			Certis USA
Jackpot™ 50WP			Certis USA
XenTari			Valent BioSciences
Agrobac	*Bt* subsp. *kurstaki*	Lepidoptera	Tecomag SRL
Bactec BT16			Plato Industries
Bactec BT32			Plato Industries
Bactosid K			Sanex Inc.
Bactospeine			Koppert
Baturad			Cequisa Agro
Biobest-Bt			Biobest
BioBit			Valent BioSciences
Biolep			Biotech International Limited
Bio-Worm Killer			Green Light Co
BMP123			Becker Microbial
Bonide			Bonide products Inc
Condor			Certis USA
Cordalene			Agrichem
CoStar			Certis USA
Crymax			Certis USA
Delfin			Certis USA
Delfin WG			Certis USA
Deliver			Certis USA
Dipel			Valent BioSciences
Ecotec			Brandt Consolidated Inc
Foray			Valent Biosciences
Forwarbit			Forward International

(continued)

Table 8.1 (continued)

Product name	Microorganism	Target pest insect order	Producer
Guardjet			Mycogen/Kubota
Halt			Wockhardt Ltd
Insectobiol			Samabiol
Javelin WG			Certis USA
Lepinox			Ecogen
Lipel SP			Som Phytopharma
Maatch			Mycogen
MVP			Mycogen
MVP II			Dow Agro Sci.
Rapax			Ecogen /Intrachem
Ringer BT			Verdant Inc
Safer's BTK™			Woodstream Canada
Scutello			Biobest
VBT			Varsha Biosci. Techn.
Spicturin	*Bt* subsp. *galleriae*	Lepidoptera	ISCB
Acrobe	*Bt* subsp. *israelensis*	Diptera	American Cyanamide
Aquabac			Becker Microbial
Bacticide			Biotech Intl
Bactimos			Valent Biosciences
Bactis			Caffaro
Bioprotec			AEF Global Inc
BTI Granules			Clarke Mos. Cont.
Prehatch SG			Meridian
Skeetal			Abbott
Summit Bactimos			Summit Chemicals
Summit Mosquito Bits			Summit Chemicals
Tekar			Thermo Trilogy
Teknar			Valent Biosciences
VectoBac			Abbott
VectoMax FG			Valent Biosciences
VectoPrime™			Valent Biosciences
Vectocide			Sanex
Novodor®	*Bt* subsp. *tenebrionis*	Coleoptera	Certis USA
Trident ®			Valent BioSciences

(continued)

Table 8.1 (continued)

Product name	Microorganism	Target pest insect order	Producer
Conserve®	*Saccharopolyspora spinosa*	Lepidoptera	Dow AgroSciences
Delegate™ WG		Lepidoptera, Coleoptera, Hemiptera, Orthoptera	Dow AgroSciences
Elector PSP®		Diptera, Coleoptera	Dow AgroSciences
Entrust® SC		Lepidoptera, Coleoptera, Hemiptera, Orthoptera	Dow AgroSciences
Exalt™		Lepidoptera	Dow AgroSciences
GF-120®		Hemiptera	Dow AgroSciences
Laser®		Lepidoptera, Coleoptera, Hemiptera, Thysanoptera	Dow AgroSciences
Naturalyte®		Lepidoptera, Coleoptera, Hemiptera, Orthoptera	Dow AgroSciences
Radiant®SC		Lepidoptera, Coleoptera, Hemiptera, Orthoptera	Dow AgroSciences
Safer®		Fire ants	Dow AgroSciences
SpinTor®		Lepidoptera, Coleoptera, Hemiptera	Dow AgroSciences
Success®		Lepidoptera*Plutella xylostella*	Dow AgroSciences
Tracer®		Lepidoptera*Helicoverpa armigera*	Dow AgroSciences
Natular™		Diptera \mosquito larvae	Clarke Biotech
Agri-Mek	*Streptomyces avermitilis*	Lepidoptera\leaf miners	Syngenta
Avicta			Syngenta
Avid			Syngenta

[a]Data from: Flexner and Belnavis (2000), Chio (2011), Koul (2011), Reddy (2013) and Chattopadhyay et al. (2017)
[b]*Bt Bacillus thuringiensis*

Entomopathogenic fungi (EPF) are extremely important for the microbial control of insect pests because almost all are prone to fungal diseases. EPF are potential biocontrol agents because of their high reproductive abilities, target-specific activities, short production times and ability to produce saprobic phases that allow them to survive longer, in the absence of an available host (Sinha et al. 2016). EPF have a wider host range compared to other biological control agents, causing infections in insect species belonging to orders Lepidoptera, Orthoptera, Homoptera, Coleoptera and Diptera. Among EPF, *Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria fumosorosea* (formerly *Paecilomyces fumosoroseus)*, *Hirsutella thompsonii*, *Nomuraea rileyi*, *Lecanicillium muscarium* and *Lecanicillium lecanii* (formerly *Verticillium lecanii*) are widely used worldwide for the control of insect pests (Deacon 1983; Wakefield 2018). Almost 1000 fungal species have been found to be pathogenic in insects, but only a few are mass produced and formulated (Wakefield 2018). Currently, six EPFs have been listed for use as insecticides in the EU pesticide database (Wakefield 2018). Faria and Wraight (2007) determined that

approximately 40% of the total myco-insecticides in the bioinsecticide market are based on *Beauveria*, but many of these products are no longer available. Unlike entomopathogenic viruses and bacteria, EPF do not require specific routes of infection. Because EPF infect arthropods by directly penetrating the host cuticle, they are primarily contact pathogens (Mascarin and Jaronski 2016). Table 8.2 lists some commercially produced EPF and their target pest insects.

Table 8.2 Commercial formulations of fungal entomopathogen insecticides[a]

Product name	Microorganism	Target pest insect order	Producer
Arysta Japan	*Beauveria bassiana*	Lepidoptera, Hemiptera, Thysanoptera	Botanigard
Bea-Sin		Diptera, Coleoptera	Agrobionsa
Beauverin		Coleoptera	USSR
Bioceres		Coleoptera, Hemiptera, Thysanoptera	Anatis Bioprotection
Bio-Power		Coleoptera, Lepidoptera	T.Stanes & Company Limited
Boverol-spofa		Coleoptera	Czechoslovakia
Conidia		Coleoptera	AgroEvo
Mycontrol-WP		Hemiptera, Thysanoptera	Mycotech Corp
Mycotrol/BotaniGard		Diptera, Lepidoptera	Mycotech
Naturalis™ L		Hemiptera, Homoptera, Coleoptera	Tray Bioscience
Ostrinil		Lepidoptera	Natural Plant Protection
Proecol		Lepidoptera	Productos Biológicos para el Agro
Beauveria	*Beauveria brongniartii*	Coleoptera	Eric Schweizer Seeds
Betel		Coleoptera	Natural Plant Protection
BioLisa		Coleoptera	Idemitsu Kosan
Engerlingspilz		Coleoptera	Andermatt Biocontrol
Melocont		Coleoptera	Kwizda
Bio-Catch	*Lecanicillium lecanii*	Lepidoptera, Coleoptera, Hemiptera	T.Stanes & Company Limited
Mycotal		Hemiptera, Thysanoptera	Koppert Biologica Systems
Vertalec		Hemiptera, Thysanoptera	Koppert Biologica Systems
Ancora™	*Isaria fumosorosea*	Diptera, Coleoptera, Lepidoptera, Thysanoptera, Hemiptera	OHP, Inc.
NoFly		Hemiptera, Thysanoptera	Futureco
PFR-97™ 20% WDG		Hemiptera, Thysanoptera, Spider mites	Certis USA
PreFeRal® WG		Hemiptera	Biobest
Priority		Acari	T. Stanes

(continued)

Table 8.2 (continued)

Product name	Microorganism	Target pest insect order	Producer
Lagenidium giganteum mycelium	*Lagenidium giganteum*	Diptera\Mosquitoes	CA Dept of Health
Laginex AS		Diptera\Mosquitoes	Agraquest Inc
Ago Bio. Metarhizium 50	*Metarhizium anisopliae*	Coleoptera, Lepidoptera	Ago Biocontrol
Bioblast		Termites	EcoScience
Biologie Bio 1020		Coleoptera	Bayer AG
Bio-Magic		Coleoptera, Orthoptera, Lepidoptera, Hemiptera	T.Stanes & Company Limited
Biopath		Blattodea	EcoScience
Green Guard®		Orthoptera	BASF
Biogreen	*Metarhizium flavoviride*	Coleoptera, Orthoptera, Blattodea	Biocare Technology Pvt. Ltd.
Green Muscle		Orthoptera	Internat. Instit of Bio. Con.

[a]Data from: Flexner and Belnavis (2000), Koul (2011), Skinner et al. (2014) and Sinha et al. (2016)

Entomopathogenic viruses (EPV) are ubiquitous, and effective biological control by viruses showed a promising potential (Prasad and Srivastava 2016). At least 16 viral families have been shown to be effective against pest insects, and RNA viruses, such as spoviruses, dicistroviruses, nodaviruses and tetraviruses, and DNA viruses, such as densoviruses, entomopoxviruses, ascoviruses, iridoviruses, nudiviruses, hytrosaviruses and baculoviruses, are among the major groups of EPV. Baculoviruses have shown potential as biocontrol agents in the control of agricultural, forest and greenhouse pest insects. In particular, these viruses are used to control pest insects from orders Lepidoptera, Diptera and Hymenoptera, and commercial formulations of these agents are produced (Table 8.3) (Payne 1986; Tanada and Kaya 1993; Inceoglu et al. 2001; Lacey et al. 2001; Prasad and Srivastava 2016).

Microsporidia have been previously accepted as protozoa, but according to recent molecular phylogenetic studies, they appear to belong to Fungi or to form a sister lineage (Han and Weiss 2017). Entomopathogenic microsporidia (EPM) are generally host-specific and play an important role in the regulation of insect populations. However, they have a slow effect because they mostly cause chronic infections. The biology of most EPM is complex because these organisms can only develop in living hosts and require intermediate hosts. *Nosema locustae* is the only commercial product developed for grasshopper control (Weinzierl et al. 1995). EPM have many disadvantages in pest control applications compared to other microbial control agents. Due to their need for a living host to breed and their rather slow effect, EPM have a limited range of applications in the area of biological control.

Table 8.3 Commercial formulations of entomopathogenic viruses[a]

Product name	Virus[b]	Target order\pest insect	Producer
Abietiv	NeabNPV	Hymenoptera*Neodiprion abietis*	Andermatt Biocontrol
Baculo-Soja	AgMNPV	Lepidoptera*Anticarsia gemmatalis*	Novozymes BioAg
Baculovirus AEE	AgMNPV	Lepidoptera*Anticarsia gemmatalis*	AEE[b]/CNPSoja
Baculovirus Soja WP	AgMNPV	Lepidoptera*Anticarsia gemmatalis*	Bosquiroli & Santos Ltd.
Biovirus-H	HearNPV	Lepidoptera*Helicoverpa armigera*	Biotech International Ltd.
Biovirus-S	SpliNPV	Lepidoptera*Spodoptera littoralis*	Biotech International Ltd.
Capex	AoGV	Lepidoptera*Adoxophyes orana*	Andermatt Biocontrol
Carposin	CpGV	Lepidoptera*Cydia pomonella*	Agrichem
Carpovirusine	CpGV	Lepidoptera*Cydia pomonella*	Fargro Ltd
Cryptex	CrleGV	Lepidoptera*Thaumatotibia* (*Cryptophlebia*) *leucotreta*	Andermatt Biocontrol
CYD-X	CpGV	Lepidoptera*Cydia pomonella*	Certis USA
CYD-X HP	CpGV	Lepidoptera*Cydia pomonella*	Certis USA
Diplomata	HearNPV	Lepidoptera*Helicoverpa armigera*	Andermatt Biocontrol
Dispavirus	LdMNPV	Lepidoptera*Lymantria dispar*	Canadian registration
Elcar	HzSNPV	Lepidoptera*Helicoverpa zea*	Novartis
Gemstar LC	HzSNPV	Lepidoptera*Helicoverpa zea*	Certis USA
Granupom	CpGV	Lepidoptera*Cydia pomonella*	Neudorff
Grap Baculovirus	AgMNPV	Lepidoptera*Anticarsia gemmatalis*	Agrocete
Gypchek	LdMNPV	Lepidoptera*Lymantria dispar*	USDA Forest Service
Heli-Cide	NPV	Lepidoptera*Helicoverpa armigera*	Pest Control India
Helicovex	HearNPV	Lepidoptera*Helicoverpa armigera*	Andermatt Biocontrol
Heliokill	NPV	Lepidoptera\Heliothis virescens	Ajai Biotech
Lecontvirus WP	NeleNPV	Hymenoptera*Neodiprion lecontei*	Canadian Forestry Service
Littovir	SpliNPV	Lepidoptera*Spodoptera littoralis*	Andermatt Biocontrol
Loopex	AcNPV	Lepidoptera*Trichoplusia ni*	Andermatt Biocontrol
Madex	CpGV	Lepidoptera*Cydia pomonella*	Andermatt Biocontrol
Madex HP	CpGV	Lepidoptera*Cydia pomonella*	Certis USA
Mamestrin	MbMNPV	Lepidoptera*Mamestra brassicae*	Natural Plant Protection
Monisarmiovirus	NeleNPV	Hymenoptera*Neodiprion lecontei*	Kemira
Multigen	AgMNPV	Lepidoptera*Anticarsia gemmatalis*	EMBRAPA
Ness-A	SeNPV	Lepidoptera*Spodoptera exigua*	Applied Chemical
Ness-E	SeNPV	Lepidoptera*Spodoptera exigua*	Applied Chemical
Polygen	AgMNPV	Lepidoptera*Anticarsia gemmatalis*	Agroggen
Protégé	AgMNPV	Lepidoptera*Anticarsia gemmatalis*	Adama Brasil
Spexit	SeMNPV	Lepidoptera*Spodoptera exigua*	Andermatt Biocontrol
Spodo-Cide	NPV	Lepidoptera*Spodoptera litura*	Pest Control India

(continued)

Table 8.3 (continued)

Product name	Virus[b]	Target order\pest insect	Producer
Spodopterin	NPV	Lepidoptera*Spodoptera* sp.	Ajai Biotech
Spod-X LC	SeNPV	Lepidoptera*Spodoptera exigua*	Certis USA
TM Biocontrol-1	OpNPV	Lepidoptera*Orgyia pseudotsugata*	USDA Forest Service
Vir-ex	SeMNPV	Lepidoptera*Spodoptera exigua*	Biocolor SA
Virin-EKS	MbMNPV	Lepidoptera*Mamestra brassicae*	NPO Vector
Virin-GYAP	CpGV	Lepidoptera*Cydia pomonella*	NPO Vector
Virosoft[CP4]	CpGV	Lepidoptera*Cydia pomonella*	BioTEPP Inc.
Virox	NeleNPV	Hymenoptera*Neodiprion lecontei*	Oxford Virology
VPN Ultra 1,6 WP	AcNPV	Lepidoptera\several pests	Agricola El Sol

[a]Data from: Flexner and Belnavis (2000) and Prasad and Srivastava (2016)
[b]*NeabNPV Neodiprion abietis* nucleopolyhedrovirus, *AgMNPV Anticarsia gemmatalis* multiple nucleopolyhedrovirus, *HearMNPV Helicoverpa armigera* multiple nucleopolyhedrovirus, *HzSNPV Heliothis zea* single nuclear polyhedrosis virus, *HearNPV Helicoverpa armigera* nucleopolyhedrovirus, *SpliNPV Spodoptera littoralis* nucleopolyhedrovirus, *AoGV Adoxophyes orana* granulovirus, *CpGV Cydia pomonella* granulovirus, *CrleGV Thaumatotibia* (*Cryptophlebia*) *leucotreta* granulovirus, *LdMNPV Lymantria dispar* multiple nucleopolyhedrovirus, *NeleNPV Neodiprion lecontei* nucleopolyhedrosis virus, *AcNPV Autographa californica* nucleopolyhedrovirus, *MbMNPV Mamestra brassicae* multiple nucleopolyhedrovirus, *SeNPV Spodoptera exigua* nucleopolyhedrovirus, *SeMNPV Spodoptera exigua* multiple nucleopolyhedrovirus, *OpNPV Orgyia pseudotsugata* nucleopolyhedrovirus

The successful biological control of insects by seven nematode families (Mermithidae, Allantonematidae, Neotylenchidae, Sphaerularidae, Rhabditidae, Steinernematidae and Heterorhabditidae) is noteworthy. Among these families, entomopathogenic families (Heterorhabditidae and Steinernematidae) are currently the most studied due to their successful use in biological control (Wakefield 2018). Approximately 250 species of insects belonging to 75 families of 10 orders have been reported to be susceptible to entomopathogenic nematodes (EPNs) (Poinar 1979; Subramanian and Muthulakshmi 2016). EPNs carry symbiotic pathogenic bacteria that cause the host death when released into its haemocoel. The bacteria carried by these nematodes belong to genera *Xenorhabdus*, associated with *Steinernema*, and *Photorhabdus*, associated with *Heterorhabditis* (Boemare 2002; Wakefield 2018). EPNs can be applied in areas where it is difficult to effectively apply other microbial insecticides. Soil acts as a barrier that prevents the dominance of predators, parasites and insecticides applied to control pest insects. However, there is no such a barrier for EPNs because the soil is their natural habitat (Kaya and Gaugler 1993). EPNs have become a commercially available product (Table 8.4) and have been used on a wide scale in greenhouses, mushroom farms (Richardson et al. 1990; Lenteren 2000), strawberry fields, blueberry orchards, grass farms (Koppenhofer and Kaya 1998), apple orchards (Wang 1990) and citrus groves.

Table 8.4 Commercially available entomopathogenic nematodes[a]

Product name	Nematode species	Target order\pest insect	Producer
Bio Safe	*Steinernema carpocapsae*	Lepidoptera, Coleoptera\Root weevils	SDS Biotech Co. Ltd.
Ecomask		Lepidoptera\Caterpillars	BioLogic
Exhibit SC-WDG		Lepidoptera, Coleoptera\Root weevils, Diptera\Fungus gnats	Novartis BCM
Guardian		Lepidoptera\Caterpillars	HydroGardens
Hortscan		Lepidoptera, Coleoptera\Root weevils, Diptera\Fungus gnats	BioLogic
J-3 Max		Lepidoptera\Caterpillars	The Green Spot
Millenium		Lepidoptera, Coleoptera	BASF Corporation
Nematac C		Lepidoptera\Cranberry girdler, Coleoptera\Black vine weevil	BASF Corporation
Savior Weevil larvae		Lepidoptera\Caterpillars	Thermo-Trilogy
Savior WG		Lepidoptera, Coleoptera\Root weevils, Diptera\Fungus gnats	Thermo -Trilogy
Termask		Termites	BioLogic
Entonem	*Steinernema feltiae*	Diptera\Fungus gnats	Koppert
Exhibit SF-WDG		Diptera\Fungus gnats	Novartis BCM
Garden Pack		Lepidoptera, Coleoptera	BioLogic
Nema-plus		Diptera\Fungus gnats	E-nema
NemaShield		Diptera\Fungus gnats	BioWorks Inc.
Nemasys		Diptera\Fungus gnats	E.C. Geiger
Nemasys-M		Diptera\Fungus gnats	MicroBio/ Biobest
Scanmask		Diptera\Fungus gnats	BioLogic
Sciarid		Diptera\Fungus gnats	Koppert
Traunem		Diptera\Fungus gnats	Andermatt Biocontrol
X-Gnat		Diptera\Fungus gnats	Thermo-Trilogy
Bio Topia	*Steinernema glaseri*	Lepidoptera\bluegrass webworm, Coleoptera\White grubs, weevils	SDS Biotech Co. Ltd.
Steinernema glaseri		Coleoptera\White grubs	Greenfire
Steinernema glaseri		Coleoptera\White grubs	Praxis
BioVector 355	*Steinernema riobrave*	Coleoptera\Citrus weevils	Certis USA
Devour		Orthoptera\Mole crickets, Coleoptera\Root weevils	Certis USA
Nematac S	*Steinernema scapterisci*	Orthoptera\Mole crickets	Becker Underwood Inc.
Otinem S		Orthoptera\Mole crickets	Ecogen

(continued)

Table 8.4 (continued)

Product name	Nematode species	Target order\pest insect	Producer
Heteromask	*Heterorhabditis bacteriophora*	Coleoptera\Weevils	BioLogic
Larvanem		Lepidoptera, Coleoptera\ Japanese beetles	Koppert
Lawn Patrol		Coleoptera\Weevils	HydroGardens
Nema-Bit		Lepidoptera, Coleoptera\ Japanese beetles	BIT
Nema-Top/-Green		Lepidoptera, Coleoptera\ Japanese beetles	E-nema
Terranem		Lepidoptera, Coleoptera\ Japanese beetles	Koppert
Dickmaulrussler-nematoden	*Heterorhabditis megidis*	Coleoptera\Black vine weevil	Andermatt Biocontrol
Nemasys-H		Coleoptera\Black vine weevil	MicroBio/ Biobest
Nemaslug	*Phasmarhabiditis hermaphrodita*	Slugs	Becker Underwood Inc.

[a]Data from: Flexner and Belnavis (2000) and Aneja et al. (2016)

8.4 Genetically Engineered Microbial Insecticides and their Efficacy against Insect Pests

8.4.1 Genetically Engineered Entomopathogenic Bacteria

Entomopathogenic bacteria (EPB) have been evaluated against agricultural insect pests and disease vectors for many years. Recombinant investigations have primarily focused on increasing the efficiency of bacterial insecticides by generating high levels of insecticidal protein and new protein combinations (Federici et al. 2003). Recombinant DNA techniques have been utilized to investigate members of the bacterial genera *Bacillus*, *Lysinibacillus*, *Pseudomonas*, *Rhizobium*, *Cyanobacteria, Photorhabdus* and *Xenorhabdus*. *Bt* is the most commonly used bacterium, and its strains can be manipulated to overexpress toxin genes or to express novel toxin combinations, to promote higher insecticidal activity or increase the host range (Jurat-Fuentes and Jackson 2012).

Electroporation technology has been used in genetic transformation studies and has allowed an improved generation of recombinant *Bt* using vegetative cells with plasmid DNA (Schurter et al. 1989). Genetic engineering studies became important after the development of the *Bt* toxin gene expression regulation approach. The expression of Cry1Fa was increased by translocating the Cry1Ac protoxin segment together with Cry1Fa. Homologous recombination and region-specific recombination systems have been successfully used. Vip3 protein expression or the alteration

of zwittermicin regulation resulted in increased insecticidal activity of *Bt* (Baum et al. 1999). The identification of the zwittermicin-encoding gene and its manipulation in large DNA fragments allowed alterations to *cry* gene synthesis (Liu 2009).

Resistant plants have been efficiently constructed using *cry* genes from *Bt*, which protect against different pest insects and have also the ability to control plant parasitic/pathogenic nematodes (Jouzani et al. 2017). Moreover, recent studies on *Bt* have confirmed that *Bt*-containing strains have potential in promoting plant growth, the bioremediation of heavy metals and other toxic compounds, anticancer activities, polymer production and antagonistic effects on plant/animal pathogenic microorganisms (Jouzani et al. 2017).

Bt chitinase genes have attracted attention due to their special functions in the digestion of shrimp waste and in increasing the pesticidal and fungicidal activity of Cry proteins against pest insects and phytopathogenic fungi. Various chitinase genes from different subspecies of *Bt* (subspp. *pakistani*, *kenya*, *colmeneri*, *canadensis*, *entomocidus*, *kurstaki*, *israelensis* and *konkukian*) have been identified and added to the gene bank. *Bt* chitinases have been used to increase the pesticidal activity of Cry toxins by digesting the insect peritrophic membrane. Many chitinase (*chi*) genes from different bacteria have been transferred into *Bt* to increase its insecticidal activity against pest insects. Chitinase genes (*chiB*/*chiC*) have been cloned from *Serratia marcescens* and inserted into *Bt*, and recombinant *Bt* strains harboring *chi* genes exhibited improved insecticidal activities (Ozgen et al. 2013; Danışmazoğlu et al. 2015; Karabörklü et al. 2018).

Other *Bacillus* spp. have also been used in recombinant studies. Several *Bt cry* genes have been inserted into other non-entomopathogenic *Bacillus* species, such as *B. subtilis*, *B. velezensis* and *B. licheniformis*, and were subsequently tested against pest insects. A recombinant *B. velezensis* strain harboring *Bt cry* genes was found to exhibit significant insecticidal activity when used against *Plutella xylostella* (Yul et al. 2009). *Bacillus subtilis* and *B. licheniformis* strains harboring the *Bt cry* gene (*cry1Ab*) exhibited similar insecticidal activity compared with the wild-type *Bt* strain LM-466 against larvae of *Tuta absoluta* (Theoduloz et al. 2003).

Lysinibacillus spp. has also been used in recombinant studies. The transfer of *cry* and *cyt* genes from *Bacillus thuringiensis israelensis* into *Lysinibacillus (Bacillus) sphaericus* yielded a strain that has been used against disease vectors such as mosquitoes. A *Lysinibacillus sphaericus* strain carrying a combination of genes encoding Cry and Cyt proteins, which exert a mosquitocidal effect, was tested on *Culex* species, exhibiting more than ten-fold higher efficacy (Federici et al. 2000).

Serratia spp. have also been used to engineer effective recombinant bacterial agents for pest management. The chitinase gene *chiABC* from *S. marcescens* was inserted into *E. coli*, and the resulting chitinase enzymes produced in *E. coli* exhibited excellent killing activity (45–80%) against *Malacosoma neustria* and *Helicoverpa armigera* (Danışmazoğlu et al. 2015). Recombinant studies have also been performed using *S. entomophila*. A 155-kb plasmid (pADAP) carrying the genes *sepABC* was targeted due to its involvement in causing amber disease symptoms. Recombinant *E. coli* and *Serratia* strains carrying a 47-kb pADAP plasmid

displayed strong anti-feeding and killing activity on infected grass grub larvae (Hurst et al. 2004).

Several pseudomonads, rhizobia and cyanobacteria have also been considered for genetic engineering studies, and the potential of using *Bt cry* genes for engineering recombinant agents from these bacteria was evaluated. The gene encoding the insecticidal toxin protein complex (TccC) from *Pseudomonas taiwanensis* was inserted into *E. coli* and overexpressed, and the resulting insecticidal toxin caused significant mortality of *Drosophila melanogaster* larvae (Liu et al. 2010).

The cyanobacterium *Agmenellum quadruplicatum* was engineered to contain the *cryIVD* gene from *Bt* subsp. *israelensis*, and the resulting strain showed significant mosquitocidal activity against *C. pipiens* (Murphy and Stevens 1992). A recombinant *Rhizobium leguminosarum* strain was constructed by introducing the *Bt* subsp. *tenebrionis* δ-endotoxin gene, and cell extracts of the recombinant strain exhibited a toxic effect against *Gastrophysa viridula* and *Sitona lepidus* larvae (Skøt et al. 1990).

Members of the bacterial genera *Photorhabdus* and *Xenorhabdus* have symbiotic relationships with nematodes of the genera *Heterorhabditis* and *Steinernema*. These symbiotic relationships have a strong pathogenicity against insect pests. The 17-kDa pilin subunit obtained from *Xenorhabdus nematophila* was cloned into *Escherichia*, and the recombinant protein was expressed, purified and used against *H. armigera*. The recombinant protein produced exhibited cytotoxicity against hemocytes of *H. armigera* larvae (Khandelwal et al. 2004). The toxin gene *tcdAt* from *P. luminescens* ssp. *akhurstii* was inserted into *Arabidopsis thaliana* plants and expressed, and the resulting transgenic plants exhibited resistance against the insect *M. sexta* (Waterfield et al. 2005a). Recently, the *Photorhabdus* insect toxin PirAB was identified and expressed in *E. coli*. The recombinant protein was injected into larvae of *Galleria mellonella*, and larval mortality was observed (Waterfield et al. 2005b).

8.4.2 Genetically Engineered Entomopathogenic Fungi

Fungal entomopathogens are one important insecticide alternative against many insects. Important fungal entomopathogens, such as *Metarhizium anisopliae*, *Beauveria bassiana*, *Lecanicillium lecanii* (*Verticillium lecanii*) and *Isaria fumosorosea* (*Paecilomyces fumosoroseus*), are produced in many countries for commercial purposes (Rath 2000). Recombinant DNA investigations focused on fungal entomopathogens to improve their efficacy and tolerance to environmental conditions (Zhao et al. 2016). Recombinant studies have primarily focused on the production of toxins, enzymes, hormones and other physiological regulators. The ability to use toxin genes from some arthropods, such as spiders and scorpions, for constructing effective recombinant fungal entomopathogens was assessed. For this purpose, the *AaIT1* toxin gene from the North African scorpion *Androctonus australis* was inserted into *M. anisopliae*. The genetically engineered *M. anisopliae* strain showed significant insecticidal activity (at very low doses) when applied to *Manduca sexta*,

and the survival time of the host significantly decreased (40%) (Wang and St. Leger 2007). Similar effects against cotton aphids (*Aphis gossypii*) were observed with a recombinant *Lecanicillium lecanii* strain harboring BmKit from *Mesobuthus (Buthus) martensii*, (Xie et al. 2015). Two insect-specific scorpion neurotoxins, LqhIT2 from *Leiurus quinquestriatus hebraeus* and *BjαIT* from *Hottentotta* (*Buthotus*) *judaicus*, were also used in recombinant studies. A genetically engineered *M. acridum* strain harboring the LqhIT2 and *BjαIT* neurotoxins exhibited significant killing activity against *Locusta migratoria manilensis* (Peng and Xia 2015). Several spider toxins, such as k-HXTX-Hv1c from *Hadronyche versuta* and u-HXTX-Hv1a from *Atrax robustus*, have also been used to improve recombinant entomopathogenic fungi (Fang et al. 2014).

Enzymes such as proteases and chitinases have also been targeted to improve the effectiveness of fungal entomopathogenic agents. Specifically, several genes, including those encoding subtilisin-like proteases, glyceraldehyde-3-phosphate dehydrogenase (promoter), chitinases, acid trehalase, sterol carriers, esterases and benzoquinone oxidoreductase, have been used to improve the insecticidal potential of fungal agents against pest insects. A genetically engineered *M. anisopliae* strain producing a subtilisin-like protease (Pr1A) caused a significant decrease in the survival time and feeding activity of *M. sexta* (St. Leger et al. 1996). Several genes expressing chitinases, such as Chit1, Chi2 and chitinase-40, were inserted into *B. bassiana*, *M. anisopliae* and *Trichoderma koningii*, respectively, and the recombinant strains exhibited improved virulence against the pest insects *Myzus persicae*, *Dysdercus peruvianus* and *Ostrinia furnacalis*, respectively (Fang et al. 2005; Merzendorfer 2013).

The overexpression of acid trehalase (ATM1) in a recombinant *M. acridum* strain caused a significant reduction in its lethal concentration (LC_{50}) against *Locusta migratoria* (Peng et al. 2015). Hybrid gene techniques have also been applied to improve recombinant fungal agents, and the production of hybrid genes, such as protease-chitinase and chitinase-chitinase, has been tested. In recombinant *B. bassiana,* hybrid gene technology (CDEP1: Bbchit1) produced better results than single genes (Fang et al. 2009). Similarly, an increase in the virulence of *B. bassiana* was observed after construction of a hybrid gene responsible for chitinase production (Fan et al. 2007). In genetic engineering studies, abiotic stresses, such as ultraviolet (UV) radiation, high temperature and low moisture, have also been used to improve fungal agents. The resistance of fungal entomopathogens to fungicides and herbicides has also been targeted in genetic engineering studies (Vega et al. 2012).

Recombinant studies on fungal entomopathogens based on hormones and other physiological regulators have been performed against pest insects. The activity of hormones, such as diuretic hormone (MSDH), and physiological regulators, such as immune-related signaling pathway inhibitors, trypsin modulating oostatic factors, serine proteinase inhibitors and pyrokinin β-neuropeptide, toward agricultural pest insects and vector insects was evaluated using RNA interference (RNAi). A genetically engineered *B. bassiana* strain (that produces MSDH) showed significant killing activity against *M. sexta*, *G. mellonella* and *Anopheles aegypti* (Fan et al. 2012). Similarly, a recombinant *B. bassiana* strain producing inhibitors of an immune-related signaling pathway exhibited significant killing activity toward *G. mellonella*

and *M. persicae* (Yang et al. 2014). A *B. bassiana* strain producing a trypsin-modulating oostatic factor also exhibited high virulence against *A. aegypti*. In addition, an *I. fumosorosea* strain was engineered to express a dsRNA targeting the gene encoding the immune-related protein TLR7, and the resulting *I. fumosorosea* strain successfully knocked down nymphs of *Bemisia tabaci* (Chen et al. 2015).

8.4.3 Genetically Engineered Entomopathogenic Viruses

Recombinant DNA techniques have been used to increase the effectiveness of entomopathogenic viruses (Karabörklü et al. 2018). Among the known entomopathogenic viruses, baculoviruses have attracted attention due to their pathogenicity against pest insects (Hails 2001). Recombinant studies have primarily focused on increasing the killing rates and speeds of baculoviruses and reducing the feeding capacities and longevities of pest insects. Many exogenous genes have been evaluated for these purposes (Slack et al. 2009), and many genes have been shown to encode substances with insecticidal bioactivity (toxins, hormones and enzymes).

Genes producing neurotoxins have been obtained from different animal groups, such as sea anemones, spiders, scorpions, mites, ants and wasps, and have been inserted into baculoviruses (Harrison and Hoover 2012). The toxins obtained after the insertion and expression of one of these genes have been used against some pest insects, and promising results have been obtained. Genetically engineered baculoviruses were found to be 10–60% more effective than wild-type baculoviruses at inducing larval paralysis and feeding cessation, and reducing larval longevity (Inceoglu et al. 2006).

Investigations on scorpion neurotoxins have been also performed. For example, genes producing the peptide neurotoxin (BeIT and AaIT) from the Asian scorpion *M. eupeus* and the North African scorpion, *A. australis* were evaluated against some insect pests (Carbonell et al. 1988; Harrison and Hoover 2012; Kroemer et al. 2015). The AaIT neurotoxin gene caused disruptions in the ion conductance of neuron axonal membranes when transferred into *Bombyx mori nucleopolyhedrovirus* (BmNPV). A recombinant baculovirus (BmAaIT) was 40% more effective than nonrecombinant strains against *Bombyx mori*. The recombinant baculovirus obtained by the insertion of the AaIT gene into *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) was 12–50% more effective than the wild-type virus against the pest insects *Helicoverpa armigera*, *H. virescens, Trichoplusia ni* and *Spodoptera exigua.*

In addition, mite (TxP)-, wasp (*Dol m*)-, spider (μ-Aga, TalTX and DTX)- and sea anemone (As and Sh)-derived toxins have been evaluated against insects (Kroemer et al. 2015). Transcription factors, gene promotors and signal peptides regulating insect development have also been investigated to determine their potential to increase the insecticidal activity of baculoviruses. Promotors from the genes *polh* and *PsynXIV* (synthetic) have been transferred into mites to drive toxin expression (Harrison and Hoover 2012).

The genes affecting hormone production in insects have also been assessed in recombinant studies. Recombinant baculoviruses that carry genes for the production of hormones, such as prothoracicotropic, eclosion and diuretic hormones, were tested on insects to evaluate their effects on pest longevity and their ability to induce larval damage (Slack et al. 2009). Recombinant baculoviruses (BmNPV) producing diuretic hormones from *M. sexta* and *H. zea* resulted in increased mortality and reduced the longevity of *B. mori* larvae (Raina et al. 2007).

Chitinolytic, proteolytic and juvenile-hormone esterase enzymes have also been used to improve the effectiveness of baculoviruses. A recombinant AcMNPV harboring a *M. sexta* chitinase gene exhibited a killing of *Spodoptera frugiperda* larvae faster than the wild-type strain (Gopalakrishnan et al. 1995). To increase the killing speed of baculoviruses, some proteolytic enzymes, such as human gelatinase A, rat stromelysin-1 and fly cathepsin-L (ScathL), have also been assessed.

The effect of gene deletions, such as the deletion of the ecdysteroid UDP-glucosyl transferase (*egt*) gene, has also been evaluated. Deletion of this gene from the AcMNPV genome increased the killing speed and reduced the feeding activity of *Trichoplusia ni* and *S. frugiperda* larvae (O'Reilly and Miller 1991).

8.4.4 Genetically Engineered Entomopathogenic Microsporidia

The microsporidia are a group of obligate intracellular parasitic fungi belonging to the genus *Microspora*. Microsporidia share a genetic relationship with fungi but carry a few distinct morphological features (Solter et al. 2012; Han and Weiss 2017). Entomopathogenic microsporidia exhibit important pathogenicity against many aquatic and terrestrial vector insects and have been evaluated in insect disease management and biological control programs (Corradi and Keeling 2009). However, their large genomes have prevented the construction of recombinant agents (Mishra 2009). Therefore, few recombinant investigations have been conducted to improve microsporidian entomopathogenic agents. Several studies have been carried out on some microsporidian proteins with important roles in spore germination and infection. The ricin-B-lectin (RBL) gene from *Nosema bombycis*, which enhances spore adhesion to BmN cells (*Bombyx mori*), was targeted. The RBL gene was extracted from *N. bombycis* and then amplified and expressed in recombinant *E. coli* BL21 (Liu et al. 2016).

8.4.5 Genetically Engineered Entomopathogenic Nematodes

Steinernema and *Heterorhabditis* are two entomopathogenic nematode genera that are used as effective biocontrol agents against many pest insects (Shapiro-Ilan et al. 2002; Karabörklü et al. 2015). Investigations on the molecular genetics of these

EPNs have lagged compared with investigations of their bacterial symbionts, *Photorhabdus* and *Xenorhabdus* (Burnell 2002). However, some studies have investigated their infectivity, persistence, and storage stability to improve their effectiveness in pest management. The hsp70 heat-shock protein gene from *Caenorhabditis elegans* was successfully transferred into *H. bacteriophora* by microinjection, and this transfer increased the heat stress tolerance of the resulting strain under laboratory conditions (Gaugler et al. 1997). Overexpression of a trehalose phosphate synthase gene, identified in *C. elegans* and transferred into *Steinernema feltiae*, increased the osmotic and desiccation tolerance of adult nematodes (Vellai et al. 1999). Stress response-related genes belonging to the insulin/IGF-1 signaling pathway or other pathways are promising candidates for the effective construction of recombinant EPNs (Lu et al. 2016). Recombinant investigations on entomopathogenic nematodes are very limited, and further studies are needed.

8.5 Major Concerns Regarding Genetically Engineered Microbial Insecticides

The use of genetically engineered microbial insecticides and their products for pest control has brought about some social concerns and debates (Karabörklü et al. 2018). The primary concern is their potential negative effects on the environment. Other concerns regarding this technology include gene flow into other microorganisms, resistance in target insect pests and undesirable effects on other non target organisms and health (Castagnola and Jurat-Fuentes 2012). However, to overcome these concerns in society, responsible research centers and scientists need to participate in education and training programs to provide better information regarding the benefits and risks associated to recombinant DNA technology.

8.6 Conclusion

The rapid advances in recombinant DNA technology led to the development of new strategies to combat pests. In addition to causing crop damage, many insect species in nature also play roles as carriers of diseases, which consequently results in economic losses. The reduction of economic losses caused by agriculture, forest and disease-carrying pests is the most important goal of insect control techniques. Currently, the use of recombinant DNA technology in pest control is a popular research topic because this technology has many advantages, such as achieving target pest control, protecting many beneficial species, increasing resistance to disease and increasing crop yield capacities.

Microbial insecticides, known as eco-friendly insecticides, have been successfully used for many years. However, genetically engineered microbial insecticides have many advantages over natural microbial insecticides, such as low dose

requirements, long-term persistence and higher efficacy. Consequently, the development of more effective strategies to control pest insects will depend on the discovery of new microbial insecticides and biotechnological advances.

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Chapter 9
Effects of Entomopathogens on Insect Predators and Parasitoids

Amin Sedaratian-Jahromi

Abstract High reliance on chemical pesticides for controlling phytophagous pests in agro-ecosystems has resulted in different negative effects, and this issue dramatically changed our attitude in pest management programs. Among different safe alternatives for combating pest populations in agro-ecosystems, biological control has considerable potential by utilization of other living organisms including predators, parasitoids and entomopathogens. Pathogenic agents are diverse group of biological operators which exhibit reliable activities in different situations and hence, their application in agro-ecosystems has significantly increased. However, to maximize the benefits and increase the effectiveness of these natural enemies, "Integrated Biological Control" (IBC) could be applied as a promised strategy. This approach not only increases the effectiveness of native natural enemies, but also has confirmed impacts on exotic agents. Furthermore, IBC could reveal actual capacity of these pathogenic agents for regulating population density of target organisms, playing a critical role for successful implementation of biocontrol programs. On the other hand, simultaneous application of entomopathogens and other natural enemies may adversely affects their biological performance, especially in the case of insect predators/parasitoids, as discussed in this chapter.

Keywords Integrated biological control · Intra-guild predation · Multi-trophic interactions · Pathogenicity

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9.1 Introduction

Currently, agricultural systems (agro-ecosystems) only partially statisfy food requirements of increasing populations. Different factors restrict successful production of these systems in different parts of the world. Among them, arthropod pests (insects and mites) are considered as most dangerous factors (Fathipour and Sedaratian 2013). Among different management strategies for suppression of such organisms, application of chemical pesticides is still practiced by farmers and growers (Nauen et al. 2001; Ditillo and Walgenbach et al. 2016). However, high reliance on chemical pesticides and their extensive usage has resulted in many deleterious effects (e.g. negative effects on non-target organisms, hazard to human health, pest resistance, resurgence and outbreak, environmental pollutions, toxic residues in agricultural products etc.). The above-mentioned disadvantageous, and also the increasing global concerns about food safety and security, revealed a need of changes in pest management programs (Mohan et al. 2008). Accordingly, organic agriculture is urgently required as this option could minimize negative effects of chemical pesticides in agro-ecosystems (Fathipour and Maleknia 2016).

Despite conventional agriculture, to achieve sustainable management and regulation of pest populations, modern agriculture relies on more eco-friendly options. These focus on integrated pest management (IPM) programs with special emphasis on non-chemical methods on host plant resistance (HPR), interference tactics by sex pheromones and biological control (biocontrol) (Sedaratian et al. 2009, 2013; Fernandez et al. 2017). Bio control is an effective strategy for management of destructive organisms (insects, mites, weeds and plant diseases) by the utilization of other living organisms known as natural enemies or biocontrol agents (e.g. predators, parasitoids and entomopathogens). This procedure typically involves deliberate human activities and is considered as an inseparable component of any IPM program, based on three basic strategies: introduction, augmentation and conservation (Rechcigl and Rechcigl 1998). However, natural enemies play a deterministic role in the success of such programs and, as a first step before application, their efficiency, together with their possible interactions with other organisms should be accurately investigated (Jervis 2005).

In comparison with other natural enemies, entomopathogens show a huge diversity. They consist of several groups of living organisms including entomopathogenic fungi, bacteria, viruses, nematodes and protists, which cause severe and often lethal infections in target organisms. Entomopathogens provide a non-chemical alternative for sustainable management of pest populations. Although our knowledge about these natural enemies steady increased in the last century, specific gaps remained on different aspects of such microbial agents. Today, many entomopathogens are commercially produced, formulated and released in agro-ecosystems for management of arthropod pests in a process similar to synthetic pesticides.

Undoubtedly, widespread use of entomopathogenic agents in natural environments has resulted in undefined effects which need to be investigated, in particular for their simultaneous interactions with other natural enemies eventually applied

(Koul and Dhaliwal 2002). In such cases, the main concern is the likelihood of detrimental interactions occurring between entomopathogens and predators/parasitoids, especially when antagonistic interactions disrupt effectiveness of pest management programs (Sedaratian et al. 2014). To develop widespread usage of these microbial agents in organic agriculture, our knowledge about such interactions should hence be extended. In fact, we need more detailed information to evaluate safety of microbial agents towards other non-target organisms in agro-ecosystems. In the current chapter, different research projects performed to evaluate possible interactions between entomopathogenic agents and insect predators/parasitoids are reviewed. Furthermore, a concise interpretation of such interactions is presented, with a discussion on future evolution of microbial pest control as well as microbial biopesticides.

9.2 Definition and Basic Principles of Biological Control

Generally, biological control could be defined as intentional practices involving the application of natural enemies (predators, parasitoids and pathogens) to reduce damage caused by phytophagous arthropods (insects and mites), weeds and plant diseases. Accordingly, the main objective of such programs is minimizing the undesirable effects of target pests and involves regulation of their populationdynamics (Crawley 1989). DeBach (1964) stated that biological control, considered as a part of natural control, could be described as *the activity of natural enemies in maintaining population density of other organisms at a lower equilibrium level than would occur in the absence of these agents* (Fig. 9.1). In fact, the concepts of "population regulation" and "equilibrium level" are inseparable parts of biological control. To regulate the population of any target organism, different factors should act (separately or in combination) in direct or inverse density-dependent manners (Huffaker et al. 1984).

Success biocontrol programs is achieved when a significant reduction in population density of a target pests occurs, with eventual maintenance below any economic threshold at non-pest status (DeBach and Rosen 1992; van Driesche and Bellows 1988). In such circumstances, stable interactions between population of pests and their natural enemies should occur, with a decline in pest population density, expected following the introduction of the biocontrol agents (Fig. 9.1). Success of biological programs may be affected by several factors (biotic and abiotic) and therefore, these programs have no similar outcomes. DeBach and Rosen (1992) stated that from 164 species of insect pests subjected to biological control programs, 75 cases resulted in "complete" success, 74 were "substantial" and 15 achieved a "partial" control. Keeping this in view, it is noticeable that among non-chemical strategies used in management programs of arthropod pests (e.g. biological, cultural, physical, mechanical, genetic, interference and etc.), biological control achieved greatest number of success (DeBach and Rosen 1992). However, any biocontrol program aims at increasing natural control of pest population. To achieve

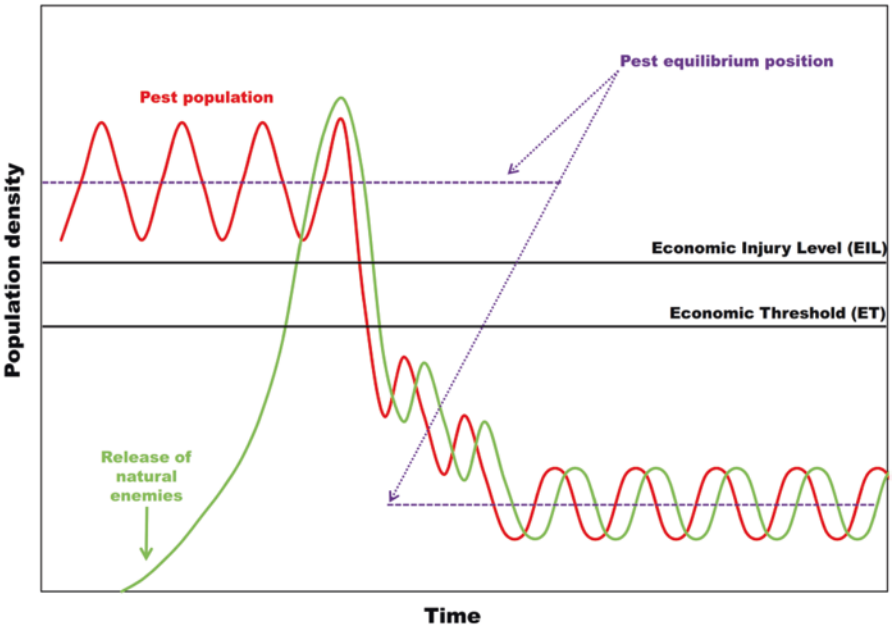

Fig. 9.1 A schematic drawing showing how the application of natural enemies in agro-ecosystems may reduce the population density of undesirable organisms

this goal, fundamental understanding of many aspects of population ecology, of both pests and their natural enemies, is urgently needed (Mills and Getz 1996).

Biological control is compatible with other management strategies for combating pest populations. This approach is considered as a reliable alternative to suppress pest damages and reduce deleterious effects of chemical pesticides. Accordingly, in modern agriculture, biological programs are strongly considered as the cornerstone of sustainability, and reliance on their applications is a key factor to guarantee food security. On the other hand, biological agents regulate population density of other living organisms at the field/greenhouse conditions. Hence, their impacts on population structure of both target and non-target organisms, as well as the environmental benefits derived, should be investigated with more accuracy.

9.3 Natural Enemies as Reliable Tools for Biological Programs

Biocontrol agents (natural enemies) have an impact on in designing biological programs and their performance affects their success rate. In tri-trophic systems (Fig. 9.2), natural enemies are placed at the top of the food chain (third level) but are limited by the abundance of the herbivorous populations (Hariston et al. 1960; Koul and Dhaliwal 2003). In these chains, direct and indirect interactions exist among

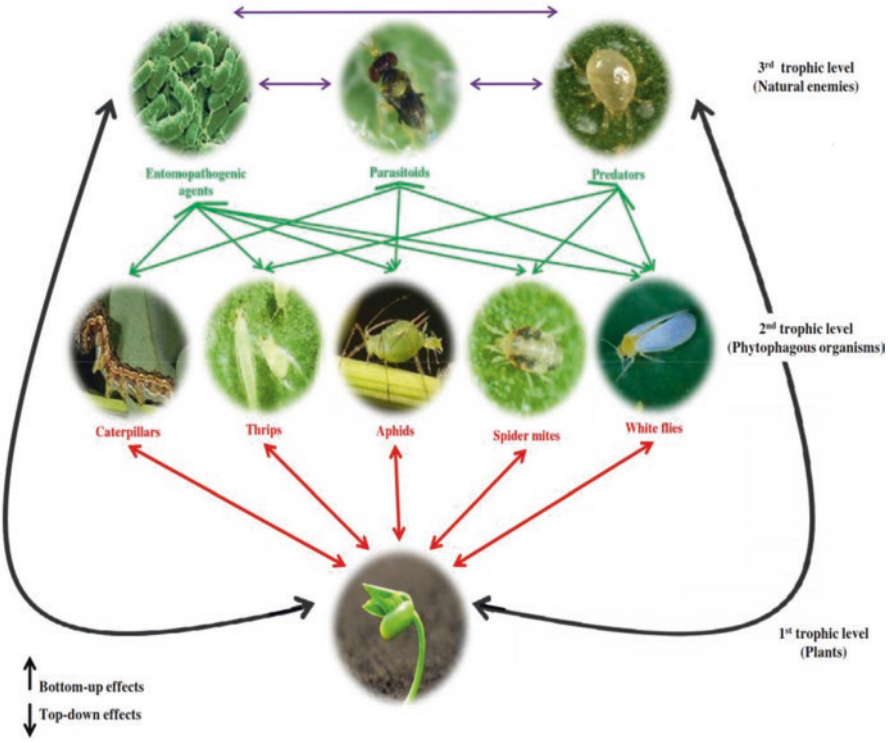

Fig. 9.2 Diagram showing tri-trophic interactions among host plants, phytophagous pests and natural enemies. (Images courtesy of ....)

different food levels and feeding activities of natural enemies, on the different herbivorous life stages (top-down effects). These interactions play a crucial role in regulating population fluctuations of undesirable organisms. On the other hand, different attributes of the first (host plants) and second (phytophagous pests) levels could significantly affect the biological performance of natural enemies (bottom-up effects) (Fathipour and Sedaratian 2013).

During recent years, considerable efforts were performed to evaluate practical and theoretical aspects of natural enemies. In this view, increasing demands for predators and parasitoids, simple life cycles of most natural enemies (particularly parasitoids), relative ease for mass rearing and investigations on these organisms in laboratory conditions resulted in an increased global attention, facilitating further research projects (Jervis 2005). Herein, brief information about different groups of natural enemies used in biological programs is presented.

Table 9.1 Some taxa of most important insect predators used for biological control of different insect pests

Order	Families	Genera
Coloeptera	Coccinellidae	*Coccinella*, *Rodalia*, *Chilocorus*, *Scymnus*, *Hipodamia*, *Stethorus*, *Cycloneda*, *Adalia*, *Cryptolaemus*, *Hyperaspis*
	Carabidae	*Calosoma*
	Staphilinidae	*Creophilus*, *Lathrobium*, *Oligota*, *Sepedophilus*
	Lampyridae	*Photuris*, *Photinus*, *Lecontea*
	Cantharidae, Dytiscidae, Gyrinidae	
Dermaptera	Forficulidae	*Forficula*, *Doru*
	Labiduridae	*Labidura*
	Labiidae	*Labia*, *Marava*
Diptera	Cecidomyiidae	*Aphidoletes*, *Feltiella*, *Tripsobremia*
	Syrphidae	*Scaeva*, *Episyrphus*
	Chamaemyiidae	*Leucopis*
	Asilidae	*Laphria*, *Efferia*, *Psilonyx*
Hemiptera	Anthocoridae	*Anthocorous*, *Orius*, *Montadoniela*
	Miridae	*Tytthus*, *Deraeocoris*, *Macrolophus*
	Nabidae	*Nabis*
	Reduviidae	*Arilus*
	Lygaeidae	*Geocoris*
	Pentatomidae	*Podisus*, *Perillus*, *Sitretrus*
	Nepidae, Belastomatidae, Corixidae, Naucoridae, Pleidae, Notonectidae, Mesoveliidae, Veliidae, Hydrometridae, Herbidae, Macrovelidae, Gerridae	
Thysanoptera	Aeolothripidae	*Aeolothrips*
	Phlaeothripidae	*Leptothrips*
	Thripidae	*Scolothrips*
Neuroptera	Chrysopidae	*Chrysopa*, *Chrysoperla*
	Hemerobiidae	*Hemerobius*
	Mantispidae	*Mantispa*
	Coniopterygidae, Myrmeleontidae	
Hymenoptera	Formicidae	*Solenopsis*
	Vespidae	*Polybia*, *Polystes*, *Vespula*
	Sphecidae	*Chlorion*, *Ammophila*, *Sphex*, *Pemphedron*, *Crossocerus*, *Philantus*
	Eumenidae	
Mantodea	Mantidae	*Mantis*, *Tenodera*
Orthoptera	Tettigonidae	*Conocephalus*, *Oecanthus*
Odonata		

9.3.1 Predators

In general, predation is defined as a biological interactions between two organisms where one of them (predator) kills and eats another ones (prey). Predators attack and kill many preys during their life span in both immature and adult stages. These natural enemies can be found in different agricultural and natural habitats. Several groups of animals have predatory behavior on insect and mite pests in agroecosystems (Koul and Dhaliwal 2003). Table 9.1 lists some of the most important groups of insect predators used in biological control programs. Feeding behavior of predators, as concerns their choice of prey, ranges from specialized to generalists (Hoffmann and Frodsham 1993). Unfortunately, although some predators are extremely useful agents, some of them have predation behaviors also on other beneficial organisms. From the view point of biology, each species presents a different life-cycle. The life history of common predators is well investigated, but our knowledge about many species is still very limited (Hokkanen 1993).

However, efficiency varies among species. Some predators have considerable impact on suppression of a prey population. For example, in the case of homopterous insects, where the insect body is covered by a waxy layer and contact chemicals have no sufficient effects, predators exhibit a reliable performance. Another success has been obtained in the case of lepidopteran pests which have borer and internal feeding behaviors (Dhaliwal and Arora 2001). It should be mentioned that some predators may have only a minor role by themselves, but contribute to overall pest mortality or provide good control at a late season.

9.3.2 Parasitoids

A parasitoid is an organism that lives, feeds and develops inside (endoparasitoid) (Fig. 9.3) or outside (ectoparasitoid) its host's body. In fact, female individuals deposit their fertile eggs in/on the body of their hosts, and the hatched larvae consume the host tissues.

In more cases, only immature stages feed on their hosts and adult individuals have a nectar-feeding behavior. Adult females of certain parasitoids, attacking scales and whiteflies, kill their hosts and provide important sources of control, causing host mortality by their parasitism activity. In nature, most insect parasitoids belong to some groups of wasps (Order: Hymenoptera) or flies (Order: Diptera) (Table 9.2).

In contrast with true parasites (fleas and ticks), feeding activity of immature stages of parasitoids kill their hosts. Furthermore, also the adult true parasites feed on their hosts. Unlike the predators, during their life span the parasitoids often consume only one host, which is not killed immediately.

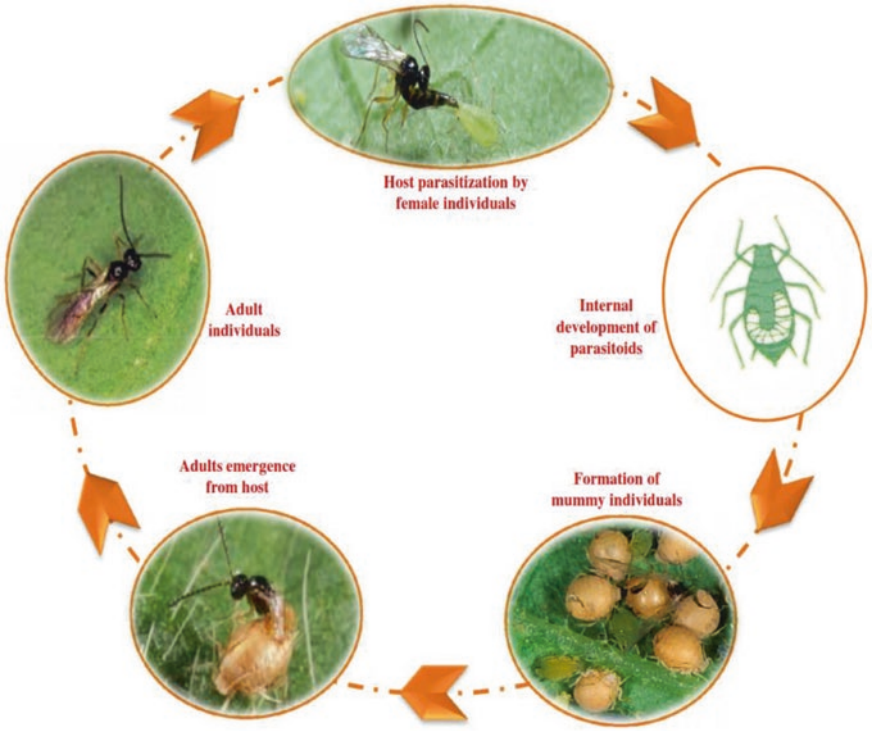

Fig. 9.3 Life cycle of *Aphidius matricariae,* an endoparasitoid of aphids. (Images courtesy of ....)

Table 9.2 The most important parasitoids used for biological control of different insect pests

Order	Superfamily	Family	Subfamily	Genera
Diptera		Tachinidae	Tachininae	*Archytas*, *Bonnetia*, *Eupleteria*, *Bigonicheta*
			Dexiinae	*Prosena*, *Dexia*, *Ptilodexia*, *Microphthalma*
			Exoristinae	*Compsilura*, *Anetia*, *Sturmia*, *Exorista*
			Gymnosematinae	*Phasia*, *Trichopoda*, *Gymnosoma*
		Sciomyzidae	–	*Sepedon*, *Sepedomerus*
		Cryptochaetidae	–	*Cryptochaetum*
		Pipunculidae	–	*Verrallia*
		Sarcophagidae	–	*Agria*

(continued)

Table 9.2 (continued)

Order	Superfamily	Family	Subfamily	Genera
		Acroceridae, Bombyliidae, Nemestrinidae, Conopidae, Phoridae		
Hymenoptera	Ichneumonoidea (Apocrita)	Orussidae (Symphyta)		*Hybrizon*
		Paxylommatidae		
		Ichneumonidae	Ichneumoninae	*Ichneumon*
			Pimplinae	*Ephialtes*, *Itoplectis*, *Pimpla*
			Porizontinae	*Devorgilla*, *Diadegma*
			Rhyssinae	*Rhyssa*, *Megarhyssa*
			Tryphoninae	*Netelia*, *Tryphon*
			Banchinae	*Banchus*, *Lissonota*
			Cremastinae	*Cremastus*, *Pristomerus*
			Ctenopalmitinae	*Hyperbatus*, *Rhorus*
			Diplazontinae	*Diplazon*, *Homotropus*
			Ophioninae	*Alophiophion*, *Ophion*
			Phygadeuontinae	*Agonocryptus*, *Phygadeuon*
		Braconidae	Alysiinae	*Aphaereta*, *Dacnusa*
			Aphidiinae	*Aphidius*, *Praon*, *Trioxys*
			Cardiochilinae	*Cardiochiles*
			Cheloninae	*Chelonus*, *Phanerotoma*, *Ascogaster*
			Euphorinae	*Microctonus*
			Macrocentrinae	*Macrocentrus*
			Microgastrinae	*Cotesia*, *Apanteles*, *Microplitis*, *Microgaster*
			Opiinae	*Opius*, *Biosteres*
	Chalcidoidea (Apocrita)	Leucospidae		*Muscidifurax*, *Spalangia*

(continued)

Table 9.2 (continued)

Order	Superfamily	Family	Subfamily	Genera
		Encyrtidae		*Comperia*, *Hunterellus*, *Ooenocyrtus*, *Epidinocarsis*, *Microterys*, *Apterencyryus*, *Anagyrus*, *Metaphycus*
		Mymaridae		*Anaphes*, *Anagrus*, *Gonatocerus*
		Trichogrammatidae		*Trichogramma*, *Megaphragma*
		Eulophidae		*Pediobius*, *Sympiesis*, *Oomyzus*, *Chrysocharis*, *Tetrastichus*, *Diglyphus*
		Aphelinidae		*Aphelinus*, *Aphytis*, *Encarsia*, *Coccophagus*
		Chalcididae		*Brachymeria*
		Pteromalidae, Torymidae, Agaonidae, Eucharitidae, Eurytomidae, Mymarommatidae, Eupelmidae, Signiphoridae		
	Proctotrupoidea (Apocrita)	Scelionidae	–	*Trissolcus*, *Telenomu*, *Scelio*
	Chrysidoidea (Apocrita)	Vanhorniidae, Proctotrupidae, Diapriidae, Platygasteridae, Plecinidae, Heloridae, Roproniidae		
		Scerogibbidae, Dryinidae, Bethylidae, Chrysididae		
	Trigonaloidea (Apocrita)	Trigonalidae		
	Stephanoidea (Apocrita)	Stephanidae		
	Evanoidea (Apocrita)	Evaniidae	–	*Evania*, *Prosevania*
		Gasteruptiidae		
		Aulacidae		
	Cynipoidea (Apocrita)	Eucoilidae		
		Ibaliidae		
		Charipidae		
		Figitidae		
	Ceraphronoidea (Apocrita)	Megaspilidae		
		Ceraphronidae		
	Vespoidea (Apocrita)	Tiphiidae	–	*Tiphia*
		Scoliidae	–	*Scolia*
		Mutillidae, Sphecidae		

Table 9.3 Most common pathogenic agents of insect pests

Type	Lineage	Taxa
Fungi	Phylum: Oomycota	Genus: *Lagenidium*
	Phylum: Zygomycota	Genus: *Entomophthora*, *Neozygites*, *Entomophaga*
	Phylum: Ascomycota	Genus: *Cordyceps.*
	Phylum: Deuteromycota	Genus: *Lecanicillium*, *Metarhizium*, *Beauveria*
	Phylum: Microsporidia	Genus: *Nosema*, *Paranosema*, *Vavraia*, *Endoreticulatus*, *Vairimorpha*, *Tubulinosema.*
Bacteria	Division: Gracilicutes (gram- negative)	Family: Pseudomonadaceae, genus *Pseudomonas*
		Family: Enterobacteriaceae, genus *Serratia*
	Division: Firmicutes (gram- positive)	Family: Bacillaceae, genera: *Bacillus, Paenibacillus, Clostridium*
	Division: Tenericutes (without cell wall)	
Viruses	Family: Baculoviridae (DNA)	Genus: Nucleopolyhedrovirus (NPV), Granulovirus (GV)
	Family: Poxiviridae (DNA)	Genus: Entomopoxvirus
	Family: Reoviridae (RNA)	Genus: Cytoplasmic Polyhedrovirus (CPV)
Nematoda	Family: Heterorhabditidae	Genus: *Heterorhabditis* sp.
	Family: Steinernematidae	Genus: *Steinernema* sp.
	Other families: Sphaerularidae, Neotylenchidae, Mermithidae, Allantonematidae, Rhabditidae	
Protista	Phylum: Apicomplexa	Classes Eugregarinorida, Neogregarinorida, Coccidia
	Other taxa: Ciliophora, Euglenozoa, Amoebozoa, Helicosporidia	

9.3.3 Pathogens

Pathogen is any microorganism (e.g., fungi, bacteria, viruses, nematodes and protista) that can infect and kill their hosts (Khetan 2001). Some of the most important entpmopathogens are shown in Table 9.3.

Deleterious impacts of chemical pesticides increased our need for safe alternatives to these compounds. This situation elicited considerable interests in entomophatogens as reliable and effective agents for suppression of insect pests in agro-ecosystems (Sedaratian et al. 2013, 2014). Under appropriate environmental conditions (e.g., extended period of high humidity or dense pest populations), entomopathogens produce an epizootic in natural populations of different arthropods, drastically decreasing their numbers (Mracek and Sturhan 2000; Udayababu et al. 2012; Haar et al. 2018). As microbial pesticides, some of these organisms such as

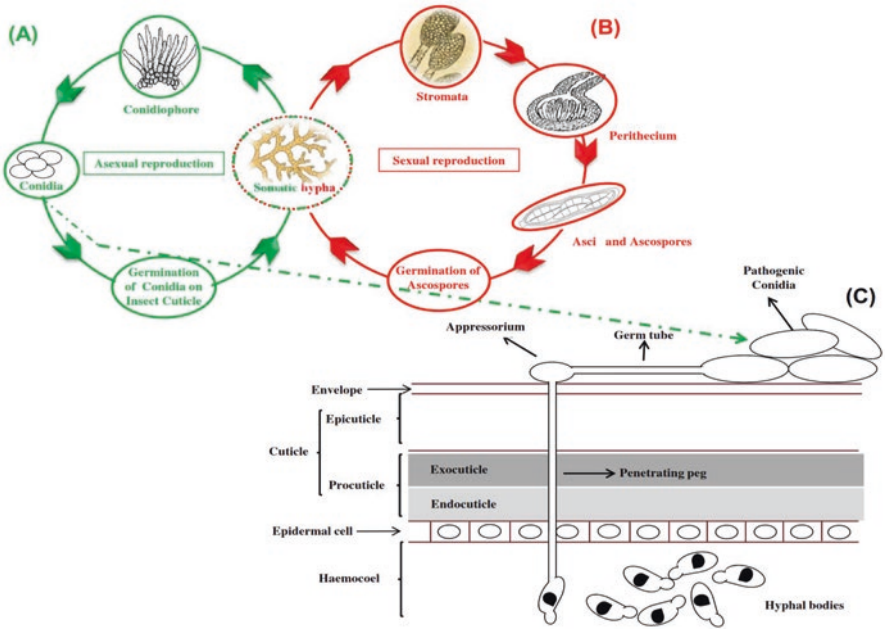

Fig. 9.4 Life cycle and infection mechanism of entomopathogenic fungi. (**a**) Asexual reproduction; (**b**) Sexual reproduction and (**c**) Infection mechanism. (Images courtesy of ....)

Bacillus thuringienis Berliner (Bt), *Metarhizium anisopliae* (Metch.) Sorok. and *Beauveria bassina* (Balsamo) are commercially available. The application of entomopathogens in management programs of different pests is favorable since their action occurs without introducing any toxic and non-biodegradable compounds to the environment, and no residue is present on agricultural products (Zimmermann 2007).

9.3.3.1 Fungi

As a diverse group of microorganisms, true fungi have about 1.5 million different species (Schmit and Mueller 2007). Among them, 700 species from 90 genera are documented with insecticidal activities (Roberts and Humber 1981). They belong to two distinct phyla: Entomophthoromycota and Ascomycota (Order: Hypocreales) (Humber 2012). The most common attribute used to consider fungi as natural groups is their sexual fruiting structure. Other characters of fungi are their feeding behavior, and structure, the unicellular (yeasts) or hyphal (filamentous) development and their reproductive strategy (both sexual [Telomorph] and asexual [Anamorph]) (Fig. 9.4).

The ability of producing sexual spores does not occur (or is rare) in many entomopathogenic fungi. Member of this group have mycelial forms that produce asexual spores (conidia). However, given their visible appearance, the hosts infected by fungal entomopathogens are very typical. In comparison with other groups, these agents directly penetrate the host cuticle and have no need for ingestion (Fig. 9.4). Having this trait enables them to parasitize phloem-feeder insects (aphids and whiteflies), which have no feeding activity on sprayed leaves of host plants (Gonzalez et al. 2016).

First attempt to use entomopathogenic fungi for control of insect pests was performed by the Russian scientist Eli Metchnikoff. In fact, he found that soils contaminated with fungal conidia could infect insect larvae. Eventually, he cultured these agents on a artificial substrate (sterilized beer mash) and tested their pathogenecity against different insect pests (Steinhaus 1975). de Faria and Wraight (2007) revealed that 170 microbial products have been developed using fungal metabolites of at least 12 species of entomopathogenic fungi.

Microsporidia

Recent molecular observations transferred the Phylum Microsporidia from Protista to Fungi (Corradi and Keeling 2009) and revealed that this group is related to Zygomycetes (Corradi and Slamovits 2010). Pathogenic activity of Microsporidia was reported both on insect pests and beneficial species. However, symptoms observed in individuals infected by Microsporidia are clearly different from those due to other fungi (Microsporidia have no fruiting bodie). *Nosema bombycis* Nageli is one of the most important species that infects silkworms, *Bombyx mori* L., producing dark spots on the larval cuticle named “pebrine”. Efforts by Louis Pasteur around 1870 resulted in strategies for controlling this disease and saved this industry in France. As previously mentioned, these microorganisms have undesirable effects on populations of beneficial insects, especially in high-density colonies. For example, *Nosema apis* (Zander 1909) and *Nosema ceranae* (Fries) are considered as dangerous pathogens of honey bees (Paxton 2010). *Nosema bombi* (Kudo) is pathogenic on bumble bees (Camerona et al. 2011). However, some species of Microsporidia — e.g. *Paranosema locustae* (Canning), *Vavraia culicis* (Weiser), *Nosema pyrausta* (Paillot), *N. portugal* and *Endoreticulatus* sp. — have a documented pathogenicity and regulate the population density of several different insect pests. These issue revealed a critical need to concentrate research projects on this group of natural enemies (Lewis et al. 2009).

In most species, infection will start by ingestion of spores during feeding activity of susceptible hosts. In the next step, ingested spores are activated in the host alimentary track and for this, several factors such as gut pH and ions (or their combination) play a main activation role (Keohane and Weiss 1998). With germination of activated spores, polar filaments are extruded and extend rapidly from the swollen spores. The emerged filament penetrates into the host cell and then, all the cellular content of the microsporidian spore (nucleus, membranes, and etc.) are injected into the cytoplasm of the host cells (Williams and Keeling 2005) (Fig. 9.5). After this stage, being deprived of mitochondria, the microsporidia vegetative stage utilizes

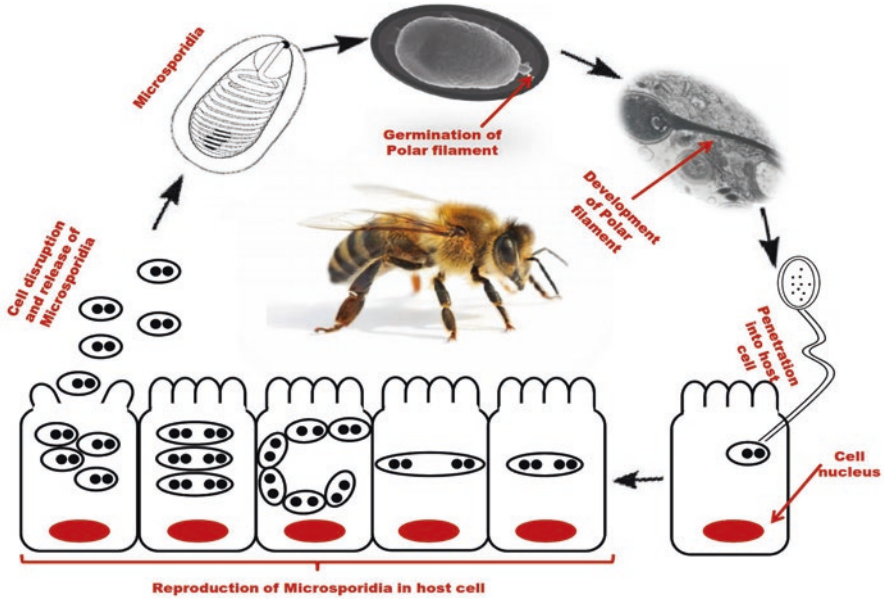

Fig. 9.5 Life cycle and infection mechanism of *Nosema apis* on *Apis mellifera*. (Images courtesy of ....)

adenosine triphosphate (ATP) from its host cells as an energy source (Keeling et al. 2010). However, through an unknown mechanism, some species could infect adjacent cells and tissues by moving from infected to healthy cells.

9.3.3.2 Bacteria

Bacteria are among the first life forms that appeared on earth. They consist of a widely diversified group of prokaryotic (deprived of a nuleus) microorganisms with different shapes, mostly spherical (cocci), rod-shaped (bacilli) or spiral (spirochaetes). Advances in microscopy during late 19th and early 20th centuries significantly increased knowledge also on the entomopathogenic bacteria. The Japanese scientist Shigetane Ishiwata conducted the first investigations on silkworm, *B. mori*, to resolve the problem known as the "*sotto-byo-kin*" disease of larvae (Aizawa 2001). Finally, his researches led to the identification of a spore-forming bacterium called *Bacillus sotto*. In 1909, German scientist Ernest Berliner found a similar case on the Mediterranean flour moth, *Anagasta kuehniella* Zeller, larvae and named the bacterium as *B. thuringiensis*. However, performed studies by another German scientist, Mattes in 1927 eventually led to the first commercial formulation of this bacterium in 1938 (Milner 1994). Currently, biopesticides with bacterial metabolites and Bt-crops which express insecticidal toxins of *B. thuringiensis* in their tissues are commonly used strategies in integrated management of insect pests.

To start its pathogenic activity, bacteria enter their host body from different routes. Although ingestion during host feeding activity is considered as the main pathway, bacteria could also infect their host from their integument, respiratory system and eggs. After ingestion, bacteria disrupt midgut epithelial cells and spread into haemolymph. In the following stage they cause bacteremia (without producing toxins and harmful factors) or septicemia (release of toxins together with bacteria reproduction). Finally, entomopathogenic bacteria kill their hosts and external symptoms appear (tissue necrosis, color changes, soft and flaccid tissues).

Entomopathogenic bacteria are classified in the groups of true bacteria (Eubacteria). Considering the presence and structure of cell walls, they are classified into three major divisions including Firmicutes, Gracilicutes and Tenericutes (Gram-positive and Gram-negative cell walls, and without a cell wall, respectively) (Jurat-Fuentes and Jackson 2012). Binary division is the usual strategy for reproduction, in which clonal copies of mother cells are produced as daughter cells. However, these organisms are present in different habitats including soil, water, acidic hot springs, deep parts of Earth's crust and even radioactive waste (Fredrickson et al. 2004).

9.3.3.3 Viruses

Also the development of insect virology is related to the silkworm industry. In fact, signs and symptoms of infected insects, caused by entomopathogenic viruses, were described by early researchers (Merian 1679; Nysten 1808). However, the study of "melting" disease in the caterpillars of silkworm resulted in the identification of refractive crystal-like bodies in the cells of infected individuals only in recent times. This was the first finding about what we actually know as *Nucleo Polyhedral Viruses* (NPVs). After this discovery, several researchers continued their studies on entomopathogenic viruses (Bergold 1947). By using an electronic microscopes, the first electron micrograph of NPVs was published by Bergold (1947).

Viruses have no free-living lifestyle and therefore, cannot be classified as true living organisms. In addition, obligate parasitism forces them to depend on the host cells for crucial physiological functions such as reproduction. Shape and size of viruses differ regarding the arrangement of their genomic and protein structures. Entomopathogenic organisms show different shapes such as rods or spheres (Rogers 2011).

Entomopathogenic viruses (Alphanodaviruses, Dicistroviruses, Flaviviruses, Iflaviruses, Tetraviruses, Cypoviruses etc.) have been reported form different insect orders such as Coleoptera, Hymenoptera, Lepidoptera, Orthoptera and Diptera (Murphy et al. 1995; Chen et al. 2012). Viral genomes (DNA or RNA) represent the most important component which conducts the infection process. Similar to entomopathogenic bacteria, infection usually began when viral occlusion bodies are ingested by susceptible hosts. After entrance, the alkaline pH of midgut environment provides a suitable conditions for the ingested bodies. Afterward, the viral genome translocates to the nucleus of midgut epithelial cells. In the next step, the basement lamina cells surrounding the tracheal system are infected and then the

infection spreads into other tissues. Entomopathogenic viruses show favourable traits, such as the narrow specificity and host range, a considerable environmental safety, a reliable virulence to target organisms, and the rapid spread of epizootics in the field conditions. These group of biological agents are hence among the promised natural enemies used for biological control of insect pests and should be considered for designing new and sustainable bio-insecticides (Chen et al. 2012).

9.3.3.4 Nematoda

Another important group of natural enemies is that of entomopathogenic nematodes. For the first time, these agents were described as "worms" on grasshoppers and then reported on bumble bees, ants and other hosts (Gould 1747). Using improved microscopes, morphological attributes were described by Kirby and Spence (1822). Several years later, the first entomopathogenic nematode, *Steinernema kraussei* (Steiner), was extracted from infected sawflies by Steiner (1923). Glaser (1931) could successfully rear *Steinernema glaseri* (Steiner) under laboratory conditions. Among different species of nematodes which are associated with insects, seven families including Sphaerularidae, Neotylenchidae, Mermithidae, Allantonematidae, Rhabditidae, Steinernematidae and Heterorhabditidae, attained more considerations (Kaya and Stock 1997; Lacey et al. 2001; Grewal et al. 2005). The majority of species used in biological programs belong to the two families Steinernematidae and Heterorhabditidae (Lewis and Clarkey 2012).

After arthropoda, the members of Phylum Nematoda show a huge diversity of habitats, in comparison with other groups of animals (Tanada and Kaya 1993). Their dependency on water is noticeable and approximately all species require this vital element for reproduction. Life style ranges from free-living to facultative or obligate parasites of other animals or plants. Pathogenic activity of some nematodes (Heterorhabditidae, Steinernematidae and some Rhabditidae) on insects is associated to the occurrence of symbiotic bacteria (Lewis and Clarkey 2012). It is documented that symbiotic bacteria from two genera *Xenorhabdus* and *Photorhabdus* have close evolutionary relations, being congruent with the entomopathogenic nematode genera *Steinernema* and *Heterorhabditis,* respectively (Ulug et al. 2014). To initiate a new infection, the nematode infective juveniles (IJs) f search their hosts and penetrate into their body. After entrance, each nematode releases its symbiotic bacteria in the haemocoel, infecting its host. These bacteria multiply and kill the hosts, becoming a food resource for growth and development of the entomopathogenic nematodes, inside the insect cadaver. Most nematodes complete up to three generations in their hosts and then spread to the environment as new IJs (Lewis et al. 2006). Some species are facultative parasites of insects (Phaenopsitylenchidae), whereas others have harmless phoretic relation (e.g., Rhabditidae, Diplogasteridae, Cephalobidae and etc.) (Poinar 1975).

9.3.3.5 Protista

Unlike previously-mentioned groups, these natural enemies have an unicellular organization, being one the most diverse groups of living organisms (Adl et al. 2005; Cavalier-Smith 2010). All species occur in aquatic and semi-aquatic environments and have an endosymbiotic lifestyle (Lange and Lordy 2012). Protista have both sexual and asexual (binary/multiple division) reproduction mechanisms. The relationships between Protista and insects range from mutualism to commensalism and parasitism. In the case of a pathogenic activity, chronic diseases may occur within the host populations (Solter et al. 1997). Accordingly, pathogenic effects on the host population may be unnoticed. Generally, the detection of external symptoms of protistan infections may be difficult. In the case of severe infections, larvae has swollen and show a whitish appearance. Furthermore, infected larvae show abnormal movements. In comparison with other entomopathogens, Protista have a larger size and their detection on cadavers of dead individuals is hence less difficult. With the aid of a light microscope, protistans are visible on special cadaver tissues, especially the midgut epithelial cells and the malpighian tubules. After infection, the reproductive phase occurs, during which resistant spores are produced to originate new infection cycles.

In this group, several taxa such as Amoebozoa, Apicomplexa (Eugregarinorida, Neogregarinorida, Coccidia), Ciliophora, Euglenozoa and Helicosporidia exhibit considerable insecticidal activity. Their potential could be trusted in management programs of different insect pests.

9.4 Integrated Biological Control and Effectiveness of Biological Control Programs

In insect pests management programs, integration of compatible strategies is one of the most reliable solutions to enhance effectiveness of control efforts (Fathipour and Sedaratian 2013). Current opinions should be revised and new approaches must be designed, to achieve the highest efficiency, due to the diversity and reproductive potential of insect populations. A review of literatures showed that the success of biological programs is affected by different factors, and that the final output may be lower than the desired expectations. Gurr and Wratten (1999) stated that among the performed classical biocontrol programs, the success rate was very low (about 10%), a disappointing statement. These researchers argued that one of the most important reasons which negatively affect the final goal of such programs is caused by ignoring the requirements of natural enemies. However, to maximize the benefits and increase the effectiveness of biocontrol programs, an attitude change is needed to achieve an “integrated biological control” (IBC) that could serve as a promised tool. To date, this term has been used to describe different types of integration. Barbagallo et al. (1982) used this term for a situation in which several natural

enemies were released into a specific agro-ecosystem to suppress populations of multiple pests. Sher and Parrella (1996) described under this term the intentional application of more than one natural enemy to control a target pest. In another statement, Gurr et al. (1998) used this term for combined application of different approaches of biological control (classic, conservation and augmentation). Gurr and Wratten (1999) indicated that the potential of classical biological control could be completely attained when basic requirements of biocontrol agents are supplied. These include nectar and pollen (Jervis et al. 1996; Riahi et al. 2016; Khanamani et al. 2017), moderated microclimate (Thomas et al. 1992) and alternative host/prey (Perrin 1975), supplied via habitat manipulation (conservation). Accordingly, they define IBC as a coupled usage of both classical and conservation strategies. Furthermore, they stated that this approach not only increase the effectiveness of native agents, but also has confirmed impacts on exotic natural enemies. However, to achieve the highest efficiency in biological programs, IBC is inevitable. This strategy could in fact reveal the actual capacity of natural enemies for regulating the density of target organisms, and plays a critical role for success implementation of biocontrol programs in future years.

9.5 Simultaneous Applications of Entomopathogens and Insect Predators/Parasitoids in IPM

Deleterious effects of chemical pesticides, used against phytophagous pests, changed our mind in pest management and elicited increasing demands for safe alternatives such as IPM programs (Kogan 1998). In modern agriculture, IPM is the main strategy for managing pest populations. As the most practicable and acceptable procedure, this strategy also appeared as the best solution to minimize undesirable effects of chemical pesticides and reach a sustainable agriculture (Fathipour and Sedaratian 2013).

In IPM programs different compatible strategies such as chemical, cultural, mechanical, physical and interference tactics, as well as biological methods, may be applied to regulate population density of herbivorous mites and insects (Metcalf and Luckmann 1994). Biological control is one of the most promised components and in some circumstances it may be considered as a cornerstone. However, limitations exist in natural conditions of agro-ecosystems, as biocontrol agents alone are often unable to minimize the population density of a target organism. To increase effectiveness of biological programs, integrated usage of natural enemies offer higher reliable options, as shown by numerous research works.

The effects of the entomopathogenic bacterium *B. thuringiensis* on biological performance of *Rogas lymantriae* Watanabe, during integrated biocontrol of *Lymantria dispar* (L.), was investigated by Wallner et al. (1983). Hilbeck et al. (1998b) found that *B. thuringiensis* has negative effects on survivorship and development of *Chrysoperla carnea* (Stephens), whose larvae are predators of aphids. Synergistic interactions between *B. thuringiensis* and *Campoletis chlorideae* Uchida

was documented by Mohan et al. (2008). Carvalho et al. (2012) evaluated interactions between *Podisus nigrispinus* (Dallas), the predatory bug of larval and pupal stages of *Plutella xylostella* (L.), and *B. thuringiensis*. Sedaratian et al. (2014) evaluated possible effects of *B. thuringiensis* on biological performance of *Habrobracon hebetor* (Say) during integrated biological control of *Helicoverpa armigera* (Hubner).

Aqueel and Leather (2013) evaluated integrated biocontrol of aphids by the fungus *Verticillium lecanii* (Zimmerman) and *Harmonia axyridis* (Pallas). Labbe et al. (2009) documented the compatibility of *B. bassiana* with two natural enemies of *Trialeurodes vaporariorum* Westwood (the predatory bug *Dicyphus hesperus* Knight and the parasitoid wasp *Encarsia formosa* Gahan). Mahdavi et al. (2013) argued that the two entomopathognic fungi *B. bassiana* and *M. anisopliae* had little negative effects on biological efficacy of *H. hebetor*. Effects of *B. bassiana* and *Metarhizium brunneum* Petch on oviposition behavior of the parasitoid wasp *Trybliographa rapae* Westwood were analyzed by Rannback et al. (2015). Bayissa et al. (2016) revealed that the simultaneous application of *M. anisopliae* and predatory ladybird *Cheilomenes lunata* (F.) could enhance the biocontrol efficiency of different aphids on crucifers and okra. The combined application of *Lecanicillium muscarium* (Petch) and the two-spotted ladybird, *Adalia bipunctata* (L.), for integrated biological control of black bean aphid, *Aphis fabae* Scopoli, was investigated by Mohammed (2018).

In the case of other entomopathogens, Murray et al. (1995) evaluated interactions between nuclear polyhedrosis virus (NPV) and larval stages of three solitary endoparasitoids *Hyposoter didymator* Thunberg, *Cotesia kazak* (Telenga) and *Microplitis demolitor* Wilkinson in biocontrol program of *H. armigera*. Furthermore, possible effects of NPV on the parasitoid wasp *H. hebetor* were studied by Stoianova (2007).

For integrated biological control of *Plodia interpunctella* Hubner, compatibility of entomopathogenic nematode, *Heterorhabditis indica* Poinar, Karunakar, and David with *H. hebetor* for coupled application was evaluated by Mbata and Shapiro-Ilan (2010). Atwa et al. (2013) assessed interactions of the koinobiont parasitoid *Microplitis rufiventris* Kokujev and two pathogenic nematodes *Steinernema carpocapsae* (Weiser) and *H. bacteriophora* (Poinar) during a biocontrol program of *Spodoptera littoralis* (Spodli). Effects of *Heterorhabditis amazonensis* Andaló, Nguyen and Moino on the predatory beetle *Calosoma granulatum* Perty, both natural enemies of *Spodoptera frugiperda* (J. E. Smith), was estimated under laboratory condition (Mertz et al. 2015).

Microsporidia also affect biological traits of natural enemies. Possible interactions between *Vairimorpha* sp. and *Trichogramma chilonis* Ishii in their simultaneous application for biological control of *P. xylostella* was studied by Schuld et al. (1999). Other authors examined combination of microsporidian entomopathogens with parasitoid wasps *Macrocentrus grandii* Goidanich (Andreadis 1980) and *Pediobius foveolatus* (Crawford) (Own and Brooks 1986).

9.6 Effects of Entomopathogens on Predators/Parasitoids

One of the most interesting combinations in IBC is simultaneous application of entomopathogens and insect predators/parasitoids. Chandler et al. (2011) stated that in situations in which other natural enemies are unavailable or have no desirable efficiency, entomopathogens could act as a reliable alternative or back-up strategy. In such circumstances different direct and indirect interactions (synergistic, antagonistic and additive) could occurr and any unpredictable outcome may also be expected (Goettel et al. 2010). Accordingly, as first step, compatibility of entomopathogens with other natural enemies (especially predators and parasitoids) should be carefully monitored as well as their possible side effects on non-target organisms including pollinators, birds, mammals, fishes etc.. In extensive applications of microbial products or wide-spread use of broad spectrum entomopathogens, such interactions were frequently observed (Zimmermann 2007). Safety of entomopathogens is crucial for other natural enemies which persist on the host plants during the cropping cycle (from planting to harvest), to minimize negative effects on their efficiency.

9.6.1 Top-Down Effects of Entomopathogens and Biological Alternations in Predators/Parasitoids

In some circumstances entomopathogens have top-down effects on predators/parasitoids as fourth trophic level. In fact, pathogenicity of these microorganisms on predators/parasitoids has different consequences and affects some key biological traits such as mortality, developmental stages, fecundity, sex ratio etc. In the following, some of the most important top-down effects of entomopathogens on predators/parasitoids are discussed. Investigating different aspects of such effects is very important, and should be emphasized for future studies.

9.6.1.1 Mortality

In some situations, widespread use of entomopathogens for managing pest populations may affect non-target organisms present in the same agro-ecosystem (Oluwafemi et al. 2009). Sedaratian et al. (2014) showed that, during integrated management of *H. armigera* by *B. thuringiensis* and *H. hebetor*, this microbial antagonist negatively decreased survivorship of *H. hebetor*. Adverse effects of *B. thuringiensis* on other two bracon wasps, *Bracon instabilis* Marsh and *Apanteles litae* Nixon, was reported by Salama et al. (1996) during the integrated management of *Phthorimaea operculella* (Zeller). Similar deleterious effects of *B. thuringiensis* were mentioned on the parasitoid wasp *Meteorus pulchricornis* (Wesmael), a biocontrol agent of *H. armigera* (Walker et al. 2007). In another case, combination of

this entomopathogen and *H. hebetor* to manage *P. interpunctella* seriously increased mortality of the parasitoid wasp (Oluwafemi et al. 2009). The same results were reported in the case of other organisms. For example, Ulug et al. (2014) stated that when predators consumed infective juvenile of entomopathogenic nematodes, severe infection could be detected in their populations. Similarly, Mertz et al. (2015) showed that when the larvae of the carabid beetle *C. granulatum* consumed infected larvae of *S. frugiperda* with entomopathogenic nematodes, a severe mortality occurred 6 days after feeding.

Studies on entomopathogenic fungi showed different outputs.. Ekesi et al. (1999) showed that one of the most important entomopathogenic fungi, *M. anisopliae*, had no adverse effects on populations of non-target organisms. is. Jacobson et al. (2001) revealed that *B. bassiana*, another entomopathogenic fungus applied for biological control of arthropod pests, had no significant effects on mortality of different life-stages of the predatory mite *Neoseiulus cucumeris* (Oedemans). Effect of this pathogenic fungus on several non-target organisms was, however, documented by Ludwig and Oetting (2001). Effects of different *B. bassiana* strains with considerable virulence on five phytoseiid mites (*N. cucumeris*, *N. californicus* (McGregor), *N. womersleyi* Xin, Liang and Ke, *Phytoseiulus persimilis* Athias-Henriot and *Amblyseius swirskii* Athias-Henriot) were evaluated by Wu et al. (2016). Results revealed that the strains tested had no pathogenecity on predatory mites and no significant mortality was recorded. Shipp et al. (2012) described that *B. bassiana* (GHA isolate) had serious negative effects on a population of the predatory bug *Orius* sp. Their results revealed that toxicity of *B. bassiana* is related to experimental conditions, as the tests performed showed a laboratory mortality higher than that observed in greenhouse conditions. Hajek and Goettel (2000) and Jaronski et al. (2003) stated that entomopathogenic fungi have wider host ranges under laboratory conditions. This issue was addressed as differences between physiological (under laboratory conditions) and ecological (in nature) host ranges (Hajek and Butler 2000). In fact, microorganisms with pathogenic activity on non-target organisms under laboratory conditions may have no infections on the same organisms in nature.

9.6.1.2 Duration of Different Life Stages

In IBC of *H. armigera* using *B. thuringiensis* and *H. hebetor*, the entomopathogenic bacterium prolonged immature development of the parasitoid wasp (Sedaratian et al. 2014). Bernal et al. (2002) observed similar findings in *Parallorhogas pyralophagus* (Marsh), a parasitoid wasp of *Eoreuma loftini* (Dyar). Similar results were reported when studying the parasitoid wasp *M. rufiventris* females developed on infected larvae of *S. littoralis* (El-Maghraby et al. 1988). Such adverse effects on growth of the parasitoid wasp *Microplitis croceipes* (Cresson) were also described by Blumberg et al. (1997).

When *M. anisopliae* (isolate M14) was applied together with *H. hebetor* to manage a population of *H. armigera*, effects on larval development prolongation were recorded and confirmed (Jarrahi and Safavi 2016). Prolonged pupal development

was observed in the parasitoid wasp *Aphidius matricariae* Haliday when developed on aphids treated with *M. anisopliae* (Rashki et al. 2009). However, in contrast with these observations, Fatiha et al. (2008) stated that *V. lecani* had no significant effect on development of the coccinelid beetle *Seranjium japonicum* Chapin. Murray et al. (1995) showed that entomopathogenic viruses (NPVs) have negative effects on larval development of three parasitoids of *H. armigera*. These researchers suggest that a time interval of at least 3 days is required between parasitization and NPV exposure to minimize such adverse effects. A similar time interval was proposed by Brown et al. (1989) to minimize NPV effects on development of the parasitoid wasp *Glabromicroplitis croceipes* (Cresson) applied for IBC of *Heliothis virescens* (F.).

In earlier study, Huger and Neuffer (1978) found a prolonged adult longevity of the braconid wasp *Ascogaster quadridentata* Wesmael when its host was infected by *Nosema carpocapsae*. Futerman et al. (2006) showed that development of the parasitoid wasp *Asobara tabida* Nees within hosts infected by the microsporidian *Tubulinosema kingi* Kramer prolonged its development. Data reported by Simoes et al. (2012) showed that immature development of *Cotesia flavipes* (Cameron) increased and its adult longevity was decreased when the parasitoids developed inside hosts infected by *Nosema* sp. Hoch et al. (2000) reported that the duration of the larval period of the parasitoid wasp *Glyptapanteles liparidis* (Bouche) was prolonged on infected larvae of *L. dispar*, when the latter were infected by the microsporidian *Vairimorpha disparis*. In another study, Hoch et al. (2002) described that infection of *L. dispar* larvae by *V. disparis* changed its carbohydrate and fatty acid contents, reducing the host nutritional quality for normal development of *G. liparidis*. In another study, effects of the microsporidia *Nosema adaliae* (Steele and Bjornson) and *Tubulinosema hippodamiae* (syn. *Hippodamia convergens* Guérin-Méneville) on development of two-spotted ladybird, *A. bipunctata*, were described by Steele and Bjornson (2014) under laboratory conditions. Results confirmed extension of larval development on preys infected by *N. adaliae*, but the other pathogen had no significant effects on the duration of life stage. Furthermore, comparison of pteromalid wasp *Muscidifurax raptor* Girault and Sanders infected and uninfected by *Nosema muscidifuracis* (Becnel and Geden) confirmed that this microsporidian prolonged the parasitoid development (Geden et al. 1995). Godfray (1994) noticed that nutritional quality of the parasitoid hosts has confirmed effects on its development. Similarly, Murugan et al. (2000) and Mohan et al. (2008) stated that induced changes in parasitoid hosts after ingestion of pathogenic microorganisms may influence the development and foraging of their parasitoids.

9.6.1.3 Fecundity

In addition to developmental periods and mortality, another direct effect of entomopathogens on insect predators/parasitoids is their possible effects on fecundity (Nielsen et al. 2005). It is documented that *Nosema bordati* Goudegnon could significantly reduce fecundity of *C. flavipes* when simultaneously applied for managing *Chilo partellus* Swinhoe (Bordat et al. 1994). Simoes et al. (2012) evaluated

possible effects of *Nosema* sp. extracted from the sugarcane borer, *Diatraea saccharalis* (Fabricius), on biological performance of theparasitoid. Their results revealed negative effects on potential of progeny parasitoid production. Geden et al. (1995) compared fecundity of the pteromalid wasp *M. raptor*, treated and untreated with *N. muscidifuracis*. Their data revealed that infection dramatically decreased the reproductive potential of this parasitoid. Jarrahi and Safavi (2016) described interactions between *H. hebetor* and *M. anisopliae* during management program of *H. armigera* confirming that the parasitoid wasp had significantly lower daily and total fecundity on infected hosts. Negative effects of *B. thuringiensis* on fecundity of *H. hebetor* were reported by Sedaratian et al. (2014). The same results were reported by other researchers (Baur and Boethel 2003; Sanders et al. 2007; Sharma et al. 2008).

However, a reduction of fecundity could be related to several factors. Roy and Pell (2000) described that fungal infection affects physiological functions of female parasitoids and this issue could directly affects their fertilization rate. Another possible reason for fecundity reduction in population of natural enemies is septicemia (Sedarataian et al. 2014). On the other hand, in circumstances in which microbial products are commercially used in large scale, other formulation components may have unknown effects on fecundity of predators/parasitoids (Flexner et al. 1986; Teera-Arunsiri et al. 2003).

9.6.1.4 Sex Ratio

One of the most important indirect effects of entomopathogens on predators/parasitoids populations is their possible impact on the sex ratio (ratio of male to female offspring) especially in the case of parasitoid wasps where the haplo-diploid mechanism allows female individuals to determine the offspring sex ratio. Considering the polygamic behavior of male individuals (fertilization of different females by one male), an increase in female progeny is so beneficial for biological control purposes and enhances the final efficiency of these programs. Different elements such as genetic factors, female wasp density, age of female and male parents, extreme temperature, relative humidity, photoperiod, host size, density, age and sex, as well as its nutritional quality could affect sex ratio of natural enemies (Legner and Badgley 1982; Kido et al. 1983; Morse 1994). Prior to oviposition, female individuals evaluate nutritional quality of their preys/hosts and then selectively decide to deposit female or male eggs. Undoubtedly, entomopathogenic agents have several effects on their hosts including reduction in size and nutritional quality and this issue could affects sex ratio of their natural enemies. However, when natural enemies detect favorable conditions, they alter their sex ratio to female-biased offsprings, in order to build up the future population (Kant et al. 2012).

It is documented that larvae of *H. armigera* infected by *B. thuringiensis* have no significant effects on offspring sex ratio of *H. hebetor* (Sedaratian et al. 2014). Similar outputs were reported by Sharma et al. (2008) when evaluating the effects of this bacterium on the sex ratio of the parasitoid wasp *C. chlorideae*. Mohammed

and Hatcher (2017) investigated sex ratio of the parasitoid wasp *Aphidius colemani* Viereck on *Myzus persicae* (Sulzer) treated with the pathogenic fungus *L. muscarium*. Results obtained showed that extension of time interval between parasitoid introduction and fungus application strongly changed adverse effects on the parasitoid sex ratio. Accordingly, they revealed that offspring sex ratio was not significantly affected when a time interval of 6–7 days was considered between application of the parasitoid wasp and pathogenic fungus. The number of emerged female faced a significant reduction (40%) when this interval was lower than 5 days. In previous study, Aqueel and Leather (2013) described that *V. lecani* significantly affected the sex ratio of *A. colemani* emerged from treated aphids.

Geden et al. (2002) observed that the sex ratio of *Tachinaephagus zealandicus* Ashmead on hosts infected by *Nosema* sp. was altered favoring the male progeny. During another study, Schuld et al. (1999) showed that ingestion of the microsporidian *Vairimorpha* sp. had no significant effects on sex ratio of the parasitoid wasp *T. chilonis*. Similar to this report, Saleh et al. (1995) explained that *N. pyrausta* did not affect the sex ratio of the parasitoid wasp *Trichogramma nubilale* Ertle and Davis, when developed on infected eggs of *Ostrinia nubilalis* Hubner. Steele and Bjornson (2012) showed that offspring sex ratio in *A. bipuctata* was not affected by the microsporidian *N. adaliae*.

9.6.2 Entomopathogen Effects on Behavioral Characters of Predators/Parasitoids

In addition to biological attributes, entomopathogens could considerably affect behavioral attributes of insect predators/parasitoids. Accordingly, this issue was subjected to different research studies. In this section, some of the most important findings are mentioned.

9.6.2.1 Pathogen Detection Strategy and Avoidance by Insect Predators/Parasitoids

Before oviposition, a female individual (predator/parasitoid) complete a sequence of steps to select the best site for construction of next generation. In the first step, it must find the habitat of its preys/hosts. Then, the female individual locates the preys/hosts in their habitats. Finally, preys/hosts are evaluated by the females to achieve the best decision for oviposition. Vinson (1976) reviewed the process of host assessment by parasitoids and argued that different factors such as size, movement, shape, sound and chemical cues (volatiles), from host feces or injured host plant tissues, were employed for host-selection. Among these factors, the volatiles emitted from host plants or preys/hosts play a key role for detecting infected patches (Afsheen et al. 2008; Nilsson et al. 2011).

However, to minimize any undesirable effect on subsequent generations and maximize immature survivorship, growth, development as well as adult fitness, it is very important that female individuals provide the best food resources. As previously mentioned, pathogenic infections seriously decrease preys/hosts quality with deleterious effects on biological performance of their natural enemies (Mesquita and Lacey 2001). Therefore, the ability of females to discriminate uninfected preys/hosts from infected ones is crucial and is considered as the first defense mechanism of predators/parasitoids against pathogenic infections (Ormond et al. 2011).

Several researchers stated that parasitoid wasps could recognize hosts infected by pathogenic fungi form healthy ones (Fransen and van Lenteren 1993; Mesquita and Lacey 2001). The ability of the tachinid parasitoid *Compsilura concinnata* (Meigen) to discriminate hosts infected with *B. thuringiensis* from healthy larvae was noticed by Erb et al. (2001). Rannback et al. (2015) concluded that when the parasitoid wasp *T. rapae* was exposed to *B. bassiana* and *Metarhizium brunneum* Petch, it could discriminate *M. brunneum*. The predatory ladybird, *C. lunata* does not prefer aphids infected by *M. anisopliae*, and this behavior provides sustainable management on crucifers and okra (Bayissa et al. 2016). Such behavior was observed in *Anthocoris nemorum* (L.) which avoids depositing its eggs on leaves treated with *B. bassiana* to decrease the risk for its progeny (Meyling and Pell 2006). However, in such situations, when predators/parasitoids discriminate infected resources and avoid them, some undesirable effects may also occur. Although avoidance of contaminated area decreases infection risks, Pourian et al. (2011) discussed that this behavior in predatory bugs increased time required for prey searching and dramatically decreases their predation rate and biological efficiency.

On the other hand, some natural enemies could not avoid contaminated preys/hosts. It is documented that the parasitoid wasp *Cephalonomia tarsalis* (Ashmed) equally parasitized hosts, *Oryzaephilus surinamensis* (L.) infected and uninfected by *B. bassiana* (Lord 2001). Hoch et al. (2000) concluded that the braconid wasp *G. liparidis* has the same parasitism rate on hosts healthy or infected by *Vairimorpha* sp. Similarly, *T. nubilale* has no ability to detect eggs infected by *N. pyrausta* from uninfected ones (Saleh et al. 1995). These findings are in agreement with those reported by Geden et al. (1992). Baverstock et al. (2005) showed that *Aphidius ervi* Haliday has no ability to recognize aphids infected by *Pandora neoaphidis* Remaudiere and Hennebert. Fransen and van Lenteren (1993) indicated that *E. formosa* could not distinguish whiteflies infected by entomopathogenic fungi. Mesquita and Lacey (2001) stated such shortcoming in the aphid parasitoid *Aphelinus asychis* Walker. As noticeable point, if natural enemies consume infected preys/hosts, efficiency of entomopathogens may be also moderately decreased (Roy et al. 2008).

9.6.2.2 Possible Effects of Entomopathogens on Foraging Behaviors of Predators/Parasitoids

Different factors (temperature, host plant, pesticide, host/prey attributes, pathogens etc.) could affect biological performance of natural enemies (Wang and Ferro 1998; Moezipour et al. 2008). Such effects are reflected in biological and behavioral changes of natural enemies. Therefore, it is very important to evaluate foraging behaviors of predators/parasitoids when these agents are exposed to infected resources both directly and indirectly. In addition to host preference, entomopathogens could seriously affect other foraging behaviors of predators/parasitoids. Pourian et al. (2011) investigated possible effects of onion thrips, *Thrips tabaci* Lindeman infected by *M. anisopliae*, on some behavioral traits of the anthocorid predatory bug, *Orius albidipennis* Reut, reporting that the searching time on infected preys significantly increased. Furthermore, *O. albidipennis* had a lower feeding time on treated individuals. Negative effects of *M. anisopliae* on the predation rate were also detected.

Alma et al. (2010) reported that when the pathogenic fungus *Isaria fumosorosea* Wize infected immature whitefly stages, the predatory bug *D. hesperus* significantly altered its predation behavior. Similarly, Pell and Vandenberg (2002) revealed that this fungus changed the predation behavior of the predatory ladybird, *H. convergens*. In another case, Sewify and El-Arnaouty (1998) stated that *V. lecanii* dramatically suppressed searching behavior and feeding capacity of the common green lacewing, *C. carnea*.

Belmain et al. (2002) and Sullivan and Berisford (2004) showed that specific cues from pathogenic fungi could act as repellents for phytophagous pests and their natural enemies. Meyling and Pell (2006) found that when *A. nemorum* encountered *B. bassiana*-infected aphids *Acyrthosiphon pisum* (Harris), it changed its predation behavior. These researchers stated that sporulating cadavers of infected hosts have repellent effects on *A. nemorum*.

Attack rates of the parasitoid wasp *A. ervi* was significantly reduced on aphids infected by the pathogenic fungus *P. neoaphidis* (Pope et al. 2002). Similar findings were reported by Baverstock et al. (2005). Another strategy is the rejection of a prey/host. Rejection behavior was observed in some natural enemies. It was observed that the parasitoid wasp *E. formosa* when locating microhabitats, searched its host and rejected those infected by pathogenic fungus *Aschersonia aleyrodis* (Webber), after probing (Fransen and van Lenteren 1993).

Effect of *B. thuringiensis* on functional response of *Trichogramma brassicae* Bezdenko was described by Vaez et al. (2013). Results obtained exhibited that exposure to infected eggs of *H. armigera* had no significant effects on functional response of this wasp. In both infected and uninfected eggs a type III response was recorded. Furthermore, infected eggs increased handling time and decreased searching efficiency of *T. brassicae*. Farrokhi et al. (2010) compared functional response of *T. brassicae* on *Wolbachia*-infected and uninfected hosts. These researchers reported that infection had no significant effects on this behavioral function. In contrast, Dong et al. (2017) studied the functional response of *Trichogramma dendrolimi*

Matsumura on eggs of the Asian corn borer, *Ostrinia furnacalis* Guenée infected and uninfected by *Wolbachia*, at three constant temperatures (20, 25 and 30 °C). Their results revealed that *Wolbachia* sp. could affect functional response of *T. dendrolimi* and its effect was temperature-dependent.

In addition to the above-mentioned alterations, entomophatogenic agents could indirectly affect behavioral attributes of insect predators/parasitoids. Wu et al. (2016) observed that the predatory mite *Neoseiulus barkeri* (Hughes) displayed self-grooming behavior to remove fungal conidia from its body surface. However, although different arthropods exhibit grooming behavior to remove undesirable agents, such as pathogenic conidia and parasitic mites (Farish 1972), Wekesa et al. (2007) explained that this behavior may reduce searching ability and predation rate.

9.6.2.3 Intra-Guild Predation Between Entomopathogens and Predators/Parasitoids

As a crucial point, it is necessary for any agricultural producer to evaluate its cropping system, as concerns how the interacting components formed food/trophic levels (Fig. 9.2). In these systems, natural enemies (predators/parasitoids and pathogens) occupy the highest position (3th level) and can regulate the population of herbivorous organisms (second level) via top-down regulatory efforts, mainly known as biological control. The success rate of biological programs highly depends on intentional manipulation of possible interactions among tri-trophic levels. However, due to lower species diversity, agro-ecosystems provide suitable conditions for such manipulations (Finke and Denno 2004).

One of the most promising procedures to optimize efficiency of biological programs is introducing new beneficial organisms (Stevens and Stuart 2008). Undoubtedly, this process may result in several interferences and cause intra-guild predation (Denno et al. 2008; Ali et al. 2013) which dramatically affects adequate control of herbivores (Rosenheim et al. 1995). Straub et al. (2008) explained that intra-guild interactions could occurr during combined application of at least two natural enemies against the same pest species. Such interactions were frequently detected in biological communities and may be observed when biocontrol agents compete and exploit the same organisms in a similar manner.

Unidirectional intra-guild interactions, i.e. between entomopathogenic fungi and insect predators/parasitoids, are asymmetric, favoing pathogenic agents. In fact, because of their wide host range, these agents may infect different life stages of insect predators/parasitoids and significantly decrease their population levels and efficiency (Brodeur and Rosenheim 2000). Fransen and van Lenteren (1993) recognized that the entomopathogenic fungus *A. aleyrodis* drastically infected the parasitoid wasp *E. formosae*, after contact with parasitized whiteflies.

In addition to contact pathogenicity, ingestion of entomopathogens by predators/parasitoids could amplify such negative effects. Pell et al. (1997) reported feeding activity of coccinellid and carabid beetles on aphids heavily infected by *P. neoaphidis*. In another study, Askary and Brodeur (1999) observed that when larval

parasitoids consumed infected aphid tissues, fungal spores were accidentally ingested. Sedaratian et al. (2014) stated that feeding activity of *H. hebetor* on larvae of *H. armigera* treated with *B. thuringinesis* caused ingestion of the entomopathogenic bacterium. In this scenario, the parasitoid biological performancewas seriously affected.

9.6.3 Other Effects

In addition to the above-mentioned effects, entomopathogens could also directly affect predators/parasitoids. Idris et al. (2001) revealed that when the parasitoid wasp *Diadegma semiclausum* Hellen consume infected larvae of *the* diamondback moth, *Plutella xylostella* infected by microsporidian *Vairimorpha* sp., emerged females have deformed wings. Such individuals faced several difficulties for their flying and searching activities, and were unable to compete with other individuals. Furthermore, results showed that infected parasitoids had smaller size in comparison to healthy ones, affecting the parasitoid fitness. In another study, Hoch et al. (2000) documented that individuals of the parasitoid wasp *G. liparidis*, emerged from host *L. dispar* infected by the microsporidian *V. disparis*, had a smaller size. Additionally, the individuals developed on infected hosts had a lower weigh. A further effect of entomopathogens concerns the egg viability of predators/parasitoids. A study by Pozzebon and Duso (2009) revealed that *B. bassiana* significantly reduced the egg hatching rate in *P. persimilis*.

9.6.3.1 Entomopathogen Effects on Immune System of Phytophagous Pests and Its Impact on Predators/Parasitoids

The insect immune system can suppress undesirable alien factors (fungi, bacteria, viruses, nematodes, protists, endoparasitoids etc.) via two different mechanisms namely humoral and cellular responses. In the humoral mechanism several antimicrobial peptides such as lectins, lysozyme, and attacin are produced and underpin insect fight *vs* introduced agents. Cellular function involves different mechanisms including phagocytosis of introduced materials by hemocytes, nodulation (trapping introduced agents by a net of hemocytes) and encapsulation (surrounding too large materials by thin layers of flatted hemocytes) (Jiravanichpaisal et al. 2006). In the case of nodulation and encapsulation, another reaction usually occurs, which is recognized as melanization. This process involves production of the pigment melanin to construct a hard and impenetrable envelope around alien factors (Cerenius et al. 2008). The role of some enzymes in the melanization process is documented by several researchers. For instance, Popham et al. (2004) stated that higher levels of phenoloxidase in *H. virescence* resulted in a higher degree of melanotic encapsulation of baculovirus-infected cells. It is documented that pathogenic infection engages immune defense of phytophagous insects and alters their vulnerability to

predators/parasitoids. In such situations, they usually try to compensate this shortcoming. Cessation of feeding on contaminated resources has been described as one of the most common responses to increased immune responses in such circumstances (Adamo et al. 2007, 2010).

Insects' immune reactions to entomopathogens affect predators/parasitoids in different manners. Appropriate immune responses could help contaminated individuals to recover from pathogenic infections. The lack of suitable responses or weak reactions will lead to the insects' death or to chronic infections, respectively. Alive individuals with chronic symptoms often have lower quality and could not supply nutritional requirements for growth and development of predators/parasitoids. This issue could indirectly affect biological performance of these beneficial organisms. As previously mentioned, predators/parasitoids, with developed detection and avoidance behaviors, could minimize such adverse complications. Otherwise, their biological performance may severely decrease. Sedaratian et al. (2014) revealed that when the ectoparasitoid wasp *H. hebetor* consumed *Bt*-contaminated food resources, its biological performance was significantly reduced. If contaminated individuals were selected for oviposition by female endoparasitoids, a higher mortality of immature parasitoids was observed (Sanders et al. 2007). In another word, if immune functions of contaminated hosts could not destroy entomopathogens, ingestion of their tissues may negatively affect biological performance of both predators and parasitoids.

Activation of immune responses in sick individuals involves energy consumption that may decrease their defensive power against predators/parasitoids. In such situations, predators/parasitoids will gain higher number of preys/hosts with a lower energy consumption. In the case of endoparasitoids, encapsulation is the most common response of the insect immune system (Blumberg 1997). This mechanism may reduce parasitoid efficiency in biological programs, prevent successful establishment of exotic parasitoids in new regions or disrupt mass rearing efforts. However, if the host immune system is engaged in the suppression of an invasive pathogen, its performance for parasitoid encapsulation will inevitably decrease. This condition may hence increase the biological performance of biological programs. It is noticeable that some parasitoid wasps have a symbiotic mutualism relationship with different microorganisms which protect their immature stages from encapsulation. This mechanism is described in next sections.

9.6.3.2 Effects of Entomopathogens on Physiological Systems of Predators/Parasitoids

Consumption of infected preys/hosts by insect predators/parasitoids has several effects on their physiological functions, especially in the case of endoparasitoids. However, exposure to entomopathogeneic agents could also affect physiological functions of predatory insects. When predators/parasitoids feed on infected haemolymph and tissues of preys/hosts, a variety of unexpected outcomes may be expected (Futerman et al. 2005). Pathogenic effects on reproductive, digestive and immune

systems of predators/parasitoids are the most important physiological involvements occurring in these natural enemies after infection.

Infection of reproductive system may result in vertical transmission of entomopathogens to subsequent generation (Mazzone 1985). It was observed that the parasitoid wasp *M. grandii*, when developing into *O. nubilalis* hosts infected by *N. pyrausta*, transmitted the entomopathogenic microsporidian to its offspring (Siegel et al. 1986). Brooks (1973) stated that some parasitoids were susceptible to the microsporidian pathogens attacking their hosts. In another study, Roy et al. (2006) showed that ingestion of pathogenic fungi significantly decreased fecundity of natural enemies. Consumption of infected preys with low nutrition quality caused detectable reduction in reproductive performance (Pozzebon and Duso 2009). In fact, such food resources could not provide the nutrients required for egg production and this issue disrupts the physiological functions of the reproductive system. Pozzebon and Duso (2009) showed that activity of the predatory mite, *P. persimilis*, on *Tetranychus urticae* Koch treated with *B. bassiana*, dramatically reduced its ability for egg production. Furthermore, the number of fertile eggs was also affected. One of the possible reasons for reduction of egg production is resources diverting from the reproductive to the immune system. In fact, to minimize mortality, also the natural enemies consume their energy resources for defense mechanisms. Seiedy et al. (2012) reported that ingestion of preys infected by *B. bassiana* seriously affected the fecundity of *P. persimilis*. These researchers assumed that the activation of the immune system and the production of secondary metabolites for suppressing aggressive agents significantly disrupted the reproductive system of the predatory mite.

In addition to reproductive and immune systems, the digestive canal, which has a vital functions in supplying required energy for growth and development of predators/parasitoids, could also affected. Moawed et al. (1997) showed that negative effects of microsporidan on endoparasitoids include the disruption of the nutritional balance in the digestive canal of parasitoid larvae, due to direct infection or aggression of undigested spores. Furthermore, this accumulation significantly decreased available space for food storage (Saleh et al. 1995). Schuld et al. (1999) showed that during feeding activity of *T. chilonis* larvae on larvae of *P. xylostella* treated with *Vairimorpha* sp., the microsporidian was detectable in the parasitoid intestinal lumen 3 days after parasitization, and then was dispersed to other tissues including flight muscles and the nervous system.

9.6.3.3 Catastrophic Synchronization Caused by Entomopathogens and Impact on Predators/Parasitoids

For the first time, the hypothesis of "catastrophic synchronization" was proposed by Godfray and Chan (1990) as an unusual output of extensive application of chemical pesticides. In fact, these researchers illustrated a specific scenario in which the population of a target organisms is synchronized at a particular stage after pesticide application. As a result, synchronized populations interrupt the biological

performance of insect predators/parasitoids that are active on other life stages of the target pests, and require food resources for their growth and development. Catastrophic synchronization shifts the multiple structure of a pest population towards a single stage one. Thus the natural enemies (especially predators/parasitoids) encounter undesirable conditions. In such situations, pest resurgence may occur as a result of unavailability of preferred stages for biological activities of predators/parasitoids. Furthermore, predators/parasitoids may reduce their reproduction potential, migrate from such environment or tolerate starvation. Pest resurgence from catastrophic synchronization was reported for coconut (Perera et al. 1988) and coffee (Waage 1989) pests.

However, although no documented information is available regarding synchronization induced in pest populations structure by entomopathogens, more attentions should be devoted to investigate this hypothesis, especially in the case of extensive application of entomopathogens in agro-ecosystems. This is especially important for large scale application of commercial formulations of entomopathogens or genetically modified host plants. Data by Sedaratian et al. (2013) revealed that commercial formulation of *B. thuringiensis* had more toxicity to first instars of *H. armigera*, whereas last instars had a relative resistance to the bacterium. Accordingly, long time application of such formulations could induce a synchronized structure in target host populations and negatively affect natural enemies such as the green lacewing, *C. carnea*, which feeds on first instars of *H. armigera*. In another case, entomopathogenic nematodes could be candidate. As previously mentioned, this group of entomopathogens infects the insect life stages in soil. With increasing population density of pathogenic nematodes, the number of infected pests in soil will increase and this issue could synchronize other stages of the pests.

9.7 Application Management of Entomopathogens Increase Their Compatibility with Predators/Parasitoids

Simultaneous application of different natural enemies is inevitable in IBC programs. As previously mentioned, synchronized application of entomopathogenic agents and insect predators/parasitoids may also have some negative outcomes on biological performance of these natural enemies. Therefore, it is very important to fully investigate different aspects of such integrations and reduce potential negative effects. One of the most reliable strategies to increase biological safety of entomopathogenic agents is their application management in agro-ecosystems, where other beneficial agents such as insect predators/parasitoids coexist. Such efforts attempt to minimize direct contact of these microbial agents with predators/parasitoids.

9.7.1 *Importance of Monitoring Population Fluctuations of Phytophagous Pests*

In modern agriculture, all control efforts must be applied at their appropriate time. In fact, ancient attitudes for calendar-based application of control strategies was changed in favor of need-base application. For this, we designed a monitoring schedule to attentively check all biological activities and population fluctuations of phytophagous hosts/preys and their natural enemies. Such monitoring activities enable agricultural producers to make accurate decisions, selecting the best strategy in an appropriate time. However, economic criteria play a basic role for implementation of control strategies in IPM (Fig. 9.2). Accordingly, each strategy is only applied when the highest performance is achieved.

Data collected during monitoring activities enable pest managers to consider a reasonable time period between intentional application of entomopathogens and release programs of insect predators/parasitoids. This period of time may considerably decrease the overall adverse effects of entomopathogens on biological performance of predators/parasitoids (Fransen and van Lenteren 1993). Furthermore, sampling target organisms during monitoring activities may reveal the level of naturally occurring infections with pathogenic agents. Consequently, when naturally infections in population of target organisms are considerable, the release of insect predators/parasitoids is not a good idea. On the other hand, if monitoring efforts revealed noticeable activities of predators/parasitoids, it is better to avoid intentional application of entomopathogens which have the same ecological niche. Such findings will help in accurate decision-making, in order to minimize direct contaminations of predators/parasitoids with pathogenic agents (Jacobson et al. 2001).

9.7.2 *Genetically Modified Plants and Their Effects on Predators/Parasitoids*

Genetically modified plants which express *B. thuringiensis* toxins in their tissues (Bt-crops) offer a reliable tool for suppressing pest populations in intensive agroecosystems, and their applications reduce pesticide usage (Lovei and Arpaia 2005). Tobacco and tomato were the first transgenic plants which express insecticidal Bt delta-endotoxins (van Frankenhuyzen 1993). Currently, these manipulated crops (tomato, cotton, potato, maize, rice and etc.) are commercially cultivated in different countries such as United States, Canada, Japan, Mexico, Argentina and Australia (Frutos et al. 1999).

O'Callaghan et al. (2005) described that one of the main benefits of Bt-crops is their insecticidal specificity. In contrast with chemical pesticides, these crops only affect target organisms. However, although Bt-crops significantly decrease pesticide

usage in agro-ecosystems, their possible effects on non-target organisms such as insect predators/parasitoids is a main, global concern. Different researchers showed that insect predators/parasitoids may receive Bt-toxins from infected preys/hosts (Obrist et al. 2006). However, researches on possible effects of Bt-crops on insect predators/parasitoids reported different outputs. Torres and Ruberson (2006) showed that Bt-cotton expressing the Cry1Ac toxin had no detectable effects on the predatory bug *Podisus maculiventris* (Say). In another study, the same findings were reported when *O. insidiosus* consumed Bt-treated preys (Al-Deeb et al. 2001). In contrast, it was observed that Bt-cotton containing the Cry1Ac toxin significantly affected survivorship of two predatory bugs *Geocoris punctipes* (Say) and *Orius tristicolor* (White) (Ponsard et al. 2002). Hilbeck et al. (1998a) found that predatory lacewing, *C. carnea*, had higher mortality and lower development rates when preys reared on Bt-crops were consumed. However, Lovvei et al. (2009) stated that insect parasitoids have more sensitivity to Cry toxins than predators. Candolfi et al. (2004) compared a population of the parasitoid wasp *Macrocentrus cingulum* Brischke in two Bt and conventional corn fields, and showed that the parasitoid had lower biological activity in the Bt-corn field. In another study, Xia et al. (1999) stated that specialist parasitoids that parasitized *H. armigera* had a lower population density in Bt-cotton fields. However, although some findings revealed that insect predators/parasitoids had lower biological activity on transgenic plants, Romeis et al. (2004) indicated that such results reflect adverse effects of feeding activity of predators/parasitoids on food resources with lower nutritional quality and were not directly related to Bt transgenic crop. On the other hand, it should be noticed that alternative food resources are more available in field conditions for insect predators/parasitoids, and this issue minimizes the adverse effects of Bt-toxins.

9.7.3 Microbial Biopesticides

Increased global demands for widespread application of entomopathogens has resulted in manufacturing commercial formulations of these microorganisms, commonly indicated as biopesticides. It is noticeable that the majority of these pathogen-based bioinsecticides was assigned to entomopathogenic bacterium *B. thuringiensis*. Koul and Dhaliwal (2002) described that commercial formulations of *B. thuringiensis* have lower undesirable effects on insect predators/parasitoids than chemical insecticides. Although these products may contain microorganisms, their metabolites or combination of both elements, only products with living organisms may be considered in biological control efforts. Considering several benefits, one of the main advantages of biopesticides is their ecological selectivity for non-target organisms. In fact, regarding monitoring outcomes, pest managers should use these products when populations of other natural enemies, especially insect predators/parasitoids, have low density, in order to minimize any adverse effects on their performance.

Among different microbial bioinsecticides, commercial formulations of entomopathogenic viruses have the lowest negative effects on insect predators/parasitoids. In the case of pathogenic viruses, commercial formulations only contain members from family Baculoviridae. Since this family has a narrow host range and its pathogenic activity is recorded on specific insects, extensive application as commercial biopesticides has the lowest negative effects on non-target organisms (Cory and Myers 2003).

de Faria and Wraight (2007) stated that over 120 fungal formulations were globally applied in management programs of different insect pests. However, most of these mycopesticide products are based on spores of *B. bassiana*, *M. anisopliae*, *I. fumosorosea*, *L. longisporum*, *L. muscarium* and *Hirsutella thompsonii* Fisher (Jaronski 2010). The wide host range of entomopathogenic fungi suggests caution when applying them in agro-ecosystems.

9.8 Changes in Environmental Conditions Alter Entomopathogen Effects on Predators/Parasitoids

Similar to all living organisms, biological activities of entomopathogens are completely dependent on environmental conditions (abiotic factors). Accordingly, unfavorable conditions significantly reduce pathogenicity of these agents. Consequently, in IBC programs, if intentional application of insect predators/parasitoids is performed when environmental conditions are unfavorable or sub-optimal, possible intra-guild interactions could be minimized.

As we know, entomopathogenic agents are a diverse group of natural enemies which have different environmental requirements. For example, high doses of ultra violet rays (UV) in field conditions negatively affects pathogenicity of *B. thuringiensis* (Sedaratian et al. 2013). Furthermore, other environmental factors, including temperature and rainfall, could affect residual life of this pathogenic agent (Frye et al. 1973; Salama et al. 1983; Pedersen et al. 1997). Soil moisture has critical impact on biological activities of entomopathogenic nematodes and dried conditions may cause significant deleterious effects on their performance. Besides, application of chemical pesticides and fertilizers in soil environments could have negative effects on biological performance of these biocontrol agents. Such conditions minimize their possible effects on target pest populations, as well as affecting other beneficial agents i.e. the predatory beetles from family Carabidae.

Among different environmental factors, relative humidity has considerable effects on biological performance of entomopathogenic fungi. Under low humidity conditions, germination of infective spores of pathogenic fungi seriously decreased, drastically suppressing fungal epizootics. In addition to relative humidity, other abiotic factors such as temperature, rain, and sunlight could also affect these fungi (Jaronski 2010). However, such limitations may hinder desirable delivery of lethal effects of entomopathogenic agents, with significant restrictions in their

pathogenicity, both on target and non-target organisms. These informations could help pest managers to manipulate negative interactions between entomopathogenic agents and insect predators/parasitoids, in order to enhance biological efficiency of IBC programs.

9.9 Symbiotic Interactions Between Entomopathogens and Insect Predators/Parasitoids

As previously stated, biological performance of predators/parasitoids may be adversely affected by the defense mechanisms of the target pests. This issue is evident in the case of endoparasitoids which deposit their eggs inside the host body, where they spend their immature development. In this time, host defense strategies activate and try to eliminate invasive factors (immature stages of parasitoids such as egg, larvae and etc.). Regarding the relatively large size of invasive particles, encapsulation is the most important mechanism employed by the host to suppress alien factors (see Sect. 9.6.3.1). On the other hand, endoparasitoids also utilize defense strategies to overcome such immune responses, and could successfully facilitate their immature development into the host haemocoel.

To achieve this goal, one known mechanisms is the mutualistic relationship detected between Ichneumonoidea wasps (Ichneumonidae and Braconidae) and polydnaviruses. However, although effects of entomopathoges on predators/parasitoids are usually negative, this mutualistic relation revealed a positive effect on the biological performance of endoparasitoids. Tan et al. (2018) defined it as obligatory mutualism. Webb et al. (2006) stated that about 30,000 species of endoparasitoid wasps from both Ichneumonidae and Braconidae families have specific mutualistic viruses. Herniou et al. (2013) revealed an approximately 100 million years evolutive background for this relation.

A symbiotic virus integrates its genome into the wasp genome with replication of the viral particles in the reproductive system of female parasitoids. However the infection process and expression of viral genes only occur in the host tissues (especially salivary glands) (Herniou et al. 2013). During oviposition, female parasitoids inject the symbiotic virus in the host body. The particles injected engage the host immune system and manipulate it to allow a successful development of the deposited eggs (Beckage 1998). Rodriguez-Perez and Beckage (2008) described that polydnaviruses injected into the haemocoel of the sugarcane borer, *D. saccharalis*, significantly reduced immune responses of caterpillars towards the eggs deposited by the parasitoid wasp *C. flavipes*. In previous studies, Rodriguez-Perez and Beckage (2006) explained that polydnaviruses reduce the adhesive attributes of the host haemocytes. Thereafter, the encapsulation process is disrupted and the eggs deposited by the parasitoids successfully complete their development.

9.10 Future Research Directions

Entomopathogens need more attention to investigate different aspects including widespread application in a large scale, pest resistance or possible interactions with non-target organisms. Even though considerable efforts were conducted to evaluate different attributes of entomopathogens in recent years, our knowledge in some areas is still restricted. One of the main gaps is our knowledge about the epizootiology of these organisms. More research projects should be designed to evaluate factors affecting epizootiology of these entomopathogenic agents in natural conditions. However, because different factors are involved, multidisciplinary efforts by different specialists should be contributed, from fields such as insect pathology, entomology, ecology, agronomy etc.Comprehensive research projects may also enhance our knowledge about possible effects of climatic changes on entomopathogens. Another directions to minimize adverse effects of entomopathogens on non-target organisms, such as pollinators, predators and parasitoids, involve the development of novel delivery tactics. To achieve this goal, Vega et al. (2012) suggested application of endophytic entomopathogenic fungi.

Our knowledge about the ecology of microsporidia, as well as their possible impacts on predators/parasitoids, is still restricted, This is a main area for future studies on this group. In addition, more taxonomic studies are also needed. Similarly, there is an obvious gap in our systematic information about entomopathogenic nematodes. In this group, our current knowledge is focused on two families, Heterorhabditidae and Steinernematidae. Therefore, future studies should deserve more considerations to other families.

To challenge chemical pesticides, efforts on commercial formulations are required. However, in contrast with chemicals, entomopathogens are living organisms and this vital point causes some difficulties for their packing, storage and application. On the other hand, commercial formulations should be ecologically selective to minimize possible adverse effects on non-target organisms. This issue is so crucial for non-specific organisms such as pathogenic microsporidia. In the case of entomopathogenic nematodes, since these agents have close symbiotic relation with *Photorhabdus* and *Xenorhabdus*, understanding their nutritional contributions will facilitate mass production efforts under *in-vitro* conditions.

In some circumstances molecular studies are needed. For example, resistance mechanisms of target organisms to different groups of entomopathogens or their metabolites are important fields that should be comprehensively pursued. Another area is the vertical and horizontal transmission of different organisms in populations of both target and non-target species. Shapiro-Ilan et al. (2012) stated that the gene flow between population of entomopathogens and target organisms represents an open field in molecular studies. In the case of entomopathogenic viruses, insect cell cultures will provide appropriate tools to evaluate different aspects of virus biology and infection, replication and transmission mechanisms. Therefore, this is a clear direction to develop our knowledge on entomopathogenic viruses. In addition, Harrison and Hoovery (2012) highlighted our gap in understanding host responses

to viral infections. These researchers suggest more studies on mass production of entomopathogenic viruses in insect cells to reduce the cost of commercial formulations. In the case of entomopathogenic bacteria, molecular screening could optimize the discovery of novel isolates as well as virulent factors. Furthermore, genetic studies could be applied to generate new toxins with higher pathogenic activity and specificity, also helpful for designing new transgenic crops.

9.11 Conclusion

Deleterious effects of chemical pesticides have changed our attitude in pest management programs, with more emphasis given to eco-friendly strategies. In recent years, entomopathogenic agents have been considered as one of the most reliable and safe alternatives. Furthermore, diversity of these biological agents allows agricultural producers to select appropriate options for controlling target organisms, in different circumstances. Considerably, our current knowledge about possible effects of these biological agents on non-target organisms, such as insect predators and parasitoids, is still limited. Therefore, before widespread application, compatibility of these microbial agents with other natural enemies (especially insect predators/parasitoids), during simultaneous applications, should be investigated. Such assessments must involve different entomopathogenic effects on predators/parasitoids, including biological, ecological, physiological, immunological and behavioral studies. Such evaluations may play a significant role in successful implementation of IBC. Although the term "success" has wide definitions, in IBC our criteria involve the intentional application of entomopathogens as a reliable tool, with the highest and lowest negative effects on target and non-target organisms, respectively. Some findings showed that entomopathogens could have adverse effects on other beneficial organisms. Therefore, comprehensive assessments are urgently needed to minimize such undesirable effects on non-target organisms, reducing the risk associated with widespread applications of these biocontrol agents.

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Chapter 10
Biological Management of Major Vegetable Insect Pests with Macro- and Microorganisms

Jaydeep Halder and Atanu Seni

Abstract Biological control is widely acclaimed due to target specificity, self-perpetuity and safety to the environment. Biological pest control is mainly achieved by using different microscopic parasitic organisms causing disease to insects. These includes bacteria (*Bacillus thuringiensis, B. papillae* etc.), viruses (Nuclear polyhedrosis virus, Granulosis virus etc.) fungi (*Beauveria bassiana, Metarhizium anisopliae, Lecanicillium* (=*Verticillium*) *lecanii, Nomuraea rileyi* etc.), or nematodes (*Steinernema*). Large size organisms, visible with naked eyes, include mainly parasitoids and predators. The role of these organisms in the management of major vegetable insect pests of tomato, brinjal, okra and cole crops are herein discussed.

Keywords Macrobials · Microbials · Biocontrol · Vegetable insect pests · Management

10.1 Introduction

Insect pests are a major biotic constraint in vegetable productions in India. Crops losses due to insect pests may reach around 30–40% of vegetable crop yields (Rai et al. 2014a). Vegetable growers largely depend on chemical pesticides for insect pests management, accounting to approximately 13–14% of total pesticides consumption in the country. The indiscriminate and excessive use of insecticides resulted in the development of resistance to insecticides in insects and their resurgence, besides various ecological problems such as the destruction of natural enemy fauna, effects on non-target organisms, residues in consumable products etc. (Halder

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et al. 2016; Roy et al. 2017). To overcome these problems in many instances alternative management methods were tested, that offer adequate levels of pest control and pose fewer hazards. One such alternative is the use of biological control agents which are especially valuable because of their extremely low toxicity to non-target organisms.

Many vegetables such as tomato, cucumber, carrot, chilli, beet, radish, garden pea etc. are often consumed fresh and serious health hazards may occur by unappropriate use of traditional synthetic insecticides. Use of different biological control agents may provide a safer and cheaper option, that can represent an important component of integrated pest management (IPM) programs. Modern insect pests control highly relied on chemical interventions, resulting in many associated problems such as pest resistance, resurgence, secondary outbreaks of minor pests in addition to other environmental hazards (Rai et al. 2014b). Therefore, utilization of biocontrol agents targeting specific pests is an eco-friendly approach with a self-perpetuating capacity, providing an alternative and viable option for most pest management purposes.

10.2 Etymology

Biological control of major insect pests relied upon microbial and macrobial management. Macrobial consist of two words; 'Macro' means 'large enough to visible in naked eyes' and 'bios' means 'life'; so macrobial means the organism which are visible in naked eyes. In insect pest management it includes mostly parasitoids and predators. So, macrobial management is the utilization of various macro-organisms i.e. parasitoids and predators to manage the insect pests in the crop ecosystem. In contrast, microbials are the organisms which are visible only under a microscope. In Integrated Pest Management (IPM) it comprises bacteria, fungi, virus, nematodes, actinomycetes that cause several diseases to insects and thereby kill them, hence, vernacularly also called them as entomopathogens.

10.2.1 Predators

Predators are large, free-living species that consume a number of preys during their life-cycle. They are not generally specific to a particular species for feeding. Some examples include the lady bird beetles, green lace wing, rove beetle, spiders etc.

10.2.2 Parasitoids

These insects, unlike predators, complete their larval stage development in a single host, whereas the adults are free-living. They generally attack hosts larger than their size. The parasitoids have a high host searching ability and high target and stage specificity, and can be used as a biocontrol agent with impact on target hosts. *Trichogramma* spp. are the widely used egg parasitoids, similarly *Bracon* spp. are larval parasitoids. *Trichospilus pupivora* is the example of pupal parasitoid.

10.2.3 Entomopathogens

These are the parasitic microorganisms that cause a disease to insects. They includes bacteria (*Bacillus thuringiensis*, *B. popillae*), viruses (Nuclear polyhedrosis virus, Granulosis virus), fungi (*Beauveria bassiana, Metarhizium anisopliae, Lecanicillium* (=*Verticillium*) *lecanii, Nomuraea rileyi*) and nematodes (*Steinernema*).

10.3 Biocontrol Agents in Vegetable Pest Management

10.3.1 Predators

Use of predaceous insects as biological control agents is very helpful in insect pest management. More than 30 families of insects are predaceous in nature. Among them, the Coccinellidae, Chrysopidae, Staphylinidae, Syrphidae, Anthocoridae, Nabidae, Reduviidae, Geocoridae, Nitidulidae, Formicidae, Cecidomyiidae and Carabidae are important from the agricultural point of view. The era of modern biological control started with predators, involving the transfer and introduction of natural enemies of insect pests. First applications began 130 years ago, with the highly successful introduction of the Vadalia beetle, *Rodolia cardinalis* (Mulsant) in California in 1888. The beetle proceeded from Australia and was introduced to control the cottony cushion scale, *Icerya purchasi* Maskell on citrus (DeBach 1964; Van Driesche et al. 2008). Mass culture and periodic release of natural enemies started in 1916 in North America with the discovery that mealybugs and black scales could be reared successfully on sprouted potato (Smith and Armitage 1931). After that, another predator, *Cryptolaemus montrouzieri* Mulsant (Coleoptera, Coccinellidae) was mass reared in an insectary and released for citrus mealybugs control (Armitage 1929).

Predators of arthropods can be divided into two broad categories, according to their food habits (Van Driesche et al. 2008):

1. Generalist predators that provide a significant, sustainable, but often unrecognized, natural control of many potential pests and can be enhanced by conservation biocontrol techniques or augmentative releases.
2. Specialized predators that, in addition to the above mentioned uses, can be introduced to new areas as part of classical biological control programs (Hagen et al. 1976).

A generalist predator feeds on preys of different insect families and on various stages e.g. eggs, larvae and/or adults. When food is scarce, generalist predators exhibit several important life history trade-offs (Valicente and O'Neil 1995; Snyder and David 2001; Van Driesche et al. 2008), particularly between survival and development, and between fecundity and survivorship, by reducing their rate of development (Wiedenmann et al. 1996; Van Driesche et al. 2008) or their reproduction rate.

Aphids are eaten by various predators such as coccinellids, i.e. *Cheilomenes sexmaculata* (Fab.), *Scymnus castaneus* Sic, *Pseudaspidemerus circumflexa* (Mots.), and syrphids such as *Peragus* sp. and *Ischiodon scutellaris* (Fab.). In general they are not species-specific (Verghese and Nagaraju 2007). Neuropterans such as *Chrysopa* spp. (Chrysopidae) are also generalists but prefer soft-bodied insects. The release of *Chrysoperla carnea* Stephen and *Mallada boninensis* Okamoto can suppress whiteflies and mealybugs in chillies (Verghese and Nagaraju 2007).

Releases of *C. carnea* at a ratio of 1:5 against *Myzus persicae* on vegetables (brinjal, peppers and tomatoes) accounted for reductions of aphids from 43% to 97%. It was observed that the combined effect of lycosid spider and carabid beetle predation on the cucumber beetles *Diabrotica undecimpunctata* (Linn.) significantly reduced beetle densities and increased yields of spring cucumbers (Snyder and Wise 2001). In Europe, *Phytoseiulus persimilis* Athias-Henriot was commercially produced and released for the control of the two spotted mite *Tetranychus urticae* Koch on cucumber in glasshouse conditions (Dhaliwal and Arora 1998, 2001). In Spain, the use of biological control agents, i.e. the predatory mite *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae) and the anthocorid bug *Orius laevigatus* Fieber (Heteroptera: Anthocoridae) successfully controlled thrips and whiteflies on sweet peppers in greenhouses, as a replacement of chemical pesticides (Calvo et al. 2012). For augmentative release purposes, *Cryptolaemus montrouzieri* Mulsant is easy to rear and may suppress mealybug insects in a sustainable way, as the predator self-perpetuates and persists in nature (Verghese and Nagaraju 2007).

Beside insects, spiders also act as predators and have a great role in insect pest suppression in vegetable ecosystem (Table 10.1). Apart from them, there are many other general predators present in nature, i.e. birds and frogs which are not amenable to mass rearing and release, but are useful in keeping a pest population under control. The importance of birds in the management of insect pests was felt in the middle of the eighteenth century (1762), when Indian mynas *Acridotheres tristis* (Linn.) were moved from India for release in Mauritius island, to control red locusts (*Nomadacris septemfasciata* Audinet-Serville). This was possibly the example of the world's first attempt at biological pest control (Verghese and Sriharan 1993;

Table 10.1 List of some predaceous spiders in vegetable crops[a]

Spider name	Crop	Prey
Lycosa pseudoannulata Boesenberg and Strand	Okra	*Cryptocephalus dodecospilus*; *Cassida indicola*
	Brinjal	*Empoasca binotata*; *Aphis gossypii*; *Henosepilachna vigintioctopunctata*; *Myllocerus subfaciaus*
Argiope pulchella Thorell	Okra	*C. dodecospilus*; *C. indicola*
	Cucumber	*Aulacophora foveicollis*; *Dacus cucurbitae*; *Mylabris pustulata*
Zygeilla indica Tikader	Okra	*C. dodecospilus*; *C. indicola*
Pardosa sumatrana Thorell	Brinjal	*E. binotata*; *A. gossypii*; *H. vigintioctopunctata*; *M. subfaciaus*
	Sweet potato	*C. indicola*; *C. dodecospilus*; *Cylas formicarius*
Pardosa milvina (Hentz)	Cabbage	*Pieris rapae*
Oxyopes lineatipes (Koch.)	Brinjal	*E. binotata*; *A. gossypii*; *H. vigintioctopunctata*; *M. subfaciaus*
	Potato	*Empoasca kerri*; *H. vigintioctopunctata*; *Myzus persicae*
	Sweet potato	*C. indicola*; *C. dodecospilus*; *C. formicarius*
Tetragnatha maxillosa Thorell	Brinjal	*E. binotata*; *A. gossypii*; *H. vigintioctopunctata*; *M. subfaciaus*
Cheiracanthium danieli Tikader	Okra	*Amrasca biguttula biguttula*, *Thrips tabaci* and *Bemisia tabaci*
Hippasa agelenoides (Simon)	Okra	*A. biguttula biguttula*, *T. tabaci* and *B. tabaci*
Leucauge celebasiana (Wlk.)	Brinjal	*E. binotata*; *A. gossypii*; *H. vigintioctopunctata*; *M. subfaciaus*
	Potato	*Empoasca kerri*; *H. vigintioctopunctata*; *M. persicae*
	Sweet potato	*C. indicola; C. dodecospilus; C. formicarius*

[a]Source: Schmaedick and Shelton (2000), Sahito et al. (2013), Verghese and Nagaraju (2007)

Verghese and Nagaraju 2007). Conserving and protecting insectivorous birds in vegetable ecosystems is helpful indeed for suppressing many insect populations. They feed on insects such as grubs, beetles, aphids, caterpillars and moths for themselves and their young ones. Birds can be attracted to field by erecting bird perches or placing water-troughs in the field. Following the later technique is quite effective for suppressing caterpillars, i.e. *Plutella maculipennis* (Curtis) (Lepidoptera: Plutellidae) in cabbage by wagtails *Motacilla* sp. Other beneficial predatory birds include the Magpie robin *Copsychus saularis* (Linn.), the Black Drongo *Dicrurus adsimilis* Bech., the Indian mynas *Acridotheres tristis* (Linn.), the Jungle babbler *Turdoides striatus* (Dumont), the house sparrow *Passer domesticus* (Linn.), that feed on various caterpillars and adult of lepidopteran insect pests (Verghese and Sriharan 1993; Regmi 2003; Verghese and Nagaraju 2007). The Indian treepie

Dendrocitta vagabunda (Latham) feeds on weevils whereas the Large-pied wagtail *Motacilla maderaspatensis* Gmelin and the Tailor bird *Orthotomus sutorius* (Pennant) feed on aphids (Verghese and Sriharan 1993; Verghese and Nagaraju 2007). Importance should be given to actions aiming at conserving and protecting the insectivorous bird diversity by providing shelter, water sources, nesting and breeding habitats by planting hedges and shrubs around the agro-ecosystem.

10.3.1.1 Predation Methods

Insect and invertebrate predators follow various predation methods to get their food. They are as follows:

Hunters, Chasers: on the ground, tiger beetles run down prey and catch it by their strong, curved jaws. In the air, dragonflies catch their prey by their spiny legs. In water, a number of beetles (e.g., diving or whirligig) and water striders (true bugs) seek and seize insect larvae, small aquatic insects and mites.

Hunters, Waiters: praying mantids get their meals by waiting. When prey comes near to them, they catch them by their strong raptorial forelegs. Their shape and color help them to camouflage in nature.

Hunters, Collective Attack: ants are a good example of collective work and use chemical signals to garner other workers for attack.

Trappers: the spiders trap their prey by making a web. Almost anything that has encountered their webs is devoured or discarded. Many families of caddisfly larvae use silk to produce different types of 'capture nets' to collect food from water. Water flows through the net, which captures suspended food particles. Ant lions dig steep-sided conical pits in sandy soil. They hide in the sand and wait for ants and other small insects to fall into the trap (Hagen et al. 1999; Van Lenteren 2003; Van Driesche et al. 2008).

10.3.1.2 Examples of Some Important Predators in Vegetable Ecosystems

Aphidoletes aphidimyza (Rondani) Cecidomyiidae: Diptera (predatory midge)

The larvae of this fly are used in vegetable and ornamental crops for aphid population suppression. Adults are weak fliers, crepuscular and eat nectar and honeydew. A maggot may eat 3–50 aphids per day and pupates in soil. The female predator lays small orange eggs near the aphids and later, the emerging maggots paralyze the aphids and suck them dry. They are sold as pupae, which are sprinkled on moist substrates. The recommended rate of release varies from 2 per m^2 to 10 per m^2, depending on the intensity of the infestation.

Chrysoperla spp. (green lacewings) Chrysopidae: Neuroptera

Almost 50 species of *Chrysoperla* are present worldwide. They eat soft-bodied insects such as aphids, mealybugs, thrips and whiteflies. They are also known as

aphid lions because of their appetite for aphids. The adults are not predaceous, but feed on honeydew and pollen that are required for egg production. Larvae are often cannibalistic. Due care should hence be taken during their mass rearing. The recommended rate of release for *C. zastrowi sillemi* ranges from 10 per m^2 in a low infestation area to 20 per m^2 in case of a severe infestation.

Cryptolaemus montrouzieri (Coleoptera: Coccinellidae)

This beetle can control the papaya mealybug, *Paracoccus marginatus* Williams and Granara de Willink, solenopsis mealybug, *Phenacoccus solenopsis* Tinsley, which lays eggs in ovisacs, but is ineffective against species such as long tailed mealybug *Pseudococcus longispinus* (Targioni-Tozzetti) that produce live nymphs, because the predator lays its eggs in ovisacs. Larvae and adults feed on all mealybug stages, but dense prey populations are required to sustain its population. In North America, the release rates are 2–5 beetles per mealybug infested plant.

Feltiella acarisuga (Vallot) (Diptera: Cecidomyiidae)

The larvae of this fly feed on all stages of the two-spotted spider mite, *Tetranychus urticae* Koch.

Harmonia axyridis (Pallas) (Coleoptera: Coccinellidae)

It feeds on aphids and tolerates low temperatures, so that it can be used in unheated greenhouses.

Macrolophus caliginosus Wagner (Heteroptera: Miridae)

This whitefly predator is widely used in European tomato crops. However, it is not approved for use in the USA, because of risk to plants. Bugs can feed on crop foliage, which allows them to establish and increase even when whiteflies are scarce. Plant feeding may cause minor damage.

Orius spp. (Hemiptera: Anthocoridae)

These anthocorids feed on thrips, mites, aphids, whiteflies, lepidopteran eggs, pollens and plant sap, but are released mainly against thrips. *Orius* multiply and act as successful thrips predators in crops such as pepper, onion and cucurbits. An adult predatory bug can eat 5–20 thrips per day. The recommended rate of release ranges from 1 to 10 individuals per m^2, depending on the level of pest infestation.

Geocoris spp. (Hemiptera: Lygaeidae)

These are generalist predators, feeding on aphids, beet army worm, Mexican bean beetle, whitefly etc. They can be reared on frozen ant pupae or on potato tuber moth larvae and string beans. In field studies, *G. pallens* was an effective predator of *Lygus* bugs, cabbage looper, mites and aphids (*M. persicae*) on sugar beets.

Phytoseiulus persimilis Athias–Henriot (Acarina: Phytoseiidae)

This predatory mite acts as a predator on the two-spotted spider mite, *Tetranychus urticae* Koch. For their optimal development, more than 75% relative humidity and temperatures above 20 °C are necessary.

Many arthropod natural enemies are effective for pest management in vegetable crop ecosystems. Tables 10.1 and 10.2 show the effective natural enemies, their families, host range, feeding site in vegetable crop ecosystem.

In addition, there are many predaceous spiders which help to check the pest population in vegetable cropping system.

10.3.2 Parasitoids

About 66% of all successful biocontrol programmes involved parasitoids (Dhaliwal and Koul 2007). Parasitoids have a great variety of lifestyles as they are free-living as adults, and parasitic as larvae. The latter feed on their hosts, which are mostly insects at various developmental stages (Kaeslina et al. 2005). They are very host specific and quite a few of them attack more than one host. The parasitoids enter their host, feed upon it, and usually kill it. They mainly include wasps, flies, beetles, but the majority are hymenopterans. It was observed that 80% of the 600,000 known species of hymenoptera are parasitoids (Sampaio et al. 2009). Among Hymenoptera, the Trichogrammatidae parasitize the eggs of several insect species. Members of Ichneumonidae and Braconidae mainly prey on larvae of Lepidoptera. The chalcid wasps also parasitize the eggs and larvae of many insects.

Some wasps insert their own eggs inside the pupae of other insects. Some hymenopterans (e.g., mud daubers) paralyze insects and seal them in a nest with an egg. The emerging larva then feeds on the paralyzed victim. Female tachinid flies lay their eggs on the bodies of other insects, mainly caterpillars, adults and grubs of coleopteran insects, and true bugs. They have an ability to find their prey, even at very low host densities, by the help of chemical cues (Vet and Dicke 1992; Godfray 1994) produced by the plants that have been damaged by the pest (Paré and Tumlinson 1997; Hajek 2004) or following kairomones emanating from the herbivores (Dhaliwal and Arora 1998; Hajek 2004).

There are some parasitoids, called idiobionts, which kill the host immediately or shortly after the initial parasitization, by permanently blocking or preventing its further development (Askew and Shaw 1986; Gordh et al. 1999; Van Driesche et al. 2008). While others are known as koinobiont parasitoids, that they do not rapidly kill their host. Typically, the idiobionts are ectoparasitic, attacking concealed hosts, and act as generalists, whereas koinobionts are endoparasitic, attacking exposed hosts, and acting like specialists (Askew and Shaw 1986; Gordh et al. 1999; Van Driesche et al. 2008).

Parasitoids are most effective at reducing pest populations when their host organisms have limited refuges to hide and escape parasitism. Generally, adult parasitoids are free-living and usually feed on honeydew, nectar, pollen, water or by piercing soft-bodied insects (i.e. whiteflies and aphids) with their ovipositor or mouthparts

Table 10.2 List of some common parasitoids, family, hosts and feeding sites from vegetable ecosystems[a]

Parasitoid	Family	Order	Host insects	Feeding site
Brachymeria spp.	Chalcididae	Hymenoptera	Flies and butterflies (larvae and pupae)	Internal or External
Trichogramma spp.	Trichogrammatidae		Moth eggs	Internal
Aenasius spp. *Copidosoma truncatellum* (Dalman), *Acerophagus papayae* Noyes & Schauff	Encyrtidae		Various insects eggs, larvae or pupae	Internal
Tetrastichus schoenobii Ferrieri *Neochrysocharis formosa* (Westwood) *Diglyphus isaea* Walker, *Pediobius foveolatus* (Crawford)	Eulophidae		Various insects eggs, larvae or pupae	Internal or External
Encarsia sp., *Aphelinus abdominalis* (Dalman), *Lysiphlebus testaceipes* (Cresson)	Aphelinidae		Whiteflies, scales, aphids mealybugs	Internal or External
Trissolcus basalis (Wollaston) *Telenomus* sp.	Scelionidae		Eggs of true bugs and moths	Internal
Campoletis chlorideae (Uchida) *Xanthopimpla punctata* Fab.; *Trathala flavoorbitalis* Cameron	Ichneumonidae		Larvae or pupae of caterpillars, beetles and wasps	Internal or External
Cotesia spp., *Bracon brevicornis* Wesmael, *Apanteles* spp., *Chelonus blackburni, Opius* spp., *Diaeretiella rapae* (McIntosh), *Microgaster* sp. *Aphidius colemani* Viereck	Braconidae		Larvae of beetles, flies, caterpillars, sawflies, aphids	Internal
Pteromalus puparum (Lin.)	Pteromalidae		Pupae of beetles, caterpillars.	External
Stethynium triclavatum Enock	Mymaridae		Cicadellid Eggs	Internal
Stermiopsis inferens Townsend *Diatraeophaga striatalis* Townsend	Tachinidae	Diptera	Beetles, butterflies, and moths	Internal

[a]Source: Hagler (2000), Srinivasan (2014), Halder et al. (2018), Seni and Chongtham (2013), Waterhouse and Sands (2001), Waterhouse (1998)

and eating the sap that comes out from the wounded tissue. In nature, it is found that the epilachna beetle, *Henosepilachna vegintioctopunctata* (Fab.), one of the major pest of brinjal, is parasitized by *Pediobius foveolatus* with a prevalence around 60–77%. The aphid *Myzus persicae* in chilli was found parasitised by *Aphidius* and *Aphelinus* spp. up to 80–98.8% of individuals, in an insecticide-free ecosystem in India (Verghese and Nagaraju 2007). In India, Manjunath et al. 1989 observed that the *Trichogramma chilonis* Ishii was responsible for up to 98% parasitism of *Helicoverpa armigera* eggs in tomato and potato. Releases of *Trichogramma pretiosum* at the rates of 100,000 per ha per week, for up to 9 weeks per season, was helpful to manage *Helicoverpa* sp. pests in tomato crops in Mexico (Van Driesche et al. 2008). The egg parasitoid, *Trichogramma achaeae* Nagaraja and Nagarkatti (Hymenoptera: Trichogrammatidae) has been recommended for control of a new invasive pest, the South American pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) in tomato in the Azores Islands (Oliveira et al. 2017). The parasitoid, *Aphelinus abdominalis* Dalman was released, at 2–4 wasps per m^2, to control the potato aphid *Macrosiphum euphorbiae* (Thomas) and the greenhouse potato aphid *Aulacorthum solani* Kaltenbach (van Lenteren 2003; van Lenteren et al. 2017). The parasitoid *Diglyphus isea* (Walker) is used for controlling leaf miners, at a release rate of 0.25 per m^2, and for preventive introductions at 2 per m^2 in heavily infested areas (van Lenteren 2003). Another species, *Encarsia formosa* Gahan, is an important parasitoid of the greenhouse whitefly *Trialeurodes vaporariorum* (Westwood) and the whiteflies *Bemisia tabaci* (Gennadius). Presently, *E. formosa* is one of the most used biological control agents in protected crops, and its recommended rate of release ranges from 1 per m^2 at the interval of 1–2 weeks for preventive use, to 9 per m^2, five times a week, for severe infestations (van Lenteren 2003).

10.4 Microbial Biocontrol Agents in Vegetable Pest Management

Entomopathogenic microorganisms are pathogenic to insects and kill the host by causing different diseases. Several species of naturally occurring bacteria, virus, fungi, protozoa and nematodes infect insect pests and play an important role in their eco-friendly regulation and management. Some of them *viz*., bacteria and fungi, can be mass-produced *in-vitro* while nematoeds and viruses can be multiplied *in-vivo*.

10.4.1 Entomopathogenic Fungi (EPF)

Fungi are important entomopathogens as they are virulent, cause mycosis by contact, persist in the environment for longer time and are mostly mass-producible *in-vitro*. Since they are considered as natural mortality bioagents and environmentally

safe, they received worldwide interest in the utilization and manipulation for biological control of insects and other arthropod pests. More than 750 species of fungi, mostly deuteromycetes and entomophthorales from about 100 genera, are pathogenic on insects (Banik and Halder 2013).

Under optimal conditions of moderate temperatures and high relative humidity, fungal spores germinate and penetrate through the insect cuticle either directly by germ tubes or by appresoria (infection pegs) to gain entry into the insect body. Once inside, the fungi multiply in the haemocoel, invading the adjacent tissues and derive the nutrients from the insect body. Subsequently, hyphae emerge from the interior through the cuticle to the exterior of the insect, and produce more spores (conidia or infective units) from the dead insect. Death takes between 4 and 10 days, depending on the type of fungus and the number of infecting spores. After death, the fungus produces thousands of new spores on the dead body, which disperse and continue their life-cycle on new hosts. Some commonly known entomopathogenic fungi such as *Beauveria bassiana*, *Metarhizium anisopliae, M. acridum*, and *M. brunneum*, *Lecanicillium* (=*Verticillium*) *lecanii*, *Hirsutella thompsonii*, *Isaria fumosorosea* are commercially sold as biopesticides in multiple formulations around the world.

10.4.2 Entomopathogenic Bacteria (EPB)

The majority of bacterial entomopathogens occur in the families of Bacillaceae, Pseudomonadaceae, Enterobacteriaceae, Streptococcaceae and Micrococcaceae. Although most of these bacteria are weak pathogens and infect insects subject to environmental stress, a few are highly virulent. Initially, the species *Paenibacillus* (former *Bacillus*) *popilliae* Dutky was introduced for management of the Japanese Beetle *Popillia japonica* Newman (Steinhaus 1975). But the most concrete and successful results were achieved with the discovery of *Bacillus thuringiensis* (*Bt*) strains showing high toxicity against specific insects at. *Bt* is a competitive alternative, compared to conventional insecticides in terms of efficacy and costs of production (Ruiu et al. 2013).

Several species of soil borne *Bacillus* and *Paenibacillus* bacteria are pathogenic to coleopteran, dipteran and lepidopteran insects. *Bt* evolved a number of subspecies, i.e. subspp. *kurstaki*, *israelensis*, *aizawai*, *sphaericus* and subsp. *tenebrionis,* that are effectively used for the management of different groups of target insect pests. *Bt* subsp. *kurstaki* and *Bt* subsp. *aizawai* are promising against caterpillars, *Bt* subsp. *israelensis* and *B* subsp. *sphaericus* target dipteran (mosquito) larvae, whereas *Bt* subsp. *tenebrionis* is effective against some coleopteran insects.

When *B. thuringiensis* is ingested by the target insects, toxic proteins (*i.e.,* delta-endotoxin) rare eleased and activate in the host midgut under the local alkaline conditions (pH 8–11). The endo-toxins then attach to the receptors sites in the midgut and create pores in the midgut cells which result in the loss of osmoregulation, midgut paralysis and finally cell lysis. Gut contents leak into hemocoel and the hemolymps leaks into the gut, thereby disrupting the pH balance. After entering the

body cavity the bacteria cause septicemia and eventual death of the host. Insects show different kinds of responses to the toxins, depending on the crystal proteins (delta-endotoxins), receptor sites, production of other toxins (exotoxins), and requirements of spores.

10.4.3 Entomopathogenic Nematodes (EPN)

Entomopathogenic nematodes are microscopic and soil-dwelling parasites of insects. Several species of *Heterorhabditis* and *Steinernema* are very popular and commercially available in multiple formulations, primarily for managing soil-dwelling insect pests. EPN infective juveniles (IJs) actively seek out their hosts and enter through their body by its natural openings *viz*., the mouth, spiracles, intersegmental membrane and anus. Once inside the host body, the EPN release symbiotic bacteria that kill the host through bacterial septicemia.

10.5 Some Successful Examples of Biocontrol Agents in Vegetable Pests Management

10.5.1 Pests of Cole Crops

Diamond back moth (DBM), *Plutella xylostella* (Lepidoptera: Plutellidae) is a serious pests of cruciferous crops, particularly cabbage and cauliflower. Damage due to this pest is reported up to 17–99% of yield by Shivalingaswamy et al. (2002a) with annual control costs estimated at one billion USD, worldwide (Talekar and Shelton 1993). One egg parasitoid, one egg-larval parasitoid and twenty nine larval parasitoids have been reported on DBM (Rai et al. 2009).

Cotesia plutellae (Kurdjumov) (Hymenoptera: Braconidae), a larval, gregarious endoparasitoid, is one of the most common parasitoid of DBM in India, causing around 10–40% larval mortality. The parasitoid is active from the first week of February to second week of April with maximum parasitization during second fortnight of March, synchronized with the maximum incidence of host larvae. The activity of parasitoid has a significant positive correlation with the incidence of the host larval population, temperature and sunshine, and a negative correlation with the relative humidity and total rainfall (Chandra and Singh 2007).

Diadegma semiclausum (Hellén) (Hymenoptera: Ichneumonidae) is one of the most important larval parasitoids of *P. xylostella* across the world, whereas *D. fenestralis* (Halmgrew) is predominant in the hilly regions of India, with parasitization varying from 73.33 to 86.67% (Devi and Raj 1995). Malathion proved highly toxic to both parasitoids, whereas fenvalerate and cypermethrin + *Bt* showed less adverse effects on parasitoid emergence (Bhardwaj et al. 2005).

Bt subsp. *kurstaki,* was found very effective for managing DBM. Commercial *Bt* formulations of *Bt* subsp. *kurstaki* can be applied in the field at 0.5–1.0 kg/ha. It was found compatible with many botanicals and their oils. Formulations with 0.2% *Btk* plus 5% neem oil was found highly effective against DBM with maximum cost/benefit ratio of (1:4.6), followed by *Btk* 0.2% plus 5% citronella oil (1: 2.9) and *Btk* 0.2% plus 5% karanj oil (1: 2.4) (Babu et al. 2008).

Aphids (*Myzus persicae* and *Brevicoryne brassicae*)

Both aphids are serious pests of cole crops such as cabbage and cauliflower, even under protected cultivations. Both the nymphs and adults suck the sap from the leaves. As a result the plants become yellow and lost their vitality. Beside sap sucking, they also secret the honeydew which is deposited on the plants and creates black sooty-moulds, inhibiting photosynthesis. Conservation of solitary endoparasitoid *Diaeretiella rapae* M'Intosh (Braconidae: Hymenoptera) is effective (Halder et al. 2014). Peak period of activity of this parasitoid is February–March, recorded under the Varanasi, India, conditions. Ladybird beetle, *Coccinella septempunctata* and *Menochilus sexmaculatus* (Coccinellidae: Coleoptera) are important polyphagous predators of aphids and other soft-bodied insects. Artificial release of 25–30 beetles per m^2 is an effective attempt to control the aphids. EPF like *L. lecanii* and *B. bassiana* were found effective against vegetable aphids like black bean aphid (*Aphis craccivora*) and mustard aphid (*Lipaphis erysimi*). Halder et al. (2013, 2017b) reported the compatibility and synergistic activities of these EPF with neem oil at half of their recommended doses. Recently, Halder and Rai (2016) reported that green lacewing larvae of *Chrysoperla zastrowi sillemi* (Esben-Petersen) preferred *M. persicae*, followed by *B. brassicae*. The highest growth index (8.31), larval survival (94.50%), larval weight (10.45 mg), pupal weight (8.78 mg), faster multiplication rate (0.051) and fecundity (183.4 per gravid female) of the predator were recorded on *M. persicae*.

10.5.2 Pests of Brinjal

The brinjal (egg plant) shoot and fruit borer, *Leucinodes orbonalis* Gunee (Lepidoptera: Pyralidae) is important among the different pests of this crop. The larvae bore the shoot as well as fruit of the plants. Damage due to this insect ranged from 11% to 93% (Shivalingaswamy et al. 2002a, b). Inundative release of *T. brassiliensis* at 250,000 parasitized eggs/ha or weekly inoculative release of 50,000 parasitized eggs/ha appeared promising (Rai et al. 2009). Conservation of the larval parasitoid *Trathala flavo-orbitalis* Cameron and pupal parasitoid *Goryphus nursei* (Cameron) (Hymenoptera: Ichneumonidae) are beneficial practices.

The Hadda beetle *Epilachna vigintioctopunctata* (Fab.) (Coccinellidae: Coleoptera) is a minor pest of brinjal. Recently, a serious proportion of its infestation was reported in cowpea in and around Indian Institute of Vegetable Research, at Varanasi (India). Conservation of parasitoid *Pediobius foveolatus* (Crowford) (Eulophidae: Hymenoptera) (Halder et al. 2011) and spraying of green muscardine fungus,

Metarhizium anisopliae at doses of 10^8 and 10^{10} conidia/ml caused mortality in first (50.0 and 64.8%) and second instar grubs (60.0 and 53.3%) 5 days after application, respectively. However, both first and second instar grubs recorded 100% mortality after 7 days following application of both doses of conidial suspension, while third and fourth instar grubs recorded mortality exceeding 70% after 7 days (Rajendran 2002).

Brinjal stem borer *Euzophera perticella* Ragonot (Lepidoptera: Pyralidae) is an oligophagous insect pest found mostly in the Indian subcontinent (Halder et al. 2017a). The Ichneumonid endoparasitoid, *Pristomerus euzopherae* Viereck was associated with *E. perticella* in and around Varanasi. The parasitization by *P. euzopherae* was recorded first during the second fortnight of April (1.91% parasitization). From April onwards, rate of parasitization gradually increased and the highest parasitization (12.48%) was recorded during July, after 7.73% scored in June (Halder et al. 2017a).

10.5.3 Pests of Tomato

Tomato fruits are attacked by *Helicoverpa armigera* (Hüb.) and *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae). Both insects are polyphagous and feed a number of vegetables. *Helicoverpa armigera* lays eggs singly on flowers, buds or tender leaves whereas *S. litura* lays eggs in masses covered by anal tuft of hairs, mostly on or under the surface of leaves. Larvae bore the immature fruit and feed its internal content that as such become unsuitable for consumption.

Campoletis chlorideae Uchida (Hymenoptera: Ichneumonidae) is an important larval parasitoid of *Helicoverpa*. Conservation of this parasitoid and mass release of 15,000 adults per ha is recommended, coinciding with 5th days after eggs mass hatching, that ensure sufficient larvae for parasitization (Rai et al. 2009). Periodical release of *Trichogramma* egg parasitoid at 50,000/ha at a 10 days interval and six releases from 45 days after transplanting, are beneficial. Spraying of HaNPV, a specific virus to *H. armigera* at 250–300 LE (Larval Equivalent) mixing with jaggery (1%), Teepol (0.1%) per ha, at evening hours, is recommended. Three applications at fortnightly interval should be done. Spraying of *B. thuringiensis* at 1 kg/ha is recommended (Table 10.3). Recently, a new solitary, koinobiont, larval endoparasitoid *Microplitis tuberculifer* (Wesmael 1837) was recorded from larvae of *S. litura* for the first time in India. The parasitoid parasitizes host larvae (most preferably first or second instar), and finally kills the larvae before reaching its pupation stage (Halder et al. 2016).

Solenopsis mealybug, *Phenacoccus solenopsis* (Tinsley) (Pseudococcidae: Hemiptera), an invasive, emerging, polyphagous pest has been observed in serious proportions on a number of solanaceous (tomato, brinjal, chillies), malvaceous (ladies finger) and cucurbitaceous (pointed gourd) vegetables, as well as on other crops including many weeds like *Parthenium hysterophorus*. Conservation of parasitoid viz., *Aenasius arizonensis* (Girault) (=*Aenasius bambawalei* Hayat) (Hymenoptera: Encyrtidae) were found promising (Halder et al. 2015). Amongst

Table 10.3 Biocontrol agents recommended in vegetables crops

Bioagents	Dose	Target pests
Trichogramma brassiliensis	2,50,000 parasitized eggs/ha (inundative release) 50,000 parasitized eggs/ha (weekly inoculative release)	Okra shoot and fruit borer Tomato fruit borer
Chrysoperla zastrowi sillemi	50,000 first instar larvae/ha (weekly release)	Okra aphid Cabbage aphid
HaNPV	250 LE/ha (10 days interval)	*Helicovserpa armigera*
SlNPV	250 LE/ha (10 days interval)	*Spodoptera litura*
Bacillus thuringiensis	500 g ai/ha (10 days interval)	Diamond back moth Shoot and fruit borer of brinjal and okra Tomato fruit borer

the entomopathogenic fungi, *Lecanicillium* (=*Verticillium*) *lecanii* at 5 g/l was found most effective followed by *Beauveria bassiana* at 5 g/l. Their corresponding median lethal time (LT_{50}) values were 112.28 and 124.04 hrs when tested against 7 ± 1 day old nymphs of *P. solenopsis* (Halder et al. 2013).

10.5.4 Pests of Okra

Naik and Shekharappa (2009) observed that *B. bassiana* and *M. anisopliae* oils, and their wettable powder formulations, recorded 96.67% mortality of leafhoppers at 10 days after treatments, whereas *V. lecanii* oil and *B. bassiana* WP formulations recorded 93.33% mortality. However, *V. lecanii* oil-based formulations showed 100% aphid mortality followed by *V. lecanii* WP (96.67%), *B. bassiana* oil and wettable powder formulations (93.33%). Jagadeesh et al. (2007) reported that plant growth promoting rhizobacterium (PGPR) *Pseudomonas* B-25 isolate was found as most efficient biocontrol agent against aphids and leafhoppers, reducing by about 79 and 81% of population, respectively, in okra. Recently, Satpathy et al. (2012) noticed that a single grub of *Chrysopela* can devour 185 leaf hoppers (*A. biguttula biguttula*) to complete its life-cycle. Suitable conservation methods should be adopted for ecofriendly management of this sucking pest. Release of the predator *C. carnea* (25,000 larvae/ha/release) plus Econeem 0.3% (0.5 l/ha) applied three times at 15-days intervals starting 45 days after sowing, were effective in reducing the population of sucking pests *viz*., leafhopper, whitefly, cotton aphid as well as the fruit-borers in okra (Praveen and Dhandapani 2001).

The potential of the rove beetle, *Paederus variicornis* Fauvel (Staphylinidae: Coleoptera) as a biocontrol agent was examined by Shivalingaswamy et al. (2002b). These authors found that the predator was active even during high summer temperatures (> 42 °C). The mean population of jassid and staphylinid predators varied from 2.04 to 21.51 and 1.55 to 3.85 individuals per plant, respectively. A solitary,

arrhenotokous, egg-larval parasitoid of the okra shoot and fruit borer, *Earias vittella* (Fab.) and *E. insulana* (Boisd.) (Lepidoptera: Noctuidae), and of a serious pest of okra, *C. blackburni* was found effective during the period August – October in and around Varanasi, with maximum 19.58% parasitization during the second week of September (Halder et al. 2018). Along with *C. blackburni* another braconid, the larval endo-parasitoid *Agathis* sp., also occurred during August–September on *E. vittella* and *E. insulana.*

Tetranychus cinnabarinus (Boisd.) (Acarina: Tetranychidae) is a serious pest of brinjal, ladies finger, beans, pointed gourd, cucumber etc., both in field as well as in green house conditions. Prolonged dry conditions favour its rapid multiplication. Both the nymphs and adults suck the sap from the lower surface of leaves and in a severe infestation from both sides of leaves. Profuse webbing is the characteristic symptom of this mite. Release of the predatory mite *Amblyseius tetranychivorus* Evans (Phytoseiidae: Acarina) at100 mite/mt^2 in greenhouse conditions was promising to control this mite.

10.6 Constraints Related to Micro and Microbial Pest Control

Some constraints in the use of natural enemies in pest management are the mass rearing difficulties and their adaptability to new weather conditions. Many researches investigated natural enemies with pesticides resistance/tolerance traits, omission of diapauses period, and enhanced temperature tolerance. However, a long way has still to be followed (Headley and Hoy 1987; Hoy 1990). Priority should be given to development of techniques for mass production on artificial diets, or searching other non-host insect species that are easy to multiply under laboratory conditions. For this, sound knowledge about taxonomy of natural enemies, environmental effect on natural enemies, releasing procedure, host-natural enemy-crop plants interactions, efficacy studies, and proper documentations arestill necessary.

10.7 Future Strategies

Biological control including macro and microbial agents is the backbone of any IPM programme. This method requires a sound understanding of the system, of the insect biology, ecology and about the constraints operating on the entomophage population. Biocontrol is currently popular among organic growers and home gardeners, but has potential in additional commercial settings, especially small acreages. Following are the strategies to be taken to further the agenda on chemicals-free crop protection.

(i) Hunt for potential biocontrol agents to reduce pesticide contamination in the environment is the need of the hour.
(ii) Development of a suitable technology for low cost mass production of a potential bioagent, and standardization of their field release method(s) need to be addressed.
(iii) Compatibility and synergistic activity of bioagents with botanicals and biorational molecules.
(iv) Temperature and insecticide tolerant/resistant strains of bioagents are the need of the hour.
(v) Promotion and validation of proven biocontrol agents among farmers must be encouraged.
(vi) Quality of the bioagents should be checked periodically to ensure trusts among end users.
(vii) Efforts are also to be directed to enhance shelf-life and potency of these bioagents, once tested under field conditions.

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Chapter 11
Biorational Approaches for the Management of Insect Pests of Vegetable Cropss

Amandeep Kaur and Ravinder Singh Chandi

Abstract Major biotic stress in vegetable production is due to insect pest attacks. In many cases, there is a total yield loss due to direct (feeding on plants) and indirect (vectors of viral disease) damages. Farmers use indiscriminate doses of pesticides that increase the pesticide load on vegetable crops and cause environmental pollution and health hazards. To deal with these excess residual load, a well planned integrated pest management (IPM) approach against insect pests is required, that could lead to higher yields and increased profits. IPM approaches involve every component of management practices that keep pest population below economic injury levels, so that they do not cause any economic loss to farmers or growers.

Keywords Vegetable · Aphid · Whitefly · Fruit borer · Tobacco caterpillar

11.1 Introduction

Vegetables are more prone to insect pests and diseases mainly due to their tenderness and softness as compared to other crops. Several abiotic and biotic factors affect their successful cultivation including a number of insect pests that attack crops from sowing to harvesting. Worldwide, a total of approx. 9000 species of insects and mites, 50,000 species of plant pathogens, and 8000 species of weeds injure crops (Zhang et al. 2011). As concerns damages, insect pests cause an estimated 14% loss, plant pathogens cause around 13% loss and weeds 13% loss (Pimentel 2009a, b).

In many cases, a 100% yield loss may be achieved, due to direct (feeding on plants) and indirect (vectors of viral disease) effects. The major insect pest attacking vegetable crops have sucking and chewing type of mouthparts. To combat these

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biotic factors, farmers use indiscriminate doses of pesticides that increase the pesticide load on vegetable crops causing environmental pollution and health hazards. Indiscriminate use of pesticide leads to poisoning, resistance and resurgence of pests, effects on non target organisms and dispersal of pesticides residues.

About 13–14% of total pesticides used in the India are applied on vegetables (Fig. 11.1). It was observed that average pesticide consumption in vegetables in India is 0.678 a.i. kg/ha with maximum pesticide usage on chilli, followed by brinjal, cole crops and okra (Rai et al. 2014).

On various crops insecticides elicited resistance at different levels. Among the polyphagous pests, whitefly resistance was found against cyfluthrin, cypermethrin, monocrotophos, quinalphos and methamidophos, while in aphids cypermethrin, deltamethrin permethrin and malathion showed resistance. Insecticides resistance in *Helicoverpa armigera* and *Spodoptera litura* have been reported *vs* cypermethrin, endosulfan, fenvalerate, quinalphos, carbaryl, lamda cyhalothrin and several other insecticides belonging to organophosphate, organochlorine, carbamate and diamide groups (Mehrotra and Phokela 2000; Rai et al. 2014; Sreelakshmi et al. 2017).

To deal with these excess residues load, a well planned IPM approach is required that could lead to higher yields and increased profits. IPM involves host plant resistance, cultural, mechanicaland physical control, biological control and biorational approaches for every part of management that keeps pest population below economic injury levels, so that they do not cause any economic loss to growers.

For sustainability of vegetable crops, insect pests need to be managed through biorational approaches supported by a required, judicious use of chemicals to achieve high economic returns, without disturbing the environmental balance. On a global scale, microbial pesticides only account for approximately 1–2% of all pesticides sold (Thakore 2006; Marrone 2007; Bailey et al. 2010). IPM is one of the economically viable and environmentally safe key technologies to increase vegetable productivity in the country. Why we go for vegetable IPM? As we consume

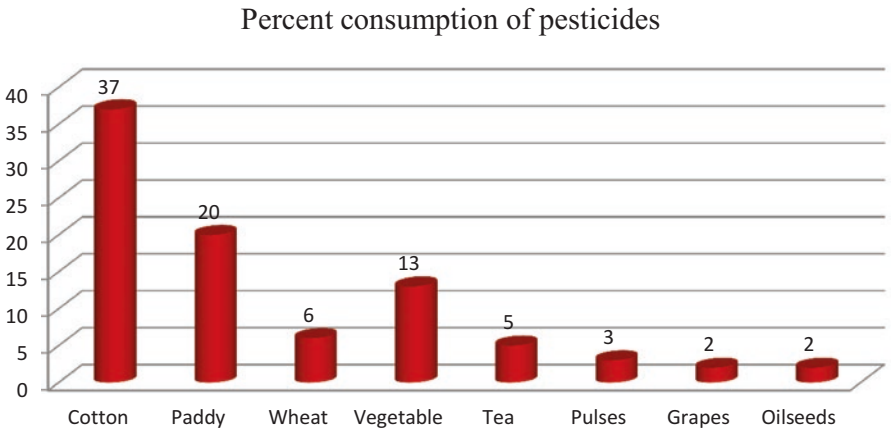

Fig. 11.1 Percent pesticides consumption on various crop under Indian conditions. (Rai et al. 2014)

vegetable as raw/semicooked or cooked or sometime we immediately consume just after harvest. So to make vegetable safe for human, adoption of IPM is a must.

Polyphagous pests cause great losses by migrating from one crop to another, and also by spreading viral diseases. In this chapter four major polyphagous insect pests have been considered i.e. aphid, whitefly, fruit borer and tobacco caterpillar. The economic importance of pest, its life cycle, nature of damage and management strategies are discussed below.

11.2 Aphids

Aphids of green peach (*Myzus persicae*), potato (*Macrosiphum euphorbiae*), mustard (*Lipaphis erysimi*), cotton (*Aphis gossypii*), cabbage (*Brevicoryne brassicae*), cabbage root (*Pemphigus populitransversus*) and other aphids play a significant role in reducing crop yields directly and indirectly, by transmitting viral disease and contaminating harvested fruit. *Myzus persicae* was found on 400 species in 40 different plant families considered as hosts (Blackman and Eastop 2000). Species complex can be seen on most of vegetable crops. Green peach aphid and mustard aphid are common and abundant species and infestations typically begin on the top of the plant at succulent places, and moves to the bottom on most leaves. Potato aphid infestation is generally scattered over the plant. The major concern, however, is the transmission of viruses through the adults and nymphs. Over 100 viruses can be transmitted by the green peach aphid. Temperature plays a great role in its influence on plant, especially cold winter in North India. As the temperature rises and become warmer, the aphids produce winged forms and migrate to the neighboring crop. Multiple generations then take place through parthenogeneis.

11.2.1 Life Cycle of Aphid

Each female produces about 50 to 100 nymphs, depending on the species. They molt about four times before becoming adults. There is no pupal stage. The life cycle can be completed within a few weeks to 2 months, depending on weather conditions. A winged adult female aphid deposits a neonate under the leaf surface and move to the next plant. These new colonies of aphids produced on leaves are capable to produce female offsprings only that appear later on in the season, during late January onwards. When food deteriorates and temperature are not favorable, winged forms appear that fly to new host in search of fresh green matter.

11.2.2 Nature of Damage

Nymphs and adult suck the cell sap by feeding on soft parts of the plant such as new leaves, buds and fruit. As a result the leaves curl upward, veins become prominent. Excess feeding leads to more honey dew secretions, followed by high sooty mould attacks that affect photosynthesis and result in lower yields.

Aphids act as carrier for viral disease. A few examples are: Potato Leaf roll Virus and Potato Virus Y to solanaceous plants (pepper, potato, tomato); Cauliflower Mosaic Virus and Turnip Mosaic Virus to crucifers, and Cucumber Mosaic Virus and Watermelon Mosaic Virus to cucurbits.

11.2.3 Management Strategies

1. Grow resistant varieties especially against viral disease.
2. Reduce overwintering populations by eradicating weeds in and around the field.
3. Monitorthe aphid vectors of potato viruses determining the optimual dates for haulm destruction, when the number of winged aphids increase, as vector of economically harmful diseases (Milošević et al. 2014).
4. Avoid split application of nitrogen, which may improve the nutrition of the phloem sap at the wrong time, resulting in high population densities of winged aphids and enhance a rapid, early crop development.
5. Remove volunteer plants as they act as reservoirs for viral disease in potatoes.
6. Use of metallized (aluminated) or reflective mulch that reduce aphid populations interfering with the ability of winged aphids to find host plants (Cannon and Bunn 2017).
7. Common predators are anthocorid pirate bug, *Orius sauteri* (Poppius), *Chrysoperla* sp., many coccinellid i.e. lady bird beetle with grub and syrphid fly larvae. *Cheilomenes* sp., *Ischiodon aegyptius*, *Lysiphlebus testaceipes,* ***Aphidius colemani*****,** *A. ervi, A. abdominalis, Diaeretiella rapae*, *Pachyneuron* sp. (hyper pararsitoid) *Syrphophagus africanus* and aphid predatory midge: *Aphidoletes aphidimyza*, *Neozygites* (entomopathogen) (Sæthre et al. 2011).
8. Banker plants can be planted along the main crop, such as barley (*Hordeum vulgare* L.) infested with bird cherry-oat aphid, *Rhopalosiphum padi* (L.) to which *A. colemani* is released (Weintraub et al. 2017)..
9. Populations of green peach aphids are reduced in winter by parasitic fungi, i.e. *Entomophthora aphidis, Lecanicillium lecanii, Beauveria bassiana* and *Metarhizium anisopliae* (Yun et al. 2017).
10. Several effective commercial formulation of *B. bassiana* like Boverin, Boverol, Mycotrop and Ostrinil may be applied.
11. Commercial formulation of *Verticillium lecanii* i.e. Vertalec may be used against aphids (Gupta et al. 2012).

12. Insecticidal soap or horticultural oil sprays can reduce aphid populations and conserve natural enemies (Table 11.1).
13. Spray the crop with rogor 30 EC (dimethoate) or metasystox 25 EC in 80–100 litres of water/acre.

Table 11.1 Commercial available entomopathogens products applied against insect pests (Kalha et al. 2014)

Strains	Product name	Targets
Bacterial *(Bacillus thurigiensis)* strains		
kurstaki	Able, bactospeina, Condor, Coatar, Dipel ES, Bactimos L., Futura, lepinox, Thuricide	Lepidoptera
aizawai	Florbac, Agree, Design, Xentari	Lepidoptera
kurstaki SA-12	Costar	Lepidoptera
kurstaki	Foil, raven	Lepidoptera/ coleoptera
kurstaki HD-1	Thuricide, Biobit, Dipel, Foray, Javelin, vaultthogenic	Lepidoptera
BACULOVIRUS		
SeNPV	Spod-X	Beet armuworm
HzNPV	Genstar, Elcar	Helicoverpa
CpNPV	Madmex	Codling moth
AgNPV	VPN	Velvetbean caterpillar
AcNPV	Gusano	Autographa californica
SINPV	Spodopterin	Spodoptera litura
ENTOMOPATHOGENIC FUNGI		
Beauveria bassiana	Mycotrol, Naturalis Conidia, Ostrinol, Boveria	Sucking pests, borers
Metarhizium anisopliae	Bio-Blast	Termite
Metarhizium flavoviride	Green muscle	Grasshoppers and locust
Verticillum lecanii	Vertalec, Mycotal	Whiteflies and thrips
Paecelomyces fumosorosus	PFRE-97, PreFeral	Whiteflies and thrips
ENTOMOPATHOGENIC NEMATODES		
Steinernema carpocapsae	Ecomask, Savior Weevil larvae, Guardian	Caterpillars
S. carpocapsae, Heterorhabditis bacteriophora	J-3 Max	Caterpillars
H. bacteriophora	Heteromask	Weevils, grubs
H. bacteriophora	Lawn Patrol	Weevils, grubs
S. feltiae	Scanmask Entonem Nemasys	Fungus Gnats

11.3 Whitefly

Bemisia tabaci (Gennadius) (Homoptera: Aleyrodidae) is a serious pest of field and horticultural crops throughout the world (Bayhan et al. 2006). At present, it is globally distributed and occurs on all continents except Antarctica (Martin et al. 2000). It is known to infest more than 600 plant species (Stansly and Naranjo 2010). Whitefly is quite serious on brinjal, cucurbits, chilli, capsicum, okra, beans, tomato and potato. Many factors are responsible for buildup of whitefly and some are listed below.

1. Round the year cultivation of preferred hosts and high reproductive rates provide optimal conditions for the development of whitefly population.
2. Weeds and volunteer plants in/around the fields during off season.
3. The non-judicious use of fertilizers and irrigation water along with susceptible cultivars further boost the pest.
4. The mild winter conditions in North India are known to elicit further carryover of whitefly.
5. Insecticide resistance in whitefly population has made the task difficult for growers.
6. The indiscriminate and non-judicious use of insecticides, particularly synthetic pyrethroids, induces the resurgence of whitefly and may affect the natural enemy complex, including general predators.

11.3.1 Life Cycle of Whitefly

Whiteflies have incomplete metamorphosis with three development stages i.e. egg, nymph and adult stage, with an additional stage designated as pseudo pupal stage. Eggs are laid either singly or in scattered clusters, usually in circular groups, on the underside of leaves, with the broad end touching the surface and the long axis perpendicular to the leaf. A female lays about 50–100 elliptical eggs on the lower leaf surface,depending on host species, temperature and humidity. Eggs are whitish when first laid but gradually turn brown. The eggs hatching takes place within a week to produce crawlers which are flat, oval, transparent, light green and scale-like. This first instar is the only larval stage of this insect which is mobile. It moves from the egg site to a suitable feeding location on the lower surface of the leaf where its legs are lost in the ensueing moult. It does not therefore move again throughout the remaining nymphal stages. The crawler may walk for few hours to cover a distance of a few millimeters before settling down on the leaf. Soon after settling, the crawler inserts its mouth parts into leaf tissues and the stylet follows an intracellular path until the phloem is penetrated and sap extraction begins. After passing through four instars, the adults emerge from the pupae. Their longevity is generally variable, depending upon the month of the year. There are 11–15 generations in a year. The adults are about 1 mm long with whitish wings and yellowish body dusted with

white waxy powder. The adults are week fliers, make trivial flights and rapidly settle down on the same plant or on the adjoining one.

11.3.2 Nature of Damage

Being polyphagous, whiteflies remain active throughout the year on a variety of hosts. Damage is caused by direct feeding and heavy infestation may reduce plant vigour and growth. Its direct feeding induces physiological disorders resulting in chlorosis. Manyvspecies produce honeydew, on which black sooty mold grow, reducing the photosynthetic capabilities of plants This situation results in stunting of plants. Bedford and Mackham (1993) reported monetary losses of 500 M USD in cotton and vegetables.

The whiteflies have piercing and sucking type of mouthparts, thus included in the category of efficient vectors like other hemipterans. They are vector of more than 100 plant viruses, which cause diseases to many commercial crops in different parts of the world (Jones 2003). Important viruses transmitted by whiteflies in vegetables include Tomato Yellow leaf Curl Virus, Tomato Torrado Virus, Sweet Potato mild mottle Virus, Cucumber Yellows Virus and Tomato Chlorosis Virus.

Bemisia tabaci has strong relationship with abiotic factors like temperature, humidity and precipitation. Extremeweather conditions appear to play an important role in population dynamics in some areas (Sharaf 1982). Upper temperature thresholds for growth and development are probably higher than 35 °C (Wang and Tsai 1996). Differences in development times of as much as 10 days have been observed on different hosts at similar temperatures.

11.3.3 Management Strategies

1. Adults should be sampled early in the morning. The crop near edges of the field is usually infested first if the adults are moving into the crop from infested areas.
2. To avoid unwanted use of insecticides, economic threshold levels suggested by different workers should adopted.
3. The use of sound cultural practices may avoid, delay, or lessen the severity of the whitefly infestation and is a good basis to begin with.
4. In crop rotations, allow a host-free period. This will prevent the continuous availability of host plants for whiteflies and ultimately reduce their build up.
5. Seedlings are a major source of spreading whitefly into new plantings. Insect free seedlings should be used as young plants are generally more vulnerable to damage. Early infestations then need to be checked.
6. Be alert for rapid population buildup when nearby host crops are in decline.
7. The removal of weed flora in/around vegetable fields during the crop season helps in checking the build up of whitefly population in subsequent sown crops.

8. A closer spacing helps in creating conditions favorable for buildup of whitefly population. It also hampers the insecticidal control operations as the insecticide fails to reach the target site.
9. The application of adequate amounts of nitrogenous fertilizers coupled with judicious use of irrigation water can check the buildup of whitefly population. As observed, the excessive use of nitrogen generally increases the population of whitefly.
10. Adults and eggs of whitefly are mostly found on the underside of young leaves while older nymphs are found on older leaves. The presence of red-eyed nymphs in large numbers means that adult numbers will increase rapidly within the next few days.
11. Conserve beneficial insects by avoiding or delaying the use of broad spectrum chemistry for as long as possible.
12. *Bemisia tabaci* is strongly attracted to the yellow color, so place yellow sticky cards or yellow plates/tins coated with grease in the field so that the attracted flies get stuck to the sticky material.
13. To deal with lower levels, place yellow sticky traps to monitor and suppress infestations.
14. Emphasis should be laid for conservation and augmentation of biological control agents by avoiding or delaying the use of broad spectrum chemistry for as long as possible. Natural predators such as ladybugs, lacewings or parasitoids of whitefly can be released.
15. In recent years many plant oils have been positively tested against whitefly, which include neem oil, cotton seed oil and castor oil. Neem oil and neem seed kernel extract is used for the control of whitefly in the initial stages of crop, and many commercial formulations are available.
16. Early season treatments for whitefly should be limited to neem based formulations and insect growth regulators (IGR).
17. Integration of available tactics to manage whitefly is the only option available and should be given due consideration. We should develop IPM strategies based on ecological approaches.
18. *Verticillium lecanii* is pathogenic to whiteflies and can be used as an effective biocontrol agent for their management.
19. *Paecilomyces fumosoroseus* can be utilized for the control of *B. tabaci* (Osborne and Landa 1992). Another fungus, *Aschersonia aleyrodis* has restricted host range and infects only whiteflies.
20. The mixture of strain IfB01 of *Isaria fumosorosea (Paecilomyces fumosoroseus)* at 2.5×10^6 conidia/L and imidaclorprid at 12.5 mg/L gave highest synergism for control of *B. tabaci* (Zou et al. 2016).
21. Spray the crop with diafenthiuron 50 WP at 200 g/ha, 80–100 litres of water/acre.

11.4 Fruit Borer

Tomato is one of the most important and widely grown vegetable crop in the world. It is a good source of nutrients especially vitamin C. Tomato ranked third after potato and sweet potato on consumption basis. Many biotic and abiotic factors are responsible for low productivity of tomato crop. It is attacked by various insect pests, but the tomato fruit borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) causes major damage to the crop. It is a polyphagous and destructive pest that infests more than 180 plant species belonging to 45 families in India (Manjunath et al. 1989).

11.4.1 Life Cycle of Fruit Borer

The female of tomato fruit borer lays several hundred eggs, on various parts of the plant. Under favourable conditions the eggs hatch within three days. The eggs are spherical, creamy white when freshly laid. The larvae are fully developed in 13–22 days and are 30–40 mm long at the final instar stage. Their color is variable, but mostly green and yellow to red-brown. Three dark stripes extend along the dorsal side. If disturbed, larvae fall from the plant and curl up on ground. Wu et al. (1993) reported that larval duration of *H. armigera* decreased as the temperature increased. The pupa is brown in colour and pupation occurs mostly in soil. The pupae develop in 10 to 15 days in soil at a depth of 4–10 centimetres. Adult moths are strong with 3.5–4 cm wing span. Females are light pale to brownish yellow stout moths, whereas males are pale greenish. Adult longevity on various host plants was 7–9 days. Singh and Sidhu (1990) reported that the total development duration varied from 26 to 168 days in different seasons on tomato, under Punjab, India conditions.

11.4.2 Nature of Damage

Females lay the majority of eggs on upper and lower surfaces of leaves. Young instars scrap the foliage whereas older larvae attack fruits and bore circular holes into the older fruits,. They thrust the head inside the fruit and eat the inner content, whereas the rest of the body hangs outside. Tomato fruit borers causeconsiderable losses up to 55 % of tomato crop (Selvanarayanan 2000). Worldwide, the annual crop losses due to *H. armigera* are about 5 billion USD (Sharma 2001). In India, yield losses around 38 % were recorded (Selvanarayanan and Narayanasamy 2006). Synthetic chemical insecticides have been used for controlling *H. armigera* but the pest could not be brought under control by using insecticides alone. The chemicals cause also negative effects to beneficial organisms/natural enemies (Bisane et al. 2009).

11.4.3 Management Strategies

1. Grow less susceptible genotypes.
2. Mechanical collection and destruction of bored fruit at periodic intervals (3–4 times) brings down the borer incidence to less than 2% (Anonymous 2012).
3. Avoid planting tomato near alternate hosts to prevent heavy infestations by *H. armigera.*
4. Deep ploughing the field will exposed the different stages of insects viz., egg, larvae and pupae to direct sunlight that helps in the reduction of pest load, and prevents the pest population build-up.
5. Collect and destroy the infected fruits and grown up larvae.
6. Grow African marigold as a trap crop for integrated management of the fruit borer.
7. Light traps can be used to attract and kill the adult moths.
8. Install pheromone traps with Helilure at 10–12/ha for its effective management.
9. Place 15–20 bird perches in 1 ha for inviting insectivorous birds for management of the fruit borer.
10. Spray the crop when density levels reach 1 larva/plant or 2% fruit damage.
11. Use of nuclear polyhedrosis virus (NPV) at 10 days interval and *Bacillus thuringiensis* at 500 g a.i. /ha (10 days interval) also give protection against fruit borer (Rai et al. 2014).
12. *Heterorhabditis bacteriophora* as an effective entomopathogenic nematode causing 74% mortality in *Helicoverpa* sp. (Vashisth et al. 2019)
13. *Metarhizium anisopliae*, *Beauveria bassiana* and *Nomuraea rileyi* were the most virulent strain and can be considered as promising agents against fruit borer (Nahar et al. 2004).
14. *Serratia marcescens* strain SRM could be suitably exploited for management of *H. armigera* (Mohan et al. 2011).
15. Natural enemies such as *Trichogramma chilonis* and *Chrysoperla carnea* can be released starting from flower initiation stage.
16. Inundative release of *T. brassiliensis* at 50,000 eggs/ha alone beginning with fruiting (Rai et al. 2014).
17. Spray neem based formulations to kill early instars larvae of fruit borer.
18. Combination of pheromone trap plus neem seed kernel extract plus *T. chilonis* and Bracon hebetor (Rahman et al. 2016).
19. Methanolic of *Vinca rosea* and *Callistemon lanceolatus* act as IGR, antifeedent plant product (Halder et al. 2009).
20. The last option for management is spraying with Coragen 18.5 SC at 60 ml, Fame 480 SL at 30 ml, and Indoxacarb 14.5 SC at 200 ml per acre, against fruit borer.

11.5 Tobacco Caterpillar

Spodoptera litura Fabricius (Lepidoptera: Noctuidae) is a polyphagous pest feeding on more than 120 species of host plant and was first described by John Christian Fabricious in 1775. It has been recorded from Afghanistan, Burma, China, Indonesia, Japan, Korea, Srilanka, Taiwan, Thailand etc. (Divya 2016). It is the most common in southern Asia. It is a severe pest during August to October and then in February to March.

11.5.1 Life Cycle of Tobacco Caterpillar

As holometabolus in nature, complete four stages of its life viz., egg, larva, pupa and adult. The adult female lays eggs on the lower surface of the leaves in clusters that are covered with shining brown hair to protect them from natural biotic and abiotic factors. The newly hatched larvae feed gregariously and later instar became solitary in nature, feeding singly on plants. There is a color variation in this insect that vary from light green to dark brown on both dorsal and ventral body sides. Larvae complete six instars and the life cycle is completed in 32–60 days. The pest completes eight generation in a year (Srivastava and Dhaliwal 2011).

11.5.2 Nature of Damage

The larvae feed gregariously for the first few days and then the instars disperse to feed individually. The older larvae feed on leaves and fresh growth and devour the leaves resulting in poor plant growth.

11.5.3 Management Strategies

1. Remove the crop debris from and around the field that harbor these pests.
2. Deep ploughing during peak summer will kill pupae.
3. For monitoring the appearance of adult, use of pheromone trap is highly effective in forecasting the crop damage.
4. Hand picking and mechanical destruction of egg masses and gregarious forms of first and second instar larvae.
5. 100 parasitoids, 50 predators and more than 12 entomopathogens have been recorded on *S. litura* in different countries. Parasitoids include *Telenomous remus* Nixon, *Glyptapenteles africanus* Cameron and *Cotesia marginiventris*. Predators are *Chrysoperla* spp., *Harpactor costalis* and *Andrallus spinidens* (Divya 2016).

6. Application of NPV at 375 LE ha^{-1} (Battu et al. 1998) or *Bacillus thuringiensis* (Bt) formulation at 1000 ml ha^{-1} (Hussain 2001).
7. Application of *Metarhizium anisopliae* FT83 at 1×10^7 conidia/ml and *Paecilomyces fumosoroseus* FG340 at 1×10^4 conidia/ml (Han et al. 2014).
8. *Serratia marcescens* strain SRM could be suitably exploited tomanage *S. litura* (Mohan et al. 2011).
9. A bacterial isolate *Enterobacter cloaca* SL11 has insecticidal potential to manage this pest and shows high mortality (Thakur et al. 2015).
10. Entomopathogenic nematodes such as *Steinernema glaseri* and *Heterorhabditis bacteriophora* at 1000 infective juveniles (IJ) /ml were found effective (Safdar et al. 2018)
11. Use poison baiting. Mix 10 kg of rice bran or wheat bran with 2 kg jaggery by adding a little water in the morning. In the evening add 250 gm of Methomyl or Thiodicarb formulation and sprinkle over the bed. Caterpillars get attracted to fermenting jaggery, feed and get killed (Kumar 2012).
12. Leaf extract of avocado (*Persea americana*) act as contact and antifeedent against this pests (Rai et al. 2014)
13. Insecticides like novaluron 10 EC at 150 ml per acre are effective against *S. litura*.

11.6 Conclusions

Indiscriminate use of pesticides leads to various adverse effects on the environment like problems of secondary pest outbreak, pest resurgence, resistance and ill effects on human health and environment. These disasters led researchers to evaluate safer, alternative ways to manage these voracious pests. Integration of methods such as early sowing or delayed sowing helps to escape the peak infestation of pests. A rational use of nitrogen fertilizers is recommended, as excess nitrogen makes the plant more succulent, as having higher sugar and water content they are more attractive to pest.s Other alternative control methods like use of pheromones, sticky and light traps also help in monitoring the pest population and forewarn its appearance in the field. Use of bird perches can be helpful to reduce the pest population as it provides space for birds to search their food easily, while sitting on the branch without any appreciable additional cost. Augmenting bioagents such as parasitoids and conserving them in the field, with additional application of microbial agents such as entomopathogenic bacteria, fungi and nematodes are promising control methods. Many of these alternative control measures are relatively cheaper. Microbes can cause horizontal transmission from infected hosts and most of them are selective in nature and safer to non target organisms. All these methods are eco-friendly and can reduce the pesticide load on vegetables and the environment. They can be easily integrated in IPM programmes, for a safer and sustainable cultivation.

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