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Introduction to Insect Biotechnology



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Dhiraj Kumar • Sangeeta Shukla
Editors

Introduction to Insect Biotechnology

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ISSN 2509-6125

ISSN 2509-6133 (electronic)

Learning Materials in Biosciences

ISBN 978-3-031-26775-8

ISBN 978-3-031-26776-5 (eBook)

<https://doi.org/10.1007/978-3-031-26776-5>

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Preface

The book “Introduction to Insect Biotechnology” is envisioned for graduate, postgraduate students, and young scholars who are interested in acquiring basic as well as updated information about insect biotechnology. The initial chapter provides a fundamental overview of insects, their origin and evolution including contribution of biotechnological and molecular biology techniques. Certainly, a new vista in insect biotechnology emerged with the development of molecular tools and methods. Understanding whether insects are friends or foes via the prism of biotechnological tools is one of the most fascinating accessible content of this book. Recent developments in insect biotechnology have been detailed, including work on insect genomes, transcriptomics, proteomics, and metabolomics as well as the development of numerous valuable products, such as antimicrobial peptides and insect-based recombinant proteins for humans and veterinary use. Biotechnological interventions leading to disease diagnosis in economically important insect model and development of disease resistant/tolerant are also highlighted. The significance of gut microbiome of insects in contemporary research is also addressed in this book, enabling students to explore in depth the relationship between gut microbiota and immune genes in detail. Insect metagenomics and its fundamental protocols are also covered. The molecular signatures of host–pathogen interactions in virus-infected Lepidopterans are briefly discussed in another chapter that also offers considerable insight into a vast repertoire of antiviral immune mechanisms. A chapter that is focused on the future of insect biotechnology towards the end of the book undoubtedly inspires academicians, scientists, and students to explore the fascinating world of insects.

New Delhi, India
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Acknowledgement

It is a great privilege to express my humble gratitude to the co-editor Prof. Sangeeta Shukla for collaboration, suggestions, and inputs in the book. I am extremely thankful to all the contributing authors for the devotion of their precious time to complete their task. This book is dedicated to the memory of my father Late Jiya Lal. When I initiated this volume, he was with me and always motivated, supported, inspired, and blessed me to frame this book. I extend my humble regards to the members of publishing team of Springer, all are always supported in the compilation of this book. I express my special thanks to my beloved daughter “Viha”, who is always a source of motivation to complete this assignment. I am thankful to “The Almighty” who has been always there to help me to sustain the endurance during my entire academics.

Dhiraj Kumar

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About the Editors



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Introduction and History of Insect Biotechnology

1

Dhiraj Kumar, Shubham Singh, Rajesh R. Kundapur, Divya Gupta,
and Sangeeta Shukla

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Keywords

Insects · Insect biotechnology · Evolution · Agriculture · Molecular techniques

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D. Kumar, S. Shukla (eds.), *Introduction to Insect Biotechnology*, Learning Materials
in Biosciences,

https://doi.org/10.1007/978-3-031-26776-5_1

What You Will Learn from This Chapter

Insects are the most successful organisms on earth in terms of their diversity and adaptability. Insect biotechnology or yellow biotechnology using these insect resources is an emerging area for biotechnology along with several claims. Insect resources have long been used to make food or functional food, feed different animals and different nutritive shakes, cosmetics as well as medicine and industrial ingredients. The insect cell lines have been used to express recombinant proteins that were thought to be very difficult to functional expression for public purposes. Only interdisciplinary research will guarantee the success story for insect biotechnology in the current situation in near future. Insects as a bioresource for new products with applications in medicine, agriculture and industry in near future. This chapter will definitely bring some basic knowledge of insect biotechnology related to their applied aspects from agriculture to modern health problems and the possible role of insect biotechnology in the development of future biotechnology using this bioresource.

1.1 Introduction

Nature has made arthropods one of the most essential species for balancing the ecosystem, flora and fauna. Butterflies are certainly one of the most attractive creatures of nature, with colours and patterns that are enjoyed by humans throughout the year. The consumption of edible insects started nearly 7000 years ago [1]. More than 2300 species of 18 orders have been reported as edible insects, of which 5 orders are with at least 100 records. These insects inhabit both aquatic and terrestrial environments [2]. The majority of them are harvested from nature though some species are farmed on a large scale. Among all, class Insecta covering about one million species of insects is the largest and most diverse group of invertebrates that evolved 400 million years ago.

Insects are involved in diverse roles in our daily life, such as pollination, soil turning and aeration, dung burial, pest control and wildlife nutrition, and form one of the important components of the ecosystem. Insects make the biological foundation for all terrestrial ecosystems. They continuously cycle nutrients, pollinate plants, disperse seeds, maintain soil structure and fertility, control populations of several other organisms and also provide a major food source for other species. Insects kept in beneficial categories provide several useful products for mankind since ancient times (Fig. 1.1). However, many harmful insects are also existing that feed on crops, fruits and seeds, but they can also destroy human properties such as clothes or wood. Blood-sucking insects such as mosquitoes, tsetse fly and sand fly can transmit several diseases such as malaria, leishmaniasis, schistosomiasis, etc.

Fig. 1.1 Products obtained from insects



Soil insects are important for the maintenance of healthy and productive agricultural ecosystems. Almost any depiction of a food web in a terrestrial or freshwater ecosystem will demonstrate insects as a major key component, although the food-web structure in these two ecosystems is quite different. Insects are having great importance as a source of food for a wide range of predators. Aquatic insect larvae serve as food for fishes and many stream fishes seem to be limited by the availability or abundance of such prey, at least on a seasonal basis. Myriads of adult mayflies are devoured at the season of their emergence by trout and this phenomenon forms the basis of the fly-fishing sport. Insects provide the major food supply for many lizards. Many amphibians are carnivorous, especially after they reach maturity, and insects form the bulk of their animal food. Birds of many families take insects as their staple food, at least during part of the year martins, swallows and swifts are almost completely dependent on flying insects for survival. Mammals such as anteaters, sloth bears, sun bears and African and Oriental pangolins are particularly tied to ant and termite colonies and a number of mammalian predators use insects as food. The British badger often digs out wasp nests to feed on the grubs and in North America black bears in north-central Minnesota feed on ants in the spring for speedy sources of protein and to obtain essential amino acids and other trace elements that are unavailable in other spring foods. Aggregations of the alpine army cutworm moth *Euxoa auxiliaris* is an important, high-quality, preferred summer and early-fall food for grizzly bears in Glacier National Park, Montana.

Class Insecta consists of around 30 orders out of which four mainly Coleoptera (beetles), Lepidoptera (butterflies and moths), Diptera (flies) and Hymenoptera (wasps, bees and ants) are dominant. Insect biotechnology or yellow biotechnology is defined as the use of whole insects, their organs, cells or molecules and their symbiotic microbes in the fields of medicine (red biotechnology), agriculture (green biotechnology) and industry (white biotechnology). The word yellow biotechnology has been chosen because of the

yellow colour of insect haemolymph, which has thus far delivered a number of chemicals and proteins used in medical, pharmaceutical, agricultural or industrial applications.

Products from insect biotechnology are applied in several fields such as agriculture, medicine, industry and several other fields. Insect biotechnology has been proven to be a useful resource in several industries, especially in the biological field including the production of enzymes, microbial insecticides, insect genes and many other substances. Insect cell culture mainly using lepidopteran cells constitutes a competitive strategy against mammalian cells for the production of biotechnology products. Among the wide range of methods and expression hosts available for the production of biotech products, they are ideal for the production of complex proteins requiring extensive post-translational modification.

The new substances resulting from this method have applications in medicine, the sustainable fortification of plants and industrial biotechnology. This means that insect biotechnology employs the overall principles of biotechnology. More specifically, insect biotechnology borrows from and is driven by scientific breakthroughs in molecular biology, particularly by the development of tools and techniques that allow genetic characterization and engineering of organisms and cells with recombinant DNA technology and plant protoplast fusion. As a basic research tool, the strategies of insect biotechnology include sequencing and annotation of insect genomes as well as scrutinizing using comparative genomics. Insect biotechnological applications to pest management include the development of resistant crops and trees that express insect-specific toxins mainly BT toxin, the design of microbial agents with enhanced insecticidal potency, and the engineering of insects that can transfer lethal genes to natural populations following their mass release in the field. Comparative genomics analyses also make it possible to identify insect-specific genes which can be targeted for rational insecticide design.

1.2 Insect Evolution

As per the red queen hypothesis, if you want to survive in nature, you have to evolve continuously with time otherwise nature will eliminate. The same concept was applied to insects, therefore those who evolve, survived and that do not change become fossils and eliminated by nature from the earth. The most recent understanding of the evolution of insects is based on studies in every branch of science including molecular biology, insect morphology, palaeontology, insect taxonomy, evolution, embryology, bioinformatics and scientific computing. It is estimated that the class Insecta originated on Earth about 480 million years ago, in the Ordovician, at about the same time terrestrial plants appeared. Insects have evolved from a group of crustaceans.

The first insects were land bound but about 400 million years ago in the Devonian period, one lineage of insects evolved to flying, the opening of flying animals. The oldest insect fossil has been proposed to be *Rhyniognatha hirsti*, which is estimated to be 400 million years old. Global climate conditions changed several times during the history

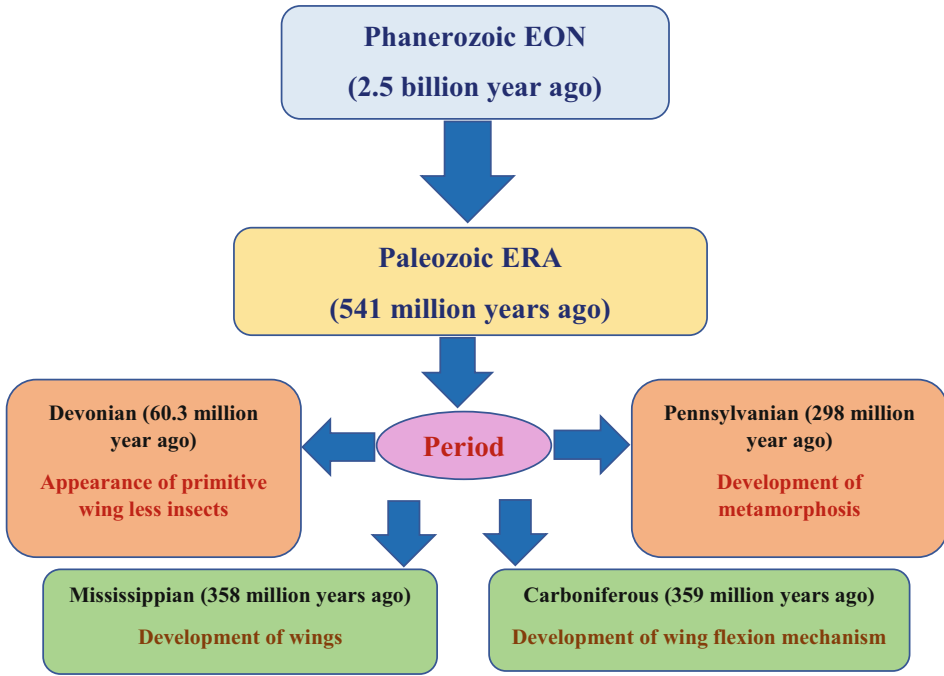


Fig. 1.2 Insect evolution and their developmental period

of Earth, and along with it the diversity of insects also changes. The Pterygotes (winged insects) underwent major radiation in the Carboniferous (356 to 299 million years ago) while the Endopterygota (insects that go through diverse life stages with metamorphosis) underwent another major radiation in the Permian (299 to 252 million years ago). Most extant orders of insects developed during the Permian period [3].

Many of the early groups became extinct during the mass extinction at the Permo-Triassic boundary, the largest extinction event in the history of the Earth, around 252 million years ago. The survivors of this event evolved in the Triassic (252 to 201 million years ago) to what are essentially the modern insect orders that persist to this day. Most modern insect families appeared in the Jurassic (201 to 145 million years ago). In an important example of co-evolution, a number of highly successful insect groups especially the Hymenoptera (wasps, bees and ants) and Lepidoptera (butterflies) as well as many types of Coleoptera (beetles) and Diptera (flies) evolved in conjunction with flowering plants during the Cretaceous (145 to 66 million years ago) [4]. Numerous modern insect genera have been developed during the Cenozoic that began about 66 million years ago; insects from this period onwards frequently became preserved in amber, often in perfect condition. Such specimens are easily compared with modern species, and most of them are members of extant genera (Fig. 1.2).

1.3 History of Insect Biotechnology

In the course of evolution, insects have developed a huge composition of active compounds, which they use to defend themselves against enemies and diseases and to explore novel food sources. Insects have overwhelmed humankind throughout history together as participants in human nutrition, and as vectors of diseases such as malaria, elephantiasis and plague. On the other hand, the fruitful part is valuable and economically important insects such as honeybee, silkworm, lac worm, etc. from ancient times. Insects also have a significant role in the biological control of pests as well as natural pollinators [5].

Fruit flies (*Drosophila*) have long been used in genetic studies and are hands-on for several studies due to similar genes related to humans and their short lifespan. However, there are many visible genetic mutations including several different eye colours, which are valuable for geneticists studying *Drosophila*. A female may lay 30–50 eggs per day throughout her lifetime. The *Drosophila* feeds and breeds on fermenting fruit or on other causes of fermenting sugar such as waste in drains or rubbish bins. In the year 1912, the first report of the mitotic chromosomes of *Drosophila* was disclosed. She observed four pairs of chromosomes in spermatogonia and oogonia of *Drosophila melanogaster* [6]. The effective application of genetic tools helped to slice up the structure and function of prokaryotic genetic material in the 50s and 60s endeavour into a new era. It is provided tools to study a systematic genetic analysis of fly behaviour and principles of inheritance, development, behavioural neurogenetics and neuronal function using identified genes [7, 8].

About 25 years back, the first milestone in the manifestation of insect biotechnology was introduced in Europe. Insect biotechnology is also called yellow biotechnology due to insects' haemolymph yellow colour which uses a variety of insects, insect-based molecules, symbionts or cells to develop products or services for humans through biotechnological techniques in the area of industrial biotechnology, agricultural biotechnology and medical biotechnology. After the discovery of the structure of chromosomes, a milestone has been established in the study of insect chromosomes, i.e. polytene. At the beginning of insect genomics, *Drosophila* is selected as an animal model which regularly used for studying the structure of chromosomes and genes, development, behaviour and genomic studies. Morgan and Muller were the first to explore polytene chromosomes in a few tissues for *Drosophila*. Nonetheless, Lewis, Nusslein-Volhard, and Wieschaus were awarded the Nobel Prize in physiology or medicine in 1995 for their work on Hox genes and embryonic development in *Drosophila* (Fig. 1.3).

Animal territorialization is the process via animals populated on the land. Arthropods show the largest majority of terrestrial biodiversity. Lozano-Fernandez and his team executed a molecular palaeobiological method, connecting biotechnology and fossil evidence to explain the long history of the terrestrial arthropods [9].

The molecular techniques are necessary tools to unveil insect biochemistry, molecular physiology, endocrinology, metagenomics, metabolomics and proteomics. The main task of insect biotechnology is to make these natural substances available to society for the betterment of mankind. Insect biotechnology, therefore, has considerable economic

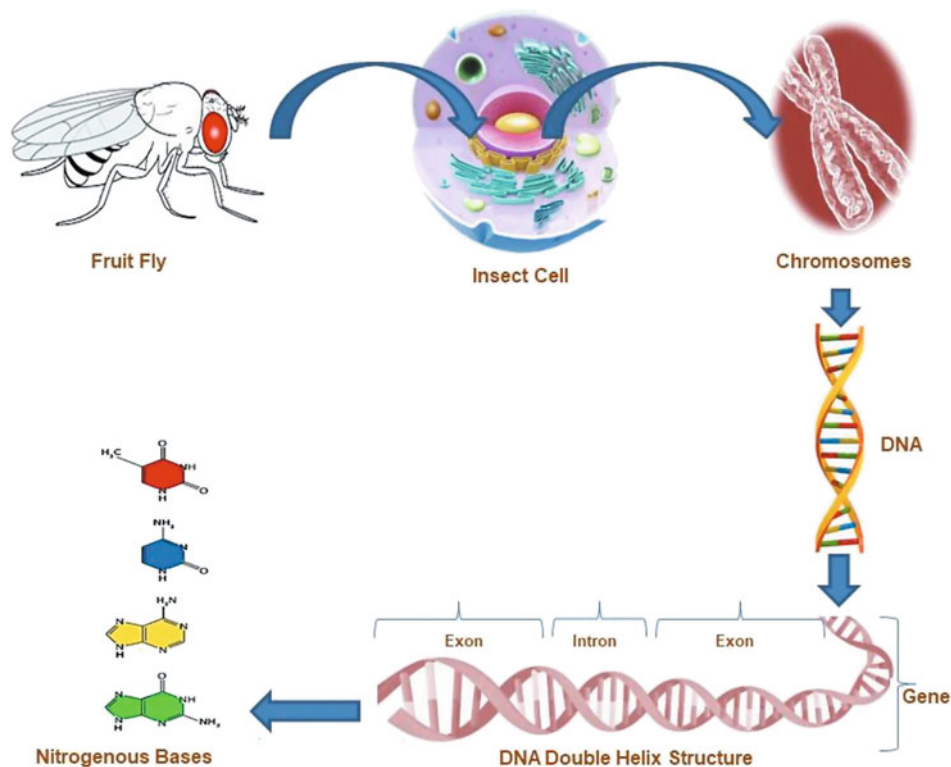


Fig. 1.3 Inside molecular structure of insect

potential and is the key factor for the spread of biodiversity and economy for humans through its various molecular and biotechnological applications. DNA and RNA are the base materials for molecular level studies similar to a higher organism. After the breakthrough in the protocol of DNA extraction and PCR amplification, millions of genes and their functions have been identified including transgenic cell development (Fig. 1.4).

The systematic development of new goods and services built on insects and their by-products has been pursued from ancient times starting from the use of honey for various purposes or the use of silk for making various colourful cloths. The greatest advances in insect biotechnology were driven by the advancement in molecular biology, particularly by the development of techniques, which allowed one to sequence the genomes of abundant several insect species. Recently developed molecular techniques are also being used, even if only partial sequence information is available because of their richness abundance. A milestone in the development of new molecular tools was the discovery of RNA interference, which allows a knocking out of individual genes and enabled scientists to study the functions of individual genes in insects.

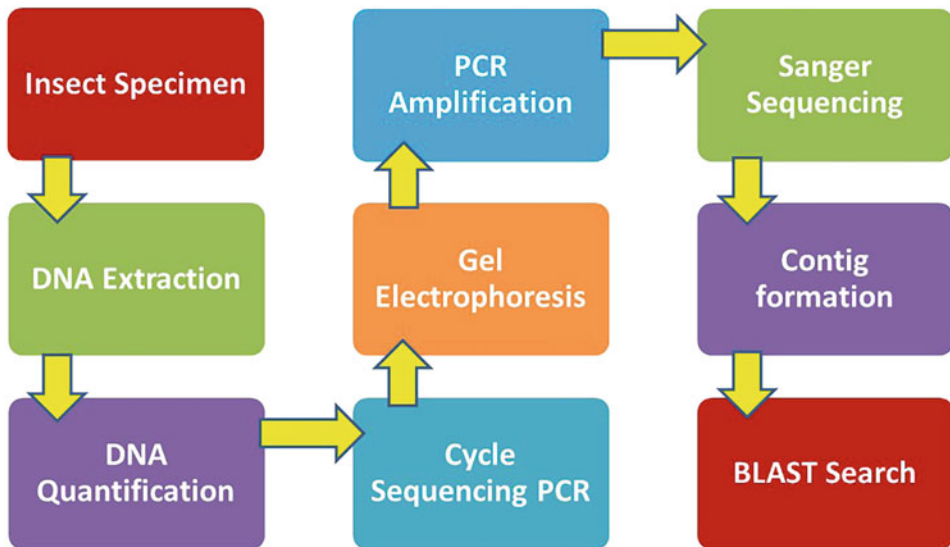


Fig. 1.4 Major steps involved in molecular techniques of insects

Insect cultivation is a novel and alternative approach for the production of protein-rich food for animals and humans as insects are having a very good amount of protein, carbohydrates, minerals and vitamins. A finding by Grau et al. reviews the mass rearing of the mealworm (*Tenebrio molitor*) for the production of food and feed for humans and other animals [10]. The paper predominantly focuses on challenges which are mainly contamination of food products with microbes from insect gut and the risk of speedily spreading pathogens and parasites found in their gut. They presented trans-generational immune priming and probiotic bacteria as alternative approaches to the use of antibiotics to prevent outbreaks of contamination among farm-produced insects. Muller et al. presented the black soldier fly (*Hermetia illucens*) as another promising source for sustainable production of proteins, lipids and bioactive substances like antimicrobial peptides and their other derivatives [11].

There are few papers that focus on the industrial applications of compounds derived from insects. Through the application of molecular techniques, Chinese scientists have reported recombinant silk as well as technical procedures to produce recombinant silk proteins into the fibre, transgenic films and hydrogels for biomedical needs. The good mechanical property of insect silk along with its non-toxic, biocompatible and biodegradable nature extracts these materials, e.g., for creating fish nets or to cover wounds and also during the spinning of skin during any type of surgical operation [12, 13]. A plasmid which is also called as vector is one important biotechnological tool. Inventors have taken the benefit of plasmids for the cloning, editing and transfer of genes. As these molecular tools and techniques reformed, insects' gene function, recombinant protein formation, application and product innovation have evolved (Fig. 1.5).

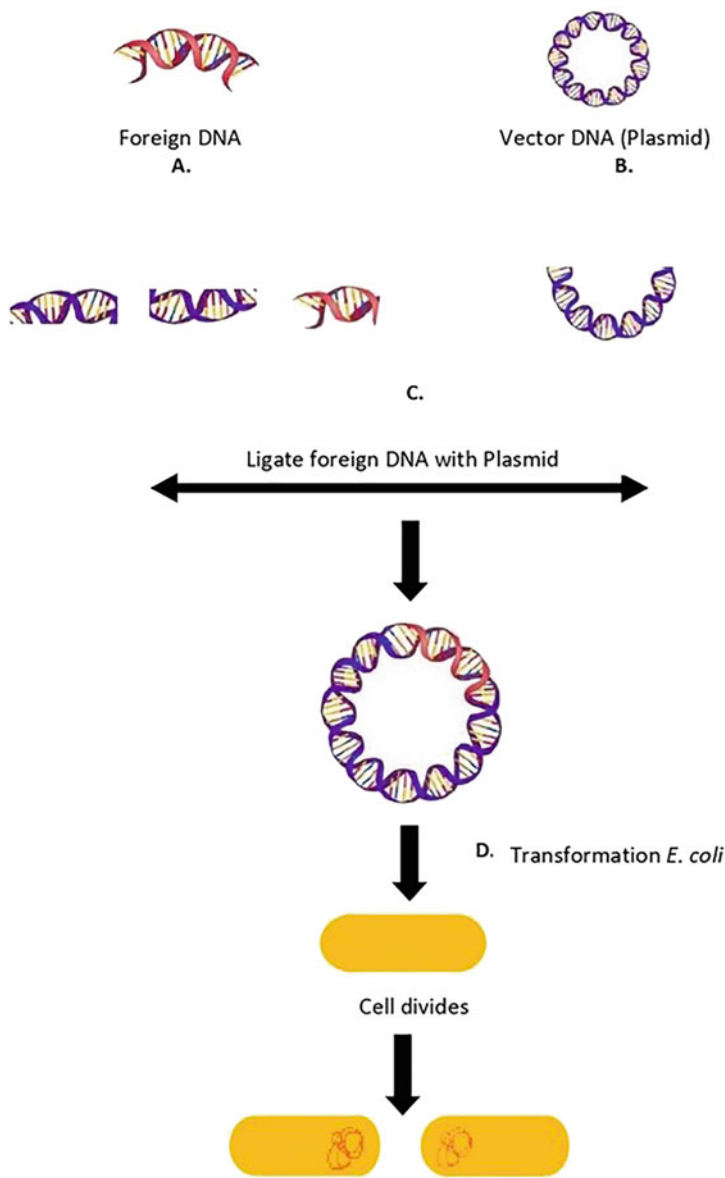


Fig. 1.5 Work flow of transgenic techniques in insects

1.3.1 Role of Biotechnology in Insect Diseases Diagnosis

Most people consider insects mostly as pests, but there are many more beneficial aspects of insects than pests. Many insects provide humans with useful materials such as honey, silk,

lac, propolis, bee venom and wax [14–16]. The pollination of crops by insects is of essential importance to mankind. The balance of nature depends on the activities of parasites and predators, following the hypothesis of co-evolution. Researchers have used this concept in biological control systems and have found it dramatically fruitful in many programs.

The recognition of silk as a valuable product dates back to China, arguably in the year 2640 B.C. Presently, China annually produces some 30,000 tons of raw silk, which accounts for 80 percent of the world's supply. Most silk is produced from the cocoons after the maturity of larvae nonetheless diseases such as pebrine, muscardine, NPV and CPV flacherie caused high economic loss. Molecular approaches are an additional advantage for the detection of specific diseases unwinding the pathogenic condition and helping to develop disease-resistant silkworm strains to increase cocoon production and economic benefits to the farmers. For the detection and characterisation of microsporidian infections, immunodiagnostic techniques such as latex agglutination and ELISA using polyclonal and monoclonal antibodies were developed [17, 18].

The value of pollination of plants by insects is almost countless. Honeybees are among the most important pollinators for the collection of honey. Bees collect pollen from different flowers, and their efforts result in an estimated 80 percent of all pollination in the world. However, many diseases occurred in honeybees which directly affect the bee farmer's returns. The microsporidia cannot divide outside their host cell. Biochemical and physiological analyses and genetic engineering were limited and hindered our understanding of the biology and pathogenesis of this pathogen. After the discovery of DNA and RNA sequencing, non-coding RNAs of honeybees including lncRNA, miRNA, piRNA and circRNA many outbreaks have been controlled using gene techniques and CAS9 protocol including molecular mechanisms underlying host–pathogen interactions [19–21]. Recently, long non-coding RNA studies have also revealed the pathogenic condition in honey and helped in the control of microsporidian diseases of the honeybee.

1.4 Insect Biotechnology and Agriculture

It has been shown by several studies that insects and nematodes are the world's most significant pests of agricultural plants and livestock. They have been shown to cause billions of dollars of losses to growers and livestock producers each year because of lowered yields, deterioration of quality and disease. As already specified earlier in several reports, chemical pesticides are a major tool for insect and nematode control. However, safer replacements to chemical pest control have become imperatives because most of these pesticides are potentially destructive to the ecosystem and human health. The beginning of biotechnology in general and insect biotechnology, in particular, is a welcome development in the fight against insect pests for the protection of crops and several other harmful impacts of insects [22, 23].

Modern agricultural biotechnology includes the management of the genetic makeup of organisms for practice in the production or processing of agricultural harvests. A key approach to increasing crop yield is the progress of pest management regimens consisting of herbicide-resistant (HR) and insect-resistant (IR) crops as well as transgenic insects. Genetically modified (GM) crops primarily became commercially available in 1996. By 2018, 90% of soybean, 78% of cotton, 72% of canola and 60% of maize hectares were cultivated worldwide with these GM crops. The most well-known case of this genetic manipulation in both plants and viruses is insertion into a plant or virus of the gene coding for the production of delta-endotoxin of *Bacillus thuringiensis* (Bt). *B. thuringiensis* is a Gram-positive, soil-dwelling bacteria, most commonly used as a biological pesticide. It is also found naturally in the environment of mosquito larvae, moth and butterfly caterpillars as well as on leaf surfaces, aquatic environments, animal faeces, insect-rich environments, flour mills and granaries. Many Bt strains produce “Cry” during the process of sporulation. Cry (Crystal proteins), also called δ -endotoxins, are proteinaceous inclusions coded by plasmid-based *cry* genes and have insecticidal action on susceptible insects. Bt has been reported non-toxic to humans and to a large extent nontarget fauna, making it a popular substitute for chemical treatments for crop protection. This has developed the use of Bt toxins as insecticides and more recently GM crops expressing Bt proteins in transgenic plants [24–28].

1.5 RNA Interference Mechanism in Insects

The post-transcriptional gene silencing strategy is a biological retort to dsRNA through transcriptional and translational repression that mediates the antiviral and innate immune system in insects known as RNA interference (RNAi). This discovery in insects was a breakthrough in the era of insect molecular biology. In insects, particularly in *Drosophila melanogaster* two RNA silencing pathways are recognized to exist which are mediated by siRNAs and miRNAs. The specificity is built on the sequence of one strand of the dsRNA corresponding to a part or all of a specific gene transcript. RNAi is a post-transcriptional switch mechanism involving sequence-specific degradation of a targeted mRNA. This degradation is simplified by the manufacture of small interfering RNAs (siRNAs) from the dsRNA, which is cleaved by dsRNA-specific endonucleases referred to as dicers gene in *Drosophila melanogaster*. The siRNAs are 21 bp dsRNA fragments carrying two nucleotide overhangs at the 3' end of each strand, one strand of the siRNA acts as a guide strand and is assembled into an RNA-induced silencing complex (RISC) in conjunction with the multi-domain protein. In insects and nematodes, RNAi has shown systemic effects on gene expression, so the gene knockout spreads throughout the organisms and persists over development. The synthesized dsRNA then acts on by the dicer enzymes to generate new siRNAs (secondary siRNAs), amplifying the silencing signal. In this way, once a dsRNA is introduced inside a cell, it will result in induction of gene silencing and persist through development as well as a unit. Nevertheless, sequence-specific gene

silencing using RNA interference (RNAi) offers enormous potential for successful pest management. Its success rate is limited due to several factors like supplied dsRNA are efficiently degraded, low amplification and spreading of the RNA signal, the target tissue is hardly permeable to dsRNAs and components of the RNAi machinery expression in the tissue. Additionally, gene efficiently overcomes RNAi by increasing mRNA expression, target mRNA is too transient and inaccessible to RNases and sequence polymorphism in resulted mRNA [6, 29–32].

1.6 Insects Role in Molecular Medicines

There are a few studies that emerge on the formation of new bacterial and virus strains and their expansion of resistance to antibacterial. In some cases perilous bacterial infections in medical clinics and, surprisingly, in day-to-day existence. A recent example to this list is Covid-19 virus. Reports of this sort of progress show the requirement of novel antiviral/antimicrobial compounds. Insects have been accounted for to create various antimicrobial mixtures which have potential as layouts for additional medication revelation and advancement [33]. In 1992, Bulet and colleagues reviewed the armoury of specialists utilized by insects to battle against attacking microorganisms. They revealed that it is nothing but a short cationic peptide which acts as an antimicrobial. Contingent upon the kind of species insect fat tissue can answer bacterial infection by the blend of a few antimicrobial substances (*Drosophila* adults produce more than 10 different molecules including drosocin, drosomycin, defensin, metchnikowins, dipteracin and cecropins) or by producing relatively few molecules (a single peptide defensin from the dragonfly *Aeshna cyanea*). Upon characterization it was observed that these peptides are universally found in various plants and animals. The industry has exploited that feature for the production of several new drugs from insects. For example, cecropins have similarities with the amphibian α -helical antimicrobial peptides, magainins found in frog skin secretions, while drosomycin from *Drosophila* and thanatin from the spined soldier bug, *Podisus maculiventris* have resemblances with plant defensins and frog skin secretion antimicrobial peptides. Bulet and his team a new discovery disclosed that insect antimicrobial peptides are helpful in overcoming the issue of acquired bacterial or viral drug resistance and of the emergence of astute pathogens especially in immunosuppressed hosts due to the capacity to kill target microorganisms quickly, had wide action spectra, not active against mammalian cells and method of activity that restricts the selection of resistant strains [34–38].

A survey of insect origin products and their development as medications revealed the status of insect biotechnology in medicine. In view of information acquired from insect folk medicines, researchers have depicted modern research into bioengineering of honey, propolis, venom from bees, silk, cantharidin, antimicrobial peptides, maggot secretions and anticoagulants from different insects into therapeutics. Recent inventions have described how modern molecular and biochemical techniques have made it feasible to manipulate and bioengineer insect natural goods into products with high medicinal value.

1.7 Conclusion

From the present chapter, it is clearly understandable that insects and their related products are involved in our day-to-day life. The current application of insect biotechnology is wide from agriculture to medicinal goods from the production of agricultural insect-resistant plants to the synthesis of insulin and other peptides. Products derived from insects are safer and without minimum side effects, so in near future, they can be used as alternatives to synthetic products which may harm our ecosystem. With the advancement of molecular techniques, insect biotechnology is still in the developing phase, in the near future a boom in insect-based products and their activities will be observed.

Take Home Message

- One of the important roles of molecular biology is to identify insect species and molecular biodiversity. Through molecular techniques, insect DNA could be isolated from ancient insect specimens, which helps to study insect evolution.
- Biotechnological methods applied to various insects are important to understanding insect genomics, proteomics, metagenomics, gene expression, signal transmission in insect populations, and immunology.
- Biotechnology has proven to disclose the complex molecular structure and their function in insects including disease diagnosis.
- Insects are a valuable resource in the production of chitinases and cellulases enzymes, recombinant proteins for biomedical applications.
- One can culture some insects in the laboratory and isolate the DNA through standard biotechnological protocols to study the basics of insect biotechnology.

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Why Biotechnology Needed in Insects?

2

Kamidi Rahul, Pooja Makwana, Suravi Ghosh, and Anil Pappachan

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Keywords

Insects · Omics tools · Silkworm diseases · Novel products of insect origin

What You Will Learn from This Chapter

Insects constitute majority of species on the planet “Earth” and contribute to diverse ecological functions. However, they are considerably understudied and underexplored for a long time. This scenario is rapidly changing with the advent of biotechnological tools that led to significant advancements in insect sciences. This chapter presents a quick review of OMICS (genomics, transcriptomics, proteomics, and metabolomics) tools that holds a great promise for gaining a better understanding of numerous biological characteristics of insects which include growth and development, physiology and biochemistry, immune responses, systematics and evolution. A section of this chapter also focuses on biotechnological approaches that led to identification of disease causing microbes and control of diseases in *Bombyx mori* L., one of the most researched lepidopteran insect models that have been exploited for millennia to produce the natural fiber, silk and finally highlights in brief about the products of therapeutic importance (antimicrobial peptides, therapeutic proteins/ vaccines) developed as a result of biotechnological interventions in insects.

2.1 Introduction

Insects are one of the most dominant and prominent living forms on Earth, having been one of the earliest inhabitants of terrestrial ecosystems around 400 million years ago [1, 2]. According to the guesstimates from the Royal Entomological Society, there could be as many as ten million species of insects inhabiting our planet of which only a meager number of one million species have been identified, documented, and characterized (URL: <https://www.royensoc.co.uk/understanding-insects/facts-and-figures/>: Accessed on 9 June 2022). Most of the described species till date belonged to the orders Coleoptera, Lepidoptera, Diptera, and Hymenoptera [3]. It is apparent from the aforementioned estimates that insects are still one of the least explored taxa with their roles and prospects still being largely undefined.

Insects play a momentous role in the functionality of eco-system [4]. They pollinate a diverse variety of plant species, operate as natural controller of pests, participate in scavenging activities, decomposition and recycling of nutrients, and serve as dietary resources to a wide range of organisms including birds, lizards, freshwater fish, mammals, amphibians as well as humans. Few insect species also inflict considerable damage to plants plummeting yields of agricultural produce, act as carriers of pathogenic agents causing diseases in plants, humans, livestock and involved in damaging infrastructure [5].

For a long time, it is considered that there were just a few commercially important insects, such as silkworms, honeybees, and lac insects, all of which were extensively researched and explored for their respective products of silk, honey, and lac. However, this trend has dramatically changed over the last 30 years with numerous products of insect origin that are beneficial to mankind were discovered with the intervention of molecular biology and biotechnological approaches in insects, thus leading to the emergence and development of a specialized field of study termed Yellow Biotechnology or Insect Biotechnology. The expression yellow represents the color of insect hemolymph which served as a potential source for the development of numerous biologically active compounds that are being employed across diverse sectors, till date [6]. This article presents a comprehensive overview of “omics” tools/techniques employed in the study of insects; biotechnological interventions leading to identification of disease-causing microorganisms and disease control in insects with silkworm as a model organism; and a brief note on products of therapeutic importance developed from insects/insect cells employing principles of biotechnology over the last couple of decades.

2.2 OMICS Tools in Insect Science

Despite how important insects are, we still know a very little about their biology. However, this trend is rapidly changing with the application of omics tools in study of insects.

2.2.1 Genomics

Although the current world witnessed tremendous developments in sequencing technologies, one would be astonished to know that only a meager number of insect genome projects (3000) have been available in the public databases (<https://www.ncbi.nlm.nih.gov/datasets/genomes/?taxon=50557>) [7]. The majority of the insect genomes that have been sequenced are those recognized to be crucial for agriculture and those that are used as model organisms in biological and biomedical research. These insects' sequenced genomes are treasure troves of knowledge since they carry the information of every protein and, consequently, every biochemical and physiological event that takes place in an insect [8].

A whole genome shotgun (WGS) approach is frequently used in modern insect genome sequencing projects which results in generation of enormous amount of short sequence segments that must be assembled. Depending on whether a reference genome is available, the process of genome assembly can be accomplished either through de novo assembly or mapping assembly. Repetitive sequences and heterozygosity are the two key elements that influence how well an insect genome is assembled. In the recent years, modern “long read” sequencing technologies (PacBio and Oxford Nanopore) are making significant improvements in the accuracy of genome assembly. The difficulty of producing a high-quality genome assembly from the raw reads (short reads of <250 bp) generated by second-generation sequencing techniques (Illumina HiSeq) is the major hurdle in insect genome sequencing. A prospective solution to these problems is the use of third-generation sequencing techniques (PacBio), which may generate lengthy reads (>10 kb) [9].

Some of the prominent insects whose genomes were sequenced first include *Drosophila melanogaster* [10], *Anopheles gambiae* [11], *Bombyx mori* [12], *Apis mellifera* [13], *Aedes aegypti* [14], *Tribolium castaneum* [15], *Nasonia vitripennis*, *Nasonia giraulti*, *Nasonia longicornis* [16], *Acyrtosiphon pisum* [17], *Pediculus humanus* [18], *Camponotus floridanus*, *Harpegnathos saltator* [19], *Culex quinquefasciatus* Say [20], *Atta cephalotes* [21], *Solenopsis invicta* [22], *Pogonomyrmex barbatus* [23], *Linepithema humile* [24], *Acromyrmex echinator* [25]. Since then, numerous insect genomes have been sequenced, and the information has been used to gain deeper insights into insect biology [8, 9].

2.2.2 Transcriptomics

Transcriptomics refers to studying the transcriptome, i.e. entire set of RNA transcripts that are produced by the genome, under particular circumstances or in a specific cell, employing high-throughput methods (microarray analysis/RNA sequencing). This branch of omics provides a detailed understanding of an organism’s biology by determining how genes are expressed in various tissues, conditions, or time intervals. Additionally, it can be used to deduce the roles of previously unannotated genes [26]. Transcriptome studies in insects are often centered to catalog all the genes expressed in a particular cell/tissue or to determine the variations in gene expression linked to specific phenotypes or in response to diverse experimental treatments [27].

Transcriptome studies involving numerous insects were carried out across the world, some of which include de novo transcriptome of budworm, *Heliothis virescens* [28]; transcriptome analysis across different developmental stages of *Hypothenemus hampei*, an arthropod pest of coffee [29], *Rhynchophorus ferrugineus* [30]; transcriptome and metabolite profiling studies of *Polypedilum vanderplanki* (anhydrobiotic insect) [31]; variations in transcriptome profile in relation to temperature stress in *Cryptolaemus montrouzieri* Mulsant [32], *Chrysomya megacephala* [33], *Monochamus alternatus* [34]; transcriptome of Pea aphids (*Acyrtosiphon pisum* Harris) mouth part cuticle [35]; transcriptome responses to *Autographa californica*, multiple nucleopolyhedrovirus

infection in *Spodoptera frugiperda* and *Trichoplusia ni* [36], *Plodia interpunctella* Granulosis Virus infection in *Plodia interpunctella* (Indian meal moth) [37]; transcriptome analysis of *Telchin licus licus* (sugarcane giant borer) [38]; transcriptome of *Anopheles gambiae* [39], locusts [40], tea aphid (*Aphis aurantii*) [41], ant (*Formica exsecta*) [42], silkworm (*Bombyx mori*) [43]; transcriptome analysis to delineate mechanisms underlying light stress response in *Mythimna separata* (Walker) [44]; transcriptome analysis of *Cryptotermes secundus*, *Macrotermes bellicosus*, *Euglossa viridissima*, *Apis mellifera capensis*, *Platythyrea punctata*, *Temnothorax rugatulus* to explain mechanisms underlying aging and fecundity in social insects [45]. All of the aforementioned studies contributed significant knowledge to our understanding of the fundamental processes underlying insect behavior, growth, reproduction and development, stress responses, immune biology, pathophysiology, and endocrinology. The data can also offer insightful information in terms of applied aspects too, most importantly the management of insect pests in agricultural crops.

2.2.3 Proteomics

Proteomics, a branch of omics involves the study and characterization of entire set of proteins present in a cell, tissue, organ, or an organism at a specified time under definite set of conditions. A cell's or an organism's proteome can change with time and in response to other variables. Proteomics also encompasses a wide range of scientific disciplines related to modifications, functions, and localization of proteins as well as protein-protein interactions. The various types of proteomic approaches include expression proteomics (quantitative and qualitative expression of proteins), structural proteomics, and functional proteomics [46].

In general, the experimental workflow includes purification or fractionation of complex proteins or peptide mixture, identification through mass spectrometry and bioinformatic analysis [47]. The extraction of protein is performed from cell/tissue in suitable buffer and proteins are confirmed on polyacrylamide gels (SDS-PAGE or native-PAGE) or 2D-gel electrophoresis. The bands or spot from the polyacrylamide gel are trypsin digested and analyzed by mass spectrometry for systematic identification of proteins/peptides on basis of mass to charge ratio [48]. Further, mass spectrum data is analyzed by search tools (SEQUEST, Mascot, and Andromeda) [49].

A number of proteome studies were performed in insects under different conditions in order to delineate various and diverse biological aspects, some of which include proteomic perspective of molecular mechanisms underlying larval and metamorphic molts in cotton bollworm, *Helicoverpa armigera* [50]; revelations into the mechanisms of *Bombyx mori* wandering behavior from integrative proteomics and metabolomics analysis of silkworm larva brain [51]; comparative proteome analysis of the ova from Rice stripe virus-infected and healthy female *Laodelphax striatellus* insects [52]; proteomic studies of insect cuticle [53]; proteome analysis of the ovarian proteins of *Aedes aegypti* [54]; proteome analysis of

gut proteins to deduce *Plodia interpunctella* resistance to *Bacillus thuringiensis* [55]; proteome studies of insect (*Trichoplusia ni*) midgut epithelial cell [56]; proteome profile of spinneret of silkworm [57]; proteome profile of *Drosophila melanogaster* infected with *Spiroplasma poulsonii* [58]; insights into transmission of begomovirus by white fly through proteome studies [59]; proteome analysis leading to identification of immune proteins elicited as a result of bacterial infection in silkworm [60]. Proteome studies are also widely employed to deduce insect vector–plant virus interactions [61] and more recently, they are also utilized to identify allergens in feed and food-grade insect species [62].

2.2.4 Metabolomics

Metabolomics which involves a comprehensive evaluation of metabolites in a biological sample (organism, biological fluids, tissues, cells) is an emerging technology with a wide range of applications. In general, untargeted (employed to determine every possible metabolite in a biological sample with a view to scan the whole metabolic network) or targeted (studies involving detection of specific/limited metabolites) metabolite profiling studies are applied to study the metabolome of a given biological sample. Of late, this branch of -omics is being widely employed in entomology studies to assess the significance of metabolites relevant to insect behavior and phenotype [63].

In general, the experimental workflow involves sample preparation (collection of insects, surface sterilization, homogenization in a suitable solvent, centrifugation and re-extraction in an appropriate solvent system) and metabolite identification as well as quantification through advanced analytical tools including gas chromatography/mass spectrometry (GC-MS), liquid chromatography/mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR). However, one must be aware that the sample preparation process and metabolite identification as well as quantification procedures vary depending on the objective of the user [64].

A number of metabolite profiling studies across various biological conditions in insect systems were performed by researchers which include juvenile hormone regulation in *Aedes aegypti* [65]; temperature stress response in *Belgica antarctica* [66]; metabolome profiling [67], temperature stress response [68–70], hypoxia [71], bacterial infection [72] in *Drosophila melanogaster*; temperature stress responses in *Drosophila montana* [73]; temperature stress response in *Sarcophaga crassipalpis* [74]; insect–bacterial symbiosis in aphids [75]; *Nosema ceranae* infection in *Apis mellifera* [76]; diapause induction in *Helicoverpa armigera* [77]; host parasitism in *Manduca sexta* [78]; metabolomic profiling in *Spodoptera frugiperda* Sf9 and *Trichoplusia ni* derived BTI-Tn5B1-4 (High Five) cell lines [79]; social behavior in *Schistocerca gregaria* [80]; venom analysis in *Anisomorpha buprestoides*, *Peruphasma schultei* [81, 82]; differences in metabolites related to growth and metamorphosis during the ontogeny of the northern damselfly [83]; metabolite differences of silkworm, *Bombyx mori* fed on normal and artificial diets [84]; metabolic

responses of silkworm infected with fungi [85]. All these studies provided valuable information in understanding various biological mechanisms underlying physiology, growth and development, responses to biotic and abiotic stress, defense mechanisms, immune responses, and social behavior in insects.

2.3 Biotechnological Interventions Leading to Disease Control in Insects: Silkworm as a Model Organism

The silkworm, *Bombyx mori* L. reared primarily for the production of silk from time immemorial is one of the most extensively studied organisms. Owing to the advantages which include relatively shorter generation times, well-defined genetic background, availability of diverse genetic resources, and substantial quantum of genes homologous to humans, researchers across the world have used this model organism to conduct numerous studies ranging from environmental monitoring to human disease models to screening of antimicrobial drugs [86]. In a completely contrasting viewpoint, another set of researchers performed studies related to various genetical, physiological, biochemical, and pathological aspects of silkworm with the primary objective of augmenting silk production.

Like any other insect, silkworms are also prone to infection by diverse microbes (bacteria, fungi, microsporidia, and viruses) resulting in cocoon crop losses, thereby hampering silk productivity. The earliest record of silkworm disease was penned by Guan Zhong (725–645 BCE) and since then countless approaches have been designed by researchers across the world to prevent silkworm diseases [87]. Although the severity of silkworm diseases declined over the years in various countries, it has not been totally eradicated for a variety of reasons. Isolation, identification, characterization, and determining the phylogenetic affiliations of the etiologic agents responsible for causing diseases in silkworm are very much imperative in designing relevant control strategies. In this context, biotechnological tools, especially the conventional PCR techniques have played a momentous role in identification of causative microbes responsible for silkworm diseases. Apart from the identification of microbial pathogens of silkworm, biotechnological as well as molecular biological tools have also played an imperative role in development of disease resistant/tolerant silkworm breeds too. This section of the chapter deals in brief about the same.

2.4 Identification of Silkworm Pathogens

2.4.1 Bacteria

In general, isolation of bacterial pathogens of silkworm is performed as per the procedure delineated as follows. Silkworms showing characteristic symptoms of bacterial flacherie or bacterial toxicosis (Table 2.1; Fig. 2.1a, b) must be collected and subjected to surface

Table 2.1 Silkworm diseases, causative agents, and symptoms

| Disease | Causative agent/s | Symptoms | Reference |
|---|---|---|------------|
| Bacterial diseases | | | |
| Bacterial flacherie/ Bacteremia/bacterial disease of digestive tract/ bacterial septicemia | <i>Aeromonas</i> sp. <i>Bacillus</i> sp. <i>Enterobacter</i> sp. <i>Pseudomonas</i> sp. <i>Serratia marcescens</i> <i>Streptococcus</i> sp. <i>Staphylococcus</i> sp. | <ul style="list-style-type: none">– Infected larvae appear inactive, turn soft, flaccid, vomit gut juices, and exhibit retarded growth– Emergence of brown specks on skin– Cephalothoracic region appears translucent– Rectal protrusion– Feces become mushy due to high moisture content– Excreta: Chain type– Rupture of skin with exudation of dark brown fluid emitting foul odor– Larvae turn black subsequent to death– If the infection is caused by <i>Serratia</i> spp., the larvae become lethargic, soften, shrink, vomit gut juices, and turn red in color (Red Septicemia) | [88]; [89] |
| Bacterial toxicosis | <i>Bacillus thuringiensis</i> var. <i>sotto</i> | <ul style="list-style-type: none">– Abrupt cessation of feeding– Vomiting of gut juices– Impairment in clasp ing power of legs– Spasm, paralysis, twisting of body, and sudden death– Larvae turn black upon death with liquefaction of internal organs– Head appears hook shaped upon death | |
| Fungal diseases | | | |
| White muscardine | <i>Beauveria bassiana</i> | <ul style="list-style-type: none">– Decreased appetite– Oily specks on the skin– Loss in elasticity– Dead larvae become flaccid, hard, and mummified– Entire body except head | [90] |

(continued)

Table 2.1 (continued)

| Disease | Causative agent/s | Symptoms | Reference |
|----------------------------------|---|---|--------------|
| | | becomes covered with white powdery material | |
| Green muscardine | <i>Nomuraea rileyi</i> <i>Metarhizium anisopliae</i> | <ul style="list-style-type: none"> – Large specks with black periphery – Loss in elasticity – Mummified larvae appear green in color | |
| Yellow muscardine | <i>Paecilomyces farinosus</i> | <ul style="list-style-type: none"> – Large specks around spiracles and small specks on skin – Mummified larvae appear yellow colored | |
| Red muscardine | <i>Sporospora uvella</i> | <ul style="list-style-type: none"> – Red colored patches on larval surface few hours before death | |
| Aspergillosis | <i>Aspergillus flavus</i> <i>A. oryzae</i> <i>A. tamarii</i> | <ul style="list-style-type: none"> – Cessation in feeding – Larvae become sluggish and settle at bottom of tray – <i>A. flavus</i> infection: Larvae turn light yellowish to green color – <i>A. tamarii</i> infection: Larvae turn brown | |
| Microsporidian disease | | | |
| Pebrine | <i>Nosema bombycis</i> <i>Microsporidium</i> sp. <i>Pleistophora</i> sp. <i>Vairimorpha</i> sp. <i>Thelohania</i> sp. | <ul style="list-style-type: none"> – Loss of appetite – Stunted growth – Inconsistent molting – Variability in size of larvae – Opaque gut – White pustules spanning the length of silk gland | [91] |
| Viral diseases | | | |
| Nuclear polyhedrosis (Grasserie) | <i>Bombyx mori</i> nuclear polyhedrosis virus (BmNPV) | <ul style="list-style-type: none"> – Integument becomes shiny and fragile – Swelling of intersegmental regions – Perpetual crawling along the edges of rearing tray – Loss in clasping power – Color of the larvae changes to light yellow – Infected larvae discharge milky white and turbid hemolymph | [92] [93] |

(continued)

Table 2.1 (continued)

| Disease | Causative agent/s | Symptoms | Reference |
|--------------------------|--|--|-----------|
| Cytoplasmic polyhedrosis | <i>Bombyx mori</i> cytoplasmic polyhedrosis virus (<i>BmCPV</i>) | <ul style="list-style-type: none"> – Loss of appetite – Retardation in growth – Larvae turn pale white in color – Transparent thorax and body atrophies – Vomiting of gut juices – Feces become soft and appear white in color | |
| Infectious flacherie | <i>Bombyx mori</i> infectious flacherie virus (<i>BmIFV</i>) | <ul style="list-style-type: none"> – Loss of appetite – Transparent cephalothorax – Larval body shrinks – Empty foregut – Diarrhea – Uneven molting resulting in larvae of different sizes | |
| Densonucleosis | <i>Bombyx mori</i> densonucleosis virus (<i>BmDNV</i>) | <ul style="list-style-type: none"> – Decrease in consumption of mulberry – Hindered growth – Flaccidity – Diarrhea – Larvae become sluggish – Translucent cephalothoracic region | |

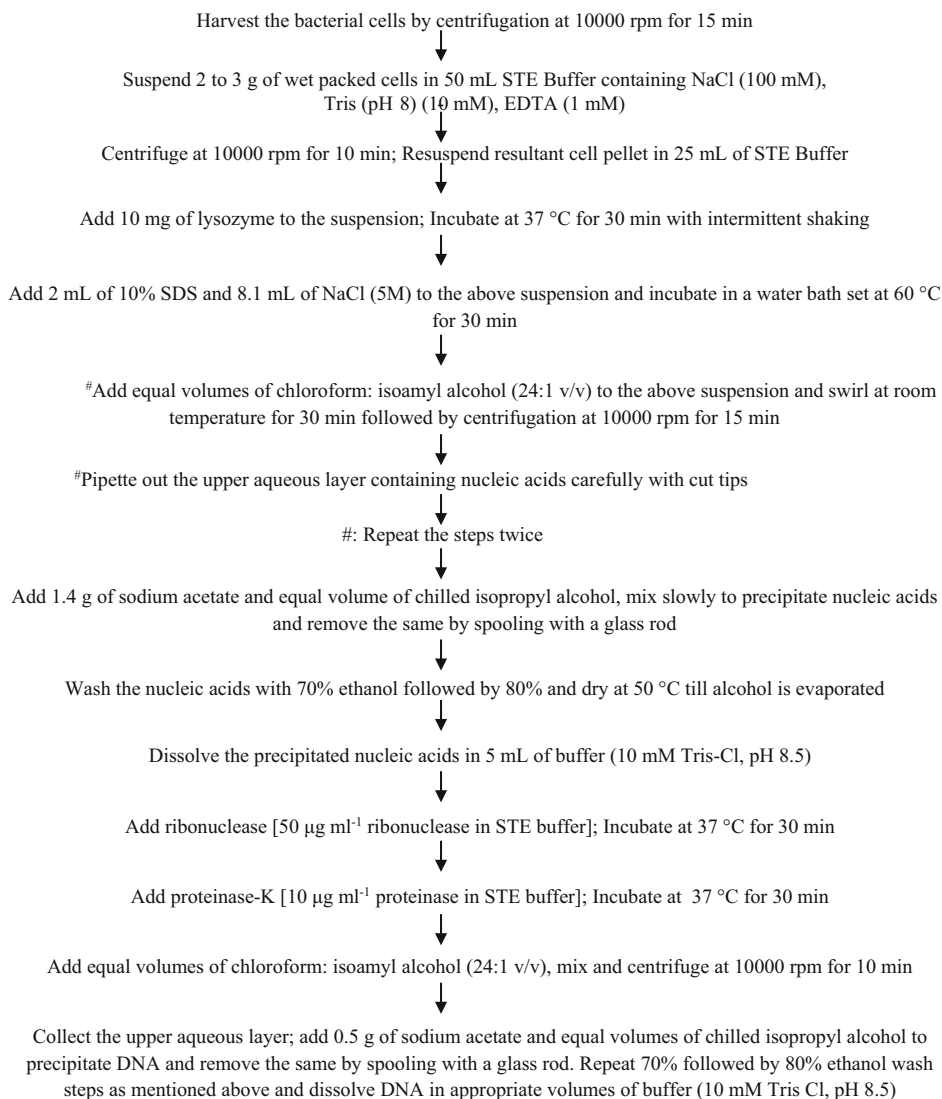
sterilization with sodium hypochlorite solution (0.5%) followed by rinsing with sterile water in order to remove any remnants of the sterilant. The midgut contents or hemolymph from the infected worms must be serially diluted in sterile saline solution. The same must be plated onto nutrient agar plates followed by incubation at appropriate temperature (30 ± 2 °C) and observed for growth at regular intervals. The bacterial isolates thus obtained must be purified by repeated streaking and the purity must be ascertained by studying the morphological features of the colony as well as microscopic observation. The pathogenicity of the isolated bacterial strains must be affirmed by performing in vivo studies following Koch postulates [89]. Koch postulates define criteria to delineate association between a causative microorganism and a disease. According to Koch postulates, the microbe responsible for a specific disease must be prevalent in all organisms suffering from the disease, but absent in healthy organisms; the microbe responsible for causing the disease must be isolated from the affected organism and grown in pure culture; the isolated microbe when inoculated to a healthy organism must inflict the same disease; and finally, it is necessary to re-isolate the microbe from the inoculated organism and confirm that it is the exact same as the original causal agent.



Fig. 2.1 Silk worm larvae exhibiting symptoms of (a) Bacterial Flacherie (b) Bacterial Toxicosis (c) White Muscardine (d) Pebrine (e) Grasserie (f) Infectious Flacherie (g) Densonucleosis

Majority of the studies performed in identification of bacterial strains causing flacherie in silkworm were based on morphological, physiological, and biochemical characters which often lead to erroneous identification and must be avoided. 16S rRNA gene sequence analysis is one of the most widely used and preferred method in bacterial identification studies. Every bacterium has at least one copy of the 16S rRNA gene, which comprises highly varied as well as conserved nucleic acid sequences. The highly conserved portions of the 16S rRNA gene can be used to create amplification primers (for PCR amplification) across taxa, whereas the nine hypervariable loci, which show significant sequence variance among bacteria, can be utilized to distinguish between taxa. Furthermore, the 16S rRNA gene's size as well as high level of functional conservation through time makes it an attractive marker for research in bacterial taxonomy and systematics [94–96].

In order to perform 16S rRNA gene sequence analysis, a well-grown bacterial colony must be selected, picked with a pre-sterilized inoculation loop and suspended in 50 μ L of sterile water devoid of nucleases. The bacterial suspension thus prepared must be lysed in a thermocycler at 92 $^{\circ}$ C for 10 min and the same can be used as a template. Alternatively, DNA can be isolated from the bacterial cultures employing standard protocols [97] as depicted in Flow Chart 2.1 or using any commercial kits following manufacturer's instructions. The PCR mixture must be prepared comprising forward (Eub27F: 5'-GAGTTTGATCCTGGCTCAG-3') and reverse (Univ1492R: 5'-GGTTACCTTGTTACGACTT-3') primers (2 μ L each),



Flow Chart 2.1 Steps involved in isolation of DNA from bacterial cultures

template DNA (5 µL), sterile water devoid of nucleases (16 µL), and PCR master mix (25 µL). The PCR conditions include initial denaturation at 96 °C for 2 min, followed by 32 cycles of denaturation at 96 °C for 40 s, annealing at 50 °C for 40 s, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min [88].

The PCR amplicons may be subjected to purification by using any commercial kits as per the manufacturer instructions and electrophoresed to observe for a desired amplicon size of ~1465 bp as well as to evaluate the quality of the amplified DNA. The PCR

amplicons must be sequenced with the same primers as mentioned above and the sequences thus obtained must be subjected to NCBI-BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine the nearest phylogenetic relative.

2.4.2 Fungi

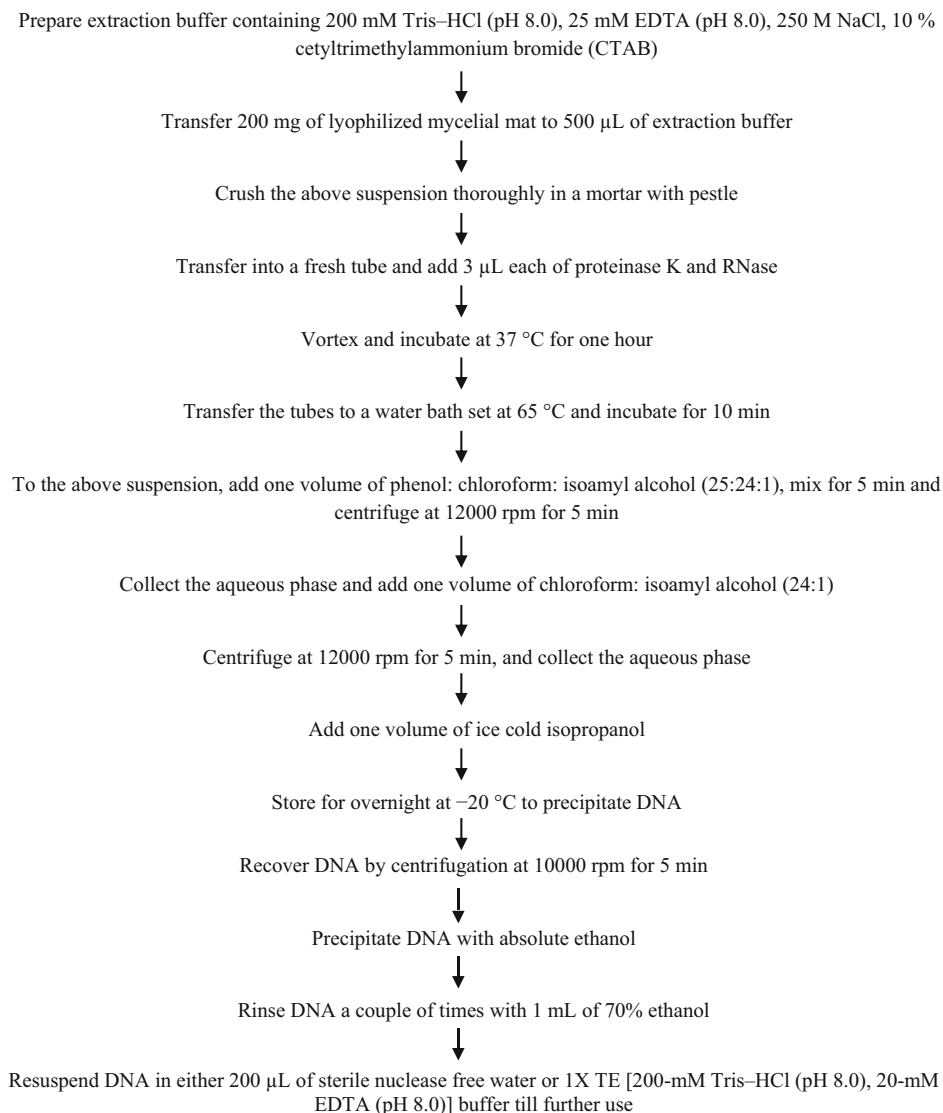
To isolate fungal pathogens of silkworm, collect the infected larvae exhibiting typical symptoms of muscardine (Table 2.1; Fig. 2.1c). The growing fungus from the mummified/infected larvae can be cultured on Sabouraud dextrose agar under aseptic conditions in a laminar air flow chamber followed by incubation at $25 \pm 2^\circ\text{C}$ for 5 to 7 days. Chloramphenicol may be added to the media to avoid bacterial contaminants. Upon visible growth, take a loop full of fungal culture onto a glass slide and observe under microscope for the study of morphological characters (conidia and conidiophores). If there are multiple strains, purification can be carried out by single spore isolation technique.

For a long time, fungal pathogens of silkworm were identified based on morphological features, just like any other fungal species. However, numerous molecular technologies for fungal identification have been developed in the recent two decades. Although molecular typing tools have a number of drawbacks, they have numerous advantages too over traditional morphological characterization and identification procedures. For fungal identification, the most preferred approach is sequencing the ribosomal region which includes ITS1 (internal transcribed spacers) and ITS2 flanking the 5.8S gene [98].

To identify fungi, first isolate DNA from the fungal strain of interest by following the steps depicted in the Flow Charts 2.2 or 2.3 [99] or alternatively by employing any commercial kits following manufacturer's instructions. The PCR mixture must be prepared comprising forward (ITS1 F: 5'-TCCGTAGGTGAACCTGCGG-3') and reverse (ITS4 R: 5'-TCCTCCGCTTATTGATATGC-3') primers [1 μL (20 pmol) each], DNA (3 μL of 80–100 ng concentration), and PCR master mix (20 μL). The PCR conditions include initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min. Purify the PCR amplicons using any commercial kit according to the manufacturer's recommendations, if necessary and electrophorese them to check for a desired amplicon size of ~650 bp as well as to assess the integrity of the amplified DNA. The PCR amplicons must be sequenced using the same primers as detailed above and the resulting sequences must be submitted to NCBI-BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to find the closest phylogenetic relative [90].

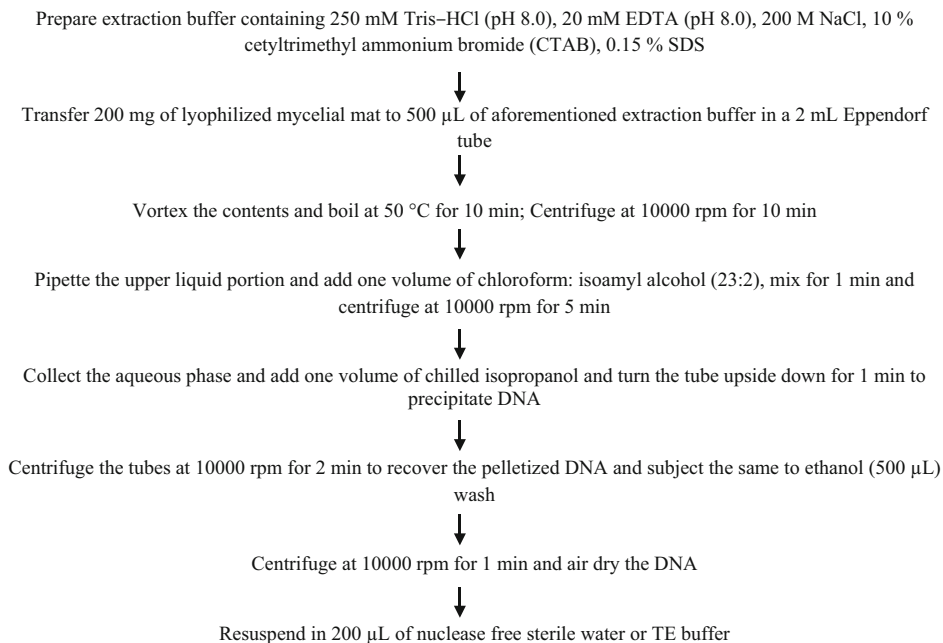
2.4.3 Microsporidia

Among all the silkworm diseases, the only transovarially transmitted disease is pebrine caused by *Nosema bombycis* Naegeli (Table 2.1, Fig. 2.1d). Although microsporidia



Flow Chart 2.2 Steps involved in isolation of DNA from fungal cultures

belonging to other genera, viz., *Microsporidium*, *Pleistophora*, *Thelohania*, and *Vairimorpha* are also known to be pathogenic to silkworm, their pathogenicity transmission through transovarial routes has not been established. Pebrine has pushed sericulture on the verge of extinction in many nations that practiced it. However, the detection of this pathogen through light microscopy (Fig. 2.2a) designed by Sir Louis Pasteur [100] and later modified by Fujiwara [101] ensured production of pebrine-free stocks enabling



Flow Chart 2.3 Steps involved in isolation of DNA from fungal cultures (alternative protocol)

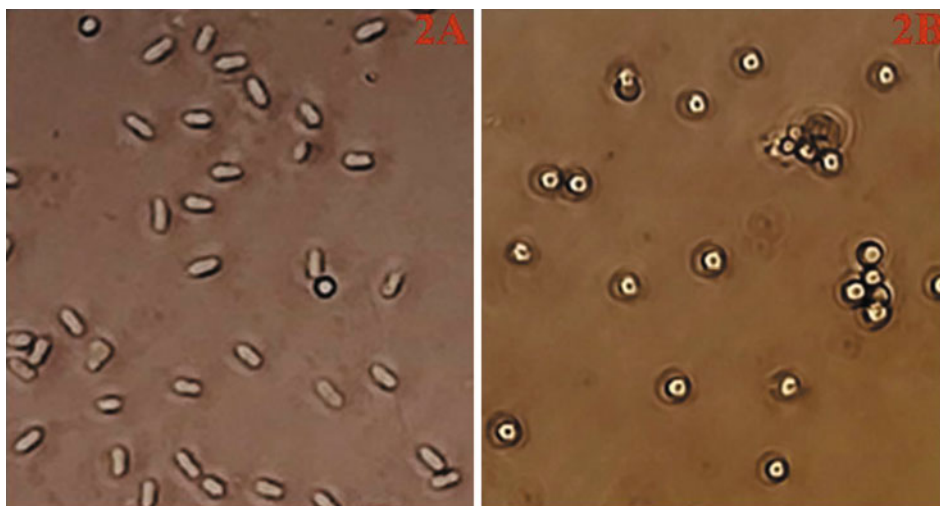
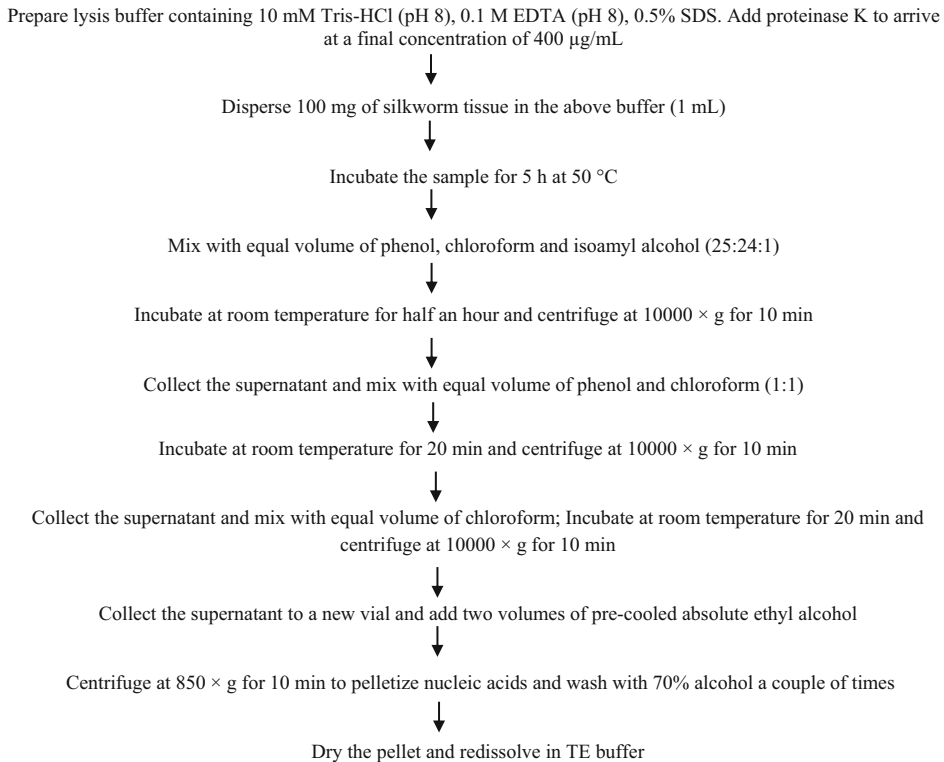


Fig. 2.2 Phase contrast micrograph of (a) Spores of *Nosema bombycis* (b) *BmNPV* polyhedra at 600x magnification



Flow Chart 2.4 Steps involved in isolation of DNA from silkworm tissue

sericulture to flourish and it goes without saying that this method is still one of the most widely used due to its ease and low cost involved. Numerous immunodiagnostic tools and nucleic acid-based detection methods have been developed [102] over the years including conventional PCR-based detection, which will be discussed here.

Isolate DNA from infected silkworm tissues following the protocol [103] as delineated in the Flow Chart 2.4 or alternatively by using any commercial kits, following manufacturer's instructions. The PCR mixture (10 μL) must be prepared comprising $1 \times$ Taq buffer, 0.25 mM dNTPs, microsporidian SSU-rRNA primers: 5-ACCAGGTTGATTCTGCCTGA-3, 5-GTTGAGTCAAATTAAGCCG-3 (0.50 μM each), 2.5 mM MgCl_2 , DNA template (125 ng), and 0.5 U Taq DNA polymerase. The PCR conditions include initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 48 °C for 45 s, extension at 72 °C for 40 s, and final extension at 72 °C for 5 min. Purify the PCR amplicons using any commercial kit according to the manufacturer's recommendations, if necessary and electrophorese them to check for a desired amplicon size of ~794 bp as well as to assess the integrity of the amplified DNA [104]. The PCR amplicons may be sequenced using the same primers as

detailed above and the resulting sequences must be submitted to NCBI-BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to find the closest phylogenetic relative.

2.4.4 Viruses

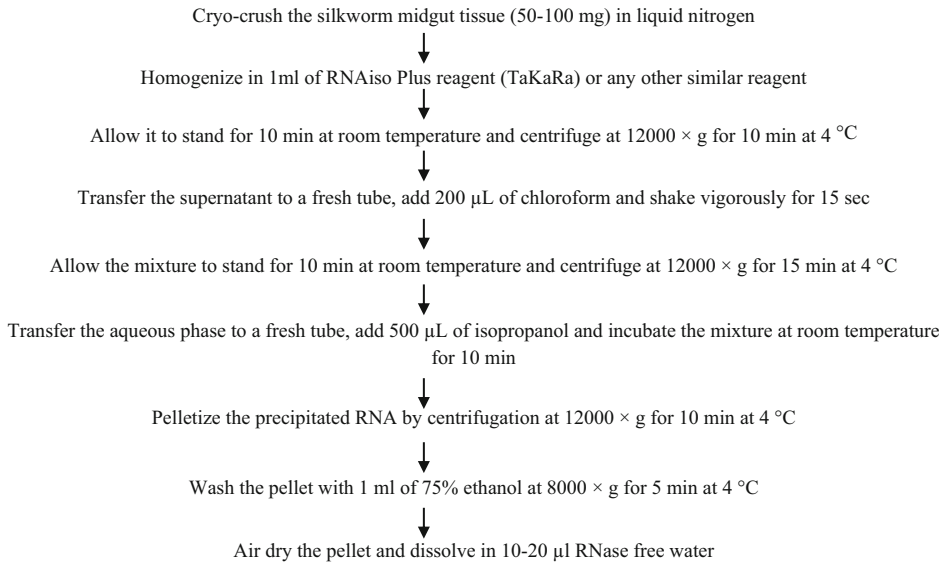
According to estimates, silkworm diseases cause ~30% of cocoon crop losses, with viral infections accounting for the majority (70 percent) [105]. Among the viral infections, damage caused by BmNPV causing grasserie disease is dominant and reported to prevail throughout the year notably during summer and rainy seasons. The other viral pathogens infecting silkworm and the symptoms inflicted are presented in Table 2.1; Fig. 2.1e, f, g. The BmNPV polyhedral bodies as observed under phase contrast microscope at 600× magnification are also depicted in Fig. 2.2b. It is also to be noted that BmNPV and BmDNV are DNA viruses that can be detected through conventional PCR techniques, whereas BmIFV and BmCPV are RNA viruses that can be detected through reverse transcription coupled with PCR.

For detection of BmNPV, isolate DNA from silkworm tissue as depicted in Flow Chart 2.4. Set up a PCR comprising 1× Taq buffer, 0.25 mM dNTPs, NPV polyhedrin: 5-GTIAARCCIGAYACIATGAA-3, 5-GCRAAYTCYTTIATYTTTAAIAC-3 (0.50 μM each), 2.5 mM MgCl₂, DNA template (150 ng), and 0.5 U Taq DNA polymerase. The PCR conditions include initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 48 °C for 45 s, extension at 72 °C for 40 s, and final extension at 72 °C for 5 min. Purify the PCR amplicons using any commercial kit according to the manufacturer's recommendations, if necessary and electrophorese them to check for a desired amplicon size of ~471 bp. BmDNV can also be detected employing the same protocol, except the following primers DNV 2 & 3: 5-AGAACC GCAAGTAATCAAGC-3, 5-CATTATATCTCCATTAGTTCC-3 must be used. The concentration of DNA template in the PCR mixture must be 50 ng. The desired amplicon size is 391 bp [104].

For detection of BmIFV and BmCPV, isolate RNA from the infected silkworm tissue using any standard protocols (Flow Chart 2.5) or kits. The protocols as delineated by [106, 107] may be followed to identify BmCPV and BmIFV, respectively.

2.5 Development of Disease-Resistant/Tolerant Silkworm Breeds

One of the strategies employed in controlling silkworm diseases involves development of silkworm varieties that are resistant/tolerant to silkworm diseases by traditional or molecular breeding techniques. Although researchers across the world have made several attempts to generate disease-resistant or tolerant silkworm breeds employing conventional breeding techniques, this strategy typically has the serious drawback of compromising the economic attributes of silkworms, which are also very crucial. Apart from the above, traditional



Flow Chart 2.5 Steps involved in isolation of RNA from silkworm tissue

breeding strategies are laborious, require much time and imprecise. So, transgenic technology, which ideally modifies only the desired/targeted trait may help to overcome the drawbacks of conventional breeding techniques. Transposon-mediated transgenesis and genome-editing tools are the predominant techniques widely employed to genetically alter *B. mori* [108, 109]. The recent advancements in electroporation and microinjection technologies also facilitated effective delivery of molecular machinery aiding in development of transgenic insects [110]. A typical transposon-mediated transgenesis experiment in silkworm involves vector construction, electroporation, and microinjection of vector and helper plasmid into the embryos and screening [111]. *piggyBac* gene vectors were widely used to carry and integrate fragments of exogenous DNA in *B. mori* [109].

Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated system (Cas) are the genome-editing tools used to genetically modify *B. mori* not only for the study of its diverse biological functions but also to develop disease-tolerant silkworm breeds. All the aforementioned techniques operate through induction of DNA double-strand breaks at target sites with the aid of either modular proteins such as zinc fingers and transcription activator-like effectors or short guide RNAs. DNA double-strand breaks can be repaired in one of two ways: either through non-homologous end-joining, which can result in the target sequence being disrupted by the insertion or deletion of nucleotides (knockout mutation), or through homology-directed repair, which can be employed to insert exogenous DNA sequences at the targeted site via homologous recombination, thereby creating a knock-in mutation [109]. Among the aforementioned tools,

CRISPR/Cas has considerable advantages over ZFNs and TALENs and is being widely used across various living forms including insects. In contrast to ZFNs and TALENs, which rely on the design and synthesis of sequence-specific DNA binding proteins, CRISPR/Cas9-based genome editing depends on a DNA endonuclease whose target-site specificity is defined by a user-designed gRNA. CRISPR/Cas9-based genome editing requires designing and synthesis of a short gRNA in contrast to ZFNs and TALENs that necessitate designing of large, target-specific proteins which is challenging as well as expensive. CRISPR/Cas9 targets DNA sequences through RNA-DNA base-pairing approach, which is more stable over protein-DNA molecular interactions that ZFNs and TALENs rely upon [109]. A typical CRISPR Cas9-based genome-editing experiment in silkworm involves selection of target site; synthesis of sgRNA; insertion of oligonucleotides bearing the sgRNA into appropriate vector and transcribing using a suitable polymerase; synthesis of Cas9 mRNA from the Cas9 expression vector; injection of sgRNA and Cas9 mRNA mixture by a microinjector into silkworm eggs; incubation of eggs at appropriate conditions, hatching and rearing of G0 individuals following standard rearing conditions; obtaining fertile eggs by sib-mating or mating with non-injected individuals; incubation of the eggs and extraction of genomic DNA (25–50 eggs); amplification of the target region by PCR; checking for the presence of mutation by sequencing the PCR product [110].

Overall, the abovementioned transposon-mediated transgenesis and genome-editing tools apart from helping in understanding a number of aspects of silkworm biology also led to development of transgenic silkworms that confer resistance against pathogens including bacteria, microsporidia (*N. bombycis*), and viruses (*BmNPV*, *BmCPV*) by overexpression of endogenous or exogenous genes or by specifically targeting pathogen-specific genes [111–124]. However, the safety of the transgenic silkworms must be elucidated before commercial exploitation [125].

Another approach is to make use of marker-assisted selection for breeding of silkworms resistant to silkworm pathogens. The best example in this category is the breeding of silkworm strains resistant to *BmDNV*. It is ascertained that nonsusceptibility to *BmDNV*1 is governed by the recessive gene *nsd-1* and the dominant gene *Nid-1*. On the other hand, resistance to *BmDNV*-2 and *BmDNV*-Z is conferred by the *nsd-2* and *nsd-Z* genes, respectively. Screening silkworm strains for detection of gene conferring susceptibility or resistance to the *BmDNV* and utilization of this information in breeding programs will ascertain development of silkworm strains resistant to *BmDNV* [126].

2.6 Novel Products from Insect Origin with Therapeutic Potential

Numerous products possessing antimicrobial, anti-angiogenic, anti-inflammatory, anti-proliferative, reno-protective, neuroactive, immunomodulatory, and analgesic properties have been discovered from diverse insect sources. Insects as well as insect derived sources were utilized from a long time as traditional medicine. However, products of insect origin

lacked the market acceptance and recognition when compared to plant or their derived products due to a wide variety of reasons [127]. This scenario is rapidly changing and numerous products of therapeutic importance developed from insect sources are being commercialized.

Biologically active compounds are abundant in insects, whose exploration and commercialization on a larger scale have been greatly facilitated by biotechnological tools. The most widely explored compounds of biomedical significance from the insect systems are antimicrobial peptides. Research efforts to investigate insects as a potential source of antimicrobials have been sparked by the escalation in microbial resistance to traditional antibiotics. Hymenoptera followed by Diptera, Coleoptera, Lepidoptera, and Hemiptera are the widely explored orders of insects for discovery of antimicrobial peptides. Antimicrobial peptides that are structurally diverse including abaecin, anoplin, apidaecin, attacin, bicarinalin, cecropin, cicadin, coleopteracin, coprisin, cryptonin, decoralin, defensin, dipteracin, dominulin, drosocin, drosomycin, eumenitin, gambicin, halictine, hinnavin, hymenoptaecin, lasiocepsin, lasioglossin, lebocin, macropin, mastoparan, melectin, metchnikowin, osmin, periplanetasin, pilosulin, poneracin, protonectin, psacothaeasin, sapecin B, scarabeicin, stomoxyn, tenecin, thaumatin, and vespa chemotactic peptides were isolated from insects. Most of the discovered antimicrobial peptides from insects showed antimicrobial activity against diverse pathogens [128].

Therapeutic proteins combating a range of human or animal anomalies have been produced in a variety of expression systems including bacterial, fungal, plant, mammalian, and insect cells. Baculovirus (host range limited to invertebrates, particularly insects)-based insect cell expression system has been widely used for the production of therapeutic proteins in significant amounts owing to numerous advantages over other expression systems. It has been thoroughly investigated for the purpose of producing viral antigens. Insect cells are capable of carrying out many of the post-translational modifications that viral antigens frequently need, including glycosylation, the creation of disulfide bonds, myristoylation, and phosphorylation. Baculovirus-based insect cell expression system was used in the development of several vaccines for human use [CERVARIX (protection against human papillomavirus (HPV)—developed by GlaxoSmithKline), PROVENGE (therapeutic vaccine to treat prostate cancer—developed by Dendreon), Flublok (immunization against influenza disease caused by influenza A and B strains—developed by Protein Sciences Corporation)] as well as veterinary use [Porcilis PCV (immunization of pigs against porcine circovirus 2 infection—developed by Merck), CircoFLEX (immunization of pigs against porcine circovirus 2 infection—developed by Boehringer Ingelheim) and Porcilis Pesti (immunization of pigs against classical swine fever—developed by Merck)] [129]. A number of vaccines are being developed employing the baculovirus-based insect cell expression system against a range of medical anomalies in humans as well as animals. Also, there are various initiatives underway to create vaccines to protect against SARS-CoV-2 using the aforementioned technology [130]. The above applications in detail as well as numerous other applications of insects will be described in upcoming chapters.

2.7 Conclusion

The arena of insect sciences has progressed significantly in recent years with the intervention of biotechnological approaches. Our understanding of insect biology is being expanded every day with fresh and fascinating facts. The integration of this knowledge with that amassed over the last few decades will open up new opportunities in formulating strategies for gaining a greater understanding of insects, their role in the ecosystem as well as in development of products beneficial to the mankind. However, considering the enormous diversity of insects, what is accomplished till date is merely the tip of an iceberg and there is much more to discover in the coming days.

Take Home Message

- Biotechnological interventions have no doubt improved our understanding of various fundamental biological mechanisms in insects as well as their interactions with other organisms and the environment
- Insects are becoming increasingly important resources of novel products with applications across diverse sectors—Thanks to biotechnological interventions
- However, there is a need to intensify studies to explore the unexplored insects

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Importance and Application of Biotechnology in Insects

3

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and Visweswara Rao Pasupuleti

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D. Kumar, S. Shukla (eds.), *Introduction to Insect Biotechnology*, Learning Materials
in Biosciences,

https://doi.org/10.1007/978-3-031-26776-5_3

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Keywords

Insect · Forensic · Entomotherapy · Resurgence

What You Will Learn from This Chapter

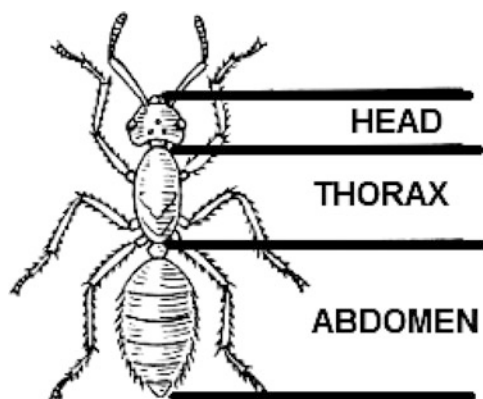
After studying this chapter, students should be able to

- define insect and describe its importance
- discuss applications in different fields
- applications of biotechnology in insect control

3.1 Introduction

The term “insect” is derived from Latin word “**insectum**,” which means “divided body” or “cut into sections .” Insect’s body consists of (a) three parts, viz., head, thorax, and abdomen, (b) three pairs of joined legs, (c) a pair of compound eyes, and (d) a pair of antennae (Fig. 3.1). They belong to arthropod group which contains chitinous exoskeleton. Insects are one of the most fascinating and diverse group of creatures and occupy around three fourths of the total organisms found on this earth [1]. Among 9.8 million estimated animals in the world, four to eight million species are known to be belonging to insects [2]. Based on the importance of insects, they are mainly classified into four groups, and they are (1) Industrially important insects, (2) Edible and therapeutically important insects, (3) Insects important for forensic investigation, and (4) Insects of ecological importance [3].

Fig. 3.1 Insect body



1. **Industrially important insects:** This group of insects produces economically important products. Examples for this group includes silk worms, lac insects, honeybees, etc.
2. **Edible and therapeutically important insects:** Using insect as a food is not a new practice. Ancient literatures from China represent the importance of insects as food and medicine. One such literature is Li Shizhen’s compendium of *Materia Medica*—a vast comprehensive books of Chinese Medicine (written during 1368–1644). These books display variety of foods used during that time which includes large number of insects. They also described the medicinal benefits of insects. Insects from ants to beetle larvae are consumed by tribes in Australia and Africa as a subsistence diet. Crispy fried locusts and beetles are relished in Thailand [4]. To quote a few examples for use of insects as medicine, a protein extracted from the insect called “Resilin” has been used to repair arteries, bee products like honey, propolis, venom are used in treatment of traumatic wounds and burns [5, 6].
3. **Insects important for forensic investigation:** An approximate interval of post-mortem can be analyzed based on observation of stage of succession of insects. A branch of entomology called “Forensic Entomology” mainly focuses on study of insects that form part of the evidence in legal cases. This is mainly associated with death enquiries. Usually insects are associated with decomposing dead body. Such insect colonization can be used to measure the time gap between death and corpse (dead body) detection, manner and cause of death[7]. Decomposing carrion found to contain five categories of insects (represented in Fig. 3.2) and they are: (1) Necrophagous insects, (2) Parasites and predators which develop on necrophagous insect species, (3) Omnivorous insects and other arthropods like wasps and ants, (4) Spring tails, pill bugs, spiders, mites. The first two among the four are very important from the standpoint of forensic entomology. They belong to the order Diptera and Coleoptera [8].

There are mainly five stages of decomposition (Fig. 3.1) of dead body and four different types of insects predominate in each stage. Usually blow flies and flesh flies



Fig. 3.2 Kind of insect and stage of decomposition of dead body

Table 3.1 Five stages during decomposition of carcasses and change with respect to insect populations and stages

| Stage | Days / hours/min | Change with respect to insect populations and stages |
|------------------|------------------|--|
| Fresh stage | 10 min to 24 h | Insects are attracted within 10 min of death, but there was no oviposition or egg laying observed. Blow flies were detected and cadaver eggs were laid |
| Bloated stage | 2–7 days | Maggots develop |
| Decay stage | 5–13 days | Conversion of carcass biomass into larval biomass of Diptera |
| Post decay stage | 10–23 | Majority of dipterans in pupal stage |
| Dry stage | 18–90 days | Decreased population of adult and larval dipterans |

arrive within few minutes following death. In the early period of decomposition of Carrion beetle adults and larvae feed on fly larvae. During early to late period of decomposition rove beetles predacious on fly eggs may occur. In the late stage decomposition, ham and checkered beetles feed on dead tissue as well as predacious on flies and beetles may occur. Skin beetles feeding on dried skin and tissue are last in the succession [9].

As per the investigations on “decomposition of exposed carrion in a tropical rainforest” conducted by Tullis and Goff [10], it was found that there are five stages in the decomposition process based on characteristics of insect populations, physical appearance, and internal temperature. The information related to this is summarized in Table 3.1.

1. Fresh stage: Around eyes, nostrils, mouth followed by anus and/or genital openings blow flies were detected and cadaver eggs were laid. The estimation of time of death in this case may be about 24 h. Insects are attracted within 10 min of death, but there was no oviposition or egg laying observed. In this stage there will not be morphological changes, but just cellular breakdown happens. The chemicals emanating due to cellular breakdown were believed to be the responsible factor for attraction of insects during early stage.
2. Bloated stage: This stage was found within 2–7 days. At this stage putrefaction starts. Gases produced by metabolism of anaerobic bacteria lead to inflation of abdomen and in the later stage there was appearance of balloon-like structure. There was raise in internal temperature of dead body due to the combined action of arthropod and anaerobic bacteria.
3. Decay stage: This stage was observed between 5 and 13 days. During this stage abdominal wall was penetrated. The internal temperature was about 14 °C above the ambient temperature. There was direct relationship between decay odor and

temperature. Important aspect observed during this stage was conversion of carcass biomass into larval biomass of Diptera. At the later stages, the larvae of Dipterans enter pupal stage.

4. Post decay stage: This stage was observed between 10–23 days. In this stage bones, cartilages, hair, and small portions of tissue are left after Dipterans larval feeding. Only by-products of decay (BOD) consisting of wet viscous material were observed.
5. Dry stage: This stage was noticed between 18–90 days. This stage contained bones and little cartilage. BOD was completely dried up during this stage. There was decreased population of adult and larval dipterans.
6. **Insects of ecological importance:** Some insects are pests on crop plants and are destructive in nature, but many are predators and parasites on these destructive pests.

In this chapter we will focus on therapeutically important insect products and use of biotechnology in controlling insect pests.

The main objective of this chapter is to delve more information about insects whether they are harmful as foes or beneficial as friends.

3.2 Insect Products with Biomedical Uses

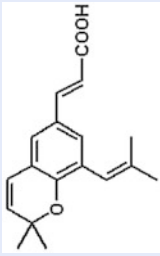
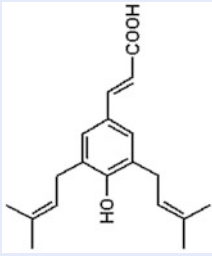
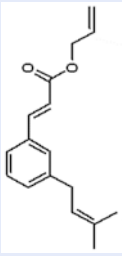
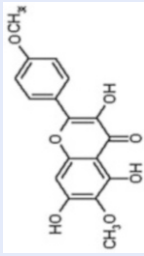
Among all the animals, insects population makes up approximately 75%. It is obvious that to withstand adverse environmental conditions which would have taken place during the past 500 million years of their evolution they must have produced many natural products [11]. During olden days insects and their natural products were used as medicines. Use of insects and the natural products produced by them as medicine is now considered as an alternative to modern therapy practiced in many parts of the world like India, China, Africa, Mexico, Spain and is called “Entomotherapy” [12]. As most of the drugs were extracted from plants, microbes, and marine organisms, insects have been literally neglected as the natural source of modern drug, although they have given many natural products like honey, beeswax, etc. [13].

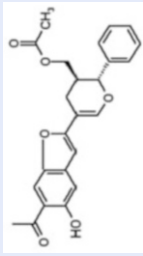
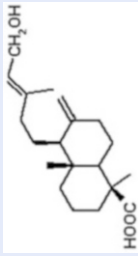
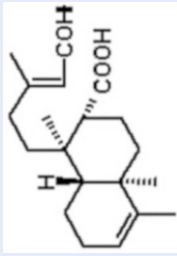
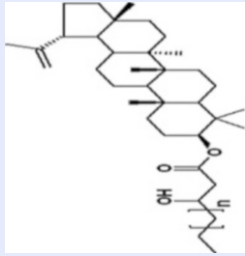
The illustrations of use of insects in folklore medicine and have subsequently proven to be potential pharmaceuticals in modern medicine [11] are listed below:

1. Use of honey in the treatment of infections and wounds
2. Bee, wasp, and ant venom for the treatment of cancer
3. In Chinese medicine use of silk is prescribed for relieving tremor and medicine for flatulence
4. Cantharidin extracted from blister beetle used for the treatment of cancer
5. As antiviral agent body extracts of many insects like bees, wasps, flies, butterflies were used.
6. Use of blood sucking insects to treat problems related to blood

A list of active compounds and their beneficial activities of honey is seen in Table 3.2.

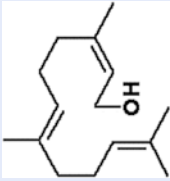
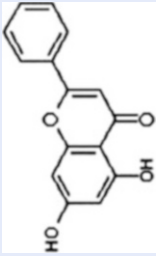
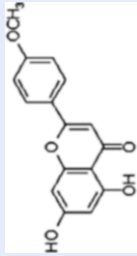
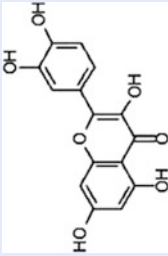
Table 3.2 Various types of insect products' bioactive compounds with chemical structure and biological activity (Reproduced from Rao et al. [14])

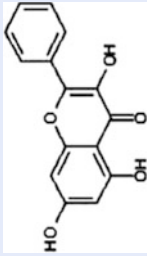
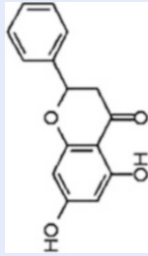
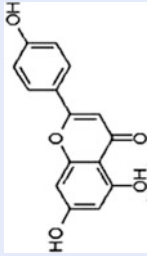
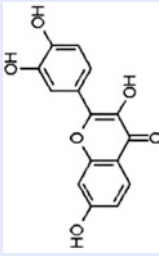
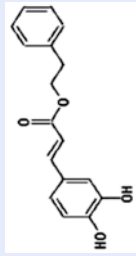
| Bee products | Bioactive compound | Chemical structure | Biological activity | References |
|--------------|--|--|--|--|
| Propolis | Phenolic compound: 2,2-Dimethyl-8-prenylchromene |  | Antimicrobial | Viuda-Martos et al. [15] |
| Propolis | Phenolic compound: 4-hydroxy-3,5-diprenyl cinnamic acid (artepillin C) |  | Antimicrobial, anti-inflammatory, anticancer | Viuda-Martos et al. [15] |
| Propolis | Phenolic compound: 3-prenyl cinnamic acid allyl ester |  | Antimicrobial | Viuda-Martos et al. [15] |
| Propolis | Phenolic compound: Kaempferide |  | Anti-tumor, anticancer | Viuda-Martos et al. [15], Khalil and Sulaiman [16] |

| | | | | |
|----------|--|--|---|---|
| Propolis | Phenolic compound: Propolis benzofuran |  | Anti-fungal | Viuda-Martos et al. [15], Khalil and Sulaiman [16] |
| Propolis | Terpenoid: Isocupressic acid, a labdane diterpenoid |  | Anti-fungal | Viuda-Martos et al. [15], Khalil and Sulaiman [16] |
| Propolis | Terpenoid: 13C-symphoretetic acid, a clerodane diterpenoid |  | Anti-tumor | Viuda-Martos et al. [15], Esquivel and Flores [17] |
| Propolis | Terpenoid: esters of long chain fatty acids (3-hydroxystearic acid $n = 11$, procrim a; 3-hydroxystearic acid $n = 13$, procrim b) and a pentacyclic triterpenoid (lupeol) |  | Antioxidant, antimicrobial, anti-tumor | Salatino et al. [18], Viuda-Martos et al. [15], Huang et al. [19] |

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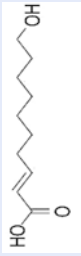
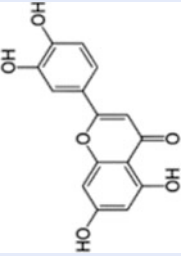
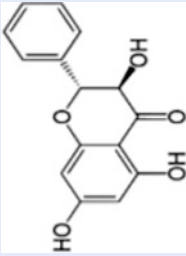
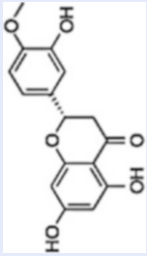
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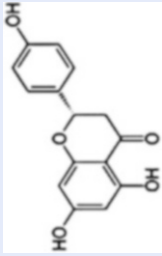
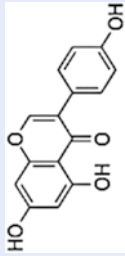
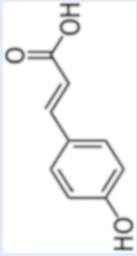
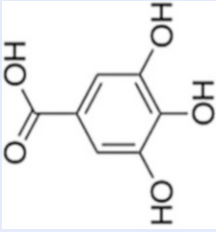
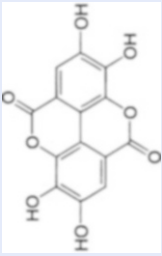
| Bee products | Bioactive compound | Chemical structure | Biological activity | References |
|-----------------|---------------------------------------|--|---|--|
| Propolis | Terpenoid: Farnesol a sesquiterpenoid |  | Anti-fungal | Viuda-Martos et al. [15], Cotoras et al. [20] |
| Propolis, Honey | Flavonoid: Apigenin |  | Antibacterial, anti-inflammatory | Viuda-Martos et al. [15], Khalil and Sulaiman [16] |
| Honey, Propolis | Flavonoid: Acacetin |  | Anti-allergic, anticancer | Viuda-Martos et al. [15], Khalil and Sulaiman [16] |
| Honey, Propolis | Flavonoid: Quercetin |  | Anticancer, anti-allergic, antibacterial, anti-inflammatory | Viuda-Martos et al. [15], Khalil and Sulaiman [16] |

| | | | | |
|--------------------|---|---|--|---|
| Honey, Propolis | Flavonoid: Galangin |  | Anticancer, antioxidant | Viuda-Martos et al. [15], Khalil and Sulaiman [16] |
| Honey, Propolis | Flavonoid: Pinocembrin |  | Antimicrobial, anticancer | Viuda-Martos et al. [15], Khalil and Sulaiman [16] |
| Honey, Propolis | Flavonoid: Chrysin |  | Antibacterial, anti-inflammatory, anticancer | Viuda-Martos et al. [15], Khalil and Sulaiman [16] |
| Honey, Propolis | Flavonoid: Fisetin |  | Antibacterial, anti-allergic, anticancer | Viuda-Martos et al. [15], Abubakar et al. [21] |
| Honey, Propolis | Flavonoid: Caffeic acid phenethyl ester |  | Anti-tumor, anticancer | Viuda-Martos et al. [15], Khalil and Sulaiman [16] |

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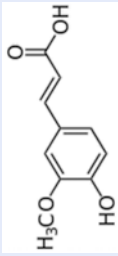
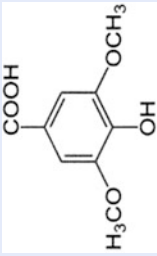
Table 3.2 (continued)

| Bee products | Bioactive compound | Chemical structure | Biological activity | References |
|-----------------------|-----------------------------|--|--|--|
| Propolis, Royal Jelly | 10-Hydroxyl-2-decenoic acid |  | Antibiotic, anti-tumor | Izuta et al. [22] |
| Honey | Flavonoid: Luteolin |  | Antioxidant, anti-inflammatory, and anti-tumor | Lin et al. [23], Mijanur Rahman et al. [24] |
| Honey | Flavonoid: Pinobanksin |  | Antioxidant | Ajao et al. [25] |
| Honey | Flavonoid: Hesperetin |  | Antioxidant, antiinflammatory | Kassim et al. [26], Mijanur Rahman et al. [24] |

| | | | | |
|-------|--------------------------------|---|---|--|
| Honey | Flavonoid: Naringenin |  | Neuroprotective, antioxidant | Badruzzaman Khan et al. [27], Mijanur Rahman et al. [24] |
| Honey | Flavonoid: Genistein |  | Anticancer | Tahir et al. [28], Mijanur Rahman et al. [24] |
| Honey | Phenolic acid: p-coumaric acid |  | Antigenotoxic, neuroprotective | Vauzour et al. [29], Mijanur Rahman et al. [24] |
| Honey | Phenolic acid: Gallic acid |  | Antianxiolytic | Mansouri et al. [30], Mijanur Rahman et al. [24] |
| Honey | Phenolic acid: Ellagic acid |  | Antioxidant, Chemopreventive, antiproliferative | Mijanur Rahman et al. [24] |

(continued)

Table 3.2 (continued)

| Bee products | Bioactive compound | Chemical structure | Biological activity | References |
|--------------|------------------------------|--|---|--|
| Honey | Phenolic acid: Ferulic acid |  | Antioxidant, anti-inflammation, neuroprotective | Mijanur Rahman et al. [24] |
| Honey | Phenolic acid: Syringic acid |  | Antioxidant, anticancer | Mijanur Rahman et al. [24], Tahir et al. [28] |

Self-Assessment Questions

1. Term insect is derived from a Latin word _____.
2. Insect exoskeleton is composed of _____.
3. A chemical extracted from the insects used in repair of arteries is _____.

3.3 Problematic Insects

Insect pests are major challenge in agricultural production. There are over six million insect species and among them only 20–30 are bringing significant damage to the major crops [31]. According to Krattiger [32], worldwide each year in agriculture billions of dollars are spent on insect pest control. Most followed method of insect pest control is use of pesticide. Extensive use of pesticides resulted in development of pesticide-resistant insects, decline in population of beneficial insects' detrimental effects on humans and environments [33–35] and also resurgence. There are over 500 species of arthropod pests that have developed resistance to one or more insecticides worldwide [36].

Pest resurgence results in increased pest population following use of pesticides. Along with this, there is another challenge wherein insect pests expand their geographic ranges due to climate change. This issue also leads to increase in numbers of generations of insect pests per year [37]. Therefore, an efficient system of pest management involves use of integrated methods involving best possible use of natural resources [31]. There is an increased importance among scientists in developing novel strategies for insect pest control. One of the contemporary approaches in controlling insect pests involves use of biotechnological approaches.

3.3.1 Biotechnological Applications in Insect Pest Control

Among the biotechnological approaches use of biopesticides and transgenic plant technologies is important.

1. **Biopesticides:** These are preparations used for controlling insect pests using naturally occurring organisms such as plants, animals, and microorganisms (mostly fungi, bacteria, and viruses) [38]. They control pests by non-toxic and eco-friendly manner. Chandler et al. [39] defined biopesticides as a mass produced agent manufactured from living microorganisms or a natural product and sold for the control of plant pests. They are gaining importance among people following eco-friendly and safe integrated crop management compatible approaches and strategies for pest management [40]. They are target specific, harmless to beneficial insects and do not cause air and water quality problems in the environment (Sengottayan and [41]). A large number of bacteria (like *Bacillus thuringiensis*, *Paenibacillus popilliae*), fungi (like *Metarhizium anisopliae*, *Nemuria releyi*), viruses (cytoplasmic polyhedrosis virus, nuclear polyhedrosis virus),

protozoa (like *Nosema*, *Vairimorpha*), and some beneficial nematodes (like *Steinernema* and *Heterorhabditis*) have been used as biopesticides in crop production [42, 43]. Among these agents, bacterial, fungal, and viral biopesticides are commonly used.

- (a) **Bacterial agents:** Insect pathogenic bacteria which do not cause air water quality are used as bacterial biopesticides. These biopesticides can be divided into four categories and they are (i) crystalliferous spore formers, (ii) obligate pathogens, (iii) potential pathogens, and (iv) facultative pathogens.
 - (i) **Crystalliferous spore formers:** Most important example for this group is *Bacillus thuringiensis*. These are bacteria capable of synthesizing cytotoxic and crystal toxins during the stationary phase and at the onset of sporulation. These toxins are called δ -endotoxins and they are found as parasporal crystalline inclusions. Upon ingestion by insects, these crystals are dissolved in the insect midgut and activated by the action of midgut proteases. This activated toxin binds to the specific receptors present on insect cell membrane, disrupt cells and collectively leading to death of the insect [44]. The crystal proteins and soluble toxins secreted by *Bacillus thuringiensis* are highly host specific [45].
 - (ii) **Obligate pathogens:** *Paenibacillus popilliae* is one of the obligate pathogens of insects. This bacterium has narrow host range that too is effective against Japanese beetle (*Popillia japonica*) only [46].
 - (iii) **Potential pathogen:** These entomopathogenic bacteria normally do not multiply in the gut of an insect, but multiply in hemocoel once they get access to it [47]. *Serratia marcescens* is an example for potential insect pathogenic bacterium.
 - (iv) **Facultative pathogens:** These are not obligate pathogens, but they damage susceptible tissue after invading susceptible tissue [47]. Important example for this group is *Pseudomonas aeruginosa*.
- (b) **Fungal agents:** These are earliest insect control agents. Of late there has been a growing interest in fungal entomopathogenic agents because of their potential of controlling insect pests. Greater than 750 species of entomopathogenic fungal species have been identified. Most of them belong to hyphomycetes and entomophthorales [48]. Most of the entomopathogenic fungi have similar mode of action. When fungal spore enters the host, they bind to cuticle. Under favorable environments, they germinate and form germ tube [49]. During this process, the fungal system produces special structures such as appressoria which helps fungi to invade insect integuments [50]. The fungal enzymes, viz., metalloproteases and amino peptidases facilitate fungal germ tube to penetrate the cuticle [51]. Once the fungi invade the body of insect, it develops hyphal bodies that spread through the hemocoel and spread across diverse muscle tissues, fatty bodies, malpighian tubules, mitochondria, and hemocytes and finally results in death of insects in 3–14 days after infection [49, 52]. These fungal agents cause

insect mortality by nutritional deficiency, destruction of insect tissues, and release of toxins [53]. Commonly used entomopathogenic fungi in control of insect pests are *Beauveria bassiana*, *Metarhizium anisopliae*, *Nemuria releyi*, and *Hirsutella thompsonii*. Biopesticides are made from naturally occurring substances that control pests by non-toxic mechanisms and in eco-friendly manner. Modern agricultural biotechnology or genetic engineering includes manipulation of the genetic makeup of organisms for use in the production or processing of agricultural products. A major strategy in increasing crop yield is the development of pest management regimens consisting of herbicide-resistant (HR) and insect-resistant (IR) crops as well as transgenic insects.

2. Transgenic Plant Technology:

A plant whose DNA is modified using genetic engineering technique is called transgenic plants. Steps involved in production of transgenic plants are as follows (refer Fig. 3.3):

- (i) Identification of gene for agriculturally important properties such as insecticidal toxin production like δ endotoxin.
- (ii) Isolation of gene of interest.
- (iii) Construction of recombinant DNA molecule using suitable vector (like plasmid).
- (iv) Introduction of gene conferring desirable properties into plant.
- (v) Selection of transformed plant.

There are two prerequisites for successful genetic manipulation of plants. They are: (1) suitable method for introduction of manipulated gene into target plant and (2) a thorough knowledge of molecular genetics of plant that is being modified. The technology of creating transgenic plant can be employed for imparting desirable properties like insect pest resistance, improvement of nutritive properties of food, etc. Development of transgenic plant for imparting insect pest resistance can be better illustrated by using transgenic Bt plants.

As we discussed earlier in this chapter, *Bacillus thuringiensis* is a bacterium that has been used as biopesticide. This bacterium produces insecticidal crystal toxin called δ endotoxin that kills larval stages of lepidopteran (moths and butterflies), coleopteran insect pests when they ingest the toxin. The gene coding for toxin production can be isolated and inserted into pest susceptible plant. As a result, δ endotoxin gene will be expressed in the plant system and plant acquires insect-resistant properties. Important transgenic plant developed include Bt cotton resistant to American bollworm, Bt Maize resistant to pests like European corn borer, Bt soybean resistant for dark black chafer.

Self-Assessment Questions

4. An example for crystalliferous spore former is _____.
5. *Paenibacillus popilliae* is used against _____ insect.

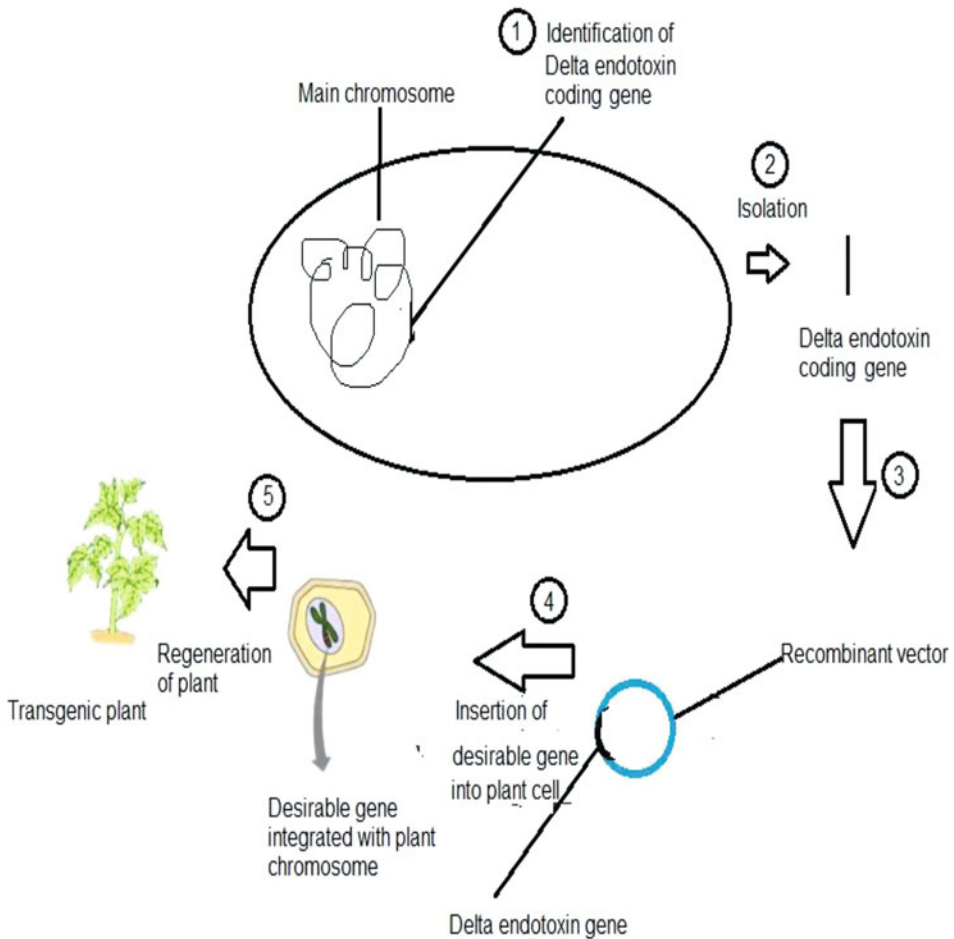


Fig. 3.3 Development of Transgenic Bt plant

6. A plant whose DNA is modified using genetic engineering technique is called_____.
7. Bt cotton is developed against _____ insect.

3.4 Conclusion

Insects are not only harmful but also beneficial to humans and environment in many ways. Insects play a major role in the agriculture, biomedical applications, pharmaceutical products, and several other fields. Insects need to be grown well with proper care for the

more production of beneficial enzymes and products which help the humans for their wellness.

Take Home Message

1. All insects are not harmful, there are many beneficial microorganisms that help us in different ways.
2. Insects play a major role in the agriculture, biomedical applications, pharmaceutical products, and several other fields.
3. Microbes living inside the human beings play an important role in gut microbiome as well.

Terminal Questions

1. Define biopesticides and add a note on different types.
2. List different insect products with their biomedical uses.
3. Write a note on edible and therapeutically important insects.
4. What are crystalliferous spore formers? Explain.
5. Write a note on fungal biopesticides.
6. Discuss the role of transgenic plant technology in insect pest control.

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Recent Advances in Insect Biotechnology

4

Sushil Kumar

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Keywords

Yellow biotechnology · Insecticides · Integrated pest management · Green biotechnology · Red biotechnology · White biotechnology

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D. Kumar, S. Shukla (eds.), *Introduction to Insect Biotechnology*, Learning Materials in Biosciences,
https://doi.org/10.1007/978-3-031-26776-5_4

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What You Will Learn from This Chapter

Insect biotechnology is the use of insects and their derivatives for agriculture, industrial and human welfare. Biotechnological tools apply to insect identification, genetic relationship, and their control for dispersal of specific biotypes of natural enemies. It plays a significant role in improving the efficacy and cost-effectiveness of bio-insecticide. It is used not only for the production of industrial enzymes like chitinases and cellulases, etc., but also for synthesized pharmaceuticals and microbial insecticides including many other substances. Insect cells especially lepidopteran cells constitute an aggressive approach toward mammalian cells for the manufacturing of biotechnological products through post-translational modifications of proteins. Insect biotechnology (yellow biotechnology) has developed within 34 years after the emergence of genetic engineering of *Bacillus thuringiensis* delta-endotoxin producer gene against the pest of the cotton plant. Now recent advances play a tremendous role in insect biotechnology to develop products or services for human use in the field of the agriculture sector (green biotechnology), health care sector (red biotechnology), and industrial production (white biotechnology).

4.1 Historical Prospective and Introduction

Insects have traditionally been used more than 5000 years ago in Asia including India, for example, silkworm and honeybees [1]. In 1911, Ernst Berliner identified and rediscovered *Bacillus thuringiensis* (Bt), a bacteria that had killed a Mediterranean flour moth. It was named after the German town of Thuringia, where the moth was discovered. The historical perspective reveals that modern insect biotechnology started with the introduction of pesticide-resistant plant production in the year of 1996 [2]. After this biological advancement up to 2008, the area of hectares cultivations increased by 90% for soybean, 78% for cotton, 72% for canola, and 60% for maize with these GM crops with the help of insect biotechnology [3]. First International Congress on Insect Biotechnology held in 2007 has become a milestone in the field of yellow biotechnology. Because of the yellow color of insect hemolymph, the term yellow biotechnology was coined. When we reached in 2011, 16.7 million farmers of 29 countries adopted GM crops and planted 160 million hectares of these yellow biotechnology-initiated crops [4]. “The Manhattan Project of Entomology” started in 2011 to sequence the genomes of 5000 insects and other arthropods over the next 5 years called the i5k for improving our lives by contributing to a better understanding of insect biology and their gene sequences against threatening our health, food supply, and economic security. In recent advances, Bt cotton has increased cotton yield by up to 60% and has reduced insecticide spray by around half of the cost per annum in India. Genetic engineering nowadays is experimenting with organisms’ genetic composition to employ them in the production or processing of agricultural goods [5]. Previous studies were only

based on strategies to kill insects including insectivores or parasitoids that are important partners in fighting against pests or vector insects. So, the recent advances in insect or yellow biotechnology have become apparent as sustainable and environment-friendly methods require the control and availability of selective insects. Herbicide-resistant and insect-resistant crops as well as transgenic insect development have now become an important technique for enhancing agricultural production [6].

Insect biotechnology or yellow biotechnology means directly insects or insect-derived products or molecules are used in agriculture, health care, and industrial production for human welfare. Global warming and climate change are already causing alarm changes in the pest and disease complexes and forcing the biologists and entomologists to make radical changes to adjust their effects. It mainly targets the growing demand for food by controlling the harmful effect of different pests and increasing the production and formulation of entomopathogenic fungi as well as proteinaceous insect toxins (scorpion toxin, mite toxin, trypsin inhibitor), hormones (eclosion hormone, diuretic hormone) and metabolic enzymes (juvenile hormone esterase) introduced into NPV and GV genome virus to increase its efficacy to kill insects. Comparative genomics helps as a research tool for insect genome sequencing and annotation. It also allows for the identification of insect-specific genes that may be targeted with rational pesticide design and recombinant DNA technology [7].

The major step of yellow biotechnology is the genetic manipulation of *Bacillus thuringiensis* (Bt) bacteria. In this method, the Bt genes, which code for insect-toxic proteins, provide a chance to create GM strains that are more potent and transgenic plants, as well as a significant increase in agricultural output [8, 9]. The use of RNA interference (RNAi) technology allows for the development of a new generation of pest-resistant GM crops [10]. Agriculture's long-term viability will be aided by insect management tactics that combine cutting-edge biotechnology with traditional wisdom and technology [11]. Insect biotechnology has shown to be a valuable resource in a variety of sectors, including industrial enzyme production such as chitinases and cellulases, medicines, microbial pesticides, insect DNA, etc. [12]. Insect cells (ICs) are ideal for the production of complex proteins requiring extensive post-translational modification [13, 14]. Recent advances in techniques especially genetic engineering of organisms have resulted in scientific breakthroughs in the field of molecular biology especially in red, green and white biotechnology (Fig. 4.1).

4.2 Advances of Insect Biotechnology in Agriculture Sector

Insects are the main competitor of the human species for food. Due to their large size and varieties, they counted as the most common enemy for pre- and post-harvested food grains. Biotechnology plays a very important role in the advances in the agriculture field through insect management. Crop pest control needs a very huge amount worldwide, including 40% expenditure only used for insect controls exclusively in developing countries

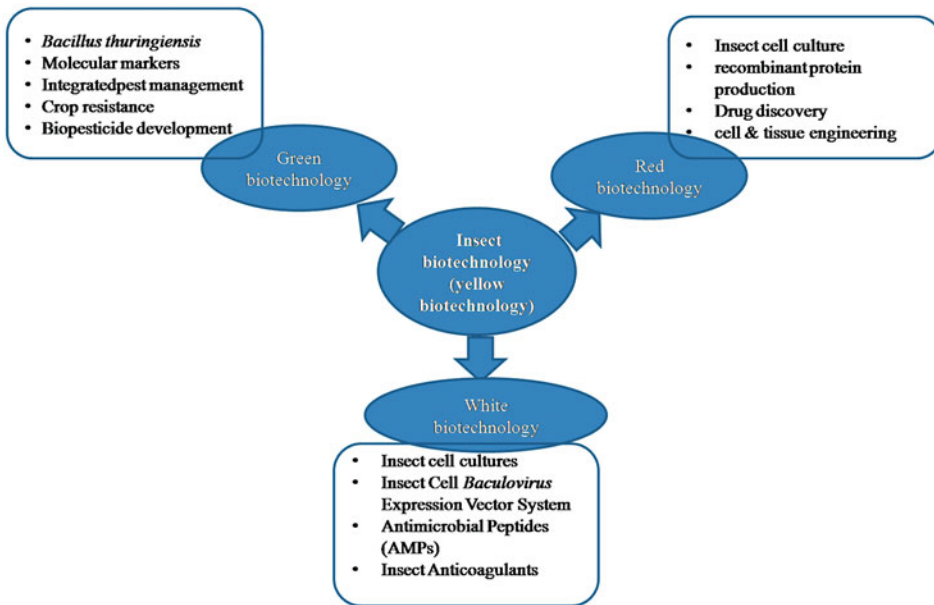


Fig. 4.1 Impact of insect (yellow) biotechnology on red, green, and white biotechnology

[15, 16]. It facilitates the development of insect-specific pest-resistant plants and trees, the creation of insecticidal microorganisms, and the engineering of insects that might spread deadly genetic material to wild populations before being released in large numbers in the field utilizing integrated pest management (IPM). However, the long-term use of pesticides has led to resistant insects and a reduction of beneficial insect populations, which has very harmful effects on humans as well as the environment [17–19]. These problems have compelled the researchers to think of a solution differently to develop different insect control strategies. The first transgenic tobacco plant was developed in 1987 that expressed an insecticidal gene that produces up to 1% soluble protein cowpea trypsin inhibitor, which enhanced the protection against the Lepidopteron pest *Heliothis virescens* (Tobacco budworm) [20, 21]. There is some major example of insect biotechnology for the agriculture field w.e.f. genetically engineered bacteria express silk genes from the mulberry silkworm (*Bombyx mori*), which are used in the production of synthetic silk. The modern advancement is majorly based on the development of *Bacillus thuringiensis* containing disease-resistant crops, various DNA molecular markers for identification of plant varieties, and integrated pest management in the agriculture sector as mentioned below:

4.2.1 *Bacillus thuringiensis* (Bt) and Their Management in Crops

Bt is a soil-dwelling gram-positive bacterium that secretes a deadly endotoxin, commonly used as a biological pesticide. It is the most well-known example of genetic manipulation

of the gene coding the delta-endotoxin protein of Bt among both plants and viruses [22]. Bt toxins are highly effective against pest organisms, mainly belonging to the Lepidoptera family, and other related species. It is less toxic to mammals and other non-target organisms. Bt cotton was commercially released in the USA in 1996, and it has since been produced in countries such as Argentina, Australia, China, Columbia, Indonesia, Mexico, and South Africa including India [14]. So, the use of genes encoding endotoxins from Bt is now a well-established technology for producing transgenic plants [23, 24]. There are two main categories of Bt toxins: Cry and Cyt. These two groups are classified further by a detailed nomenclature system that describes groups Cry1 to Cry55 including the three domain family and Cyt1 to Cyt2 [25–28]. The most affected by Bt toxins are the larvae of the insect Orders Lepidoptera (butterflies and moths), Diptera (mosquitoes), and Coleoptera (beetles) [29]. Bt toxins, on the other hand, are not poisonous to humans, wildlife, or most beneficial insects [30, 31]. For the first time in 2011, Bt cotton plantings in India reached the historic milestone of 10 million hectares, accounting for 88% of the total 12.1 million hectare cotton crop [32]. However, several findings indicated that some pests, including armyworms (*Spodoptera frugiperda*), pink ball worms (*Pectinophora gossypiella*), cotton bollworms (*Helicoverpa* spp.), and western corn rootworms (*Diabrotica virgifera virgifera*), were resistant to Bt crops [33, 34]. Insect resistance to Bt crops is managed using two major strategies: refuge and pyramiding. The refuge method entails delaying the emergence of Bt crop resistance while the pyramiding strategy means combat gene evolution [35, 36]. The introduction of insecticidal genes with entirely new mechanisms of action, such as proteinase inhibitors, is another resistance management method that is still in the research phase of development [22]. To prevent cross-resistance, integrating several Bt genes for resistance management requires that the separate toxins have diverse targets [23].

4.2.2 Role of Molecular Markers in the Agriculture Field

Insects are the most numerous and diversified of all the species that live on earth. In insects, molecular markers are essential tools for assessing genetic variations as well as similarities and subdivisions across populations. They can also help us better understand insect biosystematics and evolutionary processes that have led to some insects becoming pests quickly. DNA markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), mitochondrial DNA (mtDNA), microsatellites, and single nucleotide polymorphism (SNP) markers have aided in the understanding of the genetic basis of insect diversity.

Rather than waiting for phenotypic manifestations, DNA markers firmly connected to a gene of interest may be employed at any crop stage to screen for the existence of the gene. One of the most basic uses of genetic technologies is the simple sequence repeats (SSRs) marker. It is crucial in determining the mode of inheritance of a gene. Moussa et al. [37]

discovered a microsatellite marker connected to the BtCry1Ac resistance trait in *Helicoverpa armigera* pest. Also, the identification of mealybug pest species in Egypt and France has been investigated using a DNA barcoding approach [38]. DNA-based makers have led to tagging of several plant resistance genes and also mapping of virulence genes and their subsequent cloning for insect biotypes.

4.2.3 Integrated Pest Management (IPM) for Plants and Crops

IPM is today a widely accepted strategy to reduce overdependence on chemical insecticides and their potentially negative impact on the environment and socio-economic conditions [39–41]. It is an ecosystem-based approach to the long-term prevention of pests with a combination of techniques such as biological control, habitat manipulation, and the use of resistant varieties. The most critical stage in the effectiveness of an IPM program is the correct taxonomic identification of any insect species with their association as a pest or allied natural enemy. Physical, chemical, biological, genetic, and cultural tactics keep pest populations off-balance and avoid the development of resistance to pesticides. It also suppresses the pest population below the economic injury level of crops. Biotechnology research has now focused on improving the natural enemies of the pest. The natural enemies are bacteria, viruses, fungi, nematodes, predators, etc. Research has focused on increasing the host range and virulence of Bt by combining genes with different host specificities and properties. Emphasis has been made on stabilizing and improving the virulence of these bacteria. Biotechnological research has focused on the engineering of certain viruses to express genes whose toxins kill faster than wild-type viruses. It is the second principal area of biotechnology for pest control has been the development of crop varieties resistant to pests and diseases. The creation of crop types that are resistant to pests and diseases is the second major biotechnology pest management application. This has concentrated on incorporating insect and virus resistance into the plant genome. In addition, modification of the genome of plant-associated microorganisms has been followed as a strategy to confer insect resistance to plants.

4.2.4 Biopesticide Development and Crop Resistance

There are already over a hundred commercial biological control products on the market, with many more being manufactured and provided locally for specific production systems. Because of their relative simplicity of mass manufacturing and ability to be utilized in the same way as formulated chemical pesticides, most commercial biological control has concentrated on insect pathogens [42]. Bt is valuable in the IPM system because it kills pests (functional response) as well as reproduce at the expense of pest (numerical response) in comparison with chemical insecticides, thereby giving some control in the future pest generations. Biotechnological engineered genes for Bt toxins into plants is an ingenious

method for delivering these toxins to pests that might naturally avoid them, such as insects that feed inside plants. Qualitatively, resistance is either present or absent. However, quantitatively, horizontal resistance is exhibited in varying degrees and vertical resistance is convenient because large levels of resistance may be generated, and the strategy is consistent with breeding strategies that target important genes to improve crop performance. This includes mixed or intercropped populations of resistant and susceptible plants or genetic methods to restrict the expression of genes to certain parts of plants or at certain times. Therefore, management of resistance is very likely, although the performance of chemical pesticides is not promising.

4.3 Advances of Insect Biotechnology in the Health Care Sector

Insect vectors are responsible for many human diseases and cause millions of deaths yearly [43] in which yellow fever mosquito (*Aedes aegypti*), malaria mosquito (*Anopheles gambiae* and *Anopheles stephensi*), and pink bollworm (*Pectinophora gossypiella*) are the most important vectors of human diseases. Insect biotechnology can reduce the burden of human diseases and also utilize the insect as beneficial sources. The bioengineering of honey and wax from the bee, silk, cantharidin, antimicrobial peptides, maggot secretions, and anticoagulants from bloodsucking insects are all examples of how insect folk remedies are used today [44]. These examples demonstrate how contemporary molecular and biochemical tools have allowed manipulating and bioengineering insect natural materials into modern medications. In terms of their potential for use in medicinal applications, certain microbial symbiont compounds perform well. For example, larvae of the common queen bottle fly *Lucilia sericata* are used in traditional medicine in many cultures, and recently-developed maggot therapy is being utilized in many areas not just to treat chronic or non-healing wounds, but also to cure illnesses like diabetic foot syndrome. The active compounds generated and released into wounds by the larvae work as a disinfectant and expedite wound healing, capable of digesting necrotic tissue while leaving healthy tissues untouched. Antimicrobial molecules especially harlequin ladybird *Harmonia axyridis* in which over 50 genes have been discovered that code for antimicrobial peptides. However, current research has focused on smaller molecules that might be used as lead compounds in the creation of novel antibiotics, i.e. Hormonin derived from the harlequin ladybird, which has anti-tuberculosis and anti-malaria properties. In the case of insect-based antibiotics, it is both expensive and risky to develop such chemicals by insect biotechnologists into novel antibiotics.

4.3.1 Insect Cell Culture and Drug Discovery

Insect cells (ICs) and particularly lepidopteran cells are an attractive alternative to mammalian cells for biomanufacturing. IC culture, coupled with the lytic expression capacity of

baculovirus expression vector systems (BEVS), constitutes a powerful platform, IC-BEVS, for the abundant and versatile formation of heterologous gene products, including proteins, vaccines, and vectors for gene therapy. Such products can be manufactured on a large scale, thanks to the development of efficient and scalable production processes involving the integration of a cell growth stage and a stage of cell infection with the recombinant BEVS. ICs can produce multimeric proteins functionally equivalent to the natural ones and engineered vectors can be used for efficient expression. ICs can be cultivated easily in serum- and protein-free media. A growing number of companies are currently developing an interest in producing therapeutics using IC-BEVS, and many products are today in clinical trials and on the market for veterinary and human applications.

Several recombinant proteins, varying from membrane-bound proteins to cytoplasmic enzymes, have been effectively generated in BV-infected ICs since the discovery of the IC-BEVS in the 1980s. However, IC-BEVS is presently one of the best options for producing recombinant proteins with market demands of less than 5–10 kg/year (i.e., subunit vaccines). In practice, the majority of permitted items obtained in ICs are vaccinations rather than goods with a huge need for production, like therapeutic antibiotics. Utilizing metabolic engineering to gain a thorough understanding of the physiology of both healthy and diseased ICs, as well as ongoing advancements in insect cell media and culture methods, will undoubtedly aid in the rational design and validation of reliable, scalable, and secure production processes that will increase yields using the IC-BEVS technology.

Bone marrow mesenchymal stem cells, which are regarded as a particularly potent vehicle for cell-based treatments due to their differentiation capacity and unique immunological features, have a lot of success. BV transduction of mammalian cells does not appear to significantly affect their immunological status and behavior *in vitro* and, more importantly, *in vivo*. Due to their inherent inability to multiply in mammalian cells and their minimal toxicity, they are potentially safe options for therapeutic gene delivery. To be successful, drug and vaccine development must take into account that a large number of complex eukaryotic proteins are synthesized with the proper post-translational processing and have the requisite biological activity through various insect species with their product and application in health care sector (Table 4.1).

4.4 Advances of Insect Biotechnology in the Industrial Production Sector

Insect biotechnology is the modern usage and application of biotechnology in the industrial sector for the sustainable processing and manufacture of chemicals, minerals, and fuels. White/industrial biotechnology means the use of living cells from molds, bacteria, plants, and yeast to synthesize the easily degradable products in the industrial production sector. It may need less energy and produce fewer by-product wastes. It is solely applied to the industry to replace polluting technologies with clean ones. It also concentrates on the production of energy from renewable resources and biomasses. Starch from corn, potatoes,

Table 4.1 Uses of insect biotech products in health care sector

| S. No. | Insect species | Product | Application |
|--------|--|--|---|
| 1 | <i>Blister beetles</i> , <i>Mylabris caraganae</i> , <i>Mylabris phalerata</i> | Cantharidin | Helps in treatment of cancers, liver, pancreatic, bladder, breast, and leukemia including reduced activities of <i>Plasmodium falciparum</i> and <i>Leishmania major</i> |
| 2 | <i>Anopheles</i> salivary gland | Anopheline | Thrombin inhibition and helps developing anticoagulants especially antithrombotics |
| 3 | <i>Apis</i> spp. | Honey component | Stimulates TNF- α production through TLR4 activation for wound healing |
| 4 | <i>Apis</i> spp. | Manuka honey, buckwheat honey, honey products of Revamil, Propolis | Inhibits gram +ve MRSA, vancomycin-sensitive and vancomycin-resistant <i>Enterococci</i> , <i>Streptococcus</i> species isolated from wounds, Gram-ve bacteria associated with wounds such as <i>Pseudomonas aeruginosa</i> , <i>Stenotrophomonas</i> species, and <i>Acinetobacter baumannii</i> |
| 5 | <i>Apis</i> spp. | Quercetin, apigenin, and acacetin | Anticancer, enhances the apoptotic ability of anti-CD95 and rTRAIL in acute lymphocytic leukemia |
| 6 | <i>Asp</i> spp., <i>Vespula</i> spp., <i>Solenopsis</i> spp. and <i>Pachycondyla</i> spp | Bee, wasp, and ant venom products, e.g., melittin | Inhibits cancer cell development especially hepatic, melanoma, ovarian, osteosarcoma, leukemic, prostate, mammary gland cells, bladder, and kidney |
| 7 | <i>Lucilia sericata</i> | Maggot alloferons | Antiviral and antitumor activities |

sugarcane, and wheat is already used to produce ethanol as a substitute for gasoline—Henry Ford’s first car ran on ethanol.

4.4.1 Insect Cell Cultures (ICCs) for Large Scale Production

Perhaps one of the areas where insect biotechnology has contributed the most to basic research is the development of insect cell cultures (ICCs). Cells are frequently managed to grow in a variety of culture media created for the species being studied under conditions suitable for the propagation of stable, likely clonal lines of cells obtained from embryos, particular tissues, or whole organisms to develop cultured cells of animals, plants, insects, and fungi. The reason behind this is that individual cells are simpler to experiment on than

animals, and since the cell is the basic unit of life, it makes sense. It's also simpler to make huge amounts of metabolites in cell cultures than it is in animals, especially heterologous proteins and post-translational modification of eukaryotic proteins.

ICCs are used in a broad range of scientific domains, including the creation of recombinant proteins, viral insecticides, and vaccinations, among others. They're extremely useful in morphogenesis, genetics, virology, pathology, biochemistry, endocrinology, and molecular biology research. Several insect orders, including Diptera, Lepidoptera, and Hemiptera, have created cell lines. There are currently more than 100 insect cell lines available for recombinant protein production with lines derived from *Bombyx mori*, *Mamestra brassicae*, *Spodoptera frugiperda*, *Trichoplusia*, and *Drosophila melanogaster* being of particular insects [45]. More recently, Lepidopteran cell lines were created largely for the purpose of propagating insect viruses for use as biopesticides in the biological control of insect pests. For example, the baculovirus expression vector system coupled with insect cell cultures (IC-BEVS) has recently proven more appealing for the production of many heterologous proteins than those originating from bacteria, yeast, vertebrates, or viruses. This approach is also being used in the development of recombinant BVs for use as biopesticides, which kill insect pests more quickly than wild-type BVs. Insect cell lines are commonly used in place of prokaryotic ones because post-translational modifications are possible in insect cells. The Sf9 cell line is one of the most commonly used cell lines in ICCs [46]. ICs lines are also valuable for the study of virus–cell interaction, development of viral pesticides, production of recombinant proteins, and development of health-related products.

4.4.2 Insect Cell *Baculovirus* Expression Vector (IC-BEVS) Development and Its Use

Baculoviruses (BVs) are the most prevalent insect virus, infecting over 600 insect species worldwide especially BVs infected Lepidopteran insect caterpillars and Dipteran larvae appear to have gotten the most attention [47]. Lepidoptera, Hymenoptera, Diptera, Coleoptera, Neuroptera, Trichoptera, Crustacea, and Acarina are the insect arthropod orders with which they are most commonly linked. The virions are rod-shaped and have a lipoprotein capsid protein sheath with a DNA protein core. NPVs that infect Lepidopterans include *Helicoverpa armigera*, *S. litura*, *S. exigua*, *Amsacta Moorei*, *Agrotis ipsilon*, *A. segetum*, *Anadividia peponis*, *Thysanotus orichalcea*, *Adisura atkinsoni*, *Plutella xylostella*, *Corcyra cephalonica*, *Mythimna separata*, and *Phthorimaea operculella* [48]. Each BV has a very narrow host range, limiting itself to a particular kind of insect. The creation of the IC-BEVS, which has found widespread usage in biotechnology, was made possible by the unique properties of BVs. It produces post-translational modified, biologically active, and functional recombinant proteins. The IC-BEVS works by using homologous recombination including a transfer vector that already contains the target gene to insert a foreign gene into a portion of the genome. One of the non-essential genes in the

recombinant BV is substituted with a foreign gene producing a heterologous protein that can be produced in cultured ICs and insect larvae. IC-BEVSs have pushed the boundaries of fundamental research because of their ability to create a large number of recombinant proteins at high levels while also providing considerable eukaryotic protein processing capabilities. Furthermore, during the last 20 years, significant technological advancements have improved upon the original methodologies devised for the separation of BV expression vectors. Today, almost any researcher with a rudimentary understanding of molecular biology may extract a recombinant BV vector and utilize it to manufacture a particular protein in an ICC in a very short amount of time. IC-BEVS has become a core technology for—(1) cloning and expressing genes to study protein structure, processing, and function, (2) the production of biochemical reagents, (3) research into the regulation of gene expression, (4) commercial exploration, development, and production of vaccines, therapeutics, and diagnosis, (5) drug discovery research, and (6) the investigation and development of safer, more targeted, and environmentally friendly biopesticides suitable with sustainable farming.

The *Autographa californica* multinuclear polyhedrosis virus (AcMNPV) with Sf9, Sf21, or High Five™ cells is the most often utilized BV vector in business and research laboratories for recombinant protein synthesis [50, 51]. In recent years, IC-BEVS has acquired popularity and widespread acceptability for regular recombinant protein synthesis in ICs and larvae. Furthermore, IC-BEVS is also used to create ways for displaying foreign peptides and proteins on vitro particles, as well as the insertion of mammalian cell-active expression cassettes into BVs. They have been genetically modified to exhibit foreign peptides and proteins on their surface and have proven to be highly effective immunogens. One of the most popular cell lines for producing virus-like particle (VLP) vaccines utilizing recombinant IC-BEVS is Sf9. Other popular cell lines include BTI-Tn5B1-4 and the High Five™ cells, which are branded by Invitrogen. VLPs are multiprotein complexes that resemble the structure and replication of real native viruses but do not include the viral genome, potentially producing safer and more affordable vaccination options. Through the creation of IC-BEVS, insect biotechnology has significantly changed the biotechnology sector.

4.5 Conclusion

Insect biotechnology with sustainable agriculture tactics could be achieved not only through proper agricultural production but also through investment to achieve greater competitiveness in the world market. To decrease pest invasion and improve plant tolerance, agronomy, which includes genetic engineering, is a valuable feature across species of plants that can't be achieved by conventional techniques. Yellow biotechnology is important to provide appropriate regulatory mechanisms to ensure that products produced by new technologies are as safe as the products of traditional biotechnology. Insect control strategies that integrate advanced knowledge in biotechnology with traditional wisdom

will contribute to the sustainability of agriculture. Sustainable control methods that coexist peacefully with genetically modified plants will be provided by biological control strategies including beneficial insects, microorganisms that destroy insect pests, and pesticides generated from phytochemicals of plants. It can have a positive impact on food security from insect attacks and can contribute to the sustainability of modern agriculture.

There are many advantages of insect biotechnology for humanity, especially those driven by IC-BEVS. The generation of bio-insecticides and recombinant proteins for application in fundamental research, diagnostics, and biomanufacturing is well established. As a result of recent advances in insect biotechnology in human and animal health, including biopharmaceuticals and new generation vaccines development accepted worldwide. Additionally, the technology is perfectly adapted to the creation of viral vectors for cell treatment and the simple expression of proteins for use in fundamental biological research, tissue engineering, drug discovery, sustainable agriculture, and other applications. Building on the advancements in insect cell media quality and cultivation effectiveness is the ongoing development of tissue culture methods that support high-quality cell systems and the use of BVs as a display system. Insect biotechnology is generally regarded as a very positive advancement in contemporary scientific and biotechnological endeavors that leads to scalable and secure bio-production methods.

Take Home Message

- The use of whole insects, their organs, compounds produced by genetic manipulation of insect genes as well as their symbiotic microorganisms, in the domains of agriculture, health care, and industrial production is classified as insect biotechnology.
- Despite some reports of harmful impacts on beneficial arthropods, GM crops to express insecticidal proteins appear to have the potential to be far more ecologically friendly than insecticide-based pest-control strategies in this area.
- Beetles, moths, and mosquitoes, as well as their larvae, could be a valuable source of medication development. They are incredibly diversified at the molecular level, suggesting a treasure box full of active compounds that will assist medicine in the future.
- Insect biotechnology is the research and manufacture of novel, innovative products and processes in a cost- and eco-efficient manner, with growing use of renewable raw materials in large-scale industrial production.

Question Related to the Above Content

1. What causes the changes in the pest and diseases complexes?
 - i. Pollution
 - ii. Temperature
 - iii. Global Climate Changes
 - iv. pH
2. What are the major constraints to global production for food and fiber?
 - i. Fungi
 - ii. Algae

- iii. Protozoans iv. Insect pests
- 3. Insect Pests can be reduced utilizing—
 - i. Modern biotechnological tools ii. DNA fingerprinting
 - iii. ELISA iv. Southern Blotting
- 4. The technology enables engineering of a new generation of pest-resistant GM crops—
 - i. C DNA cloning ii. RNAi
 - iii. Blotting iv. PCR
- 5. Insect biotechnology also known as—
 - i. Green biotechnology ii. Red biotechnology
 - iii. Yellow biotechnology iv. White biotechnology
- 6. The major step of yellow biotechnology is genetic manipulation of Bacteria—
 - i. *Bacillus cereus* ii. *Bacillus thuringiensis*
 - iii. *Bacillus megaterium* iv. *Bacillus pumilus*
- 7. What are the main competitors of human species for food—
 - i. Arthropods ii. Insects
 - iii. Mollusca iv. Annelids
- 8. A long-term use of pesticides has led to—
 - i. Resistant insect ii. Reduction of beneficial insect population
 - iii. Only (i) iv. (i) and (ii) both
- 9. Bt is a bacterium—
 - i. Gram negative ii. Gram positive
 - iii. (i) and (ii) both iv. None of the above
- 10. Bt cotton was first released for commercial production in the—
 - i. USA ii. Argentina
 - iii. China iv. South Africa
- 11. The main categories of Bt-toxin are—
 - i. Only cry ii. Only cyt
 - iii. Cry and cyt both iv. All of above
- 12. One of the most fundamental applications of genetic tools—
 - i. SSRs ii. cDNA cloning
 - iii. ICs iv. RNAi
- 13. The main strategies for management of insect resistant to Bt crops—
 - i. Refuse ii. Pyramiding
 - iii. Refuse and Pyramiding iv. All of the above
- 14. The microsatellite markers linked to Bt cry1Ac resistance trait was identified in the pest—
 - i. *Helicobacter armigera* ii. *Plutella xylostella*
 - iii. *Pentalonia nigronervosa* iv. *Macrotristria dorsalis*
- 15. The first vaccine produced in ICs to be the commercialized for human use—
 - i. Provence ii. Flublock
 - iii. Chimigen iv. Cervarix

16. Recently, which cell lines have been established primarily to propagate insect viruses as biopesticide for the biological control of insect pests?

- i. Hemiptera ii. Diptera
- iii. Lepidoptera iv. Coleoptera

17. The most commonly used cell line in insect cell culture is—

- i. Sf 21 ii. Sf 9
- iii. S2 iv. BTI-Tn5B1-4

Answer of the Above Questions

iv, 2. iv, 3. i., 4. ii., 5. iii, 6. ii, 7. ii. 8. iv, 9. ii, 10. i, 11. iii, 12. i, 13. i., 14. i, 15. iv, 16. iii, 17. ii

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Insect Metagenomics

5

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and Chengliang Gong

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© Springer Nature Switzerland AG 2023

D. Kumar, S. Shukla (eds.), *Introduction to Insect Biotechnology*, Learning Materials
in Biosciences,

https://doi.org/10.1007/978-3-031-26776-5_5

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Keywords

Insect · Metagenomics · Gut · DGGE · FISH · NGS

What You Will Learn from This Chapter

Through this chapter, you will come to know about insect gut metagenomics and how it is helpful for the insect to flourish whatever the condition may be thrown in front of it. Apart from that, there are different methods of exploring the bacterial colony harboring inside the gut and finally the applications one can exploit these non-cultured bacterial species for making human life more economical and comfortable. Under the introduction part, you will learn about the link between insects and gut bacteria and the importance of the symbiotic relationship. Without culturing, one can identify the bacterial colony from the insect midgut through the use of any of the below-mentioned methods will be the most intriguing part of this chapter. Under the methods section, you will learn different methods of metagenomic analysis; this will help to interconnect methodologies and their applications. You will also learn the existing commercial applications of identified bacterial species for the production of economically important products at an industrial level.

5.1 Introduction

Insects are animals with chitinous exoskeletons with jointed appendages, omnipresent, omnivorous, hexapodal invertebrates. These creatures are a highly efficacious group of animals known for their diversity and adaptation to any environment which exists on the planet earth. The most important reason behind this success is the microbes that are harbored inside and outside of the insect body. Even though some are pathogenic, most of them are responsible for evolutionary success. The biological system like the epidermal cells of the insect gut interact with the microbes, and such interaction provides extra strength to cope up with the drastic conditions exhibited by the environment from time to time. The beneficial effects of such interactions include the change in diet and its assimilation, which acts as a shield to protect from parasites and pathogens of different nature. Over the period of time, the symbiosis between insects and microbes has grown to such an extent that the life of insects without microbes is unimaginable. As the gut of the insect is hollow, it provides a large surface area for microbes to harbor. The anatomy of the gut and the food habits both are interlinked and on close observation, one can find evolutionary traces [3, 5, 7]. In this chapter, you are going to learn different methods used to unravel the metagenome from the insect gut and the successful employment of microbiomes for industrial application.

5.2 Methods of Insect Metagenomic Analysis

The first and foremost important thing for a metagenomic analysis is to obtain a good quality of microbial DNA (deoxyribonucleic acid). Care should be taken so that the DNA must cover as much microbial species as possible. Either of the two studies can be employed for this purpose. One method is based on the isolation of DNA from the intact cell referred as the cell recovery method and the other one is the lysis of microbial cells in the presence of the environmental matrix referred to as direct lysis method. In the earlier method, the cells were obtained from any of the differentiation methods such as differential centrifugation or differential gradient. In other methods care should be taken so that nucleic acid from the host cells will not come into the final DNA isolation [1, 6].

Once we are successful in obtaining a good quality, and at the same time a sufficient amount of DNA then we can go for any of the three methods: Denaturing gradient gel electrophoresis (DGGE), Fluorescent in situ hybridization (FISH) [4], and Next-Generation Sequencing (NGS) [4].

5.2.1 Denaturing Gradient Gel Electrophoresis (DGGE)

The good quality of microbial DNA from insect midgut was the most important criterion to proceed with DGGE. The standard method of your choice can be employed. Microbial DNA Isolation Kit from any of the standard manufacturers like Qiagen, Invitrogen, Micher Nagel, etc. will give better yield; compared to conventional methods, it is time-saving. Once the purification is over, the quality assessment of DNA needs to be performed using either UV-Vis spectrophotometers, nanodrop machines, or florescent-based assays. It is advisable to perform duplicate DNA extraction for the same sample and pool them together before going for PCR amplification. The bacterial samples can be universally amplified using universal primers targeted to the 16S rRNA region. The amplicons need to be further purified with a standard PCR purification kit of any make strictly following their instructions. The purified products are now ready to be used for PCR-DGGE analysis and metagenomic sequencing. One thing that needs to be remembered at this stage is the purification and quantification to be performed back to back. The purified 16S rRNA gene amplicons were further amplified using primers spanning the V3 region of the 16Sr RNA gene. A GC clamp needs to be attached to the forward primer. DGGE analysis was performed with a vertical electrophoresis system (EX: Bio-Rad Laboratories). PCR product (~25 μ L) was loaded onto the 0.8-mm polyacrylamide gel [8% (w/v) acrylamide/bisacrylamide gel 37.5:1], containing a 30–60% urea-formamide denaturing gradient increasing in the direction of the electrophoresis (100% corresponds to 7 M urea and 40% (w/v) formamide), and run with 1 \times TAE buffer (0.04 mol/L Tris-acetate, 0.001 mol/L EDTA). The gels were run at a constant voltage of 130 V for about 4 h at 60 °C. After electrophoresis, the gels were stained, visualized under UV light, and image need to be captured. Selected DGGE bands were excised by gel cutting tips, re-suspended in sterile

water, and incubated overnight under refrigeration (4 °C) to allow DNA to diffuse out of the gel cuts. Eluted DNA was subjected to PCR amplification using only forward primer without a GC clamp. Amplicons were then purified and sequenced. The sequences thus obtained were subjected to Basic Local Alignment Search Tool (BLAST: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) search analysis. The matching GenBank data will reveal the bacterial species of interest.

5.2.2 Fluorescent In Situ Hybridization (FISH)

One of the major applications of Fluorescent in situ hybridization (FISH) is its use in insect gut metagenomic studies. This is achieved through the use of fluorescently labeled probes which are part of 16 s rRNA sequences specific to bacterial species [4]. These probes are nothing but oligonucleotide primers used in conventional PCR; the only difference is the tagging of fluorescence emitting dye. In-silico design of probe can also be performed using any of the online primer designing tools and there is an ample scope to perform online validation. Probe designing guidelines are as same as that of the primer design. In brief, an ideal length of the probe is somewhere between 15 and 25 bases with allowed 50–70% GC content and T_m should come between 50 and 60 °C. Using an open-source database, highly accurate, reliable, and species-specific probes can be designed. The intensity of the FISH signal depends on how well the probe binds to the target region within the bacterial genome. It is advisable to go for the validation of a designed probe by performing PCR where in probe as a reverse primer and in place of forward primer any of the universal forward primers can be used. Now coming to fluorescent dye, also referred to as fluorochromes, they are of different types based on the wavelength of light they emit. A multiplexing can be possible to detect more than one bacterium with distinct fluorochrome labeled probes. For detecting the emitted fluorescence, one can take the help of a high-performance laser scanning microscope (LSM). One thing we need to be cautious about is auto-fluorescence especially in the shorter region between blue and green by the insect tissue; this can be nullified if we use longer wavelength light emitting fluorescence dye in the study.

5.2.2.1 Sample Preparation

Fresh tissue is the ideal sample for the FISH analysis. Preservation can decrease the quality of the data and the overall success of the FISH. If one wants to perform FISH on the field specimens, then the whole insect body can be preserved in absolute ethanol and for tissue one can go for acetone. It is advised to perform all the dissections in an isotonic solution either Insect Ringer's solution or phosphate-buffered saline (PBS).

5.2.2.2 Fixation

Once the gut is dissected it is fixed by a freshly prepared fixative (9 vol. of PBS and 1 vol. of 40% formaldehyde) overnight at 4 °C. After washing, the gut can be stored in absolute ethanol until further use.

The primary task to continue the FISH analysis at this stage is to completely replace the water in the tissue with paraffin. This is accomplished through dehydration, clearing, paraffin infiltration, and embedding. It is a step-by-step procedure; the success of each step at the end will produce high-quality sections. It begins with replacing water with ethanol (amphiphilic solvent), then followed by xylene (hydrophobic solvent), and later it is replaced with paraffin. A scalpel is used to remove excess paraffin a few millimeters around the tissue. The paraffin block is now transferred to the specimen holder of the microtome to obtain tissue sections. The ideal thickness would be 5 μm .

5.2.2.3 Hybridization

A freshly prepared 150 μL hybridization buffer contains the probe(s) recommended for single slide. Before proceeding to the next step, a xylene is used to remove the paraffin from the sections and is followed by RNase-free water rinse. The hybridization buffer is overlaid onto the sections and without air bubbles covered with a coverslip and left overnight, preferably in dark. After a brief wash with freshly prepared PBS containing 0.3% Triton X100 [v/v], 100 μL of anti-fade solution is applied onto the slide. Any of the commercially available anti-fade solutions can be used. After covering the slide with cover slip, all the sides need to be sealed with cover slip sealant. At any stage of slide preparation, an air bubble should not be trapped between the cover slip and the slide. The samples are now ready for microscopy and analysis.

For quality control purposes each assay needs to be included at least two control experiments: (1) No probe control to measure auto-fluorescence from the tissues. (2) Competitive suppression control to observe sequence specificity and competitive suppression of hybridization signals. Combining a universal eubacterial probe with a more specific probe is also useful to detect the presence of additional bacteria not identifiable with a specific probe. The FISH signal detection and image acquisition can be performed using an epifluorescence microscope following the standard fluorescence microscopy procedure as mentioned in the instrument manual. The basic tips for FISH analysis are as follows:

1. Initially, each signal from a given probe must be acquired as a black and white image; this can be later converted into color and merged.
2. There are chances of probe dissociation from the target, so it is advisable to go for analysis as soon as mounting in anti-fade medium is performed.
3. Minimize exposure time to prevent photobleaching of fluorochromes (i.e., reduction of signal intensity).
4. To get rid of the localized photobleaching effect, images should be acquired from lower to higher magnifications.

5.2.3 Next-Generation Sequencing (NGS)

5.2.3.1 Sample Collection

The insect larvae or adult stage need to be cold anesthetized and surface sterilized with 70% ethanol. To get a sufficient amount of microbial DNA, more number of insects need to be taken for the process. The guts were dissected, pooled, and homogenized with any of the laboratory-grade homogenizer. The major contaminant sources of gut microbiome study are plant and host tissue debris. In order to get rid of this, several purification steps need to be followed. A freshly prepared 100 mL of 10 mM MgSO_4 will be added to the homogenized slurry. The mixture is ready to be passed through initially with 20 μm filters followed by 11 μm filters.

The sample thus collected needs to be spun down for about 15 min at 4000 rpm. Sufficient quantity of double distilled or deionized water is used to dissolve the pellet. The next step is to perform density gradient centrifugation. For this, 40% Percoll (3.5 mL) on the top layer and 80% Percoll (2.5 mL) on the bottom layer were created in a 10-mL centrifuge tube. The gradient should also contain cell protectants such as 0.01% bovine serum albumen, 0.01% ficoll, 0.05% polyethyleneglycol 6000, and 0.086% sucrose. All these components should be suspended in 10 mM MgSO_4 . Next, 1 mL of the sample was placed gently on the top of the 40% gradient layer. The tubes were centrifuged at 12,000 rpm at 4 °C for 10 min. The bacterial cells were collected at the interface between 40% and 80% Percoll solutions. Collected cells were washed with 1 mL of 10 mM MgSO_4 three times (4000 rpm, 5 min). The pelleted bacteria were used for DNA extraction.

5.2.3.2 DNA Extraction and Sample Preparation for NGS

It is advisable to remove the host (Insect) DNA from the samples. This can be accomplished through the use of any of the standard DNA digesting enzymes (DNases). Upon addition of DNase, the sample is allowed to rest at 37 °C for 30 min (incubation time varies with the enzyme used). After DNA digestion, 1 volume of 0.5 M EDTA as a DNA protectant needs to be added prior to heat deactivation (72 °C for 2 min) of the DNase enzyme. Bacterial DNA was extracted from the washed cells by using any of the bacterial genomic DNA isolation kits. As a quality assurance procedure, DNA extraction is followed by DNA quantification. Ideally, 3–5 μg of genomic DNA per sample will be sufficient for the sequencing library preparation. As a first step of NGS, the DNA strands need to be cut into ~300 bp fragments either by using mechanical shearing or by the aid of enzymes. Readymade Kits are used to generate libraries of DNA fragments with ligated adapters on both ends. NGS was performed using any of the sequencings by synthesis supporting instruments such as illumine platform (ex: Hiseq 2500, Illumina, San Diego, CA).

5.2.3.3 Bioinformatic Analysis of Raw Data

In brief, Metagenomics categorizes bacteria from a metagenomic sample; this is achieved through amplifying specific regions in 16S ribosomal RNA. The sequence in the form of reads matched with database of 16S rRNA data. The prokaryotic 16S rRNA gene is

~1500 bp long and contains nine variable regions interspersed between conserved regions. V3 and V4 are the regions of interest. The variable regions are frequently used in phylogenetic classifications such as genus or species in diverse microbial populations. This workflow is exclusive to Prokaryotes, which includes Bacteria and Archaea. The Metagenomics workflow generates a classification of reads at several taxonomic levels: kingdom, phylum, class, order, family, and genus or species.

Sequences with an average read length of 150 bp were introduced into SeqPrep (<https://github.com/jstjohn/SeqPrep>) to remove the adapter at 3' ends of raw reads. Raw sequences containing three or more unknown nucleotides ("N") were trimmed by Sickel (<https://github.com/najoshi/sickle>) command "sickle pe" to obtain clean reads longer than 20 bp, and to ensure that filtered reads possessed a quality threshold greater than 20. Clean reads were then assembled by SOAPdenovo at 39–47 *k*-mers. Scaffolds <500 bp were excluded. To construct a non-redundant gene set, CD-HIT was employed to cluster assembled reads at 90% coverage and 95% identity, using the longest read as the representative sequence. All high-quality reads were aligned (95% identity) against non-redundant database using SOAPaligner (<http://soap.genomics.org.cn/>) to obtain the gene abundance in each sample.

5.2.3.4 Metagenome Annotation

After extraction of the non-redundant gene set, annotation needs to be performed against Nr database by BlastP (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>) at an e-value cut-off of 10^{-10} , and the dataset was also processed by the web-based metagenomics RAST server (<https://www.mg-rast.org/>) and other bioinformatic tools are used as and when required. KEGG (<https://www.genome.jp/kegg/pathway.html>) and COG (<https://www.ncbi.nlm.nih.gov/research/co>) were employed too for the alignment of functional genes. To get information about carbohydrate-active enzymes and antibiotic-resistant genes, sequences were compared in the CAZy (<http://www.cazy.org/>) and CARD (<https://card.mcmaster.ca/>) databases, respectively. Proteins transported through the secretory pathway, which contain signal peptides, were identified through the SignalP program (<https://services.healthtech.dtu.dk/service.php?SignalP>). The ratio of annotated genes will be determined by read number of the hits to the non-redundant database. From the annotation results, contamination sequences of the host insect need to be removed. The next step is the generation of rarefaction curve; it can perform through the R package vegan (<https://cran.r-project.org/web/packages/vegan/index.html>) based on Nr annotated output file [3–4].

5.2.3.5 16S rRNA Sequencing and Taxonomic Analysis

For amplicon sequencing library preparation, the recommended reaction volume is 50 μ L containing 10 μ L of $5\times$ buffer, 2.5 U Polymerase, 250 μ M dNTPs, 200 nM of primers, 1 μ L of DNA sample and DNA-free water. The reaction needs to be performed in two sets. Following this, the sequencing adapters were linked to the PCR products. The resulting fragments will be pooled together equally and quantified by any of the DNA quantification systems; we recommend the Quantifluor dsDNA system (Promega, Madison, USA). Paired

End (PE) sequencing needs to be performed using any of the supporting instruments, as an example here we have discussed the Illumina MiSeq platform (Illumina, San Diego, CA). The raw PE reads generated through MiSeq were merged by FLASH software (<https://www.geneious.com/plugins/flash>) and trimmed with Trimmomatic (<http://www.usadellab.org/cms/index.php?page=trimmomatic>); the filter applied for trimming the read length will be $Q > 20$, N bases $< 1\%$. The next step is to remove all chimeric sequences and this is achieved through UCHIME (http://drive5.com/usearch/manual8.1/uchime_algo.html). For the taxonomic classification, RDP Classifier (<http://rdp.cme.msu.edu/>) will be an ideal choice with a confidence cut-off of 0.8. Based on the requirement, a phylogenetic tree can be drawn to exhibit the gut microbiota composition of the host insect. The longest read of each bacteria genus was picked out to generate the tree and the same will be visualized using iTOL (<https://itol.embl.de/>). Unclassified operational taxonomic units (OTUs) will be further identified with BLASTN (Fig. 5.1).

5.3 Applications of Insect Gut Microbiome

According to an estimation, less than 1% of the microorganisms are cultivable inside the laboratory; to explore the remaining large number of microorganisms, we need to depend on metagenomic analysis [1]. In the recent decade, with help of advanced technologies, metagenomics has emerged as a tool to discover novel genes, enzymes, natural products, bioactive compounds, and bioprocesses harbored inside the uncultured organisms of the insect gut [2]. On prima facie, it was believed that an enormous amount of natural products were synthesized by uncultivable microbes residing in the comfort zone of insect midgut. These natural products are with tremendous industrial and biomedical applications; some are discussed below.

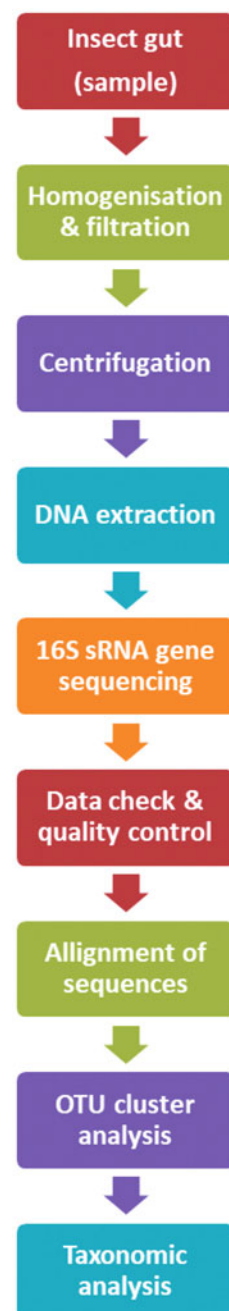
5.3.1 Various Industrially Important Enzymes

Cellulases, lipases, xylanases, amylases, proteases, and various other industrially important enzymes and their sources have been identified through metagenomics. The termites' gut was found to be the major source of wood-degrading microorganisms with the ability to generate biofuels from wood. They do so by harboring bacterial communities containing genes responsible for such conversion. Genes like RfBGluc-1 beta-glucosidase, xylanases, glycosyl hydrolase, and others are expressed as and when required.

5.3.2 Signaling Molecules

Metagenomic analysis identified signal molecules that will aid in better quorum sensing with chemically dissimilar sensing inducers. Some signal molecules are derived from

Fig. 5.1 Workflow for 16S rRNA sequencing and taxonomic analysis



bacteria such as short-chain fatty acids (SCFAs); speciality of such molecules is they can work as an energy source and also interact with G-protein-coupled receptors (GPR41/GPR43) to regulate T cell function.

5.3.3 Health Nutrients

Health nutrients or micronutrients such as different types of vitamins are synthesized by the gut microbes. Such microbes can be used for large-scale production. Most of the insects produce vitamin B complex through the help of microbial gut habitants (e.g., bedbugs, tsetse flies). Interestingly, certain insects like whiteflies have acquired bacterial genes (horizontal gene transfer) that participate in the de novo synthesis of specific B vitamins. Due to the discovery made by the metagenomics approach, the insect emerged as biomedical models of B vitamin nutrition.

5.3.4 Antibiotic Resistance Gene

The metagenomic approach has assisted in the identification of community-based antimicrobial resistance without the need for lengthy isolation and culturing of microorganisms. Antibiotic resistance genes such as novel β -lactamases or other genes were discovered through a metagenomic approach; such discovery will aid in drug development and screening. The metagenomic analysis of the gypsy moth midgut revealed an AraC family transcriptional regulator that confers tolerance to numerous antibiotics. The midgut also possesses genes, such as *sdeXY*, that encode efflux pump machinery of the RND family, which is responsible for broad antibiotic resistance.

5.3.5 Nitrogen Fixation

Nitrogen fixation through gut bacteria was observed in some insect species (ex. southern pine beetle). The novel species of bacteria thus identified through metagenomic analysis will help in developing novel bio-fertilizers. Species of cerambycid long-horned beetles harbor potential diazotrophs (e.g., *Burkholderia*, *Klebsiella*, *Enterobacter*, and *Rahnella*) for nitrogen fixation.

Take Home Message

- Insect and microbial relationship (gut metagenomics)
- Different methods such as DGGE, FISH, and NGS and their use in bacterial identification
- Students can apply logic and think about the most suitable method.
- Why study insect gut metagenomics from an application point of view

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Molecular Signatures of Host–Pathogen Interactions in Virus-Infected Lepidopterans

6

Nallabothula Triveni and Shyam Kumar Vootla

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Keywords

Insects · Antiviral · Innate immunity · Lepidoptera · Signaling pathway

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D. Kumar, S. Shukla (eds.), *Introduction to Insect Biotechnology*, Learning Materials
in Biosciences,

https://doi.org/10.1007/978-3-031-26776-5_6

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What You Will Learn from This Chapter

The role of insects as a model in innate immunity research is unquestionable. Along with *Drosophila*, Lepidopteran larvae are among the most common invertebrate models for studying antiviral immunity. Amongst insects, the order Lepidoptera includes silkworm, *Bombyx mori* a species of relevant economic value. Like any other living organism, insects are no exception to viral infections. Viruses, being obligate intracellular parasites, are intimately associated with host cells in turn favoring the diversification of host defense and virus escape mechanisms. Therefore, it is important to understand the virus–host interaction and to validate in depth diversity of antiviral strategies adopted. The RNA interference (RNAi), NF- κ B-mediated, Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway and immune deficiency (Imd) are the major antiviral defense mechanisms shown to play important roles in the antiviral immunity. Insect and virus interactions modulate several host signaling pathways such as prophenol oxidase (PPO), phosphatidylinositol 3-kinase (PI3K)/protein kinase B(Akt), and the extracellular signal-regulated kinase (ERK) pathways by viruses to elevate viral proliferation. Thus, it is noteworthy that targeting these hijacked host signaling pathways can inhibit viral proliferation. However, other innate antimicrobial pathways such as apoptosis and autophagy pathway have also been reported to play important roles in antiviral immunity. Antimicrobial peptides with antiviral activity (antiviral peptides: AVPs) show immense potential to become pharmaceutically available antiviral drugs and have become a research hotspot.

Insect–virus interactions provide interesting insight on a vast repertoire of antiviral immune mechanisms. Tapping into this diversity is challenging because of several constraints imposed by the uniqueness of each insect model. This chapter focuses on the broad overview of pathways involved in Lepidopteran insects' antiviral immunity. We discuss the challenges associated with studies on insect–virus interactions and showcase how the identification of evasion mechanisms encoded by viruses can validate antiviral pathways. We also summarize the currently validated insect AMPs with antiviral activity (AVPs), reported in the database and literature which help us to predict the antiviral activity of insect AMPs through AVP prediction software providing clues to unravel the mechanisms of insect antiviral immunity and to develop insect AVP-derived antiviral drugs.

In this review, we summarize the current knowledge of the cellular antiviral mechanisms studied in Lepidopterans, including some recent cues obtained during our research with BmNPV virus. Studies so far demonstrate a high degree of diversity in both the cellular responses against viral infections and viral responses against intracellular antiviral immunity, providing an important basis for the development of novel transmission interventions.

6.1 Introduction

With the advent of recent pandemic viruses like COVID-19 and its impact on socioeconomic aspects of society, viruses have become a matter of concern. Hence, in this direction, studying insect viruses and their mechanism of infection can definitely provide some clues on how we can tackle human viruses, as we have recently realized that it is all about their genetic makeup which decides them to be virulent, or transforming or varying strains.

Insects, one of the most evolutionarily successful organisms, occupy almost all habitats in nature. An efficient immune system is one of the attributes of their evolutionary success. Insects are constantly exposed to potentially pathogenic microorganisms and eukaryotic parasites, but only a few encounters result in infection. Insects are well known to possess a complex and efficient well-defined biological defense against pathogens and parasites, which includes: (a) the integument and gut as physical barriers to infection, (b) coordinated responses of several sub-populations of hemocytes when these barriers are breached, (c) the induced synthesis of antimicrobial peptides (AMPs) and proteins, and (d) immune signaling pathways.

A model for the sequence of events that may take place in an insect immune response is consolidated as shown in Fig. 6.1. The mechanism of viral intrusion and host resistance against viruses is very tricky and crucial. The current knowledge on insect immunity is naïve, especially in cellular and molecular details, in comparison with the overwhelming detail in which mammalian immunity is understood, but some comparisons such as insect immunity more closely resembles the innate immune system of vertebrates can be drawn.

Among the insects, only the dipterans (*Drosophila* and mosquito sps) have been widely explored for their immune response toward diverse pathogens [1, 2]. Insects have their potential use as therapeutic agents in clinical applications with the discovery of several antiviral compounds. So, a clear understanding of host–pathogen interaction and anti-defensive molecules released by pathogen to suppress host immunity is necessary before stepping into other aspects like disease control.

Lepidopterans (butterflies and moths) are one of the most ecologically diverse insect orders. Besides tapping into biodiversity, there are other reasons to particularly study antiviral immune responses in insects. For example, many important human diseases are insect-borne viruses causing dengue and zika fevers. In addition, severe economic losses are incurred when viruses infect beneficial insects such as bees or silkworms. Also, viruses can be used as potential biological control agents against pest insects. With new Lepidoptera genomes being sequenced, many of them are playing principal roles in genomics studies, especially in the fields of phylogenomics and functional genomics. The first sequenced Lepidoptera genome was derived from *Bombyx mori*, a model silkworm species economically important for silk production [3, 4].

During the natural process of the host–pathogen arms race, multiple mechanisms of attack and counterattack (host defences) have evolved. Animals defend themselves against pathogens in two major ways: innate and/or acquired immune systems. The key difference

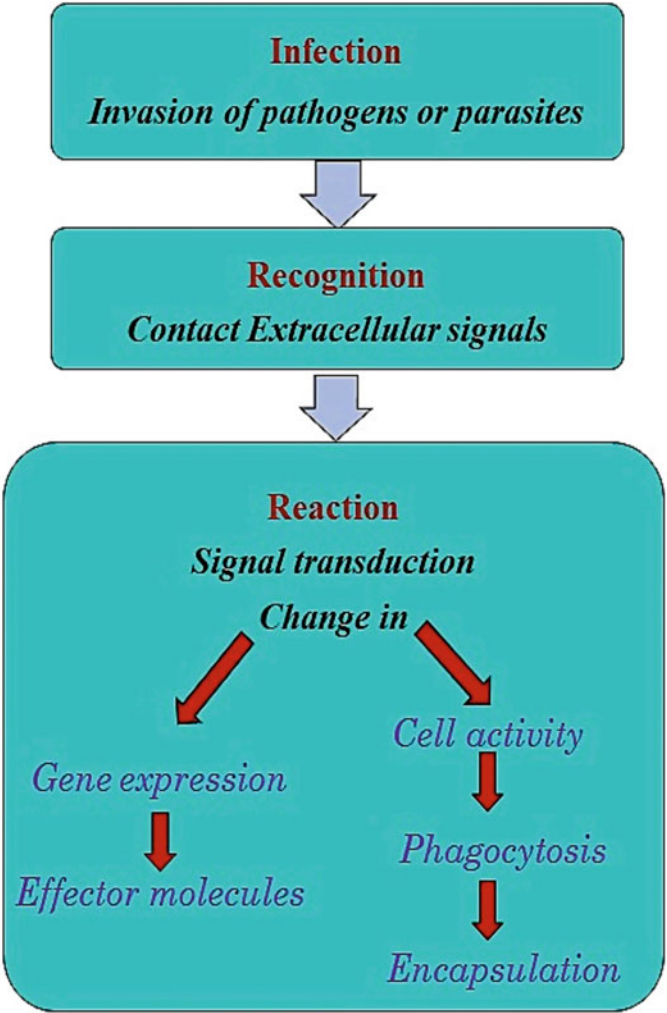


Fig. 6.1 Sequence of events after infection with a pathogen

between the two systems is the germline encoded factors involvement for recognizing and destroying pathogens in innate immunity, whereas in acquired immunity antigens recognizing effector molecules are produced leading to the development of immunological memory. The immune system in the insect world is based on innate immune responses that can be further categorized into (1) pathogen immobilization and (2) immune signaling pathways.

Pathogen immobilization is a cellular response toward microbial infections in the form of phagocytosis, melanization, coagulation, and autophagy [5, 6]. These responses aid in immobilizing pathogens, which are later destroyed extracellularly by antimicrobial

peptides (AMPs) or eliminated intracellularly. AMPs are produced and secreted in the hemolymph that directly attack microbes in response to systemic infections [7]. Upon pathogen invasion, activation of immune signaling pathways usually ensues. In insects, these are mainly the Toll, Imd, PI3K/Akt, NF- κ B, and JAK/STAT pathways.

In this review, we summarize the current knowledge of the cellular antiviral mechanisms studied in Lepidopterans. Studies so far illustrate a high degree of diversity in both the cellular responses against viral infections and viral responses against intracellular antiviral immunity, providing an important basis for the development of novel transmission interventions.

6.2 Insect Antiviral Immune Pathways

Viral infections can be basically of two types, namely acute and persistent. Acute infections are characterized by high levels of viral replication resulting in more viral particle production. Generally, these infections are limited in time either due to the death of the host or by the elimination of the virus by the host's immune system. Whereas, persistent infections usually result in lower viral replication levels, as well as lower viral load. These infections usually can last for long periods of time due to an established equilibrium between the attack and counterattack strategies of the virus–host system [8–10]. As persistent viral infections do not always cause obvious pathogenesis, their existence is often neglected. However, due to the development of genomic and transcriptomic tools, the identification of persistent viruses both *in vivo* and in cultured cells has become more recurring, with several reported cases [11–16]. Therefore, the mechanisms underlying persistent viral infections in other insects remain to be elucidated. Lepidoptera, an economically important order, is a clear example, with several persistent viral infections well reported to date [17–20].

The RNA interference (RNAi), NF- κ B-mediated pathways, and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway are the major antiviral defense mechanisms of silkworms; among these, RNAi is the major defense strategy.

6.3 RNAi Antiviral Response

RNA interference (RNAi) is a post-transcriptional gene-silencing mechanism mediated by small RNA molecules. In insects, it plays a significant role in limiting and controlling virus infection [21–24]. According to their structure and biogenesis, presently we know three well-characterized RNAi-related pathways: (1) siRNA pathway in which siRNAs are crucial antiviral effectors generated from dsRNA derived from either virus infection or encoded by the cell genome, (2) micro-RNA (miRNA) pathway in which miRNAs are effectors of endogenous gene regulation generated from cell-encoded transcripts and ultimately function to regulate gene expression generally at the level of translation, and

(3) PIWI-interacting RNA (piRNA) pathway in which piRNAs are essential defenders of genomic integrity by controlling retrotransposon activity transcribed from the cellular genome.

The pathway predominantly responsible for antiviral activity in insects is the siRNA pathway [25]. Three major lines of evidence demonstrate the significance of this pathway for insect antiviral immunity. First, impairment of siRNA leads to a compromised antiviral immune response which was shown through loss-of-function studies, both at intracellular and systemic levels [26, 27]. Second, deep sequencing studies have identified and characterized numerous virus-derived siRNAs in insects [28]. Third, viruses have evolved mechanisms through the expression of Viral Suppressors of RNAi (VSRs) to counteract the effectiveness of this pathway [29]. Long dsRNA molecules, produced mainly during the replicative cycle of viruses, are recognized and diced into siRNAs (18–24 bp) by the RNase type III enzyme Dicer2 (Dcr2) and are then incorporated into an RNA-Induced Silencing Complex (RISC). Further, they are unwound, making the guide strand available to direct target RNA recognition by complementary base pairing. At this point, to combat the viral infection, the catalytic component of RISC, Argonaute2 (Ago2), mediates the degradation of complementary viral RNA. As a result, dsRNA acts like an important pathogen-associated molecular pattern (PAMP) for viral infection, triggering a robust degradation of viral RNA via siRNAi [30].

The role of miRNAs in insect development is well known, whereas less is understood about their function in host–pathogen interactions. But, accumulating evidence in recent years has suggested their effects on host–microbe interactions. For example, several cellular as well as viral miRNAs have been found to be involved in aiding or restricting virus replication. What is commonly observed in various host–pathogen systems is the differential abundance of host miRNAs following an infection, which varies with the type of pathogen involved and at different stages of infection.

Most studies in Lepidoptera interaction with the miRNA pathway involve infection with a DNA virus, such as baculovirus. A global alteration of the microRNA profile in lepidopteran cells, such as *Spodoptera frugiperda* Sf9 cells upon infection with AcMNPV [31] as well as *Helicoverpa armigera* larvae following HaSNPV infection has also been reported [32]. Recent studies of microRNAome alterations in lepidopteran insects undergoing an infection other than baculoviral are also clearly documented [33]. One example is *Bombyx mori* larvae infection by *Bombyx mori* cytoplasmic polyhedrosis virus (BmCPV), a reovirus with segmented dsRNA genome, which causes differential expression of 35 miRNAs in libraries derived from RNA collected 3–4 days post-infection. In addition, *Helicoverpa* (*Heliothis*) *zea* nudivirus-1 (HzNV-1) large DNA genome was reported to contain the *pag1* gene which expresses a non-coding RNA and is further processed in at least two miRNAs targeting *hhi1* early gene of the virus and thus plays a crucial role for the establishment of latent infection in *S. frugiperda* Sf21 cells [34].

Elevating the expression of certain RNAi components in lepidopteran cells results in the improvement of the defence against acute viral infection. More particularly, overexpression of the key siRNAi factors Dcr2 and Ago2 in High Five cells results in a drastic reduction of

CrPV-induced cell mortality. Importantly, impressive *in vivo* improvements of antiviral capacity against the BmNPV have already been demonstrated in transgenic *Bombyx mori* strains expressing virus specific dsRNA. This focuses on the advantage of using transgenic moths with higher antiviral capacity in sericulture industry, where lepidopteran viral diseases cause significant economic losses [35–38]. Overexpression of siRNAi components is a potential alternative and/or complementary approach to obtain an enhanced antiviral response in Lepidoptera. Although the proposed strategy remains to be tested *in vivo*, it is important to note that overexpression of Ago2 in *Bombyx mori* larvae induces an enhanced RNAi response [39, 40].

6.4 Nf- κ B Innate Immune Pathways

The Imd and Toll pathways are canonical NF- κ B-dependent pathways well known to be a part of insect innate immunity, activating the downstream antimicrobial peptide (AMP) genes transcription mediated by two distinct orthologs of the NF- κ B transcription factor [41, 42]. Relish, the NF- κ B ortholog is the terminal transcription factor for the Imd pathway, wherein the Dorsal and Dorsal-related immune factor (Dif) function in the Toll pathway [43]. There have been few reports on the Toll pathway involved in antiviral immunity in silkworms. A recent report showed that BmPGRP-S2 was induced by BmCPV in the silkworm midgut [44]. Further experiments revealed that BmPGRP-S2 was a secreted protein, which may recognize a particular viral component and further transmit the signal to downstream molecules, and its upregulation increased the expression of BmImd, BmRelish, and AMPs and decreased silkworm mortality after BmCPV infection [41]. These results clearly show that the Imd pathway is involved in the defense mechanism against the RNA virus in silkworms. However, the function of this pathway in DNA virus-infected silkworms is not yet known. Also, the stimulator of interferon genes (STING) has been reported to provide antiviral immunity against BmNPV in silkworms by promoting NF- κ B activation [45]. Production of cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) is triggered upon BmNPV infection, inducing the BmSTING activation to process BmRelish, and then the activated BmRelish is translocated to the nucleus to initiate the transcription of AMP [45]. These results revealed that the NF- κ B-mediated, Imd, and STING pathways play important roles in silkworm antiviral defense, but the antiviral mechanisms of the two pathways are only partially elucidated and need more experimentation. Deciphering the roles of the Toll pathway in silkworm antiviral immunity remains a challenging task.

6.5 JAK/STAT Pathway

Apart from the RNAi pathway, several other signaling pathways also contribute to the antiviral response in insects. Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling is another significant pathway involved in multiple cellular

processes [46, 47] JAK/STAT pathway was initially characterized for its role in the development and hemocyte proliferation, and later it has been shown to also respond to bacterial and viral infections by regulating the downstream effector molecules production including antimicrobial peptides (AMPs). This pathway is activated in a paracrine fashion as a consequence of initial recognition of infection by pattern recognition receptors (PRRs) that also feed into other pathways.

This pathway comprises a diverse family of extracellular ligands such as cytokine and growth factors, transmembrane receptors, JAK tyrosine kinases that are associated with the intracellular part of the receptor, and STAT proteins [47, 48]. Following stimulation, the extracellular part of the JAK-associated receptors is bound by ligands, leading to the activation of JAKs. Subsequently, cytosolic STATs are recruited to the JAK/receptor complex, where they are phosphorylated, forming the STAT dimers, which in turn are translocated into the nucleus and bound to the DNA promoters of the target genes to regulate their expression [47, 49]. The BmNPV and BmBDV, unlike the BmCPV which is an RNA virus, induce the expression of BmSTAT in silkworms, indicating that the JAK/STAT pathway could be activated by the DNA viruses in silkworms [50]. Upregulation of BmSTAT gene expression in BmN cells increased the number of cells in the G2 phase of the cell cycle [49] enhancing host resistance to BmNPV, but not to BmCPV [51]. Additionally, inhibition of Hsp90 can cause upregulation of Bmstat expression and suppression of BmNPV replication in the BmN cell [52], but it is not clear how Hsp90 can be linked to JAK/STAT. In silkworms, the extracellular ligand and effector molecules of this pathway in response to viral infection have not been clearly identified and need further investigation.

The JAK/STAT signaling pathway is an evolutionarily conserved innate immune pathway in the insect immune response mechanism [53, 54]. Autographa californica nucleopolyhedrovirus (AcMNPV) infection in *Spodoptera frugiperda* Sf9 cells activates the key gene STAT in the JAK/STAT signaling pathway to mediate the immune response against AcMNPV [55]. BmNPV infection in silkworms led to upregulated transcription of Bmstat, indicating the activation of the JAK/STAT pathway. The mechanism by which the JAK/STAT pathway exerts its antiviral effect is not clearly known, but it is apparent that the response is specific, complex, and versatile.

6.6 Virus-Modulated Host Signaling Pathways

Several host signaling pathways including the prophenol oxidase (PPO), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), and the extracellular signal-regulated kinase (ERK) pathways have been reported to be modulated by viruses to elevate viral proliferation during the interaction between the insects and viruses. It is noteworthy that targeting these hijacked host pathways can inhibit viral proliferation in silkworms.

6.6.1 PPO Pathway

Insect prophenoloxidase (PPO) due to its involvement in cellular and humoral defense is an important innate immunity protein. PPO occurs extensively in almost all organisms, including vertebrates, invertebrates, plants, and microbes [56]. They are named differently; e.g., prophenoloxidase (PPO) in insects and crabs, tyrosinase in mammals and microbes, polyphenol oxidase (also termed as PPO) in plants, and hemocyanin in arthropods [57, 58]. The insect PPO activation pathway comprises several important proteins, including pattern-recognition receptors (PGRP, β GRP, and C-type lectins), serine proteases, and serine protease inhibitors (serpins) [57–59]. PPO activation mechanisms differ among insect species due to their complexity. Activated phenoloxidase (PO) produces melanin around invading pathogens and wounds by oxidizing phenolic molecules. Melanization is a significant immune response in insect plasma mediated by the PPO pathway which plays an essential role in the killing of microbes and wound healing. This process is initiated by the recognition of invading microbes, which activates the extracellular clip-domain serine protease (cSP) cascade to convert the zymogen PPO to active phenoloxidase (PO). PO catalyzes the oxidation of phenols to form quinones and melanin, wherein the rapid polymerization of melanin at infection sites can kill and immobilize microbes [60, 61]. Melanization can kill baculovirus *in vitro* [62, 63]. However, the PPO pathway is negatively regulated by serpins, and baculovirus can induce serpins to suppress the melanization response of host insects for survival [64].

6.6.2 PI3K/Akt Pathway

The PI3K/Akt pathway plays an important role in regulating a number of cellular processes [65–67]. Activation of PI3K can occur through the binding of a variety of ligands, including several growth factors to the receptor tyrosine kinases (RTKs). Activated PI3K then converts the substrate phosphatidylinositol 4, 5-bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-trisphosphate (PIP3), and PIP3 causes the phosphorylation of Akt (p-Akt) [67–69]. The PI3K-Akt pathway plays a significant role in cell survival, apoptosis, proliferation, migration, differentiation, and metabolic regulation, which in one or the other way decide the fate of the virus entry into the host cell and hence ultimately deciding the course of viral infection. Active PI3K-Akt signaling can fulfill many viral strategies for infection. This does not suggest that this signaling pathway only provides positive feedback for the virus. The PI3K-Akt signaling represents a two-faced player in interactions between the virus and host, able to promote both viral replication and also be an active part of the immune response that will eventually quash virus replication [70].

Xiao et al.'s study confirmed the activation of the phosphatidyl inositol 3-kinase (PI3K-Akt) pathway in sf9 cells after AcMNPV infection with increased phosphorylation of Akt. Akt is the effector of PI3K and the activation of PI3K leads to Akt activation. However, JAK/STAT and PI3K-Akt signaling pathways have been recently reported in silkworm,

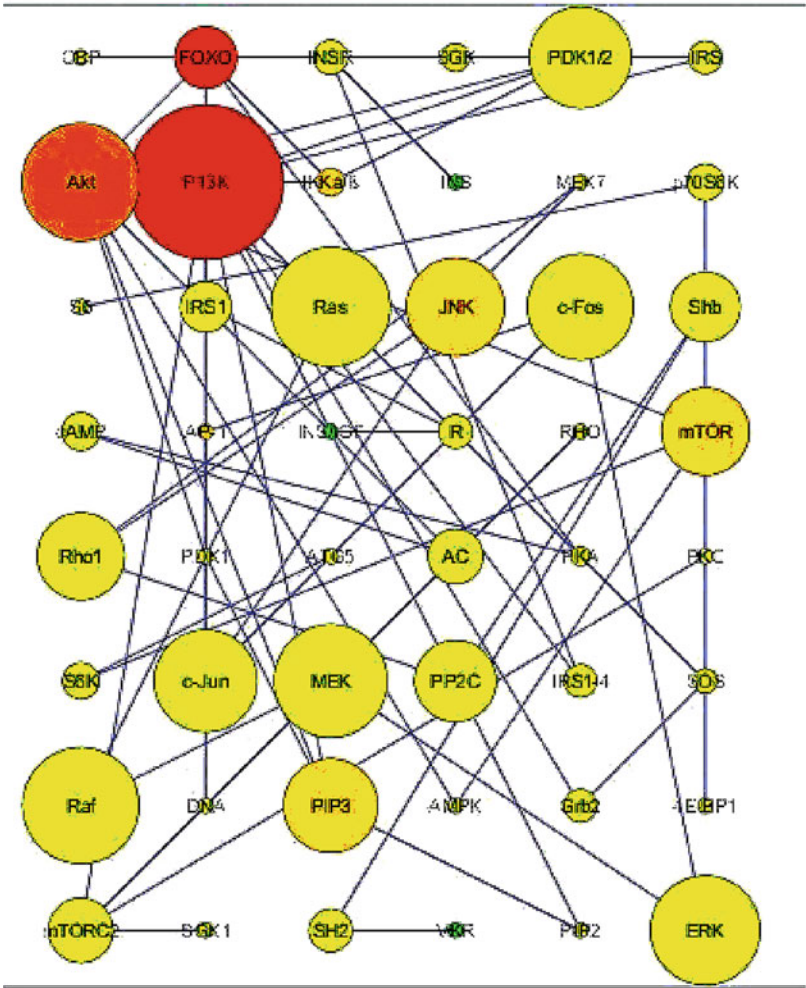


Fig. 6.2 Cystoscape analysis of FOXO signaling pathway, longevity regulating pathway, and mTOR signaling pathway (Nallabothula et al. [72])

Bombyx mori [71]. Silkworms’ resistance against BmNPV is associated with not only resistance genes but also immune signaling pathways.

The role of the PI3K-Akt pathway in NPV-infected *Antheraea mylitta* was recently explored. Bioinformatics-based preliminary studies on NPV-resistant *Bombyx mori* strain and *Antheraea mylitta* shows that PI3K-Akt pathway is the hub of all signals generated during the NPV infection (Fig. 6.2) which prompted us to analyze this pathway to test whether NPV infection modulates PI3K-Akt signaling [72]. In this study, it was demonstrated that infection of *Antheraea mylitta* with NPV elevates cellular Akt phosphorylation post-infection serving as the backbone of the antiviral response (Fig. 6.3).

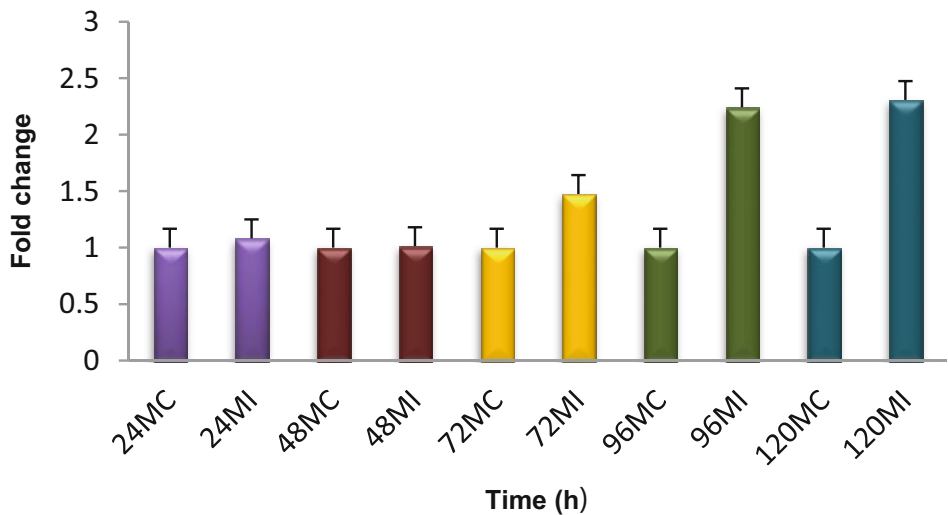


Fig. 6.3 Relative copies of Akt gene in *Antheraea mylitta* midgut samples at different time intervals post infection (6 h, 24 h, 48 h, 72 h, 96 h, and 120 h). MC: Midgut control; MI: Midgut infected (Nallabothula et al. [72])

6.6.3 ERK Pathway

A variety of extracellular stimuli such as growth factors, environmental stresses, and microbial infections can activate ERKs which are serine/threonine kinases, which can in turn transduce downstream cellular responses, including cell differentiation, survival, and apoptosis [73–76]. Activation of the ERK pathway is essential for efficient infection by many viruses. One major class of ERK regulators is the RTK family. The extracellular ligands activate RTKs upon stimulation, to promote the phosphorylation of ERK(p-ERK). The ERKs then control transcription by phosphorylating various transcription factors in the nucleus or control targets in the cytoplasm [77, 78]. The epidermal growth factor receptor (EGFR) belongs to the RTK family [69, 74]. The BmEGFR plays a significant role in BmNPV infection, which participates in the activation of ERK and PI3K/Akt pathways by the virus. Moreover, activated ERK controls the transcription of late viral genes and inhibits apoptosis [79]. Additionally, Spry is a negative regulator of the EGFR/ERK pathway and the overexpression of BmSpry suppressed p-ERK and BmNPV replication in BmE cells [77]. Further research has found that BmSpry was decreased and p-ERK was increased in silkworms after infection with BmNPV, BmCPV, or BmBDV, and the knockdown of BmSpry in transgenic silkworms caused increased p-ERK, viral content, and mortality after infection with the three viruses, revealing that both DNA and RNA viruses take over the ERK pathway to ensure viral reproduction [80]. The ERK pathway plays important roles in regulating the outcome of viral infection in silkworms, and the mechanisms remain to be fully elucidated (Fig. 6.4).

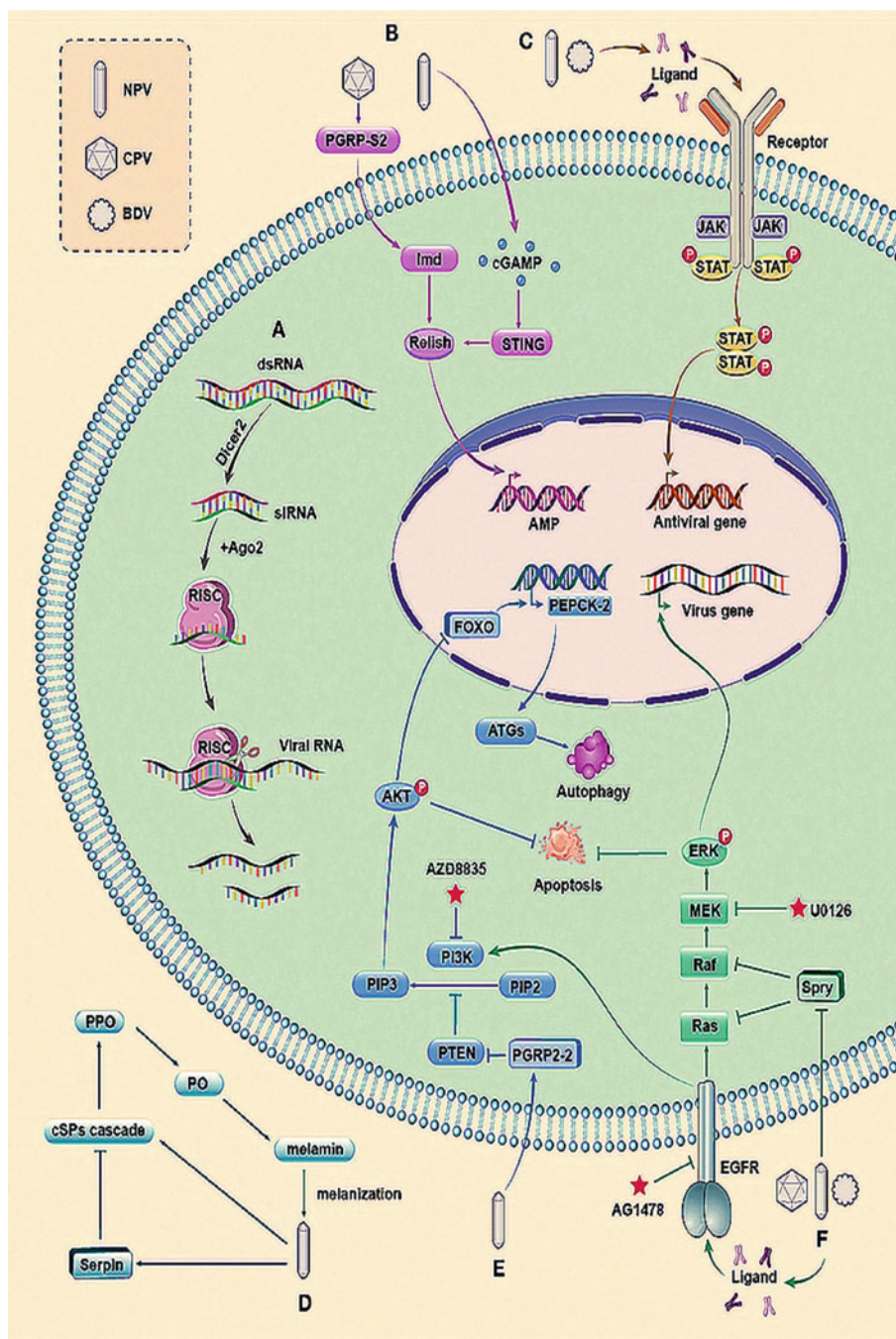


Fig. 6.4 Antiviral signaling pathways in silkworm (Jiang [81])

6.7 Autophagy

Viruses exploit multifarious mechanisms to modulate host cells to aid their replication and proliferation. Among them, autophagy, a highly conserved and regulated self-degradation process, is initiated as an adaptive response in unfavorable conditions, such as nutrient deprivation and innate immunity [82, 83]. Autophagy usually serves as a defense mechanism against viral infection (Fig. 6.4) [84, 85]; but many viruses, however, have evolved to escape, or even to exploit, this mechanism to favor their survival or replication in different ways [82, 86–88]. Thus, the role of autophagy in host–virus interactions is varied for different viruses. As per a few related reports in BmNPV infection, inhibition of autophagy by 3-methyladenine (3-MA) drastically results in a decrease of polyhedrin expression and in turn low polyhedra particle production, indicating the involvement of polyhedrin in cellular autophagy [89]. Therefore, implying that the virus may induce autophagy, which is eventually beneficial to its replication and proliferation, by elevating the expression of some autophagy-related genes. We have the following hypotheses as for how autophagy promotes BmNPV proliferation. First, immune-related proteins are specifically degraded by autophagy induced by a virus and attenuate the innate immune responses to weaken the host defense capabilities, as reported in HCV [90]; second, autophagy triggered by a virus promotes the degradation of a large number of useless proteins or organelles of the host, providing enough energy for virus proliferation; and, finally, a virus initiates autophagy and hijacks this membrane trafficking pathway to transport viral proteins to sites of viral assembly, such as rotavirus and Zika virus (ZIKV) [91, 92]. In recent years, autophagy has been proposed as an alternative antiviral mechanism in insects that is independent of the Imd, Toll, or JAK/STAT pathways [93, 94].

6.8 Antimicrobial Peptides (AMPs)

Antimicrobial peptides (AMPs) with antiviral activity are termed as antiviral peptides (AVPs) which show immense potential to become pharmaceutically available antiviral drugs. AVPs are a subset of AMPs which are considered to act as the first line of defense as an innate immune response to viral infection in many organisms. Many databases such as APD [95], AVPdb [96], and ParaPep [97], have been developed to centralize information about AMPs with increasing interest for natural AMPs as promising new drugs. Among the various AMP databases, a few databases integrate the AMPs with antiviral activity such as APD [95], AVPdb [96], DRAMP 2.0 [98], and dbAMP [99]. The information available in DRAMP 2.0 and dbAMP is updated periodically. The advantage of AVPdb is that it summarizes AVPs in accordance with the various antiviral mechanisms. Also, softwares for AVP prediction have been developed, e.g., AVPpred [100], AntiVPP 1.0 [101], and Meta-iAVP [102]. Based on the understanding of concepts relevant to insect AVP research, several user-friendly and recently released databases and websites that are suitable for insect AVP research are cataloged (Table 6.1).

Table 6.1 Databases and websites suitable for insect AVP research

| Name of the database | Websites | Function | References |
|----------------------|---|--|--------------------------|
| AVPdb | http://crdd.osdd.net/servers/avpdb/index.php | Antiviral mechanism of AVPs | Qureshi et al. [96] |
| DRAMP 2.0 | http://dramp.cpu-bioinfor.org/ | Search tool for insect AVPs | Kang et al. [98] |
| dbAMP | http://csb.cse.yzu.edu.tw/dbAMP/ | Search tool for AVPs and insect-derived AMPs | Jhong et al. [99] |
| Meta-iAVP | http://codes.bio/meta-iavp | Prediction of AVPs | Schaduangra et al. [102] |
| SignalP-5.0 | http://www.cbs.dtu.dk/services/SignalP/ | AMPs signal peptide Prediction | Almagro et al. [103] |

The domestic silkworm *Bombyx mori* is an important lepidopteran insect of high scientific and economic value. Unfortunately, viral diseases of silkworms can cause enormous economic loss in sericulture as there is currently no effective treatment [104]. Specific strains of silkworms that are resistant to some viruses do exist, but the specific underlying mechanism is still unclear [105–107]. RNAi was considered as the major defense strategy against viral infections in *Bombyx mori* like other insects [108]. In a recent study, the upregulation of the C-lysozyme of *Bombyx mori* resulted in lower *Bombyx mori* nucleopolyhedrovirus (BmNPV) production and progeny virus virulence in vivo and in vitro [109]. Further research is needed to elucidate the antiviral mechanism of lysozyme peptides. However, in silkworms, innate immune response to virus infections is not thoroughly studied although specific antiviral molecules such as PP2A [110], BmSTING [45], BmAtlastin-n [111], BmNOX [112], Bmlipase1 [113], were identified to date. Interestingly, a study reported that *Bombyx mori* peptidoglycan recognition protein S2 (BmPGRP-S2) overexpression could activate the Imd pathway and induce AMP upregulation, enhancing silkworm antiviral resistance [41]. The AVPs possess diverse structures as per different mechanisms of their action. Based on the AVPdb, a total of 45 virus-targeting strategies employed by AVPs can be distinguished such as Virus entry, Replication, Transcription, Translation, Viral assembly, Release, and Transport [96].

In future, research on insect AVPs needs to mainly focus on the following key issues: (1) Identification and characterization of insect AVPs; (2) Recognition by PRRs and downstream cascade reactions involved in insect AVPs production; (3) Molecular mechanism of action of AVPs against insect and vertebrate viruses; (4) AVPs immune escape mechanisms by viruses; and (5) Evaluation and application of insect AVPs as antiviral drugs in disease control strategy.

6.9 Apoptosis and Global Protein Synthesis Shutdown

Apoptosis and global protein synthesis shutdown (GPS) have been well documented as two of the major antiviral immune responses to control virus production in baculovirus-infected cells [114]. After entry into cells, individual baculoviruses interact diversely with different insect cell lines, bringing about various types of abortive (non-productive) as well as productive infections [115]. Abortive infections may result either from incompatibility between viral and cellular factors involved in virus replication or from activation of innate antiviral immune responses.

In a few baculovirus abortive infections, both cellular and viral protein synthesis are completely inhibited or adequately reduced after infection. Such baculovirus-induced GPS has been found in the *Lymantria dispar* cell line Ld652Y infected with *Autographa californica* multiple nucleopolyhedroviruses (AcMNPV) [116, 117]. *Bombyx mori* cell lines BmN and BmN-4 were infected with AcMNPV and *Hyphantria cunea* MNPV (HycuMNPV), respectively [118]. Ld652Y cells are non-permissive for AcMNPV, and AcMNPV-infected Ld652Y cells induce global protein synthesis shutdown approximately 12–16 h post-infection [119]. Recent observation in another Lepidopteran insect, *Antheraea mylitta* supported the phenomenon of GPS on exposure to NPV within 24 h [120].

Apoptosis is a cell death process controlled genetically in response to various apoptotic stimuli and plays an important role in the development, tissue homeostasis, and cellular defense of multicellular organisms, by eliminating unwanted, infected, or damaged cells. In virus-infected animals, apoptosis induced early in infection is utilized as an antiviral defense to inhibit viral replication and dissemination in infected animals. On the other hand, apoptosis when induced in late infection appears to act as a viral strategy to promote virus dissemination within infected animals [121]. Therefore, it seems likely that both GPS and apoptosis in baculovirus-infected cells are triggered early in infection by the same or different viral factors, and enhanced depending upon the onset of viral DNA replication [122, 123], thus suggesting that the two intracellular antiviral mechanisms operate in an overlapping manner during virus infection.

Further progress in this field requires the identification and functional characterization of cellular factors, which serve as modulators and mediators of apoptosis and global protein synthesis shutdown. Recent progress in genome-wide surveys of cellular and viral genes and their products possibly involved in baculovirus replication in lepidopteran insect cells and dynamic interactions among them may facilitate further studies on the intracellular innate antiviral immunity of lepidopteran insects [124–128].

6.10 Others Factors Involved in Antiviral Immunity

Other host factors are also being explored involved in limiting or restricting viral replication in Lepidopterans. Viral polymerases, essential enzymes for viral genome replication are also being looked into as an attractive target for antiviral drug development [129]. Also

the per os infectivity factors (PIFs), i.e., proteins required only during the primary infection of host also serve as important components to design novel antiviral drugs [130]. STING (stimulator of interferon genes), a critical signaling molecule required for the innate immune response to cytosolic nucleic acids, particularly dsDNAs derived from pathogens including viruses was recently demonstrated in *Bombyx mori*, a model organism of lepidopteran insects [131].

6.11 Conclusion

Over the past two decades, amazing progress has been made in understanding the innate immune system of insects, and the picture of the antiviral response in insects has become richer and more complex. Our current understanding of the insect immune system is still largely based on studies in *Drosophila melanogaster*. Moving from a view of signaling pathways as isolated, independent responses, it is now evident that these pathways are integrated and responsive to one another, providing a pathogen-specific response.

Considering the enormous diversity of Lepidopteran species, it will be intriguing to explore the variety of antiviral immune strategies that likely exist in these insects. These insects are equipped with various sophisticated pathways that can recognize and restrict virus infection. Lepidoptera can serve as a model for basic research on the RNAi machinery to its link with the antiviral immune response. Silkworm–virus interaction model clearly demonstrates that there are several layers of antiviral defense that depend on conserved as well as divergent pathways. Development of immune evasion mechanism is favored through modulation of the host signaling pathways by the pathogen during the co-evolution of host and viruses. When these hijacked signaling pathways are targeted using inhibitors and knocking out their key regulators via gene editing would be a promising strategy to improve silkworm resistance.

In baculovirus-infected lepidopteran cells, apoptosis and global protein synthesis shut-down are the major mechanisms of intracellular innate immunity that hinder viral replication which needs to be further explored for their modulation. Understanding the mechanisms driving the interaction between virus infection and host autophagy requires more in-depth studies. Future research needs to be directed at unraveling the complexity of antiviral immunity in Lepidopterans which promises to discover new molecular targets that can be exploited for the development of strategies to block virus transmission by insect vectors.

To conclude, one key message from this review is that the literature concerning the insect antiviral response is sparse, fragmentary, and sometimes inconsistent. The use of different insect and virus models, inoculation routes, and different experimental conditions make it difficult to compare results on the mechanisms involved in response to virus infection. Before any conclusions can be drawn, systematic and standardized approaches are needed on the antiviral activity of immune pathways in Lepidopteran insects.

Take Home Message

- Antiviral mechanisms are a worldwide problem and research hotspot. Insect–virus interactions may provide information on a vast repertoire of antiviral immune mechanisms.
- Studies on antiviral pathways would be very instructive as they would reveal original antiviral strategies for the protection of beneficial insects and the target pathways hijacked by viruses for pest control.
- Development of immune evasion mechanism is favored through modulation of the host signaling pathways by the pathogen during the co-evolution of host and viruses.
- The signaling pathways are not isolated and independent responses, but are integrated and responsive to one another, providing a pathogen-specific response.
- In baculovirus-infected lepidopteran cells, apoptosis and global protein synthesis shutdown also contribute to intracellular innate immunity that hinders viral replication.
- Insect AVPs as important components of insect antiviral innate immunity will inspire the development of effective antiviral drugs.

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Future Prospects of Insect Biotechnology

7

Deepika Divya Kadiri, P. Swarnalatha, and Sujatha Peela

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Keywords

Insecticides · Pesticides · Insect cells · Genetic modification · Agriculture · Pest management

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© Springer Nature Switzerland AG 2023

D. Kumar, S. Shukla (eds.), *Introduction to Insect Biotechnology*, Learning Materials in Biosciences,

https://doi.org/10.1007/978-3-031-26776-5_7

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What You Will Learn from This Chapter

This chapter emphasizes on future trends of insect biotechnology in protein vaccines production, future crop protection, pest management approaches, and recent biotechnological progress. The chapter also addresses the major aspects of insect biotechnology in various sectors and also its significance and associated risks in human life applications.

The finest developments in insect biotechnology were driven by the progress in present-day sub-atomic science, especially by the advancement of strategies, which permitted one to completely arrange the genomes of various insect species. The economic potential of insect biotechnology, for the exploration of biodiversity products and bioeconomy, lead to the massive growth of this field over the past 30 years.

In detailed explanation on the biological approaches to Pest Management using genetically engineered insects, self-limiting agricultural insect pests, and biorational insecticides are discussed in the chapter. Furthermore, a brief view on recent biotechnological advances in forest pest management and identification of specific pest control molecules are elaborated. In summary, the chapter attempts to show what the aspects of insect biotechnology are trying to achieve and how far these aims are currently realized. We hope that this chapter will be useful to researchers and scholars wishing to obtain an overall perspective for future research on prospects of Insect biotechnology.

7.1 Introduction and Scope

Insect biotechnology also known as yellow technology is based on the application of biotechnological techniques on whole insects, their organs, cells, or molecules, and also their associated microbes, for the development of their services and by-products [1]. These products are immensely used in the fields of agriculture (green biotechnology), medicine (red biotechnology), and industry (white biotechnology) [2, 3]. Insect biotechnology is known as a promising resource in various industries, particularly in the production of insecticides, microbial pesticides, industrial enzymes, and more. Among the various methods and expression hosts developed for the production of biotech products, Insect Cells (ICs) are designed to produce complex proteins which require extensive post-translational modification [4]. The field has evolved due to the potential importance of insects, and their derived products and services, at all stages of human undertaking. Insects are found to be the most diverse species of all the organic entities. The number of existing species is assessed at around 1.9×10^6 and conceivably addresses more than 90% of the other life forms on the earth [5]. They have a predominant impact on agriculture as well as public health. Insect biotechnology, in particular, has made scientific advances in

molecular biology, especially in the enhancement of certain biotechnological approaches which permit genetic alterations, and engineering of cells like plant protoplast fusion and recombinant DNA technology [6]. These innovative approaches are being used in medicine, plant care, and industrial biotechnology [7]. In the most recent times, bacteria are genetically engineered to express silk genes from *Bombyx mori* a mulberry silkworm and later used for the production of a natural protein fiber known as synthetic silk, some of which can be woven into the fabric [8]. Insect biotechnology techniques, as a basic research tool, include sequencing and interpretation of insect genes and analysis using comparative genetics. Pest control involves the development of advanced antimicrobial agents in plants and insect-resistant plants using pesticide biotechnology, as well as pesticide engineering that can transmit deadly genes to natural ones after their massive release. Comparative genomics analysis makes it much more likely to determine insect-specific genes that are dictated by proper pesticide development.

The development of modern technological tools and their applications in insect science has made insect biotechnology a prominent discipline in the management of insect pests and insect-borne diseases. Insect biotechnology has high economic potential, and it is considered to be a vital factor in the development of the bioeconomy and biodiversity. Scientific progress continues to prove that knowledge and skills are constantly evolving and innovative achievements are developing each day, by which people can utilize the natural resources consistently and fight food scarcity and hunger.

7.2 Protein Vaccines Produced in Insect Cells (BEVS-Baculovirus Insect Cell Expression System)

Baculovirus expression vector systems (BEVS) have been technologically advanced and have been a platform for the development of versatile and potent vaccines [9]. The baculovirus-insect cell strategies are being utilized for veterinary and human immunizations because of the capacity of developing complicated proteins. The critical utilization of this recombinant protein creation framework is a far-reaching “reprogrammable” strategy that can be utilized to deliver a wide scope of protein-based remedial and prophylactic immunizations for both human and veterinary usage, while additionally providing the chance of minimal creation costs [10]. The BEVS discussion has been depicted beforehand [11]. Figure 7.1 shows the baculovirus-insect cell articulation framework. The forum uses the natural inbuilt tendency of baculovirus to infect the insect cells. More than 500 of these species belong to a limited range of invertebrates [12]. As BEVS have the ability for high yield protein expression and are capable of holding post-translational alterations and protein oligomerization, they are considered more efficient than other expression systems like yeast and bacteria. For the generation of virus-like particle (VLP)-based vaccines, these cell expression systems are used more often. *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) is known to be the most well-known VLP component and is used in molecular pathogenesis studies of the

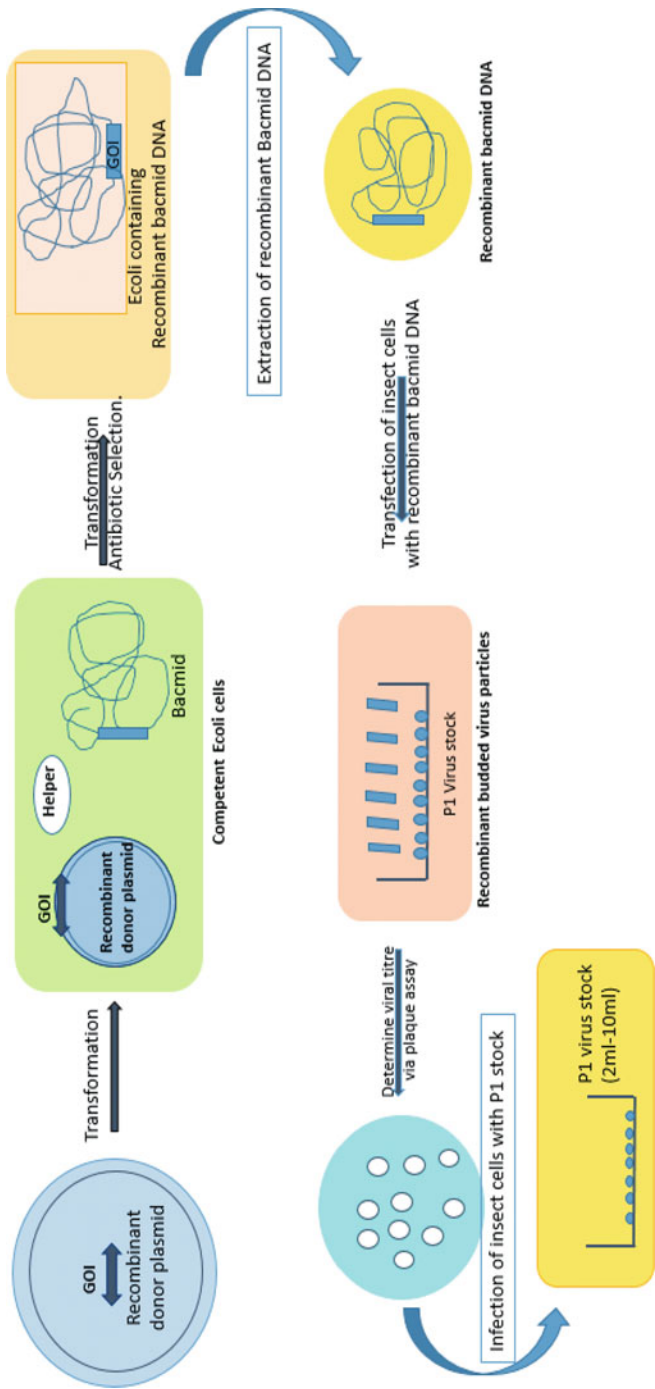


Fig. 7.1 Baculovirus-insect cell expression system

baculovirus gene [13–15]. Its genetic makeup gives Baculovirus enough energy to carry huge amounts of foreign DNA, together with many other genes, which makes it more advantageous over other vectors such as the adenovirus and vaccine [16].

To produce huge numbers and establish a working/functional viral bank (WVB) that may be exploited for protein production, the plaque is infected with insect cells numerous times. For production purposes, WVBs must stabilize and maintain integrity as the viral path develops. Laboratory kits such as Bac-to-Bac® protein expression system developed by Life Technologies use built-in bacmid technology, which allows researchers to rapidly and effortlessly create baculoviruses that interact with *E. coli* instead of homogeneous recombination in insect cells. These are useful tools for conducting small-scale research. However, recurrent genetic cassettes are not internally stable in baculoviruses found in bacteremia and can be easily lost during viral transmission [17]. When it comes to the production of large proteins, the baculovirus's long-term stability is crucial.

As per medical clinic analysts, the researchers utilized insect cells to create antibodies for cervical malignant growth and flu. Insect-cell based expression strategy has been reported to be one of the most effective platform for producing biologically active human antibodies. Among the various expression strategies, insect cell-baculovirus expression vector system (IC-BEVS) has been used in producing high yields of SARS-CoV-2 spike (S) protein which is known to be a vital antigen to target in COVID-19 vaccine. It was also reported that high yields of S protein production was first recorded from insect cells and it is greatly efficient in binding to ACE2 (angiotensin-converting enzyme2) receptor, additionally it is capable in identifying all other neutralizing antibodies headed toward various epitope clusters [18].

7.3 Self-Limiting Agricultural Insect Pests

Biologically based methods of pest control are essential, and one way is to use genetically modified insects. The “self-limiting” genus *Plutella xylostella* (OX4319L) was developed as a new way to protect cruciferous plants by allowing only male larvae to escape from the territory. Wild females paired with these restricted males do not produce viable female offspring. The greenhouse studies have shown that OX4319L self-limiting males when released into the field, the destruction of target population and attenuation of insecticide resistance genes has been observed. In a sequence of field studies on adult males and wild-type replicas, the range, perseverance, and field life of each species were monitored over an area of 2.83 ha. Overall, 95.4% of OX4319L males and 97.8% of wild-type males were scattered within less than 35 m from the re-released site. Regardless of release rate, there is no estimated consistency difference between species. With 95% certainty, 75% of the total 1500 males with OX4319L will live between 3.5 and 5.4 days, and 95% of these males will be found between 25.8 and 34.9 m from the point of release. Moth strain did not affect field survival but affected release levels. Overall, these studies suggest identical field behavior compared to the OX4319L male wild-type counterparts. Laboratory studies did not show

much differences in mating competition or internal growth rate and longevity between species. The statistical model developed from the results shows that the male release of OX4319L should provide effective pest control for *P. xylostella*. Other field studies have been suggested to illustrate the self-limiting potential of this *P. xylostella* [19] to provide insect repellent and resistance control benefits.

Pesticides are an important aspect of integrated pest management (IPM) programs, although there are unit problems associated with their unproven consequences. In addition, pest resistance may be a growing defect, with 586 species identified against one or more pests [20]. In the future, different methods will play an important role in pasture management. Over the past 20 years the use of genetically modified, insect-resistant plants (i.e., Bt plants that secrete Insects proteins from microorganisms), has been crucial in reducing the use of ancient pesticides. The major transgenic crops such as maize and cotton with insect-resistance traits have been adopted globally [21]. The use of Bt crops expanded globally when insects became highly resistant to the chemical insecticides. However, studies on field-evolved resistance in insects and pests to the Bt toxins in genetically modified crops reported decrease in the Bt crop potency [22]. The Sterile Insect Technique (SIT) is a harbinger of such strategies, during which a perfect insect field unit mediates to free wild gatherings of comparative insects [23]. Several research and development efforts are made to enhance SIT strategy for managing pest populations. Few studies also allude to the utilization of radiation that causes a deadly transformation that solely affects treated insects [24].

7.4 A Biological Approach to Pest Management Using Genetically Engineered Insects

The use of integrated pest control is crucial for food security, agricultural sustainability, and environmental protection as the world's population continues to expand. However, despite the potential benefits of GE plants in facilitating the process and improving pest and weed control, there are barriers to efficient deployment and long-term use. Numerous traits of GE crops, such as protein expression from *Bacillus thuringiensis* (Bt) that provide protection from insects and RNAi-based characteristics that provide herbicide tolerance and resistance to pathogens are known to be the vital component of the pest management system. By applying the knowledge gained from the GE plants and by understanding the technology limitations and taking the success and failure of GE facilities in Integrated Pest Management (IPM) systems for different plants and regions, the stability and flexibility of IPM systems can be improved with the future technology [25].

Innovative and applied research on genetically modified (GM) insects is being conducted to control insect vectors of human diseases such as mosquitos in the spread of dengue and malaria, and to control crop pest populations such as the olive fruit fly, diamondback moth, and Mediterranean fruit fly. When compared to the use of broad-spectrum insecticides, genetically modified insect control offers several advantages [26].

Genetic control has been a great help in recent advances in biotechnology and in the collection of successive genes to control the number of insects. Insect genes and their genetic information are found on the NCBI database, as well as on more specific sites on insects such as FlyBase, VectorBase, and BeetleBase. A program called i5k was launched in 2011 to identify the genes of 5000 insects and other arthropod species. The wasp bracovirus has genetically modified other Lepidoptera (such as monarch butterflies and silkworms).

The “Copying nature” pest management strategy is being introduced, with the objective of being relatively sustainable and environmentally safe [27]. There are several high genetics of plant-derived insect-resistant proteins in the environment that can be produced by tissue mutations in plants or by growth patterns or in response to environmental conditions. In order to boost insect resistance, these genes can be isolated or synthesized. Today’s results, mainly found in the development room, are discussed scientifically and in the context of their use. The feasibility assessment of this technology as a replacement for synthetic chemicals or as an essential element of IPM systems now needs to be tested in the fields. If proven, its long-term use will be contingent on agricultural promotion and farm management, which will reduce the accumulation of pest resistance [28]. Many pests are considered to be the largest pests in the world in the agro-ecosystems which cause the most damage to the economy. For instance, in the twentieth century, the pink bollworm *Pectinophora gossypiella* (Saunders) was a large insect species that originated in Asia and spread over America, Australia, and Africa [29]. Currently, it can be found in almost every cotton-growing country and is the primary estimate for most of them. Mediterranean fruit fly (“Medfly”) *Ceratitidis capitata* (Wiedemann) is the deadliest common species that attacks over 250 potential plants and is one of the world’s most significant economic pests [30]. Diamondback *Plutella xylostella* was developed in all stages of the brassicas problem including many vegetable plants and oilseeds. This is the first insect to modify its resistance to dichlorodiphenyltrichloroethane and Bt (a biopesticide derived from the bacterium *Bacillus thuringiensis*). The Diamondback Lab contributes US \$4–5 billion a year to the global economy, including crop waste and administrative costs [31, 32].

7.5 Biorational Pesticide

The term biorational refers to synthetic, inorganic, and organic pesticides which have less toxicity and impact on the environment. “Biorationals” have a negligible impact on species for which they are not planned (called non-target species). Plant oils, insecticidal soaps, microbial pesticides (such as Bt and entomopathogenic nematodes), and insect growth regulators are examples of biorational pesticides [33]. Effectiveness of seven biorational pesticides were appraised in laboratory and field conditions against *Bemisia tabaci* and for its parasitoid. The insecticides pyriproxyfen, buprofezin, and fenoxycarb are found to be highly effective against *B. tabaci* with 80–91% mortality and a population reduction of 66.3–84.2%. Further trials with Methoxyfenozide, Tebufenozide has resulted in mortality

rate of 50–75% and 47.8–52% of demographic decline. Additional trials with Tree oil and spice extracts has shown 25–50% mortality and 36.5–99% reduction in population. Among the experimented biorationals, Methoxyfenozide [(low-risk/class-II), induced 25–50% and tebufenozide (mildly harmful/class-III) induced 51–75% of parasite death. The post-application exposure interval (PAEIs) in the field for 7 days, and 3 days in the laboratory has ascertained that these biorationals are harmless or slightly harmful to the parasitoid (*E. formosa*). Experiments on laboratory bioassays have shown that parasitic pseudophosphate and tree oil and old germs in bitter gourd are harmless compounds. Alternatively, pyriproxyfen, buprofezin, fenoxycarb, methoxyfenozide, and tebufenozide are less biologically harmful to different species of *E. formosa* (30–79% mortality). Once within 7-day post-application exposure interval (PAEI) (168 h), it tends to conclude that almost all bioremediation studies except tebufenozide are harmless or slightly harmful to *E. formosa* and therefore often used consistently to Integrated Pest Management (IPM) in *B. tabaci* [34, 35].

Remarkable improvement has been made in the recent compilation of pesticides. Chlorinated hydrocarbons, organophosphates, and carbamates are the insecticides that are classified into several chemical classes which are used in agriculture, forestry, horticulture, and at home. Unfortunately, many of these chemicals are harmful to humans, insects and other organisms. In some cases, they are too stable in alignment and can disrupt agriculture. Despite good efforts to eliminate the pesticide's adverse effects and to improve food production and the health of humans and animals, there is a tremendous desire these days for safe and selective pesticides to focus specifically on harmful insects species, and various other creatures. In addition, the increased resistance to common pesticides provides an incentive to learn about new and natural pesticides. Attempts to develop new pesticides over the past 3 decades have been revived, as they have specific biochemical properties within a highly specific group of insects, while their properties are completely different from those of different pesticide classes. This approach has resulted in the development of various insect growth regulators (IGRs) that influence the regulation of hormones, metabolism, developmental process and metamorphosis in insects. These IGRs include ecdysone agonists (tebufenozide, methoxyfenozide, and halofenozide) that regulate lepidopteran pests and protein synthesis inhibitors (benzoylphenyl urea and buprofezin) that control the diversified range of pest. In addition, the compounds that affect chemo resistance sites (difenthiuron) and those highly interact with receptors of nervous system (imidacloprid, acetamiprid, thiamethoxam, and nitenpyram) have been developed and used effectively to fight against a diversity of insect pests [36].

7.6 Identification of Specific Pest Control Molecules

In Agriculture, ecologists and biologists are always looking for new ways to control pests and disease vectors. IPM has been the pest prevention strategy in US beekeeping industry by minimising the use of chemical pesticides and thereby reducing honey bee colony losses

and maintaining healthy colonies. In recent times IPM approach has also been the effective ways to control mosquito borne viruses such as Zika virus, dengue and chikungunya [37–39]. As the war is still raging, the bee pattern reminds us of the importance of minimizing the indirect impact of pesticides. In the fight against insects and pathogens, a new concept is emerging: compounds that affect insect behavior by modifying their odor can be improved by regulating their chemoreceptors [40]. This can be accomplished by experimenting with a large variety of natural and synthetic compounds, or by design-guided layout, as most pharmaceuticals formulations [41] have been developed. Application of satellite sensor approach for the assessment of biodiversity and progress of microbial biosensors play a major role in monitoring of the environment. Use of microbial biosensors are helpful to detect and analyse various kinds of pesticides which includes both narrow and broad spectrum pesticides. Selective pesticides, also known as narrow-spectrum pesticides, target only the specific type of insect or insect family. These compounds are toxic only to garden insects such as ants but are not naturally harmful to insect-eating insects. This unique identification helps reduce the number of unwanted pests for insect predators. However, narrow or short-spectrum pesticides may not be safe for anything other than the target. For example, Brodifacoum, a rat killer, is poisonous to dogs that accidentally eat it. It is important to read the product warning labels about the potential side effects of any diluted pesticide.

Comprehensive also called broad spectrum pesticides can destroy insects indiscriminately regardless of their species. Many of these pesticides contain neonicotinoids, organophosphates, pyrethroids, and carbamates, which are found on the labels of all pesticides for sale. Some extensive pesticides such as chlorpyrifos can be used effectively to control pest selection when used in control. It is essential to consider the consequence of extensive pesticide use on beneficial natural enemies of insects. If more natural enemies survive, they will help control the species over time and reduce the number of pesticides that need to be reintroduced.

Many pesticides contain a certain level of toxin in humans. The potential hazard level from these chemicals is defined on the product label using the words “warning,” or “hazard.” Low-toxic products have a warning word, while highly toxic products are labeled with caution and danger. There is usually no significant difference between a small amount of the toxic spectrum and a wide range of pesticides. Always check product warning labels for proper maintenance instructions. It is also a good idea to limit your exposure to any kind of pesticide by wearing protective gear and choosing a product that is less toxic if possible [42]. Biological pest control which includes predatory animals and parasites, and also entomopathogenic nematodes and microorganisms, has been active in agriculture since the beginning and farmers have a long history of increasing its efficiency. Ants are effective biological control agents in agriculture systems. Various ant species are integrated with other integrated pest management (IPM) programs. Ant colonies promote improvements in soil quality, soil nutrients and rapidly consume enormous amounts of insect pests. They also control pest

populations by interrupting pests during feeding and oviposition. Introducing ants into the citrus orchids in Asia is the first noted example of biological control, where the ants are moved between bamboo strips accommodated in between the trees and this has been followed for hundreds of years [43]. Efforts to exterminate exotic insects through “natural biological control” have largely eliminated their natural enemies since the 1800s; Despite the unintended factors, the practice has been extensively studied to prevent the effects on indigenous species. By the 1900s, farming methods had been developed in many forms of natural hostility, allowing a complete set of biological control systems against a range of targeted pests. Although the cost to farmers is much higher than the use of pesticides, the practice continues to gain popularity. Biological conservation management differs from previous forms in that it prioritizes the use of existing species in the region over the import or export of new species. The effective strategy to implement this is to maintain modified habitats with crop and farming practices that provide natural resources needed by natural enemies or to have a direct effect on nature’s pest-resistant pests [44, 45]. Green arthropods are often preferred in agricultural systems for one of the most popular crops and their lack of natural enemies [46]. Similarly, the regulation of conservation biology frequently seeks to restrict pests through crop diversification strategies by promoting the actions of carnivores and parasites [47]. An effective pest management system mainly focuses on life cycle of target pest and their interaction with its natural habitat / environment. It aims at conserving the natural enemies in the ecosystem for better enhancement of crops, agriculture area and surrounding environment. It aims at conserving the natural enemies in the ecosystem for better enhancement of crops, agriculture area and surrounding environment [48].

7.7 Recent Biotechnological Advances in Forest Pest Management

New tools have been found to fight against forest tree threats using a biological approach. Maximum environmental challenges are framed by diseases that are brought from other countries and it has been wreaked havoc on forest areas. By eating trees and other plant material, forest pests and microorganisms contribute to the healthy transformation and reproduction of the forest environment. They help restore forests by removing old or easily damaged trees, regenerating nutrients, and providing new habitats and food for wildlife. However, forest pests and diseases sometimes make world news not because of their natural benefits. Insects and local exotic diseases become “Forest Pests” when damage occurs to large areas of treasured forest land and valued forest products. These forest pests including the gypsy moth, Dutch elm disease, mountain pine beetle, and spruce budworm have caused a significant damage to commercially valued Canadian forests [49].

Canada’s Forest Pest Control Initiatives, focuses on- Preventing the entry and spread of endangered species, protecting the health of the country’s forests by limiting the existence of critically endangered, and providing forest cover for vital commercial and

related resources. To reach these goals, the Canadian provincial, local, and regional governments are taking a comprehensive approach to pest control. In integrated pest control, interventions are based on information about their short-term or long-term potential impact and also on identifying both the area and the pest. The environmental conditions, type of pests and place of outbursts are the major factors that, Canadian forest pest management is concerned and responsible for taking an initiative [50].

Generally, the responsibility is determined by forest ownership. Therefore, federal government, provincial, local, and municipal governments are responsible for pest control in their specific jurisdictions. The pests are controlled by private forest owners as well. To control pests in Canada, the responsibility owners seek scientific and advanced knowledge. The Canada Forest Service (CFS) is one of the best scientific and technical support for all the regions to rectify the forest pest issues. They also support the Canadian Food Inspection Agency (CFIA) and Environment Canada of federal agencies. CFS provides Scientific and Technical Support for the basic knowledge of forest species identification, biology and biodiversity, environmental and economic impacts, and the development of specialized tools and strategies that support evidence-based decision-making [51].

Forest pest management system basic components are basically the same as those of agricultural crop protection systems. Forests, on the other hand, are ecologically diverse and intricate. Forest ecosystems range from agriculture to natural forests, such as tree nurseries and Christmas tree planting. People benefit from forests in numerous ways, including wood and timber products, watershed protection, wildlife habitats, cattle grazing, recreation, pure beauty, and, in certain cases, food. Prices for these products and services vary accordingly. Forest management has a much longer planning horizon than agricultural systems, and it can vary greatly depending on the intended use, ownership (public or private), geographical location, and other factors relevant to specific management objectives. Legal, economic, social, political, and institutional limits all complicate forest resource management, particularly pest management. Forests are used and hunted by large numbers and by a variety of potentially destructive insects [52]. The environmental conditions, type of pests and place of outbursts are the major factors that, Canadian forest pest management is concerned and responsible for taking an initiative. Insect, pathogen, and other destructive agent interactions are diverse and complicated. They have a negative impact on forest productivity and value, as well as causing significant interruption to management programs and activities.

7.8 Entomopathogenic Fungi

Entomopathogenic fungi (EPFs) are used in pest management. Their occurrence is determined by the availability of host organisms for survival, as well as food, habitat, and atmospheric conditions [53]. This special class of fungi falls into the categories of Hypocreales and Entomophthoromycota. These groups can germinate while infecting arthropods by using spores that become attached to the epicuticle. Figure 7.2 shows the

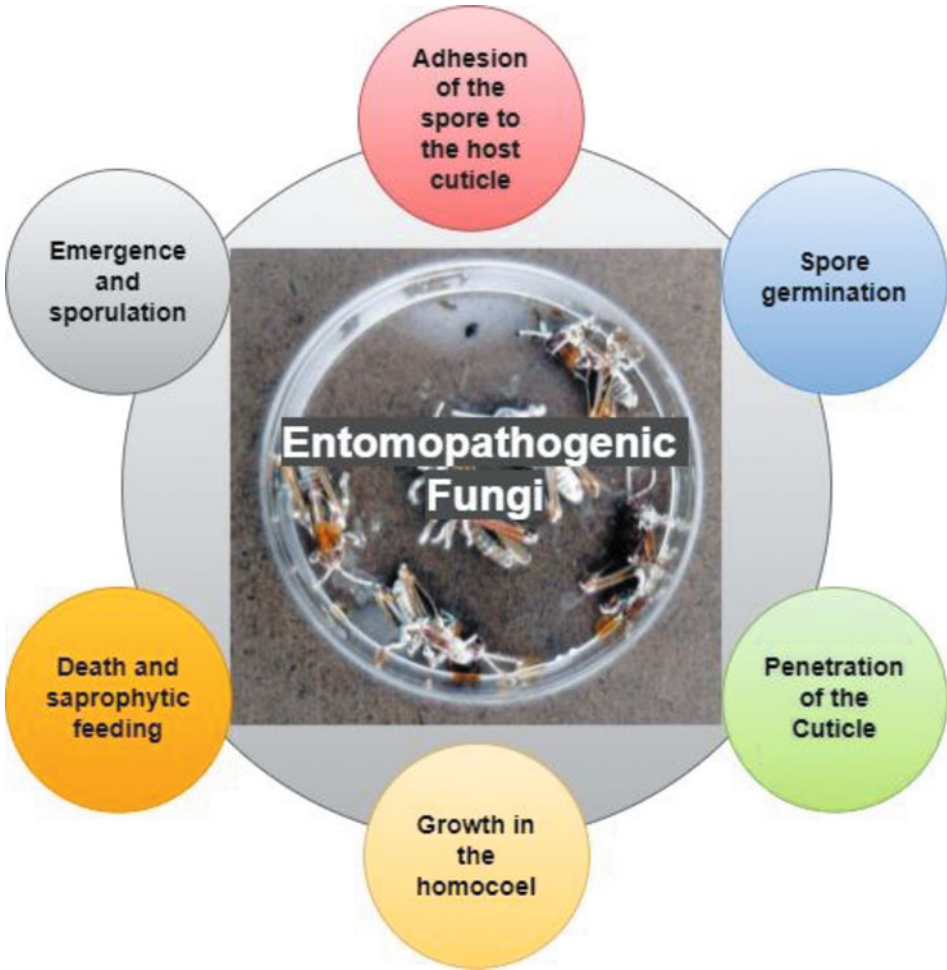


Fig. 7.2 Life cycle of entomopathogenic fungi against the insect pests

lifecycle of Entomopathogenic fungi. The proteolytic enzyme is secreted during the procuticle formation process.

Entomopathogenic fungi are a group of terrestrial fungi that allow insects to penetrate their cuticles to enter their bodies and eventually eat them [54]. These fungi are categorized into divisions, as Ascomycota, Zygomycota, Oomycota, Deuteromycota, and Chytridiomycota [55]. The insect's growth in the soil is contributed by these fungal pathogens. Among 800 fungal species identified as biopesticides, many commercial mycoinsecticides have been developed of which numerous products belong to the genera of entomopathogenic fungi such as *Isaria fumosorosea*, *Beauveria bassiana*, and *Metarhizium anisopliae* [56].

These enzymes and mechanical processes through the development of appressoria, disintegrate the insect cuticle and enter the skin to attack while the insect absorbs its nutrients from the insect tissue [57]. Entomopathogenic fungi are currently used worldwide to control many arthropod pests, especially insects on protected plants or garden plants. The study of fungal pathogens occurring naturally in communities. A survey conducted in 30 regions of Denmark, showed that the species was not infected with entomopathogenic fungi in the egg-producing environment. In comparison, a recent EU-funded study found that *D. gallinae* is one of the two most widely used fungal species, with an increased risk of infection by those found earlier in *Beauveria bassiana* and *Metarhizium anisopliae*, as well as other arthropods that were different from the species. In laboratory testing, the highest mortality rates were observed when high dosages of dry fungal conidia were administered. The fungus spreads quickly from mite to mite via contact, and at 25 °C, the time is very long (approximately 5–6 days), allowing blood transfusions to enter the female egg, which can be fungi, bacteria, or other organisms [58]. The pathogenesis of three strains of Entomopathogenic fungus, *Metarhizium anisopliae* at various phases of life on the insect *Dermanyssus gallinae* was investigated in lab research [59]. All the strains were found to be virulent to *D. gallinae* but varied in terms of their pathogenicity. Among them, the strain V245 caused an increase in death rate, at various concentrations when compared to the other two strains. The fatal concentration of different strains of entomopathogenic fungus *M. anisopliae* against *D. gallinae* was calculated based upon the exposure time of *D. gallinae* to *M. anisopliae*. The pathogenicity of *M. anisopliae* on various life phases of *D. gallinae* was found to be concentration- and time-dependent [60]. Due to environmental and food safety concerns, the use of these entomopathogenic fungi as myco-biocontrol of insects is extremely important. However, there are several drawbacks to using fungi as an insecticide, few of them are:

- (a) Short shelf life of inoculum
- (b) Insects take 2–3 weeks to get killed
- (c) The time required to get rid of the fungicide
- (d) Application also requires high humidity adherence
- (e) Availability of expensive commercial structures.

In addition, contamination of mycotoxins (aflatoxin, trichothecenes, zearalenone, fumonisins, citrine, etc.) is likely to be caused by various saprophytic fungi as they are naturally occurring and its contamination being inevitable. New methods need to be developed to help better control insects because the current pathogenic process of entomopathogens is time-consuming and requires improvement. Advanced methods in molecular biology have the capability to utilize the beneficial features of these fungi to enhance all-inclusive field activity. There is also a need to further understand the ecology of entomopathogens to develop strategies designed to improve the viability of these fungi in-field use [61]. More than 800 entomopathogenic fungi and 1000 pathogenic protozoa species have been discovered. Despite their immense potential for conservation and

biological control measures, much research on entomopathogenic fungi has tried to make them into agents for the control of insects, worms, and ticks. The earliest insect pathogenic investigations were conducted, with the goal of finding ways to reduce silkworm diseases. Named in honor of *Beauveria bassiana*, a theoretical theory was developed using the white fungus muscadine in silkworms [62], which led to the idea of using pesticide-treated molds in controlling insect pests [63]. A multi-faceted method merging all the available IPM tools into a completely comprehensive approach can be much more efficient in reducing the pests that cause economical destruction. With the increase in commercialization, entomopathogenic fungi are assured to become an important component of IPM. The usage and application of the entomopathogenic fungi remained comparatively low, despite being highly advantageous over many other biological and chemical products.

7.9 Summary

The significance of insects and their cells in all fields of human undertaking led to the advancement of insect biotechnology. Insects are found to be the most diverse species of all the organic entities. The number of existing species is assessed at around 1.9×10^6 and conceivably addresses more than 90% of the other life forms on Earth. They have a predominant effect on agriculture and the general well-being of mankind. The application of biotechnological strategies to insects and their cells contributed to the development of new biotechnological products. These products are proven to be useful resources in industrial, agricultural, medical, and modern biotechnological fields. More explicitly, insect biotechnology is driven by logical forward leaps in sub-atomic science, especially by the development of tools and strategies that permit genetic alterations and the engineering of cells/organisms. With the development of genetic transformation techniques and molecular biology, various insect-derived tools used for biotechnological research have been developed. These biotechnological applications along with the use of rDNA technology led to potential developments in pest management. The advancement of safe harvests and trees that produce insect toxins, the development of microbial agents with high insecticidal efficiency, and the mass release of genetically engineered insects that transmit lethal genes to normal populaces when released in the field, are known to be the major insect biotechnological applications to pest management. These transgenic insect biotechnological approaches could form an active resistance management strategy.

In general, insect biotechnology is the latest breakthrough in modern scientific and biotechnology innovations, which led to dangerous and safe processes for the production of bio-products. Scientific progress continues to prove that skills and information are constantly evolving and new breakthroughs emerge every day allowing people to make consistent use of natural resources and fight food shortages and starvation. Advanced scientific studies inspired by new achievements can solve the most serious environmental challenges. To this day, it has always been helpful to develop the infrastructure, skilled personnel, guiding principles, and strategies, to make the utmost use of technology and

effectively deal with the emergence of insect threats (pests), rather than scientifically suggesting confusion and negative consequences that threaten sustainable development. The new technological approaches promptly respond to the pests and fight against food scarcity and strongly contribute to sustainable development. The importance of insects in all aspects of human endeavor propelled the field forward.

Take Home Message

The development of modern technological tools and their applications in insect science has made insect biotechnology a prominent discipline in the management of insect pests and insect-borne diseases. Insect biotechnology has high economic potential, and it is considered to be a vital factor in the development of the bioeconomy and biodiversity. The new technological approaches promptly respond to the pests and fight against food scarcity and strongly contribute to sustainable development. The importance of insects in all aspects of human endeavor propelled the field forward.

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