K. Sahayaraj *Editor*

# Basic and Applied Aspects of Biopesticides

Springer

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ISBN 978-81-322-1876-0 ISBN 978-81-322-1877-7 (eBook)
DOI 10.1007/978-81-322-1877-7
Springer New Delhi Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014942322



Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

*Dedicated to all my family members*

# Foreword

I am happy to know that St. Xavier's College (Autonomous), Palayamkottai, has been organizing biennially Biopesticide International Conference BIOCICON since 2007.

Considering the intense awareness and the consciousness for the use of environmental friendly pesticides, these conferences are attracting better participation internationally. Use of bio-control agents (insect parasites, nematodes, microbes like bacteria and fungi) have become potential agents in different parts of this country and abroad and are commercially available. Every conference has given priority on certain areas and well attended and represented by all sections of academia, national labs and industries.

I congratulate the organization, and in particular Dr. K. Sahayaraj, the Organizing Secretary of these conferences, for taking the initiative for documenting the technical papers as an edited book *Basic and Applied Aspects of Biopesticides*. Though this aspect is covered in many other books, a comprehensive attempt of this kind is highly commended. I am sure that this book will help both under-graduate and post-graduate students in agriculture, zoology, and plant protection to understand the future of this subject and the existing opportunities for pest management etc.

I wish this volume is periodically re-visited and expanded to include the other aspects of regulatory procedures and other norms to be adhered in the development of products for field application.

Technology Development
& Transfer Division
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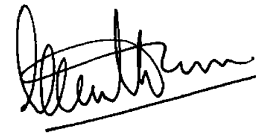
29.11.2013



**(Dr. G.J. SAMATHANAM)**

# About the Editor

**Dr. K. Sahayaraj** is Associate Professor and Director of Crop Protection Research Centre at St. Xavier's College affiliated to Manonmaniam Sundaranar University, Tamil Nadu, India. He has been awarded University Fifth Rank in M.Sc. by Madurai Kamaraj University in 1987. He has been teaching Zoology including Entomology to undergraduate and postgraduate students for more than 21 years. Dr. Sahayaraj has published four books and one proceeding on Ecofriendly Insect Pest Management. He has more than 173 publications, including original research papers, book chapters, and popular articles in insect ecology, behavior, biology and physiology, as well as numerous papers on biological control efficacy of reduviids, botanicals and fungal pathogens. Over the past 25 years, Dr. Sahayaraj's research efforts have been dedicated to multidisciplinary, integrated approaches to understanding how reduviids distribute and diversify in various ecosystems, and how their adaptive characters can be applied to pest management, especially through biointensive pest management. He has operated ten research projects funded by national (DST, DBT, CSIR, MOEs) and international (IFS) funding agencies, and now he has been operating MEFs funded project. Recently, he has been publishing an international journal, namely *Journal of Biopesticides* (ISSN 0976-0341X).

# Acknowledgements

I would like to express my gratitude to the many people who saw me through this book; to all those who provided support, talked things over, read, wrote, offered comments, allowed me to quote their remarks and assisted in the editing, proof reading and design.

I would like to thank G. J. Samathanam, Adviser & Head, Technology Development and Transfer (TDT) Division, Department of Science & Technology (DST), Govt. of India, for enabling me to publish this book.

I would like to thank Rev. Dr. Danis Ponniah S.J. Rector, Rev. Fr. R. Jesu Michael Das S.J. Secretary, and Rev. Dr. V. Guilbert Camillus S.J. Principal for their moral support and encouragement.

I thank Dr. P. Selvaraj and Dr. S. Kalidas for helping me in the process of selection and editing. Thanks to my publisher who encouraged me.

Last and not least: I beg forgiveness of all those who have been with me over the course of the years and whose names I have failed to mention.

Above all I want to thank my wife, Martin Rathi and my daughters Jesus S. Meylin and Kitheri S. Crosslin, who supported and encouraged me in spite of all the time it took me away from them. It was a long and difficult journey for them.

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# Part I

# Microbials

# *Bacillus* and Biopesticides in Control of Phytonematodes

1

Diouneia Lisiane Berlitz, Neiva Knaak, Maximiano Correa Cassal, and Lidia Mariana Fiuza

**Abstract**

Currently the major challenge of humanity is focused on population growth/increase through agricultural production in order to meet the demand for food. Overtime, different pests have emerged, with some being of great importance. Among these pests, the nematodes are noted for attacking leguminous plants, grasses, citrus, and other fruits. The main pest species are of the genus *Heterodera*, *Meloidogyne*, *Pratylenchus,* and *Globodera*. These nematodes cause losses up to 100 %, preventing agriculture of certain areas. Financially, about $100 billion annual damage is caused by nematodes. These amount to 90 % of the yield of cotton, yams, beans, and soybeans, and in citrus damage is estimated at 14 % of production. Alternative methods of control are being studied, and in this context, a bacterium of the genus *Bacillus* has prominence and importance. Besides *Bacillus subtilis*, some by pesticides are already marketed for the control of nematodes, such as Bioarc® the basis of *Bacillus megaterium*, Bio Zeid® the basis of *Trichoderma album*, and also using the brown alga, *Ascophyllum nodosum* (Algaefol®), among others. The objective of this chapter is to report the use of different *Bacillus* species and some biopesticides used to control nematodes of agricultural importance.

**Keywords**

Biopesticides • Bacillaceae • Biological control • Nematoda

## 1.1 Introduction

The biggest challenge for agriculture worldwide is to supply the demand for food, due to the ever-growing population. Data from FAO, in 2012, shows 2.2 % reduction in the world production of grains compared to 2011. It is a significant loss (52 millions of tons) and is associated, besides

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_1, 

the occurrence of agricultural pests, to the widespread and severe drought. Production of secondary grains such as soy, maize, and sorghum decreased 1.5 %. Wheat production decreased 2 % and rice, the only cereal with a lesser loss, decreased 0.2 %. The world demand for grains for 2012/2013 has been estimated in 2.3 millions of tons, a reduction of 2 %, which may result in the increase of the price for those products. Furthermore, FAO evaluates a demand of 2.4 millions of tons of those grains in 2012–2013.

In the animal kingdom, the phylum Nematoda represents about 80,000 described species, according to the Brazilian Society of Nematology. The nematodes are morphologically classified as elongated, cylindrical, or subcylindrical. They may be free-living, phytoparasites or zooparasites. The phytoparasites, also known as phytonematodes, are noted by their economic importance, attacking leguminous plants, grasses, citrus, and other fruits. The main pest species are of the genus *Heterodera*, *Meloidogyne*, *Pratylenchus,* and *Globodera*. These pests are considered the "hidden enemies" of the producers, for being hard to identify in the field. Symptoms on plant leaves may be mistaken as diseases, nutrient deficiency, drought, and soil compression. Some species may degrade plant tissues, while others may cause hormonal disorders, inducing the formation of galls, and some may release phenolic compounds. Furthermore, these parasites may transmit bacteria externally, on the plant surface, or internally, inside the alimentary canal (Moens and Perry 2009).

Phytonematodes survive over years in the soil or in vegetal remnants, being easily disseminated by agricultural implements, irrigation water, or floods, and adhered to animal feet or feces and vegetal material, such as seedlings and seeds (Oka et al. 2009). The losses may vary, from imperceptible damage to the death of a large number of plants, preventing agriculture of certain areas. Financially, nematodes cause approximately $100 billion in production losses annually (Oka et al. 2009). Researches in the biological control field aim the application of bacteria, fungi, and algae to control these pests. They are mainly focused in rhizosphere compounds with the capacity to modify this environment, affecting these parasites directly or indirectly (Machado et al. 2012). The bacteria of this genus are sporulating, are found in different habitats, and have the peculiarity of being, in many cases, entomopathogenic. Moreover, it may be deduced that *Bacillus*-based products may interfere in the nematode lifecycle, being used in the practice of biological control of these pests.

Based on this information, the general objective of this chapter is to explain the use of the *Bacillus* biopesticides and the practice of biological control of nematodes. Aspects of the biology of major nematodes will be discussed. Rrecent data of *Bacillus thuringiensis*, *Ba. subtilis*, *Ba. megaterium,* and *Ba. firmus*, especially the metabolites produced by these bacterial species, and other biopesticides based on algae and fungi for control of nematodes will be presented. Throughout the chapter, images shall be submitted illustrating the bacterial species and the phytonematodes.

## 1.2 Phytonematoda

The genus *Pratylenchus* (Pratylenchidae) has the broadest host range. Through research, this genus is recognized as a major pest of economically important crops. The *Pratylenchus*, or root lesion, is distributed in cool, tropical, and temperate environments and comprises 68 nominal species (Moens and Perry 2009). The root lesion causes internal browning in potato tubers and in the roots of corn, lettuce, peas, carrots, tomatoes, and brassicas (Guerena 2006). Therefore, the sedentary endoparasitic nematodes, which include the root-knot nematodes of genus *Meloidogyne* spp. and the cyst nematodes of genus *Heterodera* and *Globodera* spp., are the most economically important pests (Davies and Curtis 2011). The genus *Meloidogyne* has currently more than 80 species recognized (Karssen and Von Hoenselaar 1998) although most of those species are associated with particular plant species and have been little studied, in part because of their limited agricultural importance (Bird et al. 2009).

Root-knot nematodes form galls that block water and nutrient flow, stunting growth, impairing fruit production, causing foliage to yellow and wilt, and injuring plant tissue (Guerena 2006). The infective juvenile stage detects plant roots, in soil, using chemoreception and sensory perception, penetrates in the root, moves intercellularly in vascular cylinder, and forms feeding sites in the differentiation zone inducing nuclear division in host cells, giving multinucleated cells, termed giant cells (Guerena 2006; Atkinson et al. 2012). The female *Meloidogyne* lays up to 2,500 eggs, called egg mass, that hatch into larvae and develop into adults. Each adult age is characterized by phases of development of sexual and digestive systems (Dyakov and Zinovyeva 2007). The cyst nematodes, *Heterodera* spp. and *Globodera* spp., are the most important pests of soybean, potato, and sugar beet, respectively. Cyst nematodes have a small host range and their characteristic features allow regarding them as evolutionarily advanced parasites (Dyakov and Zinovyeva 2007). The genus *Heterodera* gives plants an unthrifty or malnourished appearance, foliage is liable to wilt and curl, while roots become thick and tough and take a red or brown coloring (Guerena 2006). Reproduction requires males; after fertilization, the eggs remain inside the female, gradually filling its entire body forming a bag of eggs, causing the death of the female (Williamson and Gleason 2003; Dyakov and Zinovyeva 2007). Table 1.1 shows the species of nematodes and their host plants.

Dyakov and Zinovyeva (2007) report that out of the 20,000 described species of phytonematodes, approximately 20 % or 4,000 species are connected with plants. The phytonematodes are microscopic organisms, their size varying from 300 μm to 8 mm (Fig. 1.1). The identification of the different species of nematodes may be associated to morphological identification techniques, considered as conventional techniques, and recently to the use of molecular tools. This is the case of a research of Shahina et al. (2012), which associated these techniques for the identification of *M. incognita* and *M. javanica*, in Pakistan. Furthermore, the authors associated the occurrence of these species in new cultivars, such as in olive (*Olea europaea* L.), spinach (*Basella rubra* L.), and sugarcane (*Canna indica* L.) in India, which were not previously registered.

## 1.3 *Bacillus thuringiensis*

The entomopathogen *Bacillus thuringiensis* (Berliner 1911), a Gram-positive bacterium, is naturally found in the soil (Höfte and Whiteley 1989). It is characterized by crystal production during sporulation (Fig. 1.2), containing Cry proteins, encoded by the *cry* genes, with a wide division of classes and subclasses according to their insecticide activity (Höfte and Whiteley 1989), and presently classified according to the percent identity between Cry protein sequences (Schnepf et al. 1998; Crickmore et al. 1998; Crickmore 2014).

These toxins present insecticidal activity to different insect orders, such as Lepidoptera, Coleoptera, Diptera, Hymenoptera, Hemiptera, Isoptera, Orthoptera, Siphonaptera, and Thisanoptera (Feitelson et al. 1992; Aranda et al. 1996; Cavados et al. 2001; Castilhos-Fortes et al. 2002; Pinto et al. 2003; De Maagd et al. 2001, 2003) in addition to nematodes (Marroquin et al. 2000; Jouzani et al. 2008). Currently, 735 Cry proteins, 38 Cyt proteins, and 122 Vip proteins have been described in the *B. thuringiensis* database (Crickmore 2014). Apart from those proteins, other virulence factors indentified in this bacterium comprise degrading enzymes such as phospholipase C, hemolysins, proteases, and cytotoxins (Vilas-Bôas et al. 2012).

Jouzani and coworkers (2008) identified the *cry*5, *cry*6, *cry*12 *cry*13, *cry*14, and *cry*21 genes, which presented activity against nematodes of the genus *Meloidogyne*. Beyond these genes, Höss et al. (2008) have already indicated the utilization of strains containing genes from the *cry*1 family, showing promising results against the nematode *Caenorhabditis elegans*. A recent study from Khalil et al. (2012) used the product DiPel 2x® containing 32,000 iu/mg of bacterium *B. thuringiensis* var. *kurstaki* (Valent, Canada) against *M. incognita*, in tomato plants, showing the reduction of 80 % in the nematode population. Furthermore, DiPel 2x® reduced in 60 %

**Table 1.1** Important species of genus *Pratylenchus*, *Globodera*, *Heterodera*, *Meloidogyne,* and their host plants

| Species | Culture | Citation |
|---|---|---|
| *Pratylenchus coffeae* | Coffee, citrus, yam, potato | Moens and Perry (2009), Pionner (2012) |
| *Pratylenchus goodeyi* | Banana and plantain | Moens and Perry (2009), Pionner (2012) |
| *Pratylenchus brachyurus* | Cassava, peanut, pineapple, potato, rice, tea | Moens and Perry (2009), Pionner (2012) |
| *Pratylenchus zeae* | Rice, sugarcane | Moens and Perry (2009), Pionner (2012) |
| *Pratylenchus thornei* | Broad beans, cereals, tobacco | Moens and Perry (2009), Pionner (2012) |
| *Pratylenchus penetrans* | More than 350 hosts including woody plants (e.g., fruit trees, roses) and herbaceous plants (e.g., potato, vegetables) | Moens and Perry (2009), Pionner (2012) |
| *Pratylenchus vulnus* | Woody plants – approximately 80 hosts | Moens and Perry (2009), Pionner (2012) |
| *Globodera rostochiensis* | Potato | Qin et al. (2000) |
| *Globodera pallid* | Potato | Reitz et al. (2000) |
| *Meloidogyne incognita* | Vegetables, tomato, eggplant, melon, cotton, maize, soybean, beans | Pionner (2012) |
| | | Hashem and Abo-Elyousr (2011), Collange et al. (2011) |
| *Meloidogyne hapla* | Eggplant, tomato, melon, lettuce, onion, carrot, potato, bean | Chen et al. (2000) |
| *Meloidogyne javanica* | Eggplant, tomato, melon, soybean | Pionner (2012), Collange et al. (2011) |
| *Meloidogyne exigua* | Coffee, tomato, rubber trees | Rocha et al. (2010) |
| *Meloidogyne arenaria* | Tomato, lettuce | Terefe et al. (2009) |
| *Meloidogyne hispanica* | *Ficus* sp., squash plants, grapevine, sugarcane, snapdragon | Maleita et al. (2012) |
| *Meloidogyne enterolobii* | *Psidium* spp. | Miranda et al. (2012) |
| *Heterodera glycine* | Soybean | Pionner (2012) |
| *Heterodera schachtii* | Sugar beet, potato | Madani et al. (2005) |


**Fig. 1.1** *Meloidogyne javanica* in differential interference contrast microscopy. (**a**) Immature eggs. (**b**) Juvenile stage (J2) (Source: authors)

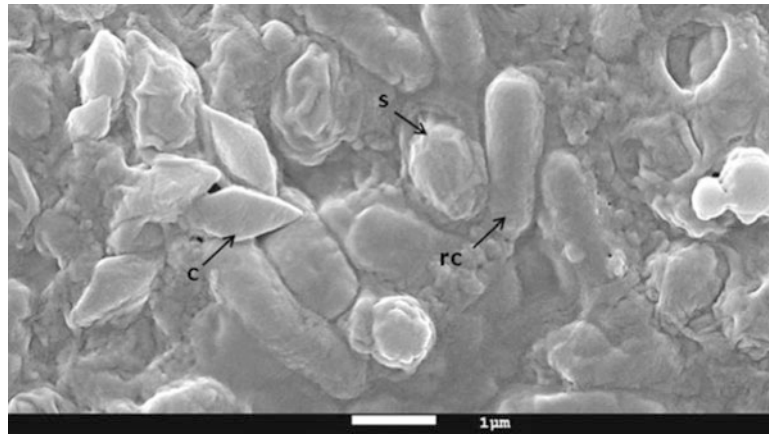


**Fig. 1.2** *Bacillus thuringiensis* in scanning electron microscopy. *s* spore, *c* protein crystal, *rc* rod cell (Source: authors)

the amount of galls/5 g of roots and in 74 % the egg mass/5 g of roots. In terms of plant development, the product significantly increased plants' length, dry weight, and fresh weight. This bacterial subspecies is known to contain a family of *cry*1 genes.

Another study shows the utilization of ten different isolates of *B. thuringiensis*, which purified Cry proteins and the supernatant, containing vegetative proteins, was applied to *M. incognita*, in laboratory and in greenhouse (Mohammed et al. 2008). The results showed that four isolates, *Bt*7, *Bt*7N, *Bt*Den, and *Bt*soto, presented 90 and 100 % mortality when utilizing Cry proteins and vegetative protein fractions, respectively. In greenhouse, these isolates were efficient, with *Bt*7N showing the best result in reducing the number of galls, the egg mass, and the number of eggs when compared to the other treatments and the control.

Soil samples from okra, eggplant, tomato, cotton, cabbage, onion, and watermelon crops were collected in Pakistan and used to isolate new strains of *B. thuringiensis* to tests against *M. javanica* (Khan et al. 2010). The authors selected five isolates to studies in greenhouse using okra plants and all the isolates reduced the infection caused by the nematodes. Different mechanisms of action may be related to the toxicity of *B. thuringiensis* against the phytonematodes, since the mode of action of the Cry proteins described by different authors is the ingestion of toxic fragments. These mechanisms may be related with the production of secondary metabolites, which reduce the attraction of the nematodes to the roots or degrade specific exudates of the roots that control the behavior of these species (Khan et al. 2010).

The review of Vachon et al. (2012) shows these theories, developed by many researchers from this field of study, about the mode of action of the nematicide and insecticide Cry proteins. The general conclusion of these authors reveals that the model mostly accepted is still the classic model described in the 1980s and 1990s, and these are protein digestion, solubilization, pore formation in the membrane, and the binding of the toxic fragments. Thereafter, occurs the cellular lysis, the intestinal paralysis and the target pest ceases its feeding and dies.

Data from Berlitz et al. (2012) show the toxicity of *B. thuringiensis* isolates of soil samples from Rio Grande do Sul state, Brazil, which presented corrected mortality of 91 % and 93 % to the isolates *Bt* 3434-2P and *Bt* 2974-11P, against second stage (J2) *M. incognita*. These results were published on the 45th Annual Meeting of Society Invertebrate Pathology, in Buenos Aires (2012), an event that gathers worldwide researchers, with specific conferences in the nematology field. These results corroborate with the results from Li et al. (2012), which isolated 3,800 bacterial strains from soil samples, with 438 belonging to the Bacilli group. One strain was identified as *B. thuringiensis* and was tested, showing 100 % mortality against juveniles of

second stage (J2) *M. incognita*. The simultaneous utilization of *B. thuringiensis* and nematicides also occurs, being tested by Khan et al. (2011a). The results of the combination of *B. thuringiensis* and the product Fertinemakil® showed an increase in the plant growth between 300 and 400 %. This product is an organic nematicide, whose main compound is neem, and its function is to increase the nutrient content of soil, providing better physical and chemical conditions to the plant.

*Bacillus thuringiensis jordanica*, serotype H71, was tested against *Meloidogyne* on concentration of $10^7$ viable spores/ml$^{-1}$ using the product Vydate® as control. Based on the results presented above, this subspecies showed 52 % reduction in the number of nematodes in the roots of tomato, thereby decreasing the number of galls per root system (Khyami-Horani and Al-Banna 2006). Researchers from Korea identified a relevant nematicidal action of *B. thuringiensis* through a screening of 114 isolates, where two of them showed a high toxicity when compared to the product Carbofuran®. After 30 days of treatment in greenhouse, Carbofuran® showed 45 % of mortality and BM2x (*B. thuringiensis*) showed 22 %. However, after 60 days, the treatments showed, respectively, 60 and 52 % of mortality (Seo et al. 2012).

The eggplant crop is also the target of attack by nematodes, mainly of the genus *Meloidogyne*. The authors Ashoub and Amara (2010) used different microorganisms in vitro assays and in a greenhouse. Among these microorganisms, three isolates of *B. thuringiensis* identified were Biovar 1, Biovar 2, and Biovar 3. Second stage juveniles of each isolate were tested separately, with a mortality rate of 70 %, 85 %, and 90 %, respectively. However the mortality was higher (92 %) when the three isolates were used simultaneously after a 24 h exposure. In greenhouse tests, the authors applied a mixture of microorganisms, and the best treatments were *B. thuringiensis*, *Mycobacterium*, and *Rhizobium,* causing total nematode mortality after 72 h of treatment application.

*Bacillus thuringiensis* has also been used in conjunction with other microorganisms with nematicidal activity, such as *Paecilomyces marquandii* and *Streptomyces costaricanus*, in the research of Chen et al. (2000) for controlling *M. hapla* in lettuce, also showing positive results in relation to reducing the number of juveniles. A more complete understanding of the relationship between the proteins of *B. thuringiensis* and the free-living nematodes can elucidate how these potential hosts and the pathogen may have co-evolved. The extent to which bacterial toxins typically affect nematodes also has environmental implications of the impact of its widespread use in programs to reduce insect. Some proteins from *B. thuringiensis* can intoxicate multiple nematode species, while other proteins cannot (Wei et al. 2003).

The biotechnological tools are also being used for the control of major species of nematodes in crops of agricultural importance. This is the case of tomato (*Lycopersicon esculentum* var. Rutgers select), which has been transformed with the *cry*6A gene to be expresses in the roots of these plants (Li et al. 2007). The authors choose the protein expressed by the *cry*6A gene for being toxic to the nematode *Acrobeloides* spp., which is phylogenetically closely related to plant parasitic nematodes. Another reason is the size of the protein Cry6A which is the smallest of the nematicidal proteins, making it easier to synthesize and to troubleshoot gene expression. The data of these authors demonstrated that *M. incognita* was capable of ingesting Cry6A protein of 54 kDa and this protein caused toxicity to the phytoparasitic nematode, indicating a decrease in the production of descendants up to four times. These results indicate that the Cry protein of *B. thuringiensis* may confer to a plant the resistance to phytoparasitic nematodes and the Cry proteins have the potential to control nematodes in transgenic plants. *Bacillus thuringiensis* serovar *roskildiensis* was used by Sato and Asano (2004) for cloning and sequencing of a new gene, *cry*21Ba1. The authors identified 67 % similarity in amino acid sequence with the gene *cry*21Aa1, which also has nematicidal activity. These data are important because they can be used as a basis for the use in plant transformation, conferring to them resistance to attack by nematodes.

## 1.4 *Bacillus subtilis*

*Bacillus subtilis*, common in nature, has been widely studied as a potential biological agent against various plant diseases worldwide (Yang et al. 2009), is nontoxic and harmless to humans and other animals, and nonpathogenic to plants (Acea et al. 1988). The bacterium produces antimicrobial compounds in vitro, including the antibiotics zwittermicin A and kanosamine (Leifert et al. 1995), lipopeptides (Pal-Bais et al. 2004), and antifungal protein bacisubin (Liu et al. 2007). The bacteria produce endotoxins and a variety of polypeptide antibiotics of the bacillomycin group, iturin, fungistatin, mycobacillin, and mycosubtilin (Kudryashova et al. 2005). On a worldwide scale, this stimulates the isolation and selection of new *B. subtilis* strains which exhibit an even broader spectrum of activity against plant pathogens. Moreover, the strains to produce hydrolytic enzymes such as proteases, lipases, b-glucanases, and cellulases (Cazorla et al. 2007) were recorded. The mechanisms of control of this bacterium involve competition, parasitism or predation, production of metabolites, and induction of structural abnormalities caused by the diffusion of volatile compounds from the bacteria (Chaurasia et al. 2005). In soil, this species interferes in the reproductive cycle of the nematodes, acting on the larvae orientation to the host plant (Sharma and Gomes 1996). On the other hand, *B. subtilis* can also act as a plant growth promoter and helps in the control of phytopathogenic fungi, such as *Rhizoctonia solani* and *Colletotrichum truncatum*, in soybean (Araújo et al. 2005).

A research from Xia et al. (2011) utilized five *B. subtilis* strains to control *M. javanica*: 69, OKB105, M1, M1-1, and M1-2, with the three last mutants from OKB105 through genetic construction. The authors tested the cultures supernatant with different dilutions and diluents. The isolates OKB105 and 69 were the most efficient, showing 100 and 89 % mortality to the nematodes, respectively. The nematicide ingredients from the supernatant of OKB105 were evaluated in SDS-PAGE showing a protein fragment of 1,000 DA, which presented high stability in in vitro tests. The authors also identified the *pur*L gene, which regulates the synthesis of intermediary metabolites of purine and may be related to the nematicide activity of *B. subtilis* OKB105. The product Stanes Sting®, containing $1 \times 10^9$cell/mL of *B. subtilis* (Ehrenberg) Cohn (Stanes Company, India), was tested against *M. incognita*, in tomato plants, and reduced in 82 % the population of this nematode species, in addition to reducing the number of galls in 54 % and the egg mass/root in 72 % (Khalil et al. 2012).

Araujo and Marchesi (Araújo and Marchesi 2009) tested the PRBS-1 strain of *B. subtilis* isolated from soil under soybean, against the gall-forming nematode, and compared it to the chemical carbofuran. The results showed that *B. subtilis* increases the aboveground biomass of the plants and reduces nematode infestation. Some formulation compounds were tested by Khan et al. (2011b) and obtained as a result the formulation composed by 1 part stock (sawdust–soil molasses mixture) and 20 parts of carrier (fly ash–soil–molasses mixture) and the *B. subtilis* cells, naming it Biocure-X®. Thereafter, the authors applied this formulate in chickpea and pigeon pea seeds to control *M. incognita*, which reduced in 39 and 43 % the incidence of nematodes in these plants, respectively. Despite these recent studies with *B. subtilis*, many studies are still necessary to correctly elucidate the mode of action of this bacterium against the phytonematoids, since the data obtained in the studies abovementioned showed efficiency of this species as nematicide.

## 1.5 *Bacillus firmus*

*Bacillus firmus* is a bacterial species that has been a subject of different studies in relation to its action against nematodes. Isolates of this species secrete some toxins that are associated to an ovicidal activity in nematodes; in other words, these toxins damage the external egg

pellicle of the gall-forming nematodes, inhibiting the hatching, in addition to acting against their juvenile stages (Terefe et al. 2009). The authors tested the product BioNem® WP, based on empiric informations about its nematicidal action. This formula, based on *B. firmus*, was produced in Israel and tested in tomato plants, in greenhouses. The authors demonstrated that, in vitro, BioNem® inhibits egg hatching of *M. incognita*, reducing in 91 % the gall formation in the plants roots when compared to the control. Important data of this study also showed the low concentration of the product used: concentrations of 0.5 %, 1 %, and 2 % reduced between 98 % and 100 % the egg hatch, 24 h after treatment application, in vitro. When utilized against juvenile stages (J2), the concentrations of 2.5 % and 3 % showed 100 % nematode mortality after 24 h. Despite the laboratory assays, these data show importance and promise in regarding the nematicide activity of BioNem®, requiring field studies to evaluate its efficiency.

According to Anastasiadis et al. (2007), the formulate BioNem® contains 3 % of lyophilized bacterial spores and 97 % of nontoxic active ingredients, such as plant and animal extracts. The authors also tested the efficiency of this product against nematodes, and on dilution of the 4 kg/ha$^{-1}$, the product presented efficiency to control the egg hatch, which is related to the production of nematotoxic substances mentioned above. Besides the mode of action of *B. firmus* elucidated by Terefe et al. (2009), it is poorly understood and few active compounds have been characterized. Another hypothesis, of the nematicidal action, is the production of toxic metabolites during bacterial fermentation, which acts on nematode survival in the juvenile stage, or J2 (Mendoza et al. 2008). A third hypothesis is focused on the competition between the bacteria and the nematodes for habitat or space on the roots, acting directly on the juveniles' death.

## 1.6 *Bacillus megaterium*

According to Huang et al. (2010), *B. megaterium* can promote the growth of plants because it increases the availability of phosphorus in soil. Some isolates of *B. megaterium* also produce volatile compounds, which are lethal to nematodes and can strongly inhibit hatching. The data of these authors show that after 6 days of incubation, the eggs hatched were completely inhibited, whereas the control showed 83 % hatching. Strains of *B. megaterium* may also produce antibiotic compounds (Vary 1992). In 2007, researchers from Germany isolated endophytic bacteria from rice roots, *Oryza sativa* (L.), and from seeds of five rice strains most cultivated in Bangladesh (Padgham and Sikora 2007). The authors performed a screening with 31 isolates, in which *B. megaterium* (isolate Ni5SO11) was the most efficient on nematode suppression. The methodology utilized was differenced because the rice plants of 3 weeks old were embedded in a bacterial solution of 6.10$^6$ CFU/ml$^{-1}$ and then transplanted. After 1 week, 900 J2 *M. graminicola* nematodes were applied around the roots and the evaluations were performed after 20 days of the nematode application. The results obtained showed that *B. megaterium* reduced the egg hatch in 4 % and in 14 % after 4 and 11 days, respectively.

Neipp and Becker (1999) reported that various isolates of *B. megaterium* were effective against *H. schachtii*, reducing between 38 and 59 % the penetration of J2 nematodes in eggplants. Another study also demonstrated that *B. megaterium* reduced in 50 % the penetration of both *M. chitwoodi* and *P. penetrans* in potato (Al-Rehiayani et al. 1999). These studies suggest that the condition created by the oxygen limitation in soil helps in the biological control of rice nematodes. The reduction in *M. graminicola* migration to the rice roots suggests a deficiency in the nematodes' ability to colonize the roots once *B. megaterium* may interfere in the chemical factors of perception that conduct the nematodes to the target plants.

Radwan et al. (2012) used the commercial product BioArc®, based on *B. megaterium* against *M. incognita* in tomato plants. The results obtained by the authors showed inhibition of 88 % in the number of galls and 98 % in the occurrence of J2 on plant roots, when the product is used at low concentration (5 g/kg soil). *Bacillus megaterium* PSB2 was isolated and

**Table 1.2** Microorganisms of the genus *Bacillus* and their main characteristics, important to the formulation of biopesticides

| Species | Characteristics | Citation |
|---|---|---|
| *Bacillus thuringiensis* | Toxic crystal production during sporulation; genes *cry*5, *cry*6, *cry*12 *cry*13, *cry*14, and *cry*21 which presented activity against nematodes; production of secondary metabolites, which reduce the attraction of the nematodes to the roots or degrade specific exudates of the roots that control the behavior of these species; phospholipase C, hemolysins, proteases, and cytotoxins | Höfte and Whiteley (1989), Jouzani et al. (2008), Vilas-Bôas et al. (2012), Khan et al. (2010) |
| *Bacillus subtilis* | Produces antibiotics zwittermicin A and kanosamine, lipopeptides, and antifungal protein bacisubin; endotoxins and a variety of polypeptide antibiotics of the bacillomycin, iturin, fungistatin, mycobacillin, and mycosubtilin groups; in soil, interferes in the reproductive cycle of the nematodes, acting on the larvae orientation to the host plant | Leifert et al. (1995), Sharma and Gomes (1996), Pal-Bais et al. (2004), Kudryashova et al. (2005), Liu et al. (2007) |
| *Bacillus firmus* | Toxin's ovicidal activity in nematodes; damages the external egg pellicle, inhibiting the hatching; acts against their juvenile stages | Anastasiadis et al. (2007), Mendoza et al. (2008), Huang et al. (2010) |
| *Bacillus megaterium* | Increases the availability of phosphorus in soil, produces volatile compounds, produces antibiotic compounds, inhibits hatching of the nematodes | Vary (1992), Terefe et al. (2009) |

tested by El-Hadad et al. (2010) showing high capacity in inhibiting the colonization of *M. incognita* and showed 100 % mortality against J2 of this species. These authors identified a high enzyme activity via the production of proteases, chitinases, and gelatinases by such isolate, and high phosphate solubilization (80 ppm). The discussion of these results is focused on the improvement of soil quality and the consequent growth of plant roots by *B. megaterium* and may be indicated for biofertilization and control of nematodes. Table 1.2 shows the data from these major Bacillus species discussed above.

## 1.7 Biopesticides

Bacilli are considered excellent candidates for formulations applied to biological pest control. The main reason is the longevity of the species belonging to the genus *Bacillus*; due to the presence of spores, structures that are formed under unfavorable physiological conditions can remain idle for many years and are able to germinate in active cells during more favorable conditions. This ability to survive through spores is a desirable feature, as it prolongs the shelf life of commercial products. Besides the pesticides mentioned above, many bacilli species, as well as algae and other microorganisms, are already used in commercial formulations and are suitable for the control of nematodes. Table 1.3 shows these formulations marketed in different countries.

Another perspective is the joint use of biological control agents, as in the case of *B. firmus* and *P. lilacinus*. These two biopesticides were used for Anastasiadis et al. (2007), where *P. lilacinus* and *B. firmus* obtained a suppression of J2 *M. incognita* of 58 % and 66 % after 7 and 14 days, respectively, compared with the control. The blue-green alga *Microcoleus vaginatus* was utilized by Khan et al. (2005) against the nematode *M. incognita* on tomato plants. The beneficial effect on plant roots increased

**Table 1.3** Other biopesticides indicated for phytonematodes

| Biopesticide | Species phytonematode | Citation |
|---|---|---|
| Bio Zeid® (*Trichoderma album*) | *M. incognita* in tomato | Radwan et al. (2012) |
| Plant Gard® (*Trichoderma harzianum*) | *M. incognita* in tomato | Radwan et al. (2012) |
| Algaefol® (*Ascophyllum nodosum*) | *M. incognita* in tomato | Radwan et al. (2012) |
| Bio-Cure-B® (*Pseudomonas fluorescens*) | *M. incognita* in tomato | Khalil et al. (2012) |
| Bio-Nematon® (*Paecilomyces lilacinus*) | *M. incognita* in tomato | Khalil et al. (2012) |
| Bionem-X® (*Pochonia chlamydosporia*) | *Meloidogyne* and other nematodes | Khan et al. (2011b) |
| Biocomp-X® (*Pseudomonas fluorescens*) | *Meloidogyne* and other nematodes | Khan et al. (2011b) |

with the increase in seaweed filtrate concentration. The nematode populations were reduced by 66 % and 97 % when used in higher concentrations. These data indicate a wide range of potential factors that may be exploited in different fields and with different technologies and products available in the market.

## 1.8 Considerations

Biological evolution brings along with it various changes in cropping systems, and the producer, for best crops, must adapt to these changes. An example of these changes are pests that previously had no great importance became increasingly detrimental to agricultural crops, such as nematodes, which come with a wide variety of crop species as hosts. Fumigant and non-fumigant nematicides are not used against parasitic nematodes since 1950, especially methyl bromide (Giannakou et al. 2004). Its biocidal effect can cause biological or soil imbalances. This chemical molecule was banned in 2005 (Oka 2010) and consumer demand for safe food has forced farmers to reduce the use of chemical pesticides. According to Oka (2010), only a small number of nematicides of the organophosphate and carbamate classes and some soil fumigants are available for nematode control in most countries. These pesticides are highly toxic for humans and soil application also causes subterranean water contamination and some of them are also absorbed by plants.

According to this author, the possible mechanisms involved in the suppression of nematodes are as follows: (i) the release of preexisting nematicidal compounds in the soil, (ii) the production of nematicidal compounds, such as ammonia and acid during the degradation process soil, (iii) the increase and/or introduction of antagonist microorganisms into the soil, (iv) the tolerance and resistance of the host plant, and (v) changes in the soil physiology that are unsuitable for development and behavior of the nematode. Combinations of these mechanisms, at once, may reduce the infestation of nematodes in agricultural soils. The presented perspectives show that the biological control can be used to protect the plant roots during brief periods, such as the establishment of the seedling or directly after the transplantation because when the root system is reestablished, it becomes vulnerable again to the nematode attack. In the different cropping systems, antagonist bacteria of nematodes may be coated on the seeds, including pre-germinated seeds or the roots, during the transplant.

Another factor is the production of toxic substances by microorganisms, which may limit the damage caused by nematodes, for example, through the production of antibiotics, siderophores, and a variety of enzymes. These microorganisms can also act as competitors for nematode colonization sites and nutrient utilization (El-Hadad et al. 2010). The mode of action studies cited in this work showed that root endophytes are capable of achieving multiple points of vulnerability in the nematode life cycle by inhibiting root penetration, thereby reducing the reproductive capacity and mobility of nematode and inhibiting the larvae hatching. Crop rotation and the use of resistant cultivars remains the primary management strategy to regulate the populations of these pests, but the

success of this technique is limited by the size of the growing area and the occurrence of mixed populations of nematodes.

The crop rotation system may be associated with the use of resistant cultivars or low reproduction factor of nematodes. In the case of the use of resistant cultivars, Pionner (2012) launched a technical communication that indicates different soybean and maize cultivars chosen according to the reproduction factor of every kind of nematode, from the most harmful to each crop. To the crop rotation, Pionner indicates the use of nonhost species, such as *Crotalaria* spp. According to Tian et al. (2007), a better understanding of the molecular basis of several pathogenic bacterial mechanisms to nematodes will assist in management decisions and could also lead to the development of new strategies for biological control. One example cited by the authors is the recognized factors of chemical attraction between the bacteria and their hosts. Knowledge of these mechanisms could be used to target or attract nematicidal bacteria to the nematodes or to regulate the populations of nematodes by these factors.

In addition to understanding the mechanisms of control of their effectiveness and environmental impacts caused by changes in organic soil, in-depth knowledge of phytochemistry, microbiology, soil chemistry, ecology, and nematology is important. In other words, a multidisciplinary research approach, involving a collaboration of the different aspects mentioned, is the best prospect in achieving the goal of research and helps in developing methods of nematode control, not only for organic farming systems or sustainable agriculture, but also for conventional agriculture (Oka 2010).

## 1.9 Future Recommendations

The demand for safe and "biologically" healthy foods has stimulated an increase in researches on biological control of pests and plant diseases. The pest control through these microorganisms, naturally found in soil, is an important ecosystem service that maintains the stability of agricultural systems and has the potential to mitigate costs for control of pests. In agroecosystems, bacteria and fungi are fairly abundant and may play a role in the regulation of pest populations. Biopesticides covered in this chapter have high application in programs of Integrated Pest Management (IPM). The relevance of such use, in an economic point of view in the agribusiness sector, refers to the reduction of production costs and a possible added value to the product. Furthermore, genetically modified plants with genes from *B. thuringiensis* have great value to be explored in the interaction with the control methods discussed in this chapter. Its importance also stands by applying cutting-edge technology, clean technology, which benefits the ecosystem by decreasing the applications of agrochemicals, which consequently reduces the toxic waste in food.

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# Biological Control of Pest and Diseases Using Fluorescent Pseudomonads

2

B. Meena

**Abstract**

Bacteria which are shown to have potential for biological control of destructive diseases are distributed in many genera. Among them, fluorescent pseudomonads are currently considered as the most effective bacteria for biological control of soil and foliar diseases. Fluorescent pseudomonads enhance the plant growth parameters, and hence, they are called plant growth-promoting rhizobacteria (PGPR). PGPR are known to control a wide range of phytopathogens like fungi, bacteria, viruses, insect pests and nematodes, and they are known to control these pathogens by biocontrol mechanism which may be by competition, or antagonism, induction of systemic resistance by these bacteria in the host plant, thereby containing the invading pathogens. For the management of pest and diseases of crop plants, applications of strain mixtures of PGPR formulations perform better than individual strains. Fluorescent pseudomonads showing various modes of action especially rhizosphere colonization, antibiotic production and induction of systemic resistance would certainly be potential biocontrol agents for the management of pest and diseases of crop plants.

**Keywords**

Biocontrol • PGPR • Formulations • Delivery systems • Mechanisms

## 2.1 Introduction

Biological control of plant diseases opened an era of new technology to manage crop diseases and received the attention of researchers throughout the world, which will enhance the sustainability of agricultural production systems, and to reduce the use of chemical pesticides. The most promising group of plant growth-promoting

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_2, 

rhizobacteria (PGPR) for biocontrol of plant diseases is fluorescent pseudomonads. Fluorescent pseudomonads associated with plants include *Pseudomonas fluorescens, P. putida, P. aeruginosa* and *P. aureofaciens*. Many of the fluorescent pseudomonads, predominantly *P. fluorescens*, have been isolated from suppressive soil for the management of soilborne and foliar diseases. *P. fluorescens* and *P. putida* are easy to isolate and grow in the laboratory, and they are not fastidious in their requirement of nutrition. They are normal inhabitants of the soil and especially of root surface of plants, so they grow well on the root surfaces when introduced artificially. Their generation time is as low as 5.2 h. Among the various biocontrol agents, fluorescent pseudomonads are known to survive both in rhizosphere and in phyllosphere (Wilson et al. 1992).

Rabindran (1994) highlighted that the culture filtrate of *P. fluorescens* isolate PfALR2 caused 100 % reduction in the germination of sclerotia and reduction in the virulence of sclerotia of *R. solani*. Development of resistance in cucumber against *P. syringae* pv. *lachrymans* as well as to the fungal pathogens *Fusarium oxysporum* f. sp. *cucumerinum* and *Colletotrichum orbiculare* by application of *P. fluorescens* 89B-27 was reported (Liu et al. 1995). Meena et al. (2000) reported that foliar application of *P. fluorescens* strain Pf1 induced accumulation of phenolics and PR proteins in groundnut. Significant increase in yield of several crops due to *P. fluorescens* had been reported (Saravanakumar et al. 2007). Uppal et al. (2008) reported that *P. fluorescens* Biotype F isolate DF37 significantly reduced the disease incidence, severity and vascular discoloration of Verticillium wilt in both cultivars Kennebec and Russet Burbank.

Several studies have indicated that PGPR may stimulate the production of biochemical compounds associated with host defence; massive accumulation of phytoalexins and phenolic compounds; increase in the activities of PR proteins, defence enzymes and transcripts; and enhanced lignification. The induction of SAR using various ISR inducers has been of recent interest with quite reasonable success. Induced resistance against Fusarium wilt of carnation caused by *Fusarium oxysporum* f. sp. *dianthi* was found by prior application of *Pseudomonas* spp. strain WCS417r (Van Peer et al. 1991). Verhagen et al. (2009) studied the ability of *P. fluorescens* CHAO to induce resistance in grapevine against *Botrytis cinerea* and highlighted the importance of salicylic acid, pyochelin and pyoverdin in priming phytoalexin responses and induced resistance. Vleesschauwer et al. (2008) demonstrated the ability of *P. fluorescens* WCS374r to trigger ISR in rice (*Oryza sativa*) against the leaf blast pathogen, *Magnaporthe oryzae*, and found that the induced resistance is regulated by an SA-independent but jasmonic acid/ethylene-modulated signal transduction pathway.

Fluorescent pseudomonads are shown to be effective against certain insect and nematode pests (Ramamoorthy et al. 2001). Tian et al. (2007) reported that fluorescent pseudomonads promote plant growth, increase rhizosphere colonization and suppressed nematodes. Pechy-Tarr et al. (2008) found that when *P. fluorescens* CHAO or Pf5 injected into the haemocoel of the tobacco hornworm, *Manduca sexta*, even low doses killed the larvae of *Manduca sexta*. Karthiba et al. (2010) reported that rice plants when treated with bioformulations containing *P. fluorescens* strains Pf1 and AH1 and *Beauveria bassiana* isolate B2 showed a greater accumulation of defence enzymes, lipoxygenase and chitinase activity against leaf folder.

## 2.2 Characteristics of an Ideal Microbial Pathogen

The ultimate aim of biological control is to achieve control over the disease by biological means. A biocontrol agent should grow and persist, or "colonize", the surface of the plant it protects. Usually colonization or even the initial population size of the biocontrol agent suppresses/modulates the pathogen from farther distances by production of allelochemicals. The ideal characteristics of a biocontrol agent include high rhizosphere competence, high competitive saprophytic ability, enhanced plant

growth, ease for mass multiplication, broad spectrum of action, reliable control, safe to environment and compatibility with other rhizobacteria and should tolerate desiccation, heat, oxidizing agents and UV radiation.

## 2.3 Plant Growth-Promoting Activity

The bacteria that provide some benefit to plants are of two general types: those that form a symbiotic relationship with them and those that are free-living in the soil but are often found near, on or even within the roots of plants. Plant growth-promoting rhizobacteria (PGPR) have beneficial effects which have been variously attributed to their ability to produce various compounds including phytohormones, organic acids and siderophores, to fix atmospheric nitrogen, to solubilize soil phosphate, to produce antibiotics that suppress deleterious rhizobacteria or to show some other unidentified mechanisms (Glick 1995).

There are several reports that PGPR have promoted the growth and reproductive parameters of plants ranging from cereals, pulses, ornamentals, vegetable crops, plantation crops and even tree species. Hofte et al. (1991) highlighted that plant growth-promoting strains of *P. aeruginosa* 7NSK2 and *P. fluorescens* ANP15 significantly increased the germination of maize seeds. The growth promotion of winter wheat by treating seeds with several strains of *Pseudomonas* spp. under greenhouse and field condition was reported by De Freitas and Germida (1992). The grain yield of wheat was increased by 46–75 % under greenhouse condition and 11 % under field condition. Dubeikovsky et al. (1993) suggested that indoleacetic acid (IAA) production by *P. fluorescens* might influence the development of blackcurrant cuttings. The strongest effect was observed as changes of root system weight and morphology. The stimulating IAA-mediated effect of bacterial inoculation on the development of the roots of the cuttings was observed. Increase in growth of rice plants by seed treatment with *P. fluorescens* was also reported (Muthamilan 1994).

Tosi and Zazzerini (1994) recorded an increase in the length of sunflower seedlings by seed treatment with *P. fluorescens* strain 14. Significant plant growth promotion with increased runner length and increased leaf number per plant in cucumber by seed and soil application of PGPR was reported (Wei et al. 1996). Williams and Asher (1996) achieved improvement in seedling emergence in proportion of healthy seedling in sugar beet by *Pseudomonas* sp. when compared to seedlings from untreated seeds. Seed coating with pseudomonad isolates like BHU1, A19 and C185 resulted in significantly greater root length, root and shoot biomass, pod yield and nodule number of groundnut compared with the control (Pal et al. 1999). This may be attributed to various factors such as ACC (1-aminocyclopropane-1-carboxylate, sigma) deaminase activity, siderophore production and increase in root length. These bacteria might have enhanced the uptake of nutrients resulting in healthier and better root system and resulting in improved plant growth.

Seed and soil application of PGPR strains showed significant plant growth promotion with increased runner length and increased leaf number per plant in cucumber (Wei et al. 1996). Meena and Marimuthu (2012) observed the boosting effect of *P. fluorescens* Pf formulation on plant growth promotion like plant height, leaf area index, root length, nodules per plant and dry matter production. The growth substrates used in micropropagation are usually devoid of beneficial microorganisms. By introducing such microorganisms to the substrates, it would be possible to lower fertilizer and pesticide inputs and grow the plants in a more sustainable way.

## 2.4 Formulation Development

Major research on biocontrol is centred with the use of cell suspensions of PGPR directly to seed. Technologies become viable only when the research findings are transferred from lab to field. Bacterial cell suspension cannot be used for large-scale field use due to difficulty in storage, transport and handling. Commercial

application of PGPR either to increase crop health or to manage plant diseases depends on the development of commercial formulations with suitable carriers that support the survival of bacteria for a considerable length of time. Carriers should be economical and easily available. The organic carriers used for formulation development are peat, lignite, talc, kaolinite, zeolite, alginate, press mud, sawdust, vermiculite, etc. The carriers with smaller particle size increased the surface area and thereby increased the resistance to desiccation of the bacteria by the increased coverage of the bacterial cells (Dandurand et al. 1994).

The major concern in commercial production systems is the achievement of adequate growth of the biocontrol agent. Mass production is achieved through liquid, semisolid and solid fermentation systems. A powder formulation with a longer shelf life would be beneficial. Talc is chemically referred to as magnesium silicate [$Mg_3Si_4O_{10}(OH_2)$] and available as powder form from industries suited for wide range of applications. It has very low moisture equilibrium, relative hydrophobicity and chemical inertness, reduced moisture absorption and prevents the formation of hydrate bridges that enable longer storage periods. Owing to the inert nature of talc and easy availability as raw material from soapstone industries, it is used as a carrier for formulation development in a large scale.

Addition of certain gums and polysaccharides as a sticker to the bacterial formulations did not reduce the viability of bacterial population (Suslow et al. 1979). Kloepper et al. (1980) described dry gum-talc formulations that permitted the successful introduction of pseudomonads onto potato seeds. *P. fluorescens* strains grown on King's B broth for 48 h were mixed with 100 g of sterilized peat soil at the rate of 50 ml broth per 100 g of soil gave good control of root rot of black gram when it was applied to soil (Samiyappan 1988).

Hofte et al. (1991) reported that *P. fluorescens* was multiplied on modified King's B medium and the cell pellet after centrifugation was resuspended in 10 ml of $MgSO_4$ (0.1 M) and 2 ml of carboxymethyl cellulose (CMC) (2 %). This suspension when treated to maize seeds improved the seedling emergence. Seed treatment of winter wheat by fluorescent pseudomonads multiplied in King's B broth for 72 h mixed with 1 % carboxymethyl cellulose and talc improved the plant growth (De Freitas and Germida 1992). Fluorescent pseudomonads multiplied on 10 % trypticase soy broth was mixed with preneutralized, sterile class 3 peat, and pH was adjusted to 6.8 by using fine-grade calcium carbonate. This peat-based inoculum when applied to cotton seeds at the rate of 110 g $kg^{-1}$ seed along with CMC (1 %) as sticker (55 ml $kg^{-1}$) controlled the seedling diseases in cotton under field conditions (Hagedorn et al. 1993).

Talc- and peat-based formulations of *P. chlororaphis* and *Bacillus subtilis* were prepared and used for the management of rhizome rot of turmeric (Nakkeeran et al. 2005). *P. putida* strain 30 and 180 survived up to 6 months in talc-based formulations. The population load at the end of 6th month was $10^8$ cfu $g^{-1}$of the product (Bora et al. 2004). The feasibility of the technique and shelf life of the product have to be evaluated to make the technology as a viable component in disease management. The commercially available formulations of *Pseudomonas* spp. are listed in Table 2.1.

## 2.5 Delivery Systems

PGPR are delivered through several means based on the nature of survival and mode of infection of pathogens. It is delivered through seed, soil, foliage, rhizomes or setts or through combination of several methods of delivery.

### 2.5.1 Seed Treatment

Seed bacterization means treating seeds with bacterial cultures that will improve plant growth; such bacterial cultures that improve plant growth are called as bacterial fertilizers. Protection against damping off of sweet corn incited by *Pythium ultimum* (Pythiaceae) was observed due to biopriming (Callan et al. 1990). They

**Table 2.1** Commercially available formulations of fluorescent pseudomonads

| Commercial product | Antagonistic bacteria | Target pathogens | Manufacturer |
|---|---|---|---|
| Bio-Save 10<br>Bio-Save 100<br>Bio-Save 1000 | *P. syringae* ESC-100 | *Botrytis cinerea*<br>*Geotrichum candidum* | Eco Science Corp, Produce Systems Div., Orlando |
| BlightBan A506 | *P. fluorescens* A506 | *Erwinia amylovora* | Plant Health Technologies, USA |
| Cedomon | *P. chlororaphis* | *Fusarium sp.* | BioAgri AB, Sweden |
| Conquer | *P. fluorescens* | *P. tolaasii* | Mauri Foods, Australia |
| Victus | *P. fluorescens* | *P. tolaasii* | Mauri Foods, Australia |
| BioJect Spotless | *P. aureofaciens* | *Pythium aphanidermatum* | Eco Soil Systems, San Diego, CA |
| Deny | *P. cepacia* | *Rhizoctonia* sp.<br>*Fusarium* sp.<br>*Pythium* sp. | Stine Microbial Products, Shawnee, KS |
| Intercept | *P. cepacia* | *Rhizoctonia* sp.<br>*Fusarium* sp.<br>*Pythium* sp. | Soil Technologies Corp, USA |
| Biocoat | *P. fluorescens* WCS374r | *Fusarium oxysporum* f. sp. *raphani*<br>*Fusarium oxysporum* f. sp. *dianthi* | S&G seeds, BV, Netherlands |

also observed that *Pseudomonas aeruginosa* strain 7NSK2 and *P. fluorescens* strain ANP15 inoculated onto maize seeds protected seeds from cold-shock damage and increased the germination. Weststeijn (1990) reported that tulip root rot caused by *P. ultimum* was suppressed by dipping tulip bulbs in *Pseudomonas* suspension of $2 \times 10^9$ cells $ml^{-1}$. Transfer of technology for commercial use could be possible if PGPR strains are available as a product. Seed bacterization with peat-based formulation of *P. fluorescens* strain 1 at the rate of 10 g $kg^{-1}$ seed reduced rice blast and sheath blight disease (Muthamilan 1994; Rabindran 1994; Rabindran and Vidhyasekaran 1996). Meena et al. (2001) reported that seed treatment with powder formulation of *P. fluorescens* resulted in significant reduction in root rot incidence of groundnut under field conditions. Gamliel and Katan (1993) reported that tomato root when inoculated with *P. alcaligenes* reduced the wilt caused by *F. oxysporum* f. sp. *vasinfectum*. In cotton, seedling disease caused by *Rhizoctonia solani* and *P. ultimum* was suppressed by *Pseudomonas* spp. under field conditions (Hagedorn et al. 1993). Treatment of tomato seeds with powder formulation of PGPR (*Bacillus subtilis, B. pumilus*) reduced symptom severity of tomato mosaic virus and increased the fruit yield (Murphy et al. 2000). Nagaraj et al. (2004) tested *P. fluorescens* strains isolated from the rice rhizosphere for their antagonistic effect towards rice sheath blight fungal pathogen, *R. solani*. Meena et al. (2006) found that when the groundnut seeds were treated with *P. fluorescens* and sown in soil, the antagonist colonized well in the groundnut rhizosphere.

### 2.5.2 Soil Application

Soil being as the repertoire of both beneficial and pathogenic microbes, delivering of PGPR strains to soil will increase the population dynamics of augmented bacterial antagonists and thereby would suppress the establishment of pathogenic microbes onto the infection court. Weststeijn (1990) found that root rot in tulip caused by *P. ultimum* was reduced by mixing *Pseudomonas* suspensions thoroughly through the soil to a concentration of $10^8$ cells $g^{-1}$ dry soil before planting the bulbs. Wilt disease of sunflower was found to be suppressed when *P. cepacia* strain N24 was applied to the seedbeds at the rate of 500 ml $m^{-2}$ under greenhouse conditions (Hebber et al. 1991). Take-all disease of wheat

was found to be suppressed by applying 120 ml of *P. aureofaciens* suspension to 13 kg of soil as atomized mist produced by use of chromatography sprayer and compressed air (Mazzola et al. 1992). Hagedorn et al. (1993) highlighted that furrow application of *Pseudomonas* spp. at the rate of 14 ml $m^{-1}$ increased the seedling stand in cotton. The improved seedling stand was due to the suppression of seedling disease in cotton by the antagonistic bacteria.

### 2.5.3 Foliar Application

The efficacies of biocontrol agents for foliar diseases are greatly influenced by microclimate. The concentration of nutrients like amino acids, organic acids and sugars exuded through stomata, lenticels, hydathodes and wounds varies highly. It affects the efficacy and survival of antagonist in phylloplane. Kelly Cartwright (1995) reported that three spray applications of *P. cepacia* to cuttings during a two-week period were more effective than either one or two bacterial sprays in the control of *Rhizoctonia* stem rot of Poinsettia. Rice blast (*P. oryzae*) can be effectively controlled by foliar spray of talc-based powder formulation of *P. fluorescens* strain Pf1 (1 kg $ha^{-1}$). The effectiveness of spraying persisted up to 15 days. When the bacterial product was sprayed on plants grown from treated seed, the effectiveness was higher than when spraying was carried out without any prior seed treatment (Vidhyasekaran et al. 1997). The dosage and frequency of application has to be standardized based on the crop value, which could be a reliable and practical approach. Selected strains from many genera of bacteria isolated from these suppressive soils have the potential to reduce plant diseases when applied to the plant root environment (Weller et al. 2002). The biological control of plant pests and diseases using a single organism has been reported to give inconsistent and poor performance. Bioformulations combining *P. fluorescens* Migula strains Pf1 and AH1 and *Beauveria bassiana* (Balsamo) Vuill. isolate B2 effectively reduced the incidence of leaf folder insect and sheath blight disease on rice plants and showed the possibility of controlling both pest and disease using a single bioformulation (Karthiba 2010).

### 2.5.4 Multiple Delivery Systems

Plant pathogens establish host-parasite relationship by entering through infection court such as rhizosphere, spermosphere and phyllosphere. Hence, protection of sites vulnerable for the entry and infection of pathogens would offer a better means for disease management. Meena et al. (2002) reported that combined application of *P. fluorescens* formulation to seed and foliage effectively controlled foliar diseases of groundnut and increased the pod yield. In rice, seed treatment followed by root dipping and foliar spray with *P. fluorescens* showed higher induction of ISR against sheath blight pathogen, *R. solani* (Radjacommare et al. 2004). Similarly, Viswanathan and Samiyappan (2001) reported PGPR-mediated ISR against red rot disease in sugarcane. Application of PGPR strains showed enhanced resistance to bacterial speck and spot of tomato (Kavitha and Umesha 2007).

The influence of plant growth promotion and induced systemic resistance (ISR) resulted in enhancing the disease resistance in tea plants against blister disease by PGPR bioformulations (Saravanakumar et al. 2007). Delivering of rhizobacteria through combined application of different delivery systems will increase the population load of rhizobacteria and thereby suppress the pathogenic propagules. PGPR formulations comprising of bacterial strain mixtures having the capability to induce chitinase in plant play an important role in hydrolyzing chitin, the structural component in gut linings of insects, and would lead to better control of insect pest (Broadway et al. 1998). In addition, certain PGPR strains also activate octadecanoid, shikimate and terpenoid pathways. This in turn alters the volatile production in the host plant leading to the attraction of natural enemies. Identification of entomopathogenic PGPR strains that have the capability to colonize

phylloplane in a stable manner will be a breakthrough in the management of foliar pests (Otsu et al. 2004). Combined application of entomopathogenic strains with compatible PGPR strains which have the ability to suppress plant diseases has to be developed for broad-spectrum action.

## 2.6 Mechanisms of Biocontrol

Besides the capacity to colonize roots intensively for an extended period of time, other mechanisms are involved that make the fluorescent pseudomonads an effective biocontrol agent. PGPR that indirectly enhance plant growth via suppression of phytopathogens do so by a variety of mechanisms. These include the ability to produce siderophores that chelate iron, making it unavailable to pathogens; the ability to synthesize antifungal metabolites such as antibiotics, fungal cell wall-lysing enzymes or hydrogen cyanide, which suppress the growth of fungal pathogens; the ability to successfully compete with pathogens for nutrients or specific niches on the root; and the ability to induce systemic resistance. Multiple interactions occur between the bacteria and between bacteria and other microorganisms involving competition, antibiosis, parasitism and predation. Various interactions also occur between bacteria and plant roots that can be beneficial, neutral or harmful to the plant. Biochemical and molecular approaches are providing new insight into the genetic basis of these traits, the biosynthetic pathways involved, their regulation and importance for biological control in laboratory and field studies (Nelson 2004). The beneficial effects of these bacteria, in most cases, have been related to their ability to produce plant growth hormones and/or antimicrobial substances and to protect growing roots from deleterious root microbes present in the rhizosphere (Harish et al. 2008).

### 2.6.1 Antibiosis

Antibiosis is now often implicated as an important mechanism of biological control, resulting from the fact that it is an attractive mechanism to study and can provide a highly effective mode of action. Single strains of *Pseudomonas* produce several different antibiotics. Since PGPR being a potential candidate in disease management through multiple modes of action, it becomes highly imperative to know about the role of antibiotics in the management of plant pathogens. Production of antibiotics by rhizosphere bacteria is controlled by complex regulatory networks, in which plant, bacterial and environmental signals are involved. Environmental factors have a significant influence on the production of specific metabolites by fluorescent pseudomonads. Inhibition of pathogens of several crops by the release of diffusible or volatile metabolites such as pyrrolnitrin, pyoluteorin, phenazine or cyanide was confirmed by application of DNA technology and biochemical reaction techniques (Gutterson 1990).

The compound 2,4-diacetylphloroglucinol (DAPG) is a phenolic molecule produced by certain plant-associated fluorescent pseudomonads of worldwide origin (Thomashow et al. 1997). It has antifungal, antibacterial, antihelminthic and phytotoxic properties. DAPG is synthesized by condensation of three molecules of acetyl coenzyme A with one molecule of malonyl coenzyme A to produce the precursor monoacetylphloroglucinol, which is subsequently transacetylated to generate DAPG by a biosynthetic route utilizing chalcone synthase (CHS)-type enzyme (Shanahan et al. 1992). *P. fluorescens* strain CHAO suppressed black root rot of tobacco caused by *Thielaviopsis basicola* and take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici*. The suppression was due to the production of 2,4-diacetylphloroglucinol (Keel et al. 1992). Phenazine comprises a large family of heterocyclic nitrogen containing brightly coloured pigments with broad-spectrum antibiotic activity. Several strains of fluorescent pseudomonad produce antifungal metabolites, namely, phenazines (Thomashow et al. 1997). Suppression of take-all of wheat by *P. fluorescens* strain 2-79 was mainly due to the production of antibiotic phenazine-carboxylic acid (Thomashow and Weller 1990). Though phenazine plays a vital role in the management of soilborne pathogens, the chemotaxis

and motility of the bacteria decides the antifungal action of the antibiotic producers.

Pyrrolnitrin (3-chloro-4-(2′-nitro-3′-chlorophenyl)pyrrole) is a broad-spectrum antifungal metabolite produced by many fluorescent strains of the genus *Pseudomonas*. The biological control agent *P. fluorescens* BL915 contains four gene clusters involved in the biosynthesis of antifungal molecule. Pyoluteorin is an aromatic polyketide antibiotic consisting of a resorcinol ring, which is derived through polyketide biosynthesis. It is produced by several *Pseudomonas* spp. that suppress plant diseases incited by phytopathogenic fungi (Maurhofer et al. 1994). It mainly inhibits the oomycetous fungi, including *P. ultimum* against which it is strongly active. Paul and Sharma (2006) reported the production of two antibiotics—pyoluteorin and pyrrolnitrin by *P. fluorescens*—which inhibits the growth of *Phytophthora capsici*, the pathogen on black pepper. Sreenivasulu et al. (2006) reported that the volatile metabolite of *P. fluorescens* completely inhibited the pathogen of basal stem rot of coconut, *Ganoderma lucidum*.

### 2.6.2 Hydrogen Cyanide Production

Hydrogen cyanide production by certain fluorescent pseudomonads was found to influence the plant root pathogens. Suppression of black root rot of tobacco (*Thielaviopsis basicola*) by *P. fluorescens* CHAO was mainly due to the production of hydrogen cyanide (Stutz et al. 1986). Voisard et al. (1989) reported that mutants of CHAO deficient in HCN production were less suppressive than the parental strain to *T. basicola* in tobacco. Defago et al. (1990) highlighted that cyanide secreted by *P. fluorescens* strain CHAO played a role in the suppression of take-all (*G. graminis* var. *tritici*) and root rot (*R. solani*) of wheat. Root rot suppression by *P. fluorescens* strain E-11-3 was due to the production of HCN which influences the pathogen or the host or both. Wei et al. (1991) reported that four PGPR strains of *P. fluorescens*, namely, G8-4, *P. aureofaciens* 28-9 and 36-5 and *P. putida* 34-13, produced HCN in vitro, whereas two strains *P. aureofaciens* 25-33 and *Serratia plymuthica* 2-67 that induced resistance in the host showed no HCN production. There are suggestions that the biocontrol of pathogens through HCN production by certain fluorescent *Pseudomonas* may be due to the induction of plant resistance against certain pathogens.

### 2.6.3 Competition for Nutrients and Space

Competition between pathogenic and saprophytic microorganisms for organic materials released from the roots can reduce growth and/or pathogenic activity of the pathogens. Many successful antagonists that do not produce antibiotics are able to grow rapidly at the wound sites and are better to extreme nutrients and environmental conditions compared with postharvest pathogens. Unless an organism can compete favourably with other organisms and effectively scavenge and utilize favourable nutrients, it will not constitute a significant proportion of the rhizosphere population. The involvement of competition for nutrients in biological control by fluorescent *Pseudomonas* spp. was suggested in several studies. It was found that in vitro antagonistic activity is based on competition and correlated with disease suppression. Moreover, addition of specific substrates to the plant pathogen system reduced biological control (Elad and Chet 1987). All disease-suppressive mechanisms exhibited by fluorescent pseudomonads are essentially of no real value unless these bacteria can successfully establish themselves at the root environment. Nutrient competition varies in different rhizospheres, depending on the available sources of carbon, nitrogen, sulphur, phosphate and micronutrients. It is not yet very clear whether a superior ability utilizes a particular type of nutrient or nutrients provide advantage to fluorescent pseudomonads.

### 2.6.4 Siderophore Production

Siderophores are low-molecular-weight molecules that are secreted by microorganisms to take up iron

from the environment, and their modes of action in suppression of disease were thought to be solely based on competition for iron with the pathogen. These siderophores have been classified as either pyoverdins or pseudobactins. The production of these siderophores has been linked to their disease suppression ability (Loper and Buyer 1991). Production by certain fluorescent pseudomonads of extracellular, water soluble, yellow and green pigments in KB medium that fluoresce in UV light is known for a hundred years. All such pigment (or siderophore)-producing pseudomonads *P. aeruginosa, P. fluorescens* and *P. putida* belong to one intrageneric homology group. Pseudobactin, the first siderophore, was isolated, purified and characterized by X-ray crystallography from *P. fluorescens* strain B10. The involvement of pseudobactin production by fluorescent pseudomonads in biological control was reported by several workers (Weller 1988; Loper and Buyer 1991). Lemanceau et al. (1992) indicated that the production of pseudobactin 358 by *P. putida* WCS358 appeared to be responsible for the suppression of Fusarium wilt of carnation. Bhavani and Abraham (2005) found that two strains of *P. fluorescens* antagonists of *Phytophthora palmivora* causing pod rot of cocoa produced appreciable quantities of siderophores to suppress the pathogen. The production of siderophores by bacterial antagonist as one of the mechanism of antagonism against *Fomes lamaoensis* in tea was reported (Chakraborty et al. 2006).

### 2.6.5 Induced Systemic Resistance

Induced resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated (Audenaert et al. 2002). Induced resistance results from perception of rhizobacteria by plant roots which give rise to an increased level of resistance which expressed upon subsequent infection by a pathogen. Localized induction of resistance at the site where eliciting bacteria are present on the roots is difficult to demonstrate, because a challenging pathogen will also be subject to bacterial antagonism at this same location. In contrast, no direct interaction between inducing bacteria and a challenging pathogen is possible when each organism is present at spatially separated sites and no contact between the two is established. Enhanced resistance due to ISR by PGPR is achieved by induction of defence compounds of phenylpropanoid pathway and PR proteins (pathogenesis-related proteins). Induced systemic resistance triggered in some rhizobacterial strains depends on salicylic acid (SA) signalling in the plants. Induced resistance by *P. aeruginosa* 7NSK2 was found to be iron regulated and involved three siderophores, pyoverdin, pyochelin and salicylic acid. Salicylic acid is also a precursor in the production of SA-containing siderophores, such as pseudomonine in *P. fluorescens* WCS374 (Audenaert et al. 2002). The transcriptome of rhizobacteria-induced systemic resistance in *Arabidopsis* revealed that root colonization by *P. fluorescens* WCS417r did not lead to transcriptional changes in the leaves, whereas in the roots there is a large set of genes that are differentially transcribed (Verhagen et al. 2004).

Several studies have indicated that PGPR may stimulate the production of biochemical compounds associated with host defence; massive accumulation of phytoalexins and phenolic compounds; increase in the activities of PR proteins, defence enzymes and transcripts; and enhanced lignification. Peroxidase (PO) catalyzes the last step in the biosynthesis of lignin and other oxidative phenols, and it is associated with disease resistance in plants. In groundnut, increased activity of PO was observed due to application of *P. fluorescens*, and PO isoforms were expressed at higher levels (Meena et al. 2000). Phenylalanine ammonia lyase (PAL) is the first enzyme involved in phenylpropanoid pathway and plays a key role in the biosynthesis of phenolics and phytoalexins. When cucumber roots were treated with *P. corrugata* 13 or *P. aureofaciens* 63-28, PAL activity was stimulated in root tissues in 2 days, and this activated accumulation lasted for 16 days after bacterization (Chen et al. 2000).

Induction of higher PPO activity was noticed in tomato and hot pepper pretreated with fluorescent pseudomonads strain against *Pythium* diseases (Ramamoorthy et al. 2002). Phenolics are fungitoxic in nature and increase the physical and mechanical strength of the host cell wall. M'Piga et al. (1997) reported that application of *P. fluorescens* strain 63-28 brought about cell wall thickening, deposition of phenolic compounds and formation of callose resulting in restricted growth of *F. oxysporum* f. sp. *radicis-lycopersici*. Such rapid defence reactions at the site of fungal entry delay the infection process and allow sufficient time for the host to build up other defence reactions to restrict pathogen growth. Recently reports on mechanism of biological control revealed that several microbial strains protect plants from various pests, diseases and phytonematodes in several crops by activating defence genes; encoding chitinase, glucanase, peroxidase and synthesis of phytoalexins; and inducing physiological changes (Kavino et al. 2007; Rajendran et al. 2007; Saravanakumar et al. 2007; Harish et al. 2008).

Pathogenesis-related proteins are designated as PRs and are defined as proteins coded by the host plant but induced specifically in pathological or related situations. They are not only accumulated locally in the infected leaves but also induced systemically associated with the development of systemic induced resistance against further infection by pathogens (van Loon et al. 1994). Induced resistance by PGPR is associated with the accumulation of PR proteins (Radjacommare et al. 2004). Application of PGPR strains showed enhanced resistance to bacteria speck and spot of tomato (Kavitha and Umesha 2007) and anthracnose disease in mango (Vivekananthan et al. 2004). The influence of plant growth promotion and ISR resulted in enhancing the disease resistance in tea plants against blister disease by PGPR bioformulations (Saravanakumar et al. 2007).

## 2.7 Conclusion

PGPR have gained worldwide importance and acceptance for agricultural benefits. These microorganisms are the potential tools for sustainable agriculture and the trend for the future. Scientific researches involve multidisciplinary approaches to understand adaptation of PGPR to the rhizosphere, mechanisms of root colonization, effects on plant physiology and growth, biofertilization, induced systemic resistance, biocontrol of plant pathogens, production of determinants, etc. The technology of commercial use of biocontrol agents has tremendous potentials. The inconsistency in performance of these PGPR strains is a major constraint to their widespread use as biocontrol agent in commercial agriculture. However, genetic manipulation of PGPR has the potential to construct significantly better strains with improved biocontrol efficacy. The applications of mixture of biocontrol agents may be a more ecologically sound approach because it may result in better colonization and better adaptation to the environmental changes occurring throughout the growing season.

## 2.8 Future Recommendations

Future strategies are required to clone genes involved in the production of antibiotics, siderophores and other metabolites and to transfer these cloned genes into the strains having the good colonization potential along with other beneficial characteristics. PGPR offer an environmentally sustainable approach to increase crop production and health. The application of molecular tools is enhancing our ability to understand and manage the rhizosphere and will lead to new products with effectiveness. Trends in research include the increased use of biorational screening processes to identify microorganisms with potential for biocontrol, increased testing under semicommercial and commercial production conditions and increased emphasis on combining biocontrol strains with each other and with other control methods, integrating biocontrol into an overall system. Research should be initiated on the development of improved formulations, adjuvants and protectants for microbial fungicides. Critical studies on the field survival of the antagonists based on biochemical and molecular methods have to be strengthened.

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# Role of Entomopathogenic Fungus in Pest Management

3

J. Alice R.P. Sujeetha and K. Sahayaraj

**Abstract**

Insect pests cause enormous damage to our crops. However, insects like other living things have their enemies in nature. Microorganisms like fungi, bacteria, and viruses also play a vital role in checking the insect population. Among which fungi parasitize the insects and cause severe epizootics than bacteria and viruses, and it is distributed worldwide. During the late nineteenth and early twentieth centuries, many entomopathogenic fungi were examined as possible control agents for various insect pests. Entomopathogenic fungi belong to 750 species that cause infections in insects or mites. Fungus is very specific and belongs to the superkingdom Eukaryota. During the recent years, there has been a resurgence of interest in entomopathogenic fungi caused by factors such as increasing insecticide resistance and environmental concerns over pesticide use. This has led to the isolation and identification of native isolates of fungi from a wide range of hosts. Many entomopathogenic fungi are relatively common and often induce epizootics and are therefore an important factor in regulating insect populations. The research has to be focused in mass multiplication and formulations of fungi using locally available cheap and cost-effective materials.

**Keywords**

Entomopathogenic fungus • Distribution and diversity • Pest management

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## 3.1 Introduction

Insect pests cause enormous damage to our crops. There are a majority of insects like beetles, aphids, hoppers, scale insects, white flies, thrips, plant bugs, moths, borers, etc., that are inimical when they reach a high population level. However, insects like other living things have their

K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_3, 

enemies in nature. Microorganisms like fungi, bacteria, and viruses also play a vital role in checking the insect's population. Among which fungi parasitize the insects and cause severe epizootics than bacteria and viruses. During the late nineteenth and early twentieth centuries, many entomopathogenic fungi were examined as possible control agents for various insect pests. During the recent years, there has been a resurgence of interest in entomopathogenic fungi caused by factors such as increasing insecticide resistance and environmental concerns over pesticide use. This has led to the isolation and identification of native isolates of fungi from a wide range of hosts. Increasing awareness about abuse of chemical pesticides had stimulated renewed interest in the development of alternative and environmentally compatible materials for insect pest control. In this regard, entomopathogenic fungi have been considered as one of the components of IPM. Before the advent of the "Green Revolution", our farmers largely relied on organic manure and cultural method of pest management, which were helpful in promoting biocontrol agents. Diversified fungal species occur on insect pests in different ecosystem, thus maintaining a biotic tolerance to keep the pest population below economic injury level.

Fungi have been suggested as agents for the biological control of insects for over a century, but their use remains extremely limited. Under natural conditions, fungi are a frequent and often important natural mortality factor in insect population. Some species are facultative generalist pathogens, such as *Aspergillus* and *Fusarium*. However, most species are obligate pathogens; they are quite specific. These fungi often have a wide host range although there is considerable genetic diversity within species (Driver et al. 2000). Many entomopathogenic fungi are relatively common and often induce epizootics and are therefore an important factor in regulating insect populations. Unlike other potential biocontrol agents, fungi do not have to be ingested to infect their hosts but invade directly through the cuticle so that they can be effectively used for the control of all the insects including sucking insects.

Over 100 different fungal genera have been shown to parasitize living insects (Roberts and Yendol 1971). Both saprotrophs and primary pathogens have been isolated from dead or diseased insects in most ecological niches. They tend to be more common in tropical areas where temperature and humidity favour their growth, but they are also important in the regulation of insect number in temperate areas. Fungal pathogens flourish abundantly in the rice ecosystem with its prevailing high humidity and infection occurs naturally (Narayanasamy 1994). Epizootics of fungus *Fusarium pallidoroseum* (Cooke) Sacc. (Manisegarane and Letchoumanane 1996) and *Beauveria bassiana* (Bals.) Vuill. (Ambethgar 1996) were noticed at Karaikal, India. Epizootics caused by naturally occurring viral and fungal pathogens are often responsible for spectacular crashes of insect pest populations (Evans 1986). Introduction of fungal pathogens into the host population initiates epizootics and prevents or reduces damage by the pest. The initiation of artificial epizootics has been accomplished for long-term control especially in areas where high humidity condition prevails (Sandhu et al. 2012). To date, the use of entomopathogenic fungi has been considered as one of the potential tools in biological control. This chapter outlines the current state of knowledge of insect fungal pathogens as it relates to their present use and future potential as mycoinsecticides.

## 3.2 Distribution and Pathogenicity of Entomopathogenic Fungi

The entomopathogenic fungi include taxa from several of the main fungal groups. Many common and/or important entomopathogenic fungi are in the order Hypocreales of the Ascomycota: the asexual (anamorpha) phases (e.g. *Beauveria*, *Metarhizium, Nomuraea*, *Paecilomyces* = *Isaria*, *Hirsutella*) and the sexual (teleomorph) state (e.g. *Cordyceps*, *Entomophthora*, *Zoophthora*, *Pandora*, *Entomophaga*) which belong in the order Entomophthorales. Entomopathogenic fungi are an important and widespread component of most terrestrial ecosystems. Furthermore,

entomopathogenic fungi are distributed in a wide range of habitats including aquatic forest, agricultural, pasture, desert, and urban habitats. It seems they are not only in places where there are no victims – insects nor other arthropods. Of course, the spread of individual species of entomopathogenic fungi is different. However, some of them can be found practically throughout the world. The effects of factors such as geographical location, climatic conditions, habitat type, cropping system, and soil properties, as well as the effects of biotic factors on the occurrence and distribution of entomopathogenic fungi, have been broadly studied elsewhere by many microbiologists.

Entomopathogenic fungi have been also recorded north of the Arctic Circle. They have been *Tolypocladium cylindrosporum*, *B. bassiana*, and *Metarhizium anisopliae* in Norway (Klingen et al. 2002) and *B. bassiana*, *M. anisopliae*, and *Isaria farinosa* (=*Paecilomyces farinosus*) in Finland (Vänninen 1995). In addition, entomopathogenic fungi have been reported also from Arctic Greenland (Eilenberg et al. 2007) and Antarctica. In the latter location including endemic Antarctic species, *Paecilomyces antarctica* isolated from the Antarctic springtail *Cryptopygus antarcticus* in the peninsular Antarctic (Bridge et al. 2005). It is associated in variety of habitats. India is bestowed with a rich biodiversity of entomopathogens, and the exploitation of these natural and renewable resources is essential in a successful biocontrol strategy. Performance of a bait matrix treated with the entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) Sorokin, Strain Saravan (DEMI 001), against termite *Microcerotermes diversus* Silvestri was evaluated. The highest rate of mortality occurred at concentrations of $3.7 \times 10^7$ and $3.5 \times 10^8$ conidia per mL (Cheraghi et al. 2013).

Entomogenous fungi are potentially the most versatile biological control agents, due to their wide host range that often results in natural epizootics. These fungi comprise a heterogenous group of over 100 genera with approximately 750 species, reported from different insects. Many of these offer a great potential in pest management. The most important fungal pathogens are *Metarhizium* spp., *Beauveria* spp., *Nomuraea rileyi, Verticillium lecanii*, and *Hirsutella* spp. (Table 3.1).

**Table 3.1** Occurrence and pathogenicity of entomopathogenic fungi in different insect hosts in India and various countries

| Crop | Common name | Scientific name | Location | Entomopathogenic fungi |
|---|---|---|---|---|
| **Different localities in India** | | | | |
| Rice | Green leafhopper (GLH) | *Nephotettix virescens* Dist. | Ludhiana | *Penicillium* spp., *Alternaria tenuis*, *Curvularia* spp., *Fusarium oxysporum* Schlecht |
| | Brown plant hopper (BPH) | *Nilaparvata lugens* (Stal.) | Karaikal | *Pandora delphacis* (Hori) Humber |
| | BPH and GLH | *N. lugens* and *N. virescens* | Annamalai Nagar, Chidambaram | *P. delphacis* |
| | BPH and WBPH | *N. lugens* and *Sogatella furcifera* (Horv.) | Coimbatore | *Metarhizium anisopliae* (Metsch.) Sor. |
| | BPH, GLH, and WBPH | *N. lugens, N. virescens* and *S. furcifera* | Ludhiana | *Hirsutella* spp. |
| | BPH, GLH, and WBPH | *N. lugens*, *N. virescens* and *S. furcifera* | Ludhiana | *M. anisopliae* |
| | BPH and GLH | *N. lugens* and *N. virescens* | Ludhiana | *Aspergillus flavus* Link, *A. niger, Mucor* spp., *Rhizopus* spp. |

(continued)

**Table 3.1** (continued)

| Crop | Common name | Scientific name | Location | Entomopathogenic fungi |
|---|---|---|---|---|
| | BPH and GLH | *N. lugens* and *N. virescens* | Ludhiana | *Beauveria bassiana* (Bals.) Vuill. |
| | Stem borer | *Chilo auricilius* (Dudgn) | Ludhiana | *B. bassiana* |
| | Yellow rice stem borer, leaf folder, yellow hairy caterpillar, and cutworm | *Scirpophaga incertulas* (Walk.), C*naphalocrocis medinalis* (Guenee), and *Psalis pennatula* (Fab.) | Annamalai Nagar, Chidambaram | *M. hiemalis* Wehmer., *F. moniliforme* Shield, and *Scopulariopsis* spp. |
| | Leaf folder | *C. medinalis* | Karaikal | *B. bassiana* |
| | Leaf folder | *C. medinalis* | Karaikal | *Aspergillus* spp. |
| | Leaf folder | *C. medinalis* | Karaikal | *Fusarium pallidoroseum* (Cooke) Sacc. |
| | Leaf folder | *C. medinalis* | Karaikal | *Fusarium pallidoroseum* (Cooke) Sacc. |
| | Leaf folder | *C. medinalis* | Karaikal | *Zoophthora radicans* (Brefeld) Batko |
| | Leaf folder and stem borer | *C. medinalis* and *S. incertulas* | Amritsar | *B. bassiana* |
| | | | | *A. parasiticus* Speare |
| | Leaf folder | *C. medinalis* | Ludhiana | *B. bassiana* |
| | | | | *Penicillium* spp. |
| | | | | *A. niger* var. Tieghem |
| | Leaf folder | *C. medinalis* | Karaikal | *B. bassiana* |
| | Leaf folder | *C. medinalis* | Karaikal | *F. pallidoroseum* |
| | Leaf folder | *C. medinalis* | Karaikal | *Z. radicans* |
| | Leaf folder | *C. medinalis* | Punjab | *Nomuraea* spp. |
| | Leaf folder | *C. medinalis* | Arunachal Pradesh | *B. velata* Samson and Evans |
| | Leaf folder and skipper | *C. medinalis* and *Pelopidas mathias* (Fab.) | Ludhiana | *B. bassiana* |
| | Leaf folder and ear head bug | *C. medinalis* and *Leptocorisa acuta* (Thumb) | Coimbatore | *B. bassiana* |
| | Skipper | *Marasmia patnalis* Bradley | Karaikal | *B. bassiana* |
| | Skipper | *P. mathias* | Cuttack | *B. bronbniartii(= tenella)* |
| | Hispa | *D. armigera* Oliv. | Assam | *B. bassiana* |
| | Hispa | *D. armigera* | Ludhiana | *B. bassiana* |
| | Green horned caterpillar | *Melanitis leda ismene* Cramer | Ludhiana | *F. oxysporum* |
| | Grasshopper | *Oxya nitidula* (Walk.) | Karaikal | *B. bassiana* |
| | Grasshopper | *O. nitidula* | Karaikal | *Aspergillus* spp. |
| | Cutworm | *Pseudaletia unipuncta* (Haworth) | Karaikal | *N. rileyi* (Farlow) Samson |
| | Ladybird beetle | *Coccinella septempunctata* L. | Lucknow | *B. bassiana* |
| | Grass hopper | *Hieroglyphus banian* | Coimbatore | *M. anisopliae* |
| | Grass hopper | *Oxya nitidula* | Coimbatore | *M. anisopliae* |
| Sorghum | Ear head caterpillar | *Helicoverpa armigera* Hubner | Bangalore | *N. rileyi* |
| Red gram | American boll worm | *H. armigera* | Bapatla | *B. bassiana* |
| | American boll worm | *H. armigera* | Bangalore | *N. rileyi* |
| Soybean | Tobacco caterpillar | *Spodoptera litura* (Fab.) | Vridhachalam | *N. rileyi* |
| Pea | Aphids | *Aphis craccivora* Koch | Vellayani | *F. pallidoroseum* |
| Black gram | Spotted pod borer | *Maruca testulalis* | Karaikal | *B. bassiana* |

(continued)

**Table 3.1** (continued)

| Crop | Common name | Scientific name | Location | Entomopathogenic fungi |
|---|---|---|---|---|
| Groundnut | Yellow hairy caterpillar | *Amsacta albistriga* Walker | Karnataka | *B. bassiana* |
| | Tobacco caterpillar and American boll worm | *S. litura* and *H. armigera* | Guntur | *N. rileyi* |
| | Tobacco caterpillar and American boll worm | *S. litura* and *H. armigera* | Hyderabad | *N. rileyi* |
| | Groundnut pests | *S. litura* | Palayamkottai | *B. bassiana* |
| | | *Aproaerema modicella* (Deventer) | | *Paecilomyces fumosoroseus (Wize)* |
| | | *Aphis craccivora* (Koch) | | *Verticillium lecanii (Zimm.)* |
| | | *Mylabris pustulata* Faust | | |
| Sugarcane | Shoot borer | *Chilo infuscatellus* Snell | Coimbatore | *B. bassiana* |
| | White grub | *Holotrichia serrata* (Fab.) | Coimbatore | *M. anisopliae* |
| Cotton | Red cotton bug | *Dysdercus cingulatus* | Thoothukudi | *M. anisopliae* |
| Mango | Leaf webber | *Idioscopus* spp. | Basti (UP) | *B. bassiana* |
| Coconut | Rhinoceros beetle | *Oryctes rhinoceros* L. | New Delhi | *M. anisopliae* |
| | Rhinoceros beetle | *O. rhinoceros* | Coimbatore | *M. anisopliae* |
| Castor | Hairy caterpillar | *Pericallia ricini* Fab. | Palayamkottai | *B. bassiana* |
| | | | | *P. fumosoroseus* |
| | | | | *V. lecanii* |
| Teak | Leaf skeletonizer | *Eutectona machaeralis* (Walker) | Jabalpur | *B. bassiana* |
| | | | | *F. oxysporum Schlecht* |
| **Other parts of the world** | | | | |
| Rice | GLH | *N. bipunctatus* | China | *B. bassiana* |
| | GLH | *N. virescens* | Taiwan | *B. bassiana* |
| | BPH | *N. lugens* | Manila | *B. bassiana* and *M. anisopliae* |
| | WBPH | *S. furcifera* | China | *B. bassiana* |
| | Black bug | *Scotinophara lurida* B. | Philippines | *B. bassiana* |
| | Black bug | *S. coarctata* B. | Korea | *B. bassiana* |
| | Leaf folder | *C. medinalis* | USA | *Z. radicans* |
| | Leaf folder | *C. medinalis* | Philippines | *N. rileyi* |
| | Leaf folder | *C. medinalis* | Philippines | *Paecilomyces farinosus* (Holm ex S.F. Gray) |
| | Leaf folder | *C. medinalis* | Philippines | *B. bassiana* |
| | Leaf folder | *C. medinalis* | Philippines | *B. bassiana* |
| Rice | Leaf folder | *C. medinalis* | Fiji | *M. anisopliae* |
| | Leaf folder | *C. medinalis* | Fiji | *Spicaria rileyi* (Farlow) Charles |
| | Zigzag leafhopper | *Recilia dorsalis* (Mot.) | Philippines | *B. bassiana* |
| | Small plant hopper | *Laodelphax striatellus* | China | *B. bassiana* |
| Maize | Stem borer | *Chilo partellus* (Swinhoe) | Kenya | *B. bassiana* |
| | Stem borer | *Eldana saccharina* W. | Ghana | *Verticillium aboatrum* |
| | | | | *Aspergillus flavus* |
| | | | | *Fusarium oxysporum* |
| | Western corn Rootworm | *Diabrotica virgifera* LeConte | Hungary | *Metarhizium anisopliae* |
| | | | | *Beauveria bassiana* |

(continued)

**Table 3.1** (continued)

| Crop | Common name | Scientific name | Location | Entomopathogenic fungi |
|---|---|---|---|---|
| Soybean | Cabbage looper | *Trichoplusia ni* Hubner | Columbia | *N. rileyi* |
| Crucifers | Diamond back moth | *Plutella xylostella L.* | England | *Z. radicans* |
| | Cabbage looper | *T. ni* | USA | *S. rileyi* (Farlow) Charles |
| Coconut | Rhinoceros beetle | *O. rhinoceros* | France | *M. anisopliae* |
| | Rhinoceros beetle | *O. rhinoceros* | New Zealand | *M. anisopliae* |
| Guava | Scale | *Icerya seychellarum* | Egypt | *V. lecanii* |
| | | | | *M. anisopliae* |
| | | | | *B. bassiana* |
| Potato | Ladybird beetle and Colorado potato beetle | *Coleomegilla maculata lengi, Leptinotarsa decemlineata* (Say) | Canada | *B. bassiana* |
| – | Aphids | *Myzus persicae* Sulzer | Britain | *Verticillium lecanii* (Zimm.) Viegas |
| – | – | Lepidoptera, Coleoptera | UK | *B. velata, B. amorpha* |
| Elm | Bark beetle | *Scolytus scolytus* (Fab.) | England | *B. bassiana, M. anisopliae, P. farinosus* |
| Peas | Aphid | *Acyrthosiphon pisum* Harris | England | *Entomophthora* sp. |
| – | Bug | *Lygus hesperus* (Knight) | California | *B. bassiana* |
| – | Grasshopper | – | Argentina | *Fusarium verticillioides* |
| Pine | Bark beetle | *Ips* spp. | Bulgaria | *Beauveria bassiana* |
| | | | | *Isaria farinosa* |

## 3.3 Isolation and Identification of Entomopathogenic Fungi

Most of the entomopathogenic fungi are found within the deuteromycetes and entomophthorales. Entomopathogens such as *M. anisopliae* and *B. bassiana* are well characterized in respect to pathogenicity to several insects, and they have been used as agents for the biological control of agriculture pests worldwide. The entomopathogenic fungi can be collected from naturally infested dead cadavers and also from soil.

### 3.3.1 Dead Cadavers

The insects were found either sticking to the leaf sheath or floating on standing water, being overgrown by a chalky white mass of conidia. The cadavers were collected in sterile glass tube for isolating the causal organism in the laboratory. The insect cadavers were surface sterilized with 0.5 % sodium hypochlorite in 75 % alcohol and rinsed in three changes of sterile water for 1–2 min. Washed specimen was cut into pieces and placed on Sabouraud's dextrose agar (SDA) with yeast extract medium. The fungal colonies appeared were observed. Tip of the mycelial growth was transferred aseptically into the SDA slants. The cultures were purified and maintained in SDA slants (Dayakar and Kanaujia 2001).

### 3.3.2 Soil

Sixteen rice moth *Corcyra cephalonica* (Stainton) larvae were placed in a plastic container with the soil collected from four layers of soil at four larvae per layer. Baited plastic container was left at room temperature for 3–5 days (Zimmermann 1986). In this technique, *Corcyra* was used instead of *Galleria*. After 3 days, the dead larvae were examined for the fungal

infection under microscope. The fungus associated with mycosed larvae were isolated as mentioned above. Sahayaraj and Borgio (2009) isolated *Metarhizium anisopliae* from the soils collected from various regions in Tirunelveli district of Southern Tamil Nadu, India. Very recently, Sánchez-Peña et al. (2011) used *Tenebrio molitor* (Coleoptera: Tenebrionidae) larval baits for the collection of selective entomopathogenic fungi from soil in four adjacent habitats (oak forest, agricultural soil, pine reforestation, and chaparral habitat) in Saltillo, México. The persistence of fungal pathogens was found to be higher in soil than the phyllosphere indicating that they can be naturally favoured for the control of pests in groundnut (Sahayaraj and Karthick Raja 2011). The major entomopathogenic fungi recovered from samples were *Beauveria bassiana*, *Metarhizium anisopliae*, *Paecilomyces* spp., *B. brongniartii*, *P. chlamydosporia*, and *Lecanicillium attenuatum*. The diversity of entomopathogenic fungi was greater in soil samples from forests compared to crop fields, vegetables, and fruits, respectively, in Pakistan (Waqas et al. 2013).

## 3.4 Identification

Entomogenous fungi can be identified by ARS Collection of Entomopathogenic Fungal Cultures, US Department of Agriculture, Agricultural Research Service, US Plant, Soil, and Nutrition Laboratory, Tower Road, Ithaca, NY.

### 3.4.1 *Beauveria* sp.

*Beauveria bassiana*, a filamentous fungus, belongs to a class of insect pathogenic deuteromycetes also known as imperfect fungus. An example of such species may be *Beauveria bassiana* which is reported from tropical rainforest (Aung et al. 2008) and has been found in Canada as far north as latitude 75° (Widden and Parkinson 1979). Strains of *Beauveria* are highly adapted to particular host insects. Broad ranges of *B. bassiana* spp. have been isolated from a variety of insects worldwide which are of medicinal or agricultural importance. *Beauveria bassiana* is a fungus that grows naturally in soils throughout the world and acts as a pathogen on various insect species, causing white muscardine disease. The colonies of the fungus are white in colour with cottony aerial mycelium. Conidiophores are single, or branched, oblong, cylindrical, or flask shape bearing laterally or at extremity vesicles giving rise to sporogenous cells (phialides). Phialides generally globose, sometimes cylindrical, flask like, and curved or straight. Conidia are globose (1–4 μ) to oval shape (1.5–5.5 × 1–3 μ).

### 3.4.2 *Metarhizium anisopliae*

This is commonly called as green muscardine fungus. *Metarhizium anisopliae* author family *is* also a very potential pathogen on insect pests and is explored for mycobiocontrol of notorious insect pests. This fungus is widely distributed and recorded in more than 300 hosts. The affected cadavers show typical green aerial mycelia. Initially the colony appears white in colour and later turns to green in colour. Mycelium is composed of hyaline, septate, branched hyphae. Conidiophores are short, erect, hyaline, septate, simple, or branched, terminating in single or a cluster of phialides. Conidia are single celled, hyaline, smooth, and long ovoid to cylindrical (4.8 × 1.6 μ). Based on the size of the conidia, *M. anisopliae* is divided into two varieties, namely, *M. anisopliae var. anisopliae* and *M. anisopliae var. major*. The former has short conidia, of 3–9 μ length, and the latter has long spores, of 9–18 μ length. Scanning electron microscope studies revealed that naturally fungi *B. bassiana* and *M. anisopliae* infection begins when conidia (asexual spores, the seeds of a fungus) attach to insect's cuticle, the spores germinate and penetrate the insect's skin and enter the host. Once the fungus penetrates the host; it produces toxins that overcome the insect immune system. Thereafter, the hyphae penetrate through the cuticle to the outside and cause white (*B. bassiana*) or green (*M. anisopliae*) sporulation on the insect's body (Kabarty et al. 2014).

### 3.4.3 *Verticillium lecanii (=Lecanicillium lecanii)*

Another entomopathogenic fungus *Verticillium lecanii* is a widely distributed fungus, which can cause large epizootics in tropical and subtropical regions, as well as in warm and humid environments. It is known primarily as a pathogen of aphids, scales, white flies, thrips, and red spider mites. It is also called as white halo fungus. The fungus is characterized by the presence of conidiophores in verticillate whorls and on which conidia are borne in slime or mucus balls.

### 3.4.4 *Nomuraea* sp.

*Nomuraea rileyi*, another potential entomopathogenic fungus, is a dimorphic hyphomycete that can cause epizootic death in various lepidopteran and coleopteran insects. It has been shown that *Spodoptera litura* and some belonging to *Coleoptera* are susceptible to *N. rileyi*. The host specificity of *N. rileyi* and its ecofriendly nature encourages its use in insect pest management. The colonies of the fungus are white initially and later turn to green in colour (malachite green). Hyphae are 2–3 μ in diameter, smooth, septate, hyaline, and slightly pigmented. Conidiophores are long (160 μ) and consist of dense compacted clusters of phialides and branches in whorls on the upper section. The branches are short and swollen. Phialides are short and cylindrical to globose, with very swollen base tapering abruptly to a narrow neck. The conidia are 3–4 × 2.5 μ and pale green in colour.

### 3.4.5 *Paecilomyces* sp.

*Paecilomyces* is a genus of nematophagous fungus which kills harmful nematodes by pathogenesis, causing disease in the nematodes. Thus, the fungus can be used as a bio-nematicide to control nematodes by applying it to soil. *Paecilomyces lilacinus* principally infects and assimilates eggs of root-knot and cyst nematodes. The fungus has been the subject of considerable biological control research following its discovery as a biological control agent in 1979. *Paecilomyces fumosoroseus* (Wize) *P. farinosus* causes a sickness called "yellow muscardine" in white flies. The colonies of these fungi are white, red, or yellow in colour, and phialides occur as divergent loose groups.

### 3.4.6 *Hirsutella thompsonii*

*Hirsutella thompsonii* Fisher is a well-known fungal pathogen which is commonly associated with the acarines. This parasitic fungus was originally described by Fisher, who isolated it from the citrus rust mite, *Phyllocoptruta oleivora*, in Florida. It has a grey, fluffy mycelial growth and a brownish to greyish-green substratum colour. The hyphae are 1.5–2.0 μm wide and smooth. The conidiogenous cells arise singly at intervals from the vegetative hyphae, mono- or polyphialide, unevenly verrucose, with a conical to flask-shaped base and a narrow neck. The neck may be unbranched or branched, bearing enteroblastic conidia singly at the tip of the branch.

## 3.5 Mode of Action of Entomopathogenic Fungus (EPF)

In contrast to bacteria and viruses that pass through the gut wall from contaminated food, fungi have a unique mode of infection. They reach the haemocoel through the cuticle. Fungi invade insects by penetrating their cuticle or "skin". Once inside the insect, the fungus rapidly multiplies throughout the body. Death is caused by tissue destruction and, occasionally, by toxins produced by the fungus. The fungus frequently emerges from the insect's body to produce spores that, when spread by wind and rain or contact with other insects, can spread infection. Attachment of a fungal spore to the cuticle surface of a susceptible host represents the initial event in the establishment of mycosis. When the pathogen reaches and adheres to the host surface, it proceeds with rapid germination and growth which are profoundly influenced by the availability of water, nutrients, oxygen as well as pH, and temperature and by the effects of toxic host-surface compound. Fungi invade their hosts by

penetration of the host cuticle or put pressure on cuticle by making appressorium and then penetrate by penetration peg (Sandhu 1995).

In many areas of the cuticle, the chitin is organized helically giving rise to a laminate structure. Conidia germinate on the host surface and differentiate an infection structure termed appressorium (St Leger 1991). Entomopathogenic fungi need to penetrate through the cuticle into the insect body to obtain nutrients for their growth and reproduction. Entry into the host involves both enzymatic degradation and mechanical pressure as evidenced by the physical separation of lamellae by penetrated hyphae. A range of extracellular enzymes that can degrade the major components of insect cuticle, including chitinases, lipases, esterases, and at least four different classes of proteases, have been suggested to function during the fungal pathogenesis. The fungi begin their infective process when spores are retained on the integument surface, where the formation of the germinative tube initiates; the fungi starts excreting enzymes such as proteases, chitinases, quitobiases, lipases, and lipoxygenases after the successful penetration; and the fungus is then distributed into the haemolymph by formation of blastospores (Bhattacharyya et al. 2004). The entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* are ubiquitously distributed in soils. As insect pathogens, they adhere to the insect cuticle and penetrate through to the insect haemocoel using a variety of cuticle-hydrolysing enzymes. Once in the insect haemocoel, they are able to survive and replicate within, and/or evade, phagocytic haemocyte cells circulating in the haemolymph (Michael et al. 2010; Sahayaraj et al. 2013). Shaukat Ali et al. (2014) reported that lipases play an important role in the infection process of entomopathogenic fungi by hydrolyzing the ester bonds of lipo proteins, fats and waxes present on the insect surface and in the body. This was studied in the scale insect *Isaria fumosorosea* and confirmed that extra cellular lipase enzyme produced by this insect can be exploited for enzyme based insect management.

## 3.6 Mass Multiplication

Culture conditions influence the virulence of any fungi (Ortiz-Urquiza et al. 2013); hence, it is imperative to study about the culture of entomopathogenic fungi. Hussey and Tinsley (1981) observed that wheat bran was considered to be a suitable substrate for mass production of *B. bassiana*. Silva and Loch (1987) reported that *N. rileyi* had been multiplied on polished rice grain. Soybean chunk (Pandit and Som 1988), carrot medium (Deva Prasad 1989), and rice grain (Rama Mohan Rao 1989) were recommended for the mass production of *B. bassiana*. Recently grains, vegetable wastes, seeds, rice husk, sawdust, and liquid media such as coconut water, rice and wheat washed water, and rice cooked water recommended for the mass production of *B. bassiana* (Sahayaraj and Karthick Raja 2008). However, par-boiling, water, and washing regimes of the rice substrate were recommended by Taylor et al. (2013). Mathai et al. (1998) studied the use of different locally available cheaper substrates for the mass multiplication of *F. pallidoroseum* and found that wheat bran alone and rice bran with tapioca bits gave maximum spore count followed by wheat bran with straw bits. Vyas et al. (1989) showed that crushed maize was shown to be an ideal substrate for culturing *B. brongniartii*. Liu et al. (1990) observed that chitin-supplemented rice grain medium gave the highest germination of *B. bassiana* in the laboratory. Devi (1994) reported that crushed sorghum was effective for the conidia production in *N. rileyi*. Narayanasamy (1994) observed that *P. delphacis* was mass produced on the cereal grain of *Sorghum vulgare* and *Pennisetum typhoides* and it sporulated enormously in few days.

Mazumder et al. (1995) evaluated the locally available industrial waste substrates for culturing *B. bassiana* and revealed that husk supplementation with 2 % dextrose was found to be more suitable. Udayaprabhakar (1995) tested various natural substrates and noticed sorghum-impregnated media was found to be efficient for the mass production of the fungi *P. delphacis* and

*M. hiemalis*. Faizal and Mathai (1996) suggested that wheat bran and rice bran were found to be suitable substrates for the mass production of *F. pallidoroseum* for the control of aphids. Puzari et al. (1997) reported that *B. bassiana* could be mass multiplied with cellulose wastes, namely, rice hull, sawdust, and rice bran either alone or in combination. Parthasarathy (1997) reported that sorghum and maize grain media excelled among the various diets evaluated with respect to biomass production, radial growth, and spore germination of *Z. radicans*. Reji Rani et al. (2000) found that the fungus *F. pallidoroseum* can be mass multiplied well on the naturally available substrates like cowpea leaves and cotton seed cake and gave maximum spore count and mycelial growth, followed by rice bran, gingelly cake, and fallen *Pongamia* leaves. Among the liquid media tried, coconut water was found to yield maximum spore count.

Green muscardine fungus *M. anisopliae* could be mass multiplied using tapioca chips method and coconut water method (Mohan and Gopal 2002). Sharma et al. (2002) observed that *M. anisopliae* produced maximum sporulation on molasses yeast broth; the highest conidia of *Beauveria* species were obtained with molasses yeast broth and bajra followed by sorghum and maize grains for *M. anisopliae* and cowpea grains for *B. bassiana* and *B. brongniartii*. Several techniques for the mass production of entomogenous fungi were mostly designed to yield infective conidia in large numbers. Wadyalkar et al. (2003) reported that potato dextrose broth was found to be the best in spore production for *M. anisopliae*. Sahayaraj and Karthick Raja (2008) reported that wheat grains supported maximum spore production for *B. bassiana*, while sorghum recorded maximum spore production in *P. fumosoroseus* and *V. lecanii*. Similarly carrot, jack seeds, and lady's finger also supported good growth and sporulation of all the three tested fungi. Coconut water supported maximum growth and sporulation. Paddy chaffy grains support maximum growth for all the three *Fusarium* isolates. Paddy chaffy grains support maximum sporulation for *B. bassiana* isolates and *F. moniliforme* GLH isolate. Similarly, rice bran recorded maximum sporulation for *F. pallidoroseum* leaf folder isolate (Senthamizhlselvan and Alice 2008). Jagadeesh Babu et al. (2008) reported that the highest spore count was recorded in the broken rice and followed by that broken jowar.

*Nomuraea rileyi* is a well-known fungal pathogen thriving in a range of obligate hosts, particularly the polyphagous species of *Heliothis*, *Spodoptera*, *Pseudoplusia*, *Trichoplusia*, *Plutella*, and *Rachiplusia* (Boucias et al. 1984; Chen et al. 2012). However, the lack of reliable cost-effective protocols for mass production of this entomopathogenic fungus limits its commercialization. Thakre et al. (2011) studied various agricultural products like rice, sorghum, and wheat and agro wastes, namely, refuse raw potatoes and refuse raw bananas for mass scale cultivation of entomopathogenic fungus *Nomuraea rileyi*. Among the grains, rice ($5.53 \times 10^7$ spore/g) supported the maximum spore production of fungus followed by refuse raw bananas ($4.2 \times 10^7$ spores/g) and sorghum ($4.01 \times 10^7$ spores/g) on the 11th day after inoculation of spore suspension.

## 3.7 Formulations

Next to isolation of virulent fungi, the important step is to identify the use of appropriate formulations against insect pests. Formulations can greatly improve the efficacy of entomopathogens. A substantial number of mycoinsecticides and mycoacaricides have been developed worldwide since the 1960s. Products based on *Beauveria bassiana* (33.9 %), *Metarhizium anisopliae* (33.9 %), *Isaria fumosorosea* (5.8 %), and *B. brongniartii* (4.1 %) are the most common among the 171 products. Examples of few commercial formulations are given in Table 3.2 (de Faria and Wraight 2007). The key components were active ingredient, carrier, stickers, surfactants, UV protectants, etc. The principal ingredients in most biopesticide products based on insect pathogenic fungi (mycopesticides) are dry spores called conidia, and viability of these conidia is

**Table 3.2** Examples of some of the fungal formulations used for the management of various pests in different localities

| Fungus | Country | Product name | Target insect pests |
|---|---|---|---|
| *Beauveria bassiana* | Czech Republic | Boverol | Coleoptera (Chrysomelidae) |
| | Czech Republic | Boverosil | Coleoptera (Curculionidae) and stored pests |
| | France | Ostrinil | Lepidoptera (Crambidae) |
| | Hawaii | BotaniGard ES | Coleoptera |
| | | Mycotrol | |
| | Spain | Trichobass | Coleoptera (Curculionidae, Scarabaeidae), Lepidoptera (Castniidae, Pieridae), Hemiptera (Aleyrodidae), Thysanoptera (Thripidae) + Acari (Tetranychidae) |
| | South Africa | Bb Plus | Hemiptera (Aphididae) + Acari (Tetranychidae) |
| | South Africa | Bb Weevil | Coleoptera (Curculionidae) |
| | India | BioGuard | Coleoptera (Curculionidae, Scarabaeidae), Hemiptera (Aleyrodidae, Aphididae), Lepidoptera (Crambidae), Thysanoptera (Thripidae) |
| | | Rich | |
| | India | Bio-Power | Coleoptera (Curculionidae, Scarabaeidae), Hemiptera: Auchenorrhyncha (Cicadellidae, Delphacidae), Lepidoptera (Plutellidae) |
| | India | Racer | Lepidoptera (Noctuidae) + |
| | Russia | Boverin | Hemiptera (Aleyrodidae), Thysanoptera (Thripidae) + Acari (Tetranychidae) |
| | Mexico | Bea-Sin | Coleoptera (Curculionidae, Scarabaeidae), Hemiptera (Aleyrodidae) |
| | Mexico | Bio-Fung | Orthoptera |
| | USA | Balence | Diptera (Muscidae) |
| | USA, Mexico, Denmark, Italy, Spain, Sweden, Japan | BotaniGard 22 WP | Coleoptera (Curculionidae, Scarabaeidae), Hemiptera (Miridae, Cicadellidae, Fulgoridae, Aleyrodidae, Aphididae, Pseudococcidae, Psyllidae), Thysanoptera (Thripidae) |
| | USA, Mexico, Denmark, Italy, Sweden | Mycotrol ES | Coleoptera (Chrysomelidae, Curculionidae, Scarabaeidae), Hemiptera (Miridae, Cicadellidae, Fulgoridae, Aleyrodidae, Aphididae, Pseudococcidae, Psyllidae), Lepidoptera (Crambidae), Orthoptera (Acrididae, Tettigoniidae), Thysanoptera (Thripidae) |
| | USA, Mexico, Greece, Italy, Spain, Switzerland | Naturalis L | Coleoptera, Diptera Lepidoptera, Hemiptera, Hymenoptera, Orthoptera, Thysanoptera and Acari |
| | USA | Organigard | Lepidoptera, Hemiptera,, Orthoptera, Thysanoptera |
| | Costa Rica, Panama | Beauvedieca | Coleoptera (Curculionidae) |
| | Costa Rica | Nativo 2 SC | Coleoptera (Curculionidae) |
| | Egypt | Bio-flay Biosect | Hemiptera |
| | Brazil | Boveriol | Isoptera (Rhinotermitidae, Termitidae) |
| | Colombia | Ago Biocontrol Bassiana 50 | Coleoptera, Diptera, Hemiptera, Lepidoptera |
| | Colombia, Dominican Republic | Bauveril | Coleoptera (Curculionidae, Scarabaeidae), Lepidoptera (Castniidae) |
| | Colombia | Conidia | Coleoptera (Curculionidae) |

(continued)

**Table 3.2** (continued)

| Fungus | Country | Product name | Target insect pests |
|---|---|---|---|
| *Beauveria brongniartii* | Austria, Italy | Melocont-Pilzgerste | Coleoptera (Scarabaeidae) |
| | Switzerland | *Beauveria brongniartii* | Coleoptera (Scarabaeidae) |
| | | Myzel | |
| | Reunion Island | Betel | Coleoptera (Scarabaeidae) |
| | Japan | Biolisa Kamikiri | Coleoptera (Cerambycidae) |
| *Hirsutella thompsonii* | India | MeteHit | Acari |
| | India | Mycohit | Acari (Eriophyidae) |
| | USA | Mycar | Acari (Eriophyidae) |
| *Isaria fumosorosea* (formerly *Paecilomyces fumosoroseus*) | Europe | PreFeRa | Hemiptera (Aleyrodidae) |
| | India | Priority | Acari (Eriophyidae,Tetranychidae) |
| | Mexico | Pae-Sin | Hemiptera (Aleyrodidae) |
| | Mexico | *P. fumosoroseus* | Hemiptera (Aleyrodidae) |
| | USA, Mexico | PFR-97 20 % WDG | Hemiptera (Aleyrodidae, Aphididae), Thysanoptera (Thripidae) + Acari (Tetranychidae) |
| | Colombia | Ago Biocontrol *Paecilomyces* 50 | Coleoptera + Nematoda |
| | Venezuela | Bemisin | Hemiptera (Aleyrodidae) |
| | India | PaciHit Rich | Hemiptera (Aleyrodidae), Thysanoptera (Thripidae) + Nematoda |
| *Lecanicillium* sp. (formerly *V. lecanii*) | Spain | Trichovert | – |
| | India | Bio-Catch | Hemiptera (Aleyrodidae, Aphididae, Pseudococcidae) |
| | India | Biovert | "Insects" + Nematoda |
| | | Rich | |
| | India | Mealikil | Hemiptera: Sternorrhyncha ("scales") + others (not specified) |
| | Colombia | Ago Biocontrol *Verticillium*50 | Hemiptera, Diptera |
| | Scandinavia | MicroGermin | Hemiptera (Aleyrodidae, Aphididae) |
| *Metarhizium anisopliae* | Germany, Switzerland | BIO 1020 | Coleoptera (Curculionidae) |
| | Switzerland | *Metarhizium* | Coleoptera (Scarabaeidae) |
| | | Andermatt | |
| | | C + H/TK | |
| | | *Metarhizium* | |
| | | Schweizer | |
| | | C + H/TK | |
| | India | Pacer | Isoptera |
| | Australia | BioCane | Coleoptera (Scarabaeidae) |
| | | Granules | |
| | | Biological | |
| | | Insecticide | |

(continued)

**Table 3.2** (continued)

| Fungus | Country | Product name | Target insect pests |
|---|---|---|---|
| | Mexico | Fitosan-M | Coleoptera (Scarabaeidae), Orthoptera |
| | Mexico | Meta-Sin | Coleoptera (Curculionidae, Scarabaeidae), Hemiptera (Cercopidae), Orthoptera |
| | USA, Mexico | Bio-Blast | Isoptera (Kalotermitidae, Rhinotermitidae, Termopsidae) |
| | | Biological | |
| | | Termiticide | |
| | USA | Bio-Path | Blattodea (Blattellidae, Blattidae) |
| | | Cockroach | |
| | | Control | |
| | | Chamber | |
| | USA | MET 52 | Thysanoptera |
| | | | Hemiptera(Aleyrodidae) |
| | Guatemala | Salivase | Hemiptera (Cercopidae) |
| | Brazil | Biocontrol | Hemiptera (Cercopidae) |
| | Brazil, Panama | Biotech | Hemiptera (Cercopidae) |
| | Brazil | Metaquino | Hemiptera (Cercopidae) |
| | Colombia | Ago Biocontrol *Metarhizium* 50 | Coleoptera, Hemiptera, Lepidoptera, Orthoptera |
| | Venezuela | Cobican | Coleoptera (Scarabaeidae), Hemiptera (Cercopidae, Aphididae) |
| *Nomuraea rileyi* | Colombia | Ago Biocontrol *Nomuraea* 50 | Lepidoptera |

one of the most commonly reported indicators of product quality (de Faria et al. 2010). The first attempt to control a pest with a fungal agent was carried out in Russia in 1888, when the fungus now known as *Metarhizium anisopliae* (Metschn.) Sorokın was mass produced on beer mash and sprayed in the field for control of the beet weevil *Cleonus punctiventris* (Germar) (Lord 2005). Recently a new mycoinsecticide was developed from *M. anisopliae* for greenhouse and vegetable growers in the USA (Thomas Ford 2013). Boverin, a *Beauveria bassiana*-based mycoinsecticide for control of the Colorado potato beetle and codling moth in the former USSR, was developed in 1965 (Kendrick 2000). The common formulation that must be ready to use is wettable powder, "A powder formulation to be applied as a suspension after dispersion in water". Another one is suspension concentrate, "A stable suspension of active ingredient in water, intended for dilution with water before use". Oil-miscible flowable concentrate is also becoming popular everywhere during recent time. Effectiveness of oil-based conidia formulation of three indigenous fungal isolates such as *Beauveria bassiana*, *Verticillium lecanii*, and *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) was evaluated against fourth instar larvae of *Pericallia ricini* Fab. (Lepidoptera: Arctiidae) under laboratory conditions and found that oil-based formulations of *V. lecanii* caused the highest mortality of 97.33 % at $3.9 \times 10^9$ spores per $mL^{-1}$(Sahayaraj and Borgio 2012).

## 3.8 Future Thrust Area

Selection of virulent strains through genetic manipulation will be the most important advancement in the use of entomopathogenic fungi. Use of agricultural wastes can be employed for the cultivation of fungus in large

scale. Awareness must be created among the farming community to prepare cheaply available, inexpensive substrates for their use. Sufficient funding can be directed to carry out research in this line. Further, hands-on training can also be initiated in this area of research to scientists and farmers. The main bottleneck in this line of research is lack of quality products. Hence, this area can be strengthened.

## 3.9 Conclusion

At present this area of research is least exploited, even though the entomopathogenic fungi are considered to be one of the important, potential candidates as there are several advantages of using these fungal pathogens as pest control agent. Most of these are host specific and less toxic to mammals having a wide host range, and moreover these mycoinsecticides are ecofriendly too. Survey work should be undertaken in different geographical locations to collect and identify virulent fungal pathogens. The use of mycoinsecticides has to be popularized.

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# Microbial Viral Insecticides

4

Aparna S. Kalawate

**Abstract**

Microbial viral insecticides are pathogens that attack insects and other arthropods. Baculoviruses (BV) are parasitically replicating microscopic elements. Baculoviruses are extremely small and are composed primarily of double-stranded DNA required for the virus to establish itself and reproduce. The genus Baculoviruses contains three subgroups of viral types: nuclear polyhedrosis viruses (NPVs), granulosis viruses (GVs) and nonoccluded viruses. NPVs and GVs differ in the number and structure of the protective protein coat and are both relatively large and complex in structure in comparison to many other types of viruses. While little information is available for viruses from the third subgroup, several aspects of the infectivity and mode of action of NPVs and GVs have been studied. The most common route of entry into an insect is by ingestion. The primary site of infection is the midgut cells by membrane function. However, two distinct mechanisms of virus uncoating occur among the baculovirus, that is, NPVs uncoat within the nucleus, whereas GVs uncoat within the nuclear pore complex. NPVs may pass through the intestinal epithelium immediately after ingestion, thereby establishing a systematic infection of the haemocoel prior to virus replication in the midgut cells. The GVs do not appear to pass through midgut cells as rapidly as NPVs, and the developmental cycle of GVs is longer than that of NPVs.

The NPVs are mass produced in larval hosts grown on artificial diet or host plant. Usually third to fourth instar larvae of *Helicoverpa armigera* are infected with the viral food. The definitive phase of viral disease occurs over a period of 5–10 days. Once the complete infection of the virus in the larvae is completed, the larvae start 'putrefying' releasing billions of polyhedra. In commercial production, larvae are

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_4, 

being harvested before purification to keep bacteria at a low level in the final product. After the larval production phase is complete, the larvae are collected and formulated. NPVs are ideal candidates for use where a single lepidopteran species is the major pest. NPVs are being used against *H. armigera* and *Spodoptera litura* on cotton, corn, sorghum, tomatoes and chrysanthemum. It is also being used against *Anticarsia gemmatalis* of soybean. One of the most important successes in commercial production and use of a GV is *Cydia pomonella* GV (CpGV) on apples and pears. Advantages in using microbial viruses are safety for humans and other nontarget organisms, reduction of pesticide residues, little or no development of resistance by the target organism, no secondary pest outbreak and no preharvest interval is required. Though there are many advantages, some disadvantages are also there, for example, host specificity is a double-edged sword; it is an advantage as well as a disadvantage. Moreover, long period of lethal infection is required, and the virus gets inactivated by environmental factors like ultraviolet light, extreme temperature, etc. In this chapter, an attempt has been made to cover the commercially available BVs for the control of agricultural pest particularly in India. The objective of this chapter was to briefly cover the aspects like importance of baculoviruses in pest control, history, genome and the products available in the Indian market.

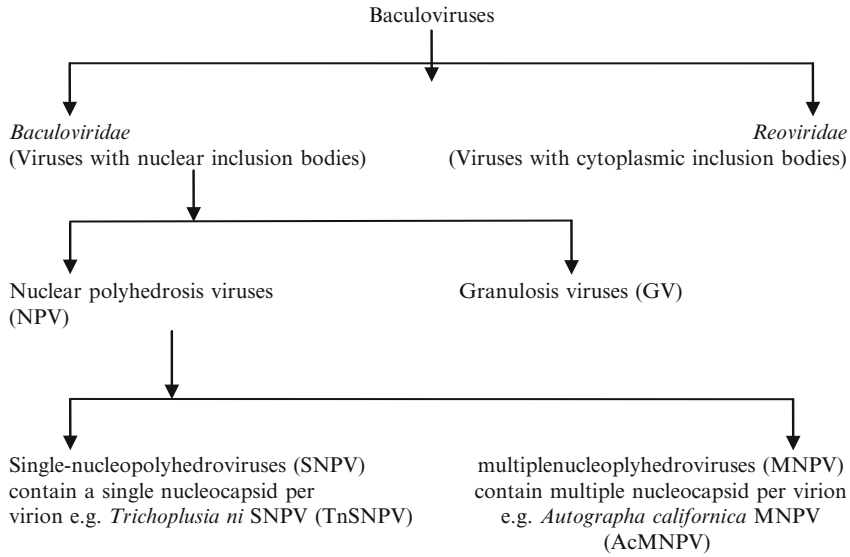
**Keywords**
Baculovirus • Viral insecticides and microbial control • Biopesticides and entomopathogenic viruses

## 4.1 Introduction

Biopesticides are certain types of pesticides derived from such natural materials as animals, plants, bacteria and certain minerals. The EPA separates biopesticides into three major classes based on the type of active ingredient used, namely, microbial, biochemical or plant-incorporated protectants (GMOs). All aspects of the utilisation of microorganisms or their by-products in the control of insect pest species are called microbial control. A virus is a small infectious agent that can replicate only inside the living cells of organisms. Viruses infect all types of organisms, from animals and plants to bacteria and insects. The study of viruses is known as virology, and the study of viruses causing diseases in insects is known as insect pathology. Inclusion viruses are submicroscopic, obligate, intracellular and pathogenic organisms. Seven families of viruses, namely, *Baculoviridae*, *Reoviridae*, *Iridoviridae*, *Poxviridae*, *Parvoviridae*, *Picornaviridae* and *Rhabdoviridae*, cause diseases in insects. But the viruses of families *Baculoviridae* and *Reoviridae* are the most important for their role as biopesticides because of their high virulence.

## 4.2 Classification

Baculoviruses are different from vertebrate viruses and therefore safe to humans and other vertebrates. The classification of baculoviruses is represented in the following flow chart.




### 4.2.1 Baculoviruses

The divisions viz., NPV and GV were recently challenged because the comparison of 29 fully sequenced baculoviral genomes indicated that virus phylogeny followed more closely the classification of the hosts than the virion morphological traits, but the traditional division into two genera is still widely used (Boguslaw Szewczyk et al. 2011). Baculoviruses have double-stranded genome with rod-shaped nucleocapsids. The infectious virus particles or virions are occluded in protein bodies called polyhedra (NPV) or granules (GV). NPV polyhedra are larger than the virions (usually 1–15 μm) and may contain many virions. The infection occurs after a susceptible host eats the polyhedra or granules, which are dissolved in the basic digestive gut juices. The virions are released when the protein matrices dissolve. The virions enter the nuclei of midgut cells and eventually infect many of the tissues and organs in the insect, primarily the fat body, epidermis and blood cells. The baculoviruses which are not occluded in polyhedra have recently been removed from the Baculoviridae group. The infection caused by baculoviruses is called 'wilting disease' because the larva becomes wilted and tissues of the host liquefy; infection of the epidermis causes the host to appear to melt, releasing virus particles into the environment. Often just before death, the larvae climb to the highest part of the substrate and attach themselves by their prolegs.

Baculoviruses are considered to be the most beneficial of the insect viruses to man, because of their utility in insect control, their specificity to the arthropods and their more recent use in fundamental biological studies using molecular techniques. Nevertheless, they also cause diseases in beneficial insects, and, therefore, the use in the environment as biological control agents requires an understanding of host range and the mechanisms that control host specificity (Miller 1997).

## 4.3 History

The virus family Baculoviridae have been known for hundreds of years. The earliest record of baculovirus infection was in Chinese silkworms. Paralysis and subsequent liquification occurring in the larvae affected with baculoviruses were found in many ancient literature. It wasn't until the early twentieth century that it was established that the virus particles were embedded in proteinaceous crystals of polyhedrin. This crystalline

matrix allows the virus to survive in the environment. It was at this stage that baculoviruses were suggested as a method of natural control of pest insect populations. In the 1930s and 1940s, rod-shaped virions were identified within the crystalline polyhedrin. During the same period, baculoviruses were observed to be an effective biological control agent of an insect pest. It was discovered that the spruce sawfly (accidentally introduced into North America) could be effectively controlled by the subsequent introduction of a baculovirus.

The first baculovirus to be registered as a pesticide (in 1975) was a commercial failure. However, the use of a baculovirus as a pest control agent that was a nucleopolyhedrovirus used to control the Douglas-fir tussock moth in 1984 was a notable success. This has attracted many investigators to understand the molecular biology of the baculovirus and has led industrial interest in the commercialisation of it in the 1990s. Major achievements have been made in the field of baculovirology in the past two decades. These viruses now have a major role in the field of biomedical research as well as contributing to our understanding of the complex virus-host interactions. Baculoviruses are now being used for making them recombinant by utilising the genetic engineering for insect control.

The first viral insecticide Elcar™ was introduced by Sandoz Inc. in 1975 (Ignoffo and Couch 1981). Elcar™ was a preparation of *Heliothis zea* NPV which is relatively a broad-range baculovirus and infects many species belonging to genera *Helicoverpa* and *Heliothis*. HzSNPV provided control of not only cotton bollworm but also of pests belonging to these genera attacking soybean, sorghum, maize, tomato and beans. In 1982, the production of this biopesticide was discontinued. The resistance to many chemical insecticides including pyrethroids revived the interest in HzSNPV, and the same virus was registered under the name Gemstar™. HzSNPV is a product of choice for biocontrol of *Helicoverpa armigera* (Mettenmeyer 2002). Countries with large areas of such crops like cotton, pigeon pea, tomato, pepper and maize, for example, India and China, introduced special programmes for the reduction of this pest by biological means. In Central India, *H. armigera* in the past was usually removed by shaking pigeon pea plants until caterpillars fell from the plants onto cotton sheets. This technique is now used to obtain caterpillars which are fed on virus-infected seeds. Baculovirus preparations obtained in this way are used by farmers to prepare a bioinsecticide spray applied on pigeon pea fields. Another baculovirus, HaSNPV, is almost identical to HzSNPV. It was registered in China as a pesticide in 1993 (Zhang et al. 1995). It has been used for large-scale biopesticide production and has been extensively used on cotton fields (over 100,000 ha of cotton in the last decade). Broad-spectrum biopesticide based on Ha NPV is also used in India (Srinivasa et al. 2008).

The forests of temperate regions are very often attacked and defoliated by the larvae of Lepidoptera (most common pest species are *Lymantria dispar*, *Lymantria monacha*, *Orgyia pseudotsugata* and *Panolis flammea*) and some Hymenoptera species (mainly *Neodiprion sertifer* and *Diprion pini*). *L. dispar* MNPV formulations marketed under trade names Gypchek, Disparivirus and Virin-ENSH and *O. pseudotsugata* MNPV under trade names TM BioControl-1 and Virtuss (Reardon et al. 1996) are sometimes used for forest protection. Forest ecosystems tend to be more stable than agricultural systems, allowing for natural or applied baculoviruses to remain in the environment for long periods of time increasing the chance of natural epizootics by these agents. Caterpillars of moths belonging to *Spodoptera* genus are of primary concern for agricultural industry in many countries of the world.

Two commercial preparations based on *Spodoptera* NPV have been available. These are SPOD-X™ containing *Spodoptera exigua* NPV to control insects on vegetable crops and Spodopterin™ containing *Spodoptera littoralis* NPV which is used to protect cotton, corn and tomatoes. About 20,000 ha of maize annually was controlled with *Spodoptera frugiperda* NPV in Brazil (Moscardi 1999), but at present it has not been used due to technical problems in

the virus production under laboratory conditions. The use of *Spodoptera litura* NPV has been tested on cabbage crops in India (Kumari and Singh 2009). Many other species belonging to the *Noctuidae* family are economically important pests of sugarcane, legume, rice and others. *Autographa californica* and *Anagrapha falcifera* NPVs were registered in the USA and were field-tested at a limited scale. These two NPVs have relatively broad host spectrum and potentially can be used on a variety of crops infested with pests belonging to a number of genera, including *Spodoptera* and *Helicoverpa*.

Granulovirus CpGV is the active component of a number of biopesticides used for the protection of apple and pear orchards against the codling moth *Cydia pomonella*. Some of the trademarks of CpGV-based products are: Granusal™ in Germany, Carpovirusine™ in France, Madex™ and Granupom™ in Switzerland and Virin-CyAP in Russia. Annually up to 250,000 ha of orchards has been protected with Madex™ in different European countries (Vincent et al. 2007). Considering application of all trade names of the CpGV, this may be the most important worldwide viral insecticide currently applied in terms of treated area.

Other important viruses that are currently employed to control insects include the tea tortricids *Adoxophyes honmai* and *Homona magnanima* granuloviruses (GV) in Japan. The area sprayed with GVs comprised 5,850 ha in Kagoshima in 1995, equivalent to 80 % of all the tea fields in the prefecture (Nishi and Nonaka 1996). The GVs of *H. magnanima* and *A. honmai* were registered in 2003; however, the use of GVs has recently declined. One reason for the reduction in use of GVs in Japanese tea fields is the changing pattern of occurrence of other pests. Mulberry scale, for example, has been increasing recently, and chemical treatment is required to control this insect and at the same time GVs are sprayed. The spray also kills *H. magnanima* and *A. honmai*. Furthermore, GVs have been applied in Kagoshima for more than 10 years, and the populations of *H. magnanima* and *A. honmai* have been reduced (Nakamura 2003). In China, 12 baculoviruses have been authorised as commercial insecticides (Sun and Peng 2007), including *H. armigera* NPV (the most widely used virus in China for cotton, pepper and tobacco protection), *S. litura* NPV (vegetables), *S. exigua* NPV (vegetables), *Buzura suppressaria* NPV (tea), *Pieris rapae* GV and *Plutella xylostella* GV (vegetables). The use of baculoviruses in China is the greatest worldwide, regarding the number of viruses being registered for insect control. Sun and Peng (2007) also reported a cypovirus (CPV) produced in China for the control of *Dendrolimus punctatus*, an insect pest of pine forests. The well-known success of employing baculovirus as a biopesticide is the case of *Anticarsia gemmatalis* nucleopolyhedrovirus (AgMNPV) used to control the velvet bean caterpillar in soybean (Moscardi 1999). This programme was implemented in Brazil in the early 1980s and came up to over 2,000,000 ha of soybean treated annually with the virus. Recently this number dropped down, mainly due to new emerging pests in the soybean complex. The use of AgMNPV in Brazil brought about many economical, ecological and social benefits. At the soybean grower level, the financial savings from the use of the virus may reach up to ca. U$ 7/ha/season, including product cost and application cost. The annual savings at the grower level, in the total area sprayed with the virus, was over US$ 11,000.000. Since the beginning of the programme, more than 17 million litres of chemical insecticides was not sprayed in the environment. The protection of soybean fields in Brazil has proven that baculoviral control agents can be effectively produced on a large scale and they may be an alternative to broad spectrum chemical insecticides. On the basis of this spectacular success of a baculovirus pesticide, it is needless to say that the advantages of biopesticides over chemical pesticides are numerous. Safety for humans and nontarget organisms, preservation of biodiversity in the environment and reduction of toxic residues in agricultural end products are just the examples of potential benefits. However, the cost of biopesticide production has been usually higher than the cost of conventional pesticides (Boguslaw Szewczyk et al. 2011).

Genomic variability has been described for many wild-type viruses including *A. californica*

MNPV, *S. frugiperda* MNPV, *S. litura* MNPV, *P. flammea* MNPV and *Mamestra configurata* NPV. Genotypic variants can be recognised by the presence of submolar fragments in the electrophoretic patterns of restriction endonuclease digestion products of a viral genome. Genotypic variation in baculovirus genomes can include point mutations, both small and large deletions and insertions (Krell 1996). Though mutations can occur in any place of the genome, the presence of some hot spots was observed for certain genomic alterations such as insertions due to transposable elements or deletions in the hypervariable DA26 gene region (Kamita et al. 2003). AgMNPV genomic variability has been also carefully studied because the selection pressure due to the application of AgMNPV in the field during subsequent years could lead to alterations in virus stability. The method of choice was the technique of restriction endonuclease analysis. Viral DNAs were initially purified from diseased larvae collected during several crop seasons and compared to AgMNPV-79, a wild-type virus that was used originally and subsequently in this programme (Souza et al. 2001). These results indicated that the virus maintains considerable stability, even with the existence of some genetic changes shown in the DNA restriction profiles. It has been also observed that the virus retains its virulence to the host insect throughout the years of its application.

## 4.4 Future Use of Baculovirus Pesticides

Large-scale application of AgMNPV in Brazil has proven that the baculovirus protection can be done at relatively low cost. It is very likely that the growing awareness of the benefits of the environment-friendly pesticides will result in the re-evaluation of the prospects for biological protection with baculovirus preparations. However, until today, baculovirus insecticides have not met their full potential to control pest insects worldwide. The development of recombinant baculovirus was efficiently completed by researchers in several countries, but the in vitro commercial technology still lags, due to technical problems. Future development of baculovirus pesticides will probably depend on the attitude towards genetically modified organisms. In countries where use of genetically modified organisms is restricted, only naturally occurring baculoviruses will be used for protection of crops. In this case the improvements will be at the level of diagnostics of infection, development of the in vitro cultures and changes in the formulations of the biopesticide. In countries which favour the introduction of genetically modified organisms, the improvements will be achieved by introduction of exogenous genes into baculovirus genome, thus greatly enhancing the killing activity of bioinsecticide formulations.

Reliable assays for the progress of infection with baculovirus are necessary because the major problem in using biopesticide for crop protection is their slow action and lack of morphological changes in larvae in first stages of baculovirus propagation. The lack of such assays may incline agricultural services to use subsequent chemical means of protection which, from the ecological point of view, may be redundant. Fast and sensitive methods in diagnostics based on baculovirus genome detection will probably play a predominant role in future. For strictly quantitative assays, real-time PCR is the best method.

The in vitro production is still a strong requirement on a commercial perspective of baculoviruses use as insecticides. However, the accumulation of genotypic variations by serial passage in cell culture prevents its large-scale production. One of the most important effects of the viral passage is the change from the parental, many polyhedra per cell (MP) phenotype, to the few polyhedra per cell (FP) phenotype. The major problem of the passage effect is the reduced occlusion and loss of virulence of the occluded virus (Krell 1996). Frequent mutations have been identified within a specific region in the few polyhedra (FP) mutants that contains the 25 k *fp* locus (Harrison and Summers 1995; Lua et al. 2002). This gene encodes a 25 kDa protein that is essential for virion occlusion and polyhedron formation. Another type of mutants generated during serial passage of baculovirus is the formation of defective interfering particles (DIPs).

These mutants have lost the ability to be replicated in the host cell without the aid of a helper virus, and large sizes of their genome are usually deleted (Pijlman et al. 2001). These particles replicate faster because they are smaller and inhibit the replication of a standard virus. The challenge to make in vitro commercial production of baculoviruses a viable initiative depends on the development of new techniques to sustain MP production through passages in cell cultures from small flasks to large-scale commercial fermentors.

The stability of baculoviruses is influenced by temperature, pH, humidity and the presence of additives, but ultraviolet light is probably the most detrimental factor to viral survival. Under field conditions, little activity is left when the virus is not shaded by plant canopy; therefore, much effort has been devoted to the development of UV protectants (Shapiro and Dougherty 1994; Zou and Young 1994; Morales et al. 2001). The best results were obtained for stilbene fluorescent brighteners which are marketed under many trade names (e.g. Phorwite AR, Blankophor and others). Future developments in the formulations of brighteners may lead to the reduction of cost of baculovirus production. Inactivation of baculoviruses may be also caused by plant metabolites such as peroxidases which generate free radicals (Hoover et al. 1998). The inactivation can be reduced by addition of free radical scavengers such as mannitol or enzyme superoxide dismutase to baculovirus preparations (Zhou et al. 2004). The inactivation of Ha NPV was found to be reduced when it was sprayed in combination with adjuvants like *Leucaena* leaf extract, eucalyptus leaf extract and Ranipal in the morning and evening (Kalawate and Nachane 2006). The activity of baculoviruses against their natural hosts may be enhanced by introduction of insect-specific toxins or by interference with insect physiology (Bonning and Hammock 1996; Inceoglu et al. 2001). Baculovirus genome modifications by introduction of exogenous toxin genes were extensively studied in many laboratories. Most of the research was devoted to the studies of arthropod toxin genes isolated from the scorpion or spiders (Bonning and Hammock 1996; Inceoglu et al. 2007). The most potent insect-specific toxin gene used for construction of baculovirus recombinants was the gene coding for a toxin from scorpion *Androctonus australis*. The feeding damage caused by larvae infected with this modified baculovirus was reduced by about 60 % in comparison to a wild-type baculovirus (Inceoglu et al. 2001). Toxin genes isolated from other scorpions, for example, *Leiurus quinquestriatus hebraeus* (Froy et al. 2000), straw itch mite *Pyemotes tritici* (Burden et al. 2000), ants (Szolajska et al. 2004) or spiders (Hughes et al. 1997) have been intensively studied as potential enhancers of baculovirus activity. Arthropod toxins usually attack insect sodium channels producing final effect similar to the chemical insecticides of the pyrethroid group. However, the specific target in sodium channels is different, so there is a potential possibility to produce synergistic effect by biopesticide/chemical pesticide application (McCutchen et al. 1997). Baculovirus recombinants that produced occlusion bodies incorporating *Bacillus thuringiensis* toxin were constructed by making a fusion protein consisting of polyhedron and Bt toxin (Chang et al. 2003). The pathogenicity of the recombinant was remarkably increased compared to wild-type virus. These studies proved that it is possible to construct a biopesticide which combines the advantages of the virus and the bacterial toxin.

The changes to host physiology were done by introducing genes coding for some insect hormones or hormone-modifying enzymes into baculovirus genome or by deletion of the baculovirus-encoded ecdysteroid glucosyltransferase (*egt*) gene. The former approach was employed by cloning juvenile hormone esterase gene into baculovirus genome which overexpressed decreases the concentration of the juvenile hormone which is a signal for a caterpillar to stop feeding and pupate. This line of research is being pursued in some laboratories (Hammock et al. 1990; Inceoglu et al. 2001). The deletion of the baculovirus-encoded *egt* gene was used first by O'Reilly and Miller (1991). The product of the *egt* gene interacts with larval moulting and indirectly increases the time of feeding of

infected caterpillars. The *egt* deletion from baculovirus genome resulted in 30 % faster killing of caterpillars. Another advantage of this genomic modification is the fact that the *egt* gene is not essential for viral replication and can be replaced with an exogenous gene, the product of which may enhance the insecticidal activity of the recombinant virus (Sun et al. 2004).

In the future, genetically modified baculoviruses will contribute to the expansion of baculovirus use worldwide, as these GMOs are considered safe through extensive research conducted over many years. The scientific data indicate that baculoviruses pose no hazard to other animals than their hosts, and this was documented by a number of studies from different laboratories. Recombinant baculoviruses were not pathogenic to bees and all vertebrate species (Sun et al. 2004) as well as to the natural enemies of larvae such as parasitoids and predators (Boughton et al. 2003). However, in spite of this sound evidence, preliminary field trials of genetically modified baculoviruses raised massive public protests which put on hold further trials for a long time. The slow progress in application of genetically modified baculoviruses as pesticides may be in part due to the choice of toxin genes used for modifications of the baculovirus genome which were isolated from highly dangerous invertebrates. Taking into account the origin of these social conflicts, the choice of toxin genes used for genome modifications should be restricted to genes coding for ecologically natural insect toxins, for example, the genes coding for toxic polypeptides of parasitoid wasps occurring in regions infested by a particular pest. The more rational approach is also needed in the social perception of dangers associated with genetically modified baculoviruses by educating the public on risks and benefits of recombinant baculovirus pesticides (Boguslaw Szewczyk et al. 2011).

## 4.5 Genome

Circular and double-stranded DNA genome has been found in baculoviruses. The genome size of these viruses ranges in size from 80 to 180 kbp. Of the fully sequenced baculovirus genomes, the number of open reading frames (ORFs) ranges from approximately 120 to 160 (Fig. 4.1). In addition to the genes encoded in the genome, there are also a number of small repeated sequences known

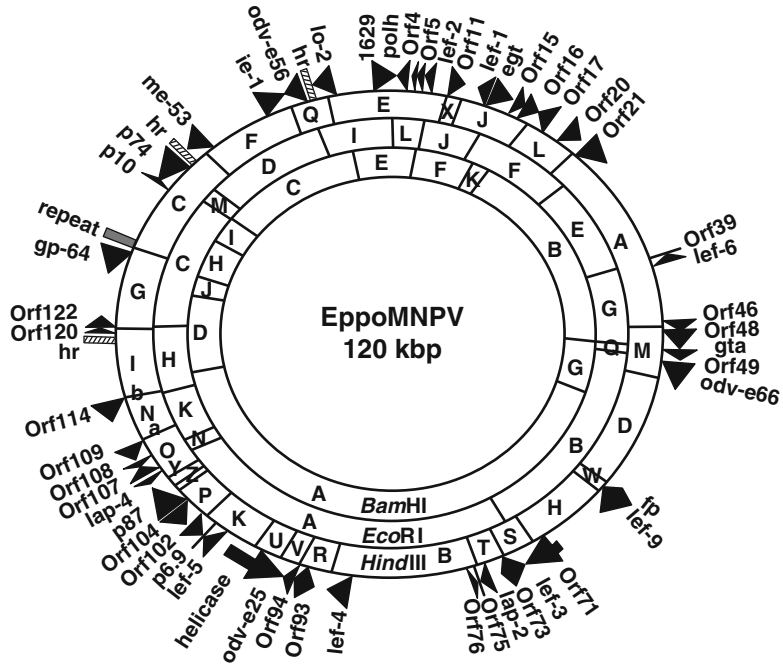



**Fig. 4.1** *Eppo* MNPV genome map (Kalmakoff and Ward 2007)

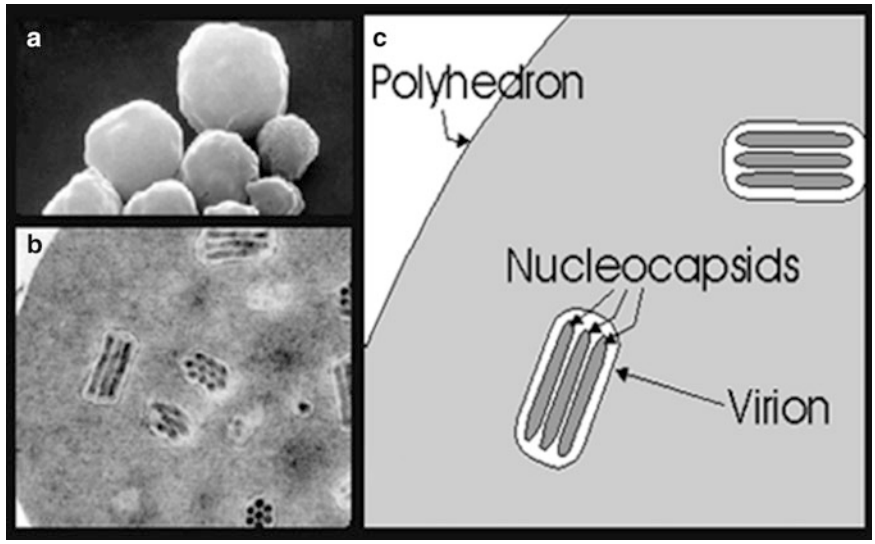



**Fig. 4.2** Baculovirus particles or polyhedra (**a**); cross section of a polyhedron (**b**) and diagram of polyhedron cross section (**c**); electron micrographs (**a** and **b**) by Jean Adams, graphic © by V. D'Amico)

as homologous regions (hrs) interspersed in the genome. These regions have been shown to enhance early gene transcription and also to act as origins of replication. Many of the genes in a baculovirus genome have overlapping ends allowing a large number of genes to be encoded in a smaller amount of DNA (Kalmakoff and Ward 2007).

Baculoviruses have gained great attention in molecular biology laboratories because they are very versatile genetic engineering tools (Van Oers 2006). Current knowledge about the biology of AcMNPV is to a large extent a consequence of the developments of baculovirus-based expression vectors. Baculovirus system of expression of foreign genes has many advantages over other systems because high level of foreign gene expression is usually achieved compared to other eukaryotic expression systems (Boguslaw Szewczyk et al. 2011). Baculovirus genome can accommodate large pieces (up to 50 kbp) of foreign DNA, so it is possible to express more than one foreign gene. Additionally, the insertion of specific signal sequences in front of a foreign gene leads very often to the export of the gene product outside of the infected cell (Boguslaw Szewczyk et al. 2011).

## 4.6 Structure

A distinctive rod-shaped nucleocapsid which is 30–60 nm in diameter and 250–300 nm in length is present in baculoviruses. GVs are occluded with dimensions of about 0.3 × 0.5 μm. The occluded NPVs are polyhedral in shape, and the size of it is approximately 0.15–15 mm. The occluded form of both the baculoviruses (GVs and NPVs) can clearly be seen using a light microscope. The occlusion-derived virus (ODV) is produced in the later stages of viral infection and is enclosed in a proteinaceous occlusion body. The spread of the virus from insect to insect is horizontal, and the virus persists for long periods in the environment. Baculoviruses also have a second morphology. This second form of the virus is found within an infected insect. This form is known as budded virus (BV). BVs generally contain a single nucleocapsid and are enclosed in an envelope obtained as the nucleocapsids bud out through the cell wall. Prior to the budding of the virus, the cell wall is modified by the addition of the viral protein GP64. This protein has been shown to be required for effective spread of the virus within the host (Fig. 4.2).

## 4.7 Life Cycle

The infection of baculovirus starts with the ingestion of the virus-infected material by the insect larvae (Fig. 4.3). Death of the larvae occurs in 3–8 days depending on the larval species and instars (Table 4.1). The life cycle of baculovirus involves two forms of virus, that is, occlusion-derived virus (ODV) and budded virus (BV). The ODV is responsible for the primary infection of the host and is present in a protein matrix of polyhedron or granulin. The BV is released during the secondary infection from the host cell (Fig. 4.4). When a susceptible insect feeds on the virus-contaminated plants, the initial infection occurs. The protein encapsulating the baculovirus DNA dissolves in the alkaline midgut of the larvae releasing ODV. These ODVs then fused with the columnar epithelial cell membrane of the midgut and are taken into the cell in endosomes. Nucleocapsids are then transported to nucleus. Baculovirus DNA is then replicated in the cell nucleus until the rupture of midgut cells takes place. The development of BV occurs and the secondary infection starts. The infection spreads throughout the body in the haemolymph and infects the cells of haemocoel, fat bodies, trachea and hypodermis of the larvae. At this stage, the larvae stop feeding and die eventually (Fig. 4.4). There are different types of proteins present in baculoviruses which are required to carry the infection in the host. The different types of proteins and their functions are presented in Table 4.2.

## 4.8 Relative Effectiveness

It is widely acknowledged that baculoviruses can be as effective as chemical pesticides in controlling specific insect pests. However, the expense of treating a hectare of land with a baculovirus product invariably costs more than an equally efficacious chemical treatment. This difference in price is due primarily to the labour-intensive nature of baculovirus production. Some viruses can be produced in vitro (within cell cultures in the laboratory, not requiring whole, living insects). These are less expensive than those that can only be produced in vivo, that is, inside of living insects. The cost of rearing live hosts adds greatly to the final cost of the product. It is to be hoped that insect cell culture systems currently being developed for other uses may ultimately make viral pesticides more cost-effective.

### 4.8.1 Appearance

The insects that are killed with baculovirus have a characteristic shiny-oily appearance and are often seen hanging limply from vegetation. They are extremely fragile to the touch, rupturing to release fluid filled with infective virus particles. This tendency to remain attached to foliage and then rupture is an important aspect of the virus life cycle. As discussed above, infection of other insects will only occur if they

**Table 4.1** Phases of baculovirus infection

| Phase(s) | Description |
|---|---|
| Early (0–6 h postinfection) | Expression of genes involved in the replication of the virus and manipulation of the host. Delayed early genes often require the presence of viral transregulators (e.g. IE-0, IE-1, PE38) for efficient transcription |
| Late (6–24 h postinfection) | Transition from early to late is characterised by shutdown of the host cell DNA replication and protein synthesis. Nucleocapsids are produced. Budded virus is produced and disseminates the virus throughout the host |
| Very late (or occlusion) (18–24 to 72 h postinfection) | Advanced stage of virus infection. Virions become occluded in the protein polyhedrin. Viral proteases liquefy the host and degrade the chitinous exoskeleton. Occluded progeny virus is disseminated onto surrounding material for horizontal spread. The extensive lysis of cells frequently causes the host insect to literally melt, and this is called 'wilting disease' |

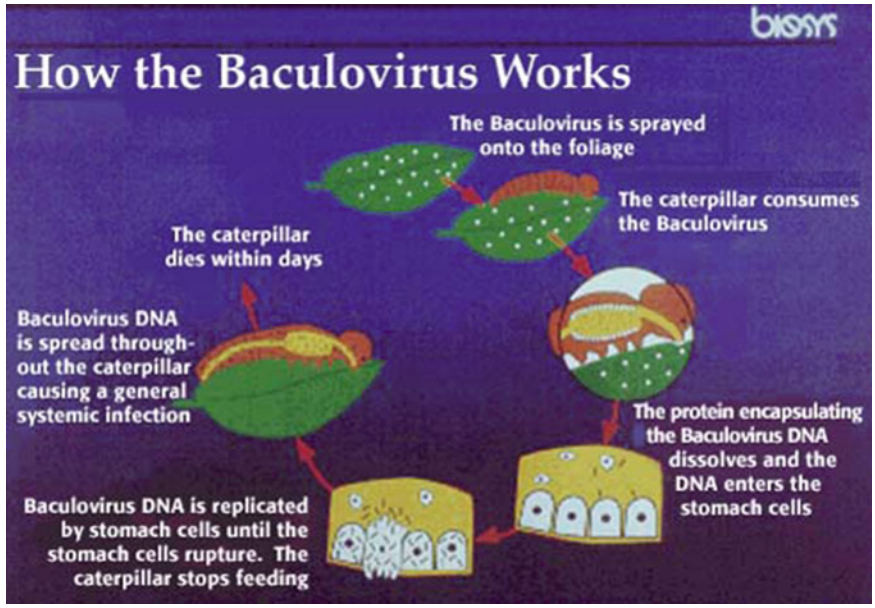



**Fig. 4.3** The mode of action of baculovirus (Ramon Georgis 1996)

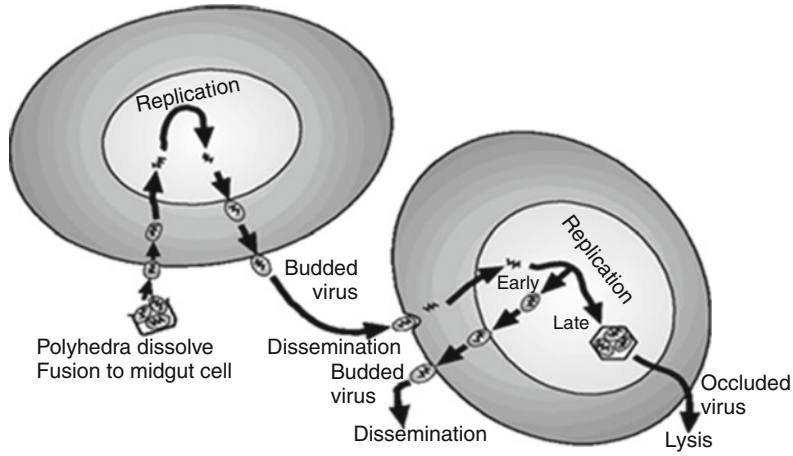



**Fig. 4.4** General overview of the replication cycle of baculoviruses (Kalmakoff and Ward 2007)

eat foliage that has been contaminated by virus-killed larvae. It is interesting to note that most baculoviruses, unlike many other viruses, can be seen with a light microscope. The polyhedra of many viruses look like clear, irregular crystals of salt or sand when viewed at 400× or 1,000×. The fluid inside a dead insect is composed largely of virus polyhedra – many billions are produced inside of one cadaver.

#### 4.8.1.1 Habitat

Baculoviruses can be found wherever insects exist. Because rain and wind readily carry baculoviruses from place to place, it is likely that every piece of land and body of water contains some virus particles. It is widely accepted by researchers that most produce currently on the shelves is 'contaminated' by baculovirus particles (Heimpel et al. 1973). In fact, the pervasiveness of

**Table 4.2** The important role of proteins in baculovirus infection

| Protein | Function |
|---|---|
| Polyhedrin/granulin | Hyper-expressed protein which produces the crystalline matrix of the occlusion bodies. Provides protection from environmental damage |
| GP64/F-protein | Present on budded virus only; envelope fusion protein required for efficient entry of the budded virus into cells |
| EGT | Enzyme for inactivating the host moulting hormones, ecdysteroids |
| P35, IAP-1, IAP-2, IAP-3, IAP-4 | Inhibitors of apoptosis – prevent or delay cells from undergoing programmed cell death |
| DNApol | Viral DNA polymerase – required to replicate the viral genome |
| IE-0, IE-1, IE-2, PE38 | Transactivators produced early in the replication cycle. Regulate the activity of other genes especially early in the replication cycle |
| LEFs (at least 18) | Late expression factors – required for the expression of late genes. Some also act to downregulate host cell activities |
| P6.9 | Dephosphorylation of this protein is required for DNA packaging. Phosphorylation on viral entry into the cell leads to the DNA unwinding |
| Ubiquitin | Has similarity to eukaryotic ubiquitin. May act by blocking the degradation of selected proteins during viral infection |
| Cathepsin and chitinase | Possible role in damaging peritrophic membrane to aid initial infection. Required for liquefaction of the host and hence dissemination of the progeny virus |

baculovirus particles, along with the results of tests performed in conjunction with registration, may be considered both indirect and direct evidence for the safety of these agents.

#### 4.8.1.2 Baculovirus Hosts

Over the years, baculoviruses have been reported from a variety of different species of invertebrates. However, the only well-documented hosts belong to the order of Diptera, Hymenoptera and Lepidoptera. In some of the literature, it has been reported that occluded virions resemble NPVs in a caddis fly (Trichoptera) (Hall and Hazard 1973) and a shrimp species (Couch 1974). An occluded baculovirus-like virus was also reported for a thysanuran, but it did not appear to affect its host and transmission studies failed (Larsson 1984). Baculoviruses have also been reported from Orthoptera (Henry and Jutila 1966), but later these were classified as pox viruses, and from Coleoptera, but these are normally not occluded and were later placed in an unassigned category. Reports of infection of other insects, for example, a coleopteran (Ryel and Cline 1970), could not be confirmed. However, the infection occurred under laboratory conditions, where neuropterans were fed on Lepidoptera that had died of an NPV infection. Consequently, the neuropterans were likely heavily contaminated from their food source, and although they appeared to die of an NPV infection, they were probably exposed to an unusually high virus dose. Naturally infected Neuroptera have not been documented.

## 4.9 Pesticide Compatibility

Viruses particles per se are generally unaffected by pesticides, although some chlorine compounds should be expected to damage or destroy viruses if applied at the same time. Baculovirus efficacy, however, can be altered in many ways by the effects of chemical pesticides on the host insect. A review by Jacques and Morris (1981) showed that of 10 pesticide-virus combinations, 9 resulted in an additive effect on insect mortality. However, some of the pesticides included in that review have since been banned. More work is needed to explore the effectiveness of insecticide 'cocktails' consisting of environmentally friendly chemical agents and baculoviruses in India.

## 4.10 Recombinant Baculoviruses

Recombinant baculoviruses are usually constructed in two steps. Initially, a heterologous gene is introduced into a baculovirus transfer

vector. It consists of a bacterial replicon of a multicopy plasmid, a selection marker gene, promoter and terminator regions along with flanking baculovirus sequences from a nonessential locus and a multiple cloning site (or a single unique restriction site) downstream from a viral promoter. Most often the promoters and the flanking DNA originate from one of the late genes: polyhedrin or p10 gene. The latter is another viral gene coding for a protein which is produced in large quantities late in the infection. It is the main component of the fibrillar structures which accumulate in the nucleus and in the cytoplasm of infected cells. For some purposes, weaker early promoters, such as basic protein promoter (p6.9), may be preferred (Boguslaw Szewczyk et al. 2011).

Around 400 insect cell lines are known which potentially can be used for in vitro propagation of baculoviruses. Only a few of them support the growth of AcMNPV. These lines were obtained from two parental organisms: *Spodoptera frugiperda* and *Trichoplusia ni* (Lepidoptera: Noctuidae). The most widely used line is Sf9 which grows well in suspension. BTI-Tn5B1-4 derived from *T. ni*, known as High Five cells, has been also largely used for viral growth (Granados et al. 1994). Cell lines which can be used for the propagation of *Lymantria dispar* nucleopolyhedrovirus (LdMNPV), *Heliothis zea* nucleopolyhedrovirus (HzSNPV), *Bombyx mori* nucleopolyhedrovirus (BmNPV), *Anticarsia gemmatalis* nucleopolyhedrovirus (AgMNPV) and a few other baculoviruses are also currently available (Boguslaw Szewczyk et al. 2011).

## 4.11 Baculovirus Production Technology

At present, commercial production of baculoviruses has been carried out only in vivo, either by applying the virus against the host insect in the field and collecting diseased or dead larvae or by producing the target insect in the laboratory on an artificial diet. The latter is the most commonly used method for producing baculoviruses in many countries, but both methods have been used successfully for the commercial production of the *Anticarsia gemmatalis* baculovirus (AgMNPV) in Brazil (Moscardi 1999, 2007). For some insects, there are no available artificial diets, and, therefore, the commercial production of baculoviruses of these baculovirus biopesticides 27 insects is generally too difficult or impossible under laboratory conditions. In such cases, field production of baculovirus stocks may be sometimes a method of choice, also from financial point of view (Moscardi 1999).

In laboratory culture, the production of occlusion-derived virions (ODV) is not necessary for the survival of the virus. The budded virus (BV) particle is the form used for cell-to-cell transmission in cell culture. The main protein of the BV particle is the GP64 (Blissard 1996), essential for virus budding and responsible for entrance of the virus into the next host cell. Various culture conditions are known to influence infection of lepidopteran cells by baculoviruses and include temperature, pH, dissolved oxygen concentration, osmolality and nutrient composition of the culture medium. The investigation on factors associated with loss of genetic stability and the use of new strategies such as isolation of more stable variants, as well as the reduction of costs of cell culture medium components, are important requirements for process optimisation of in vitro baculovirus production.

The requirements for productive insect cell lines (Jem et al. 1997) and for highly productive culture media (Chakraborty et al. 1999) are other challenges for in vitro production of baculovirus. Many cell lines are available for production purposes and are derived from various sources, thus exhibiting a wide variety of growth and production characteristics. Careful screening or formulation of media must be performed for a particular virus isolate cell line combination, as different media can greatly affect polyhedra yields (Pedrini et al. 2006). Recently, a new strategy for in vitro production was proposed based on many polyhedra (MP) variants. These are clones selected using the plaque assay technique after several passages of the virus in cell culture. MPs maintain the wild-type features such as formation of many polyhedra in the cell

nucleus and budded virus high titre (Slavicek et al. 2001; Pedrini et al. 2005) which allow them, in principle, to compete with the population of few polyhedra mutants accumulated in cell culture.

## 4.12 Baculoviruses: Indian Scenario

Biopesticides fall under the Insecticide Act (1968) under which any microbial organism manufactured or sold for pest and disease control should be registered with the Central Insecticides Board (CIB) of the Ministry of Agriculture. The national agricultural research system, comprising of the many ICAR institutes as well as state agricultural universities, plays a leading role in promoting biopesticides. The Project Directorate of Biological Control is involved in testing the quality of biopesticides and training the officers of the state department of agriculture in quality control protocols. The National Centre for IPM routinely incorporates the use of biopesticides in its IPM validation programmes and demonstrations, as do the IPM centres of the Directorate of Plant Protection, Quarantine and Storage. Commodity research boards have also played a role in researching and developing biopesticides for pest control in key crops such as cotton, coffee, tea and cardamom. Other biopesticides currently under development include *Hyblaea puera* NPV for controlling teak defoliator (Biji et al. 2006) and *Amsacta albistriga* NPV for controlling this pest on groundnuts.

Baculovirus group has a very narrow host range and generally infests the larvae of crop pests. The research aimed at insect pest control is, therefore, confined to nuclear polyhedrosis viruses (NPVs) and granular viruses (GVs). In India, extensive research has been conducted on the use of NPVs for tackling two major pests, namely, *Spodoptera litura* and *Helicoverpa armigera*. Nuclear polyhedrosis viruses like Ha NPV and Sl NPV are increasingly being used as alternatives to chemicals. These viruses have distinct advantages over other methods of pest control. NPVs are virulent pathogens of insect characterised by the polyhedral occlusion bodies (POB). These viruses are highly specific and do not affect beneficial insects like parasitoids and predators and are safe to fish, birds, animals and man. Considering the usefulness of NPVs, there has been a growing demand amongst the farmers for these bioagents.

The Government of India allocates funds for IPM programmes for all major crops, but these funds are mainly implemented at the state government level, through programmes promoting the use of biopesticides to farmers. Major national research programmes such as the National Agricultural Technology Project (2000–2006) and the current National Agricultural Innovation Project also contain important biopesticide research and development components. At the state level, 50 % of the plant protection budget is allocated to eco-friendly agriculture (Singhal 2004) to cover both the training of farmers and the procurement of biopesticides for distribution. A website on 'biocontrol strategies for eco-friendly pest management' has been launched recently by the Department of Biotechnology (DBT). The DBT has had a substantial funding programme for the research and development of microbial pesticides since 1989, with over 200 projects funded (Wahab 2004). This encourages the development of new technology and academic industrial links. The DBT also provides financial support for the generation of toxicological data to promote registration of microbials; data generation has been completed for almost all the currently registered biopesticides. The state governments play the main role in implementing IPM. Their IPM programmes for purchasing and distributing biopesticides to farmers have been vital to creating a market for and encouraging private commercial production of microbial pesticides. States such as Tamil Nadu, Gujarat, Andhra Pradesh and Maharashtra have been particularly active in promoting microbial pesticide use. The State Universities of Agriculture have played important roles in biopesticide research and in a few cases are also producing biopesticides themselves and are advising companies in production. The State Agricultural Universities and other stakeholder agencies, through the Agricultural Sciences Centre (Krishi Vigyan Kendra), are encouraged to take up initiatives to promote local production of microbial pesticides. Indian companies have

formed a biopesticide supplier's association, the All India Biotech Association, to coordinate the commercial sector's voice in developing government policy. Other organisations actively promoting biopesticides include nongovernmental organisations (NGOs) such as the M.S. Swaminathan Research Foundation and international research centres based in India such as the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and the International Rice Research Institute.

### 4.12.1 Major Equipment Required

The major equipments like centrifuge, laminar flow, magnetic shaker, microscopes, autoclave, coolers, refrigerators, incubator, distillation units, etc., are required in addition to glassware, plastic trays, basins and iron racks for mass production of Ha NPV and Sl NPV.

***Spodoptera litura* (tobacco caterpillar)**: *Spodoptera litura* commonly known as tobacco caterpillar is a polyphagous pest. It is a serious pest of tobacco nurseries and also a sporadic pest of cauliflower, cabbage, castor, cotton, groundnut, potato and lucerne. It causes serious crop losses.

**Sl NPV**: The virus is specific and infects only tobacco caterpillar. NPV can be successfully multiplied on tobacco caterpillar, and the viral extraction can be applied in the field to control the caterpillar. For continuous production of Sl NPV, it is necessary to rear tobacco caterpillar larvae continuously in a lab condition.

**Gram pod borer (*Helicoverpa armigera*)**: It is widely distributed in India and infests/damages a variety of cultivated and wild plants throughout its distribution range. It is a serious pest on commercial crop like cotton; pulses like red gram and Bengal gram; vegetables like tomato, bhendi and dolichos bean; oilseeds like sunflower, soybean and safflower; and cereals like sorghum and maize.

**Ha NPV**: Ha NPV is a highly infective microbial biopesticide which can be used to control gram borer. It is being made from naturally diseased or under laboratory conditions artificially infected larvae of gram borer.

### 4.12.2 Mass Production of Ha NPV and Sl NPV

The mass production of Ha NPV and Sl NPV involves 3 steps: (1) rearing of adult gram pod borer and tobacco caterpillar for mass production of eggs, (2) rearing of larvae of the above species either on the host plants like chickpea and castor under seminatural condition or on the synthetic diet in the laboratory conditions. In the model only the latter is considered for large-scale commercial production of NPV and (3) inoculation of Ha NPV and Sl NPV into the larvae of gram pod borer and tobacco caterpillar, respectively, for mass multiplication of viruses and extraction of polyhedral occlusion bodies (POBs) from the diseased larvae, which are used as biopesticide on the crop plants.

#### 4.12.2.1 Details of Mass Production

*Diet preparation*: The larvae of gram pod borer and tobacco caterpillar can be multiplied by using chickpea-based semisynthetic diet. The composition of the diet for rearing larvae is as follows:

| | Item | Quantity |
|---|---|---|
| *'A' fraction:* | Chickpea (Kabuli chana) flour | 105.00 g |
| | Methyl para-hydroxy benzoate | 2.00 g |
| | Sorbic acid | 1.00 g |
| | Streptomycin sulphate | 0.25 g |
| | 10 % formaldehyde solution | 2.00 ml |
| *'B' fraction:* | Agar-agar | 12.75 g |
| *'C' fraction:* | Ascorbic acid | 3.25 g |
| | Yeast tablets | 25 tablets |
| | Multivitaplex | 2 capsules |
| | Vitamin E | 2 capsules |
| | Distilled water | 780.00 ml |

Three hundred ninety ml of water is mixed with fraction 'A' of the diet in the blender which is run for 2 min. Fractions 'A' and 'C' are mixed, and the blender is run again for 1 min. Fraction 'B' is boiled in the remaining 390 ml water, added to the mixture of A and B, and the blender is run for a minute. Formaldehyde solution is added at the end, and the blender is again run for a minute.

#### 4.12.2.2 Mass Production of Eggs

*Tobacco caterpillar*: The culture of tobacco caterpillar is initiated by collecting eggs from the fields

of castor, cauliflower, lucerne, tobacco, etc. These field-collected eggs are reared in isolation to eliminate the emerging parasitoids and diseases, if any. The culture can also be established by collecting the gravid females with the help of light traps. Once the pure culture is established, the mass production is commenced under laboratory conditions after the first generation established.

Pairs of newly emerged moths of tobacco caterpillar are placed in well-ventilated plastic containers. The inner wall of the containers is lined with paper to enable the adults to lay eggs. The bottom of the container is lined with sponge covered over by blotting paper. The moths are provided with 50 % honey solution and water on two cottons swabs placed in small plastic cups. The eggs which are generally laid in batches on the paper are cut out. Freshly laid egg masses are sterilised by dipping in 10 % formalin for 30 min, washed in running water for 30 min, dried on blotting paper and kept for hatching in sterilised glass vials.

The freshly laid eggs can also be surface sterilised in 0.05 % solution of sodium hypochlorite for 5 min. These eggs are washed several times in running tap water to remove the traces of sodium hypochlorite. The traces of sodium hypochlorite could be neutralised by dipping the eggs in 10 % sodium thiosulphate solution, and again the eggs are washed thoroughly under running tap water. The surface-sterilised eggs are kept in plastic tubes (7.5 × 25 cm) on moist tissue paper for continuing the stock culture. After 3 days, the newly hatched larvae are transferred to bouquets of castor leaves and kept in a plastic container with stand for pupation. The pupae are collected 3 days after all the larvae enter the sand. The pupae are sexed and kept on a lid over a wet sponge in adult emergence cage. After 10 days, freshly emerged males and females are collected from their respective emergence cages. Tobacco caterpillar larvae can be multiplied on a chickpea-based semisynthetic diet composition and preparation of which has been mentioned above.

**Gram pod borer** (***Helicoverpa armigera***): The culture of gram borer is initiated by collecting the adults with the help of light traps. It could be by collection of larvae on a large scale from its host crops in endemic areas. Nucleus culture can also be obtained from the established laboratories. The material thus obtained is reared in the laboratory in aseptic conditions, and the healthy progeny is selected and established. The production starts with the availability of 250 pairs of adults every day, which will yield 10,500 eggs daily. The adults are kept at 100 pairs in each oviposition cage with a cloth enclosing the frame. A circular plastic mesh (on which cotton swabs soaked in water and honey solution are placed in small containers) rests on a support above the base of the frame. The cloth cover is open at both ends with a 20 cm vertical slit in the centre which can be closed with a zip or cloth clips. The cloth cover enclosing the frame is tied with rubber bands at both ends. It is placed on tray with a sponge at the bottom soaked in water. The temperature inside the cage is maintained at 260 C and humidity at 60–90 %.

The eggs are laid all over the inner surface of the cloth cover. The egg cloth is removed daily. This cloth is surface sterilised in 10 % formalin for 10 min; the eggs could also be surface sterilised using 0.2 % sodium hypochlorite solution for 5–7 min and treated with 10 % sodium thiosulphate solution to neutralise the effect of sodium hypochlorite and rinsed in distilled water. The eggs are later placed on paper towel under laminar flow for drying. The dried cloth pieces containing eggs are kept in 2 l flasks containing moist cotton. Flasks are plugged with cotton wrapped in muslin cloth, and the bottom of the flask is wrapped with aluminium foil.

#### 4.12.2.3 Rearing of Larvae on Semisynthetic Diet

##### 4.12.2.3.1 Tobacco Caterpillar

*Stage I (rearing of early instar larvae)*: The rearing unit is prepared by placing a sponge piece on a glass sheet. The sponge is covered with a single layer of soft tissue paper. A small plastic container containing 200 surface-sterilised eggs of tobacco caterpillar is placed in the centre over the tissue paper. A Petri dish containing about 200 ml of diet is placed inverted over the tissue paper. The eggs hatch within 25 h, and neonate larvae crawl and spread out on the diet.

*Stage II (rearing of late instar larvae)*: Late instar larvae are reared in modified plastic boxes. One window each on the four sides of the box is cut and covered with a fine plastic mesh to provide sufficient ventilation and to prevent moisture accumulation inside the box. A thick layer of sterilised sand is spread at the bottom of the box. A small piece of tissue paper is kept at the centre over the sand.

The diet in the Petri dish (containing 200 larvae) is divided into five equal pieces. One piece of diet bearing 40 larvae is kept in plastic box over the tissue paper so that the sand does not soil the diet. In this way, five boxes are charged with larvae from 1 Petri dish. A plastic grill is fitted into the box in such a manner so that it forms a crest higher than the brim of the box. Thick cake of diet (about 500 g) in a Petri dish is divided into two equal pieces. One such piece is kept on the top of the crest, and the lid of the box is then fixed so that the diet and grill crest are opposed to each other just beneath the lid. After consuming the small quantity of diet on tissue paper, the larvae crawl and perch on the grill and feed from the ceiling of the box. The boxes are stacked and left intact for 3 days. During this time, the diet is almost completely consumed. Now another piece of fresh diet (about 250 g) is kept on the crest in each box, and the boxes are closed and stacked again. During the last 3–4 days of larval stage, the food consumption is higher and so is the faecal matter accumulation on the sand layer. After 20 days from hatching, the larvae move into the sand and start pupating. In a period of 25 days, all the larvae pupate and the chitinisation of pupae is also completed. The boxes are now ready for the pupal harvest. The pupae are collected, cleaned, sterilised and placed in adult emergence cages. The freshly emerged moths are then placed in oviposition cages.

*Gram borer*: The larvae of gram borer can also be reared on a chickpea-based semisynthetic diet as mentioned above. The diet is poured as per the requirement either on the nylon mesh for rearing 5–7-day-old larvae or in tray cells for rearing the older larvae or poured into sterilised Petri plates and allowed to solidify. The diet could be stored in the refrigerators for up to 2 weeks. For preparing large quantities of diet, the quantity of diet ingredients to be used should be calculated accordingly, and industrial-type waring blenders could be used. The larvae are removed from the top of the aluminium foil-wrapped flasks with a brush and then transferred to the diet. Two hundred twenty larvae are transferred to diet impregnated on nylon mesh and placed in plastic containers or sterilised glass vials. 100 such containers are maintained daily for 5–7 days. Multicellular trays with semisynthetic diet are advantageous for rearing a large number of larvae. Starting with 10,500 eggs, the total number of larvae available is 10,000 considering an estimated 5 % mortality in initial 5 days of emerging and 10 % mortality up to first 5–7 days. The total number of larvae available for virus production is 8,000 (80 %). The rest of 20 % will be utilised for maintenance of host culture continuously.

The diet requirements at various stages of production of larva are as follows: for the young larvae, up to 5–7 days will be 2 g/larva; for 5–7-day-old larvae, for Ha NPV production will be 4 g/larva; for 5–7-day-old larvae, for continuation of host culture will be 6 g/larva; and for rearing the field-collected larvae for augmenting the nucleus stock will be about 1 kg.

In host culture units, larvae start pupating when they are 18–19 days old, and the pupation will be over within 2–3 days. The harvested pupae are surface sterilised using 0.2 % sodium hypochlorite solution followed by washing with 10 % sodium thiosulphate solution to neutralise sodium hypochlorite and then washed thoroughly with distilled, sterilised water. After washing, the eggs are dried by rolling over blotting paper. The male and female pupae are separated out and placed over moist sponge in adult emergence cages. The egg, larval, pupal and adult stages of gram borer last 3–4, 18–29, 7–8 and 7–9 days, respectively. The oviposition period of the females is about 5 days.

#### 4.12.2.4 Production of *Helicoverpa armigera* NPV (Ha NPV) and *Spodoptera litura* NPV (Sl NPV)

For Ha NPV and Sl NPV production, the synthetic diet prepared in the laboratory is poured at 4 g/cell in the multi-cavity trays, and the diet surface is uniformly sprayed with virus prepared in distilled

**Table 4.3** Commercially available products in India

| Virus | Products (company name) | Targets |
|---|---|---|
| *Helicoverpa armigera* NPV | Helicide (Pest Control India Ltd., India) | *Helicoverpa armigera* |
| | Virin-H | |
| | Helocide | |
| | Biovirus-H (Biotech International Ltd., India) | |
| | Helicop | |
| | Heligard (Margo Biocontrols Pvt. Ltd., India) | |
| *Spodoptera litura* NPV | Spodo-Cide (Pest Control India Ltd., India) | *Spodoptera litura* |
| | Spodoterin | |
| | Spodi-Cide | |
| | Biovirus-S (Biotech International Ltd., India) | |

Note: Some of the above-mentioned products are locally made, and hence the formulator name is not known and has not been registered

Source: CIB and RC website, minutes of the Registration Committee meetings, June 2003 – March 2009. Other products should be included

sterilised water at 18 × 106 POBs/ml. Eighty percent of the total 5–7-day-old larvae can be utilised for Ha NPV and Sl NPV production. The trays are incubated at 26 °C for 7 days. In case of virus-infected larval trays, the diseased larvae die after attaining their maximum size of 6th instar, where the dead caterpillar will have 2–6 billion polyhedral occlusion bodies (POBs), in terms of larval equivalent (LE). One LE of *H. armigera* NPV = $6 \times 10^9$ POBs; 1 LE of *S. litura* = $2 \times 10^9$ POBs. The dead larvae have to be harvested, macerated in distilled/sterilised water and filtered through muslin cloth to get the crude suspension of the virus. The extraction is centrifuged to further clarify the solution (Table 4.3).

### 4.12.3 Other Important Aspects

General precautions to be followed while maintaining host cultures are the following: (a) In production units, keep the host culture in a separate room, and the virus production and storage facility should be located in a different facility. (b) In the NPV production units, in spite of best care, 100 % larvae are not infected; the larvae which do not turn inactive after 4–5 days and keep consuming the normal diet should be culled out regularly from the NPV production unit. (c) Utmost care should be taken to prevent the break in the chain of the production system. This could be achieved only if highly dedicated and disciplined workers are engaged for such production units. (d) Strict hygiene should be maintained in different facilities. The equipments used should be either heat sterilised or sterilised using steam or chemicals. The workplace should be thoroughly disinfected with sodium hypochlorite solution. (e) The host culture should be initiated from a batch of healthy adults. (f) Microbial infection could be avoided if good insect husbandry practices are followed. If infection is detected, the culture or infected part should be destroyed immediately. Besides hygienic conditions, optimum temperature (24–26 °C) and humidity (65–70 %) should also be maintained. (g) The texture and quality of the natural/semisynthetic diet should be good. (h) entry to host culture unit after visiting virus production unit should be avoided.

### 4.12.4 Mechanism of Action

The virus acts as a stomach poison. The NPV particles are called as polyhedral inclusion bodies (PIBs). When these PIBs present on the plant foliage, the insect larvae will eat the contaminated food, and the virus enters the midgut of the insect larvae. Then the proteinaceous polyhedra come in contact with the alkaline pH of the midgut. The proteinaceous covering rapidly dissolves, thereby releasing the infectious virions. After the

liberation of virus particles, the nucleocapsid envelop fuses with microvillar membrane of the gut wall cells. The nucleocapsids are released to enter the nucleus where viral DNA replicates and produce secondary infections which invade fat body and the haemolymph. The massive destruction of body tissue eventually kills the insect.

### 4.12.5 Field Application and Dosage

Ha NPV is used for controlling *H. armigera* attacking cotton, red gram, Bengal gram, tomato, okra, sunflower, groundnut, chillies, maize, sorghum, etc., whereas Sl NPV is used for controlling tobacco caterpillar attacking tobacco, groundnut, soybean, sunflower, cotton, cabbage, beetroot, cauliflower, etc.

#### 4.12.5.1 Directions for Use of NPV

The recommended dosage is 200 ml of NPV/acre or 500 ml/ha containing 100 and 250 larval equivalent (LE) of NPV, respectively, as active infective material (one LE $= 6 \times 10^9$ POBs), or 100 ml of NPV could be diluted in 200–400 l of water when high volume sprayer is used and in 50–70 l of water in case of power sprayers or preferable to spray using high volume knapsack sprayer. Virus should be sprayed during evening hours. Spray should be initiated as soon as some newly hatched larvae are observed or 3–5 days after a trap catch of 5 months per pheromone trap. Subsequent sprays should be made at 7–10 days intervals depending upon the pest population.

#### 4.12.5.2 Compatibility with Other Insecticides

The viral pathogens seem to be less sensitive to chemical pesticides. When the combination of pathogen and pesticide is used, sometimes synergistic action is noticed. But in recent years, mixing of NPV with insecticides is not advisable due to cross resistance problem.

#### 4.12.5.3 Environmental Factors Affecting the Action of NPV

The environmental factors which affect the action of NPV are ultraviolet component of sunlight, rainfall, temperature, humidity and leaf surface compounds. The application of Ha NPV in the evening hours provides better efficacy than the morning hours in the field. NPV degrades in the sunlight quickly, and hence adjuvants have to be added along with it while spraying. Solar radiation affects the field persistence of baculovirus. Ultraviolet radiation in the range of 280–310 nm inactivates baculovirus. Fluorescent brighteners can be used to increase its persistence in the field. Leucaena leaf extract, eucalyptus leaf extract and Ranipal can be used with Ha NPV to enhance its efficacy (Kalawate et al. 2005). Temperature in the range of 70–80 °C inactivates the viruses for the exposure of 10 min. But the temperature in the field will not reach to this extent, and hence it is less important in the field persistence. Washing of virus from the leaves by rain is a major factor affecting the persistence, and hence stickers have to be added with the viruses. Organic and inorganic substances can be leached from the foliage surface which can have positive or negative effect on the viruses.

### 4.12.6 Advantages in Using Viral Pesticide

Advantages are (1) control of target pests; (2) a high degree of specificity, which makes them especially valuable for use in integrated pest management programmes; (3) safe to humans and other warm-blooded animals; (4) residues present after application of viral pesticides pose no hazards to humans or other animals; and microbial insecticides can be applied even when a crop is almost ready for harvest.

### 4.12.7 Disadvantages in Using Viral Pesticide

Disadvantages include the following: (1) Toxic to only a specific species or group of insects, each application may control only a portion of the pests present in a field, garden or lawn. If other types of pests are present in the treated area, they

will survive and may continue to cause damage. Conventional insecticides are subject to similar limitations because they too are not equally effective against all pests. Nonetheless, the negative aspect of selectivity is often more noticeable for microbials. (2) Inactivation by heat, UV and rain can wash out the virus present on the foliage. (3) special formulation and storage procedures are necessary.

## 4.13 Future Focus

In India, the potential of baculovirus has not been utilised fully to control the economic insects. The new developments in this field depend upon the development of recombinant baculoviruses and its commercial production. The most important aspect is to educate the farmers about the benefits of the NPVs. The inclusion of baculoviruses in organic farming and integrated pest management has to be made understood by the farmers.

## 4.14 Conclusions

Baculoviruses provide a promising alternative approach to pest control. Available data suggest that the viruses are effective against insects and do not pose any deleterious effects on other components of the ecosystem (other invertebrates, plants and vertebrates including man). In India, some preliminary work has been done in molecular characterisation of certain indigenous baculoviruses and expression of mostly foreign gene products of medical and veterinary importance utilising baculoviruses of certain alien origin; no work has been done in the field of agricultural plant protection, especially towards genetic improvement of the baculoviruses.

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# Entomopathogenic Nematodes as Biopesticides

5

Atwa A. Atwa

**Abstract**

Entomopathogenic nematodes (EPNs) are microbial control agents which have become important in biological control or integrated pest management of insect pests as biopesticides. EPNs are widespread all over the world and are found in almost all places where there is agricultural land and forests, and in the desert where there are desert plants. Where insects are present in the environment, they may help the spread of EPNs of a number of species of the genera *Steinernema* (more than 61 species) and *Heterorhabditis* (more than 14 species). The factors responsible for aggregated distribution of EPNs may include behavior and the spatial and temporal variability of the nematodes' natural enemies, such as nematode trapping fungus. Nematodes also have limited dispersal ability. Many infective juveniles are produced from a single host, which can also produce aggregates. Patchy EPN distributions may also reflect the uneven distribution of the host and nutrients in the soil. The metapopulation as a whole can persist as long as the rate of colonization is greater than or equal to the rate of population extinction.

EPNs infect only insects and live inside the body of their insect host, so they are designated endoparasitic. EPNs infect many different types of soil insects, including the larval and pupal forms of butterflies, moths, beetles, and flies, as well as adult crickets and grasshoppers. EPNs have been found in all inhabited continents and a range of ecologically diverse habitats, from cultivated fields to deserts, yet are safe for plants and animals. Most biopesticides require days or weeks to kill their host, yet nematodes, working with their symbiotic bacteria (*Xenorhabdus* for the family Stienernematidae and *Photorhabdus* for the family

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_5, 

Heterorhabditidae), can kill their insect hosts within 24–48 h. Dozens of different insect pests are susceptible to infection, yet no adverse effects have been shown against beneficial insects or other nontarget organisms in field study experiments. Nematodes are amenable to mass production and do not require specialized application equipment as they are compatible with standard agrochemical equipment, including various sprayers and irrigation systems. Although the biological control industry has acknowledged EPNs since the 1980s, today thousands of researchers representing more than 50 countries are working to develop nematodes as biological insecticides. Nematodes have been marketed on every continent except Antarctica for control of insect pests in high-value horticulture, agriculture, home gardens and garden niche markets. In this chapter, we focus on EPNs as biopesticides in insect control. Isolation and distribution, application techniques, and field application models of EPNs as biopesticides throughout the world are discussed. The chapter closes with a discussion of mass production of EPNs, the safety of EPNs, and quality control of EPN production.

**Keywords**
Entomopathogenic nematodes • Insect host • Mass production • Safety • Quality control

## 5.1 Introduction

Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae possess impressive attributes for biological control. EPNs are recognized as insect-parasitic nematodes, beneficial nematodes, biocontrol agents, biological control agents, biological insecticides, or biopesticides. These nematodes are also recognized as pathogens or microbial control agents because of their symbiotic association with bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp.) that are mainly pathogenic to insects. Because of a mutualistic relationship with pathogenic bacteria, these nematodes are named "entomopathogenic nematodes" (EPNs). They have a worldwide distribution as they have been isolated from every inhabited continent and many islands (Hominick et al. 1995). They have been isolated from different soil types, from sea level to high altitudes, and from natural habitats of disturbed agroecosystems. Because EPNs are obligate parasites in nature, they need to recycle in their hosts to maintain their presence in the environment. The distribution of the nematode population is patchy at any given site (Campbell et al. 1997) and may depend on various abiotic and biotic factors, including their seasonal variations and foraging strategies. From a practical point of view, after inundative release of the nematode, recycling is a highly desirable attribute because it can provide additional and prolonged control of the pest and avoid or reduce the need for further applications. Numerous studies have shown that nematode recycling in the soil environment occurs after inundative release (Kaya 1990), but factors that influence survival and infectivity also affect nematode recycling. Until we understand nematode behavior thoroughly, the practical approach will be to use these nematodes as biological insecticides.

EPNs contribute to the regulation of natural populations of insects. However, the population of naturally occurring EPNs is normally not high enough to manage soil-dwelling plant pests. Therefore, during the last four to five decades, these live nematodes have been commercially

mass-produced and inundatively applied to control many garden insects, turfgrass insects, nursery insects, greenhouse insects, and insects that feed on different field crops. These biopesticides (EPNs and their symbiotic bacteria) are safe to produce and are not harmful to humans, other mammals, most beneficial insects, or plants. EPNs do not pose any health risk to consumers of nematode-treated agricultural produce and do not cause any damage to the environment, and they are exempt from registration requirements in most countries. EPNs also have no detrimental effect on other beneficial nematodes, including bacterial feeders, some fungal feeders (*Aphelenchus* sp.), predatory nematodes, and other soil microbial communities. But EPNs can be detrimental to plant-parasitic nematodes that are responsible for causing a tremendous economic loss to the agriculture industry throughout the world. EPNs can suppress the populations of many economically important plant-parasitic nematodes, including foliar nematodes, potato cyst nematodes, ring nematodes, root-knot nematodes, root-lesion nematodes, sting nematodes, stubby root nematodes, and stunt nematodes.

Most recent publications on EPNs have focused on their potential use as biocontrol agents, but little is known about the structure and dynamics of their natural populations. Accordingly, a soil survey is conducted to assess the occurrence of EPNs and to find new isolates, across seasons, habitats, and geographic regions. Although the results from many laboratory tests with EPNs have been promising in regard to controlling insect pests, field evaluation results have often been highly variable, particularly in regard to well-hidden insects of cryptic habitats such as soil (scarabs) and tunnel-living (leopard moth and red palm weevil) insects. They are well protected from chemical insecticides, with a high rate of survival. Thus, these insect hosts are capable of producing large populations and new generations that subsequently disperse or migrate or both to more susceptible plant hosts, where more control measures are required. Therefore, field trials have been conducted to validate laboratory findings.

However, one species, *Steinernema scapterisci*, has been successfully introduced as a classic biological control agent against mole crickets in Florida (Parkman and Smart 1996), suggesting that suitable conditions prevail for this nematode to recycle. In this respect, EPNs belonging to the families Heterorhabditidae and Steinernematidae have already been successfully used throughout the world for the control of important agricultural insect pests. The qualities that make EPNs excellent biocontrol agents are their broad host range, their ability to search actively for their hosts, and to kill them relatively quickly, their economic mass-production, and their being noninjurious to vertebrates, easily applied, compatible with most chemical insecticides, and environmentally safe.

The main goal of this chapter is to illustrate the use of EPNs as bioinsecticides. This goal will be achieved through five main sections related to each other as follows: (1) isolation and distribution of EPNs, (2) techniques for application of EPNs as biopesticides, (3) field application models of EPNs as biopesticides throughout the world, (4) mass production of EPNs, and (5) safety of EPNs and quality control of EPN production.

## 5.2 Isolation and Distribution

EPNs from the families Heterorhabditidae (Poinar 1976) and Steinernematidae (Travassos 1927) are obligate insect parasites which can infect and kill a broad range of insect hosts (Kaya and Gaugler 1993). These nematodes are symbiotically associated with entomopathogenic bacteria of the genera *Photorhabdus* and *Xenorhabdus* (Boemare et al. 1993). These nematodes have been used successfully as bioinsecticides against insect pests. EPNs have a global distribution; the only continent where they have not been found is Antarctica. However, biotic and abiotic factors cause the distribution of EPNs to differ across different regions. Factors such as soil texture, moisture content, temperature, ultraviolet (UV) light, seasonal variation, dominating vegetation, host-finding ability, and dispersal agents are thought to be important in determining their distribution (Griffin et al.

1991). The goal of this section is to discuss the survey of EPNs and factors affecting the natural occurrence and distribution of EPNs around the world. The isolation of EPNs is the first step to establish EPNs as bioinsecticides for controlling insect pests.

### 5.2.1 Survey and Taxonomy

Nematodes belonging to the families Heterorhabditidae and Steinernematidae (Nematoda: Rhabditida) that are entomopathogens have been isolated from soil-inhabiting insects throughout many parts of the world (Poinar 1990). Several taxonomical publications (Liu and Berry 1996) have indicated that if some morphological characters of the infective juvenile (such as the body length and the distance from the head to the base of the esophagus) are combined with some other characters of the male (the shape of spicules, bursa, and genital papillae), most EPNs can be separated. However, other diagnostic methods, such as starch gel electrophoresis (Akhurst 1987), DNA restriction fragment length polymorphisms, restriction enzyme analysis (Smits et al. 1991), cross-mating, isoelectric focusing (Joyce et al. 1994), and randomly amplified polymorphic DNA PCR methods (Liu and Berry 1996), have been used to identify species and strains of EPNs. Also, the use of both molecular and classical methods can overcome the difficulties of extensive overlap in morphometric characters among EPN species and/or strains (Waturu et al. 1997).

The identification of EPNs by standard morphological criteria alone is rarely straightforward (Liu et al. 1999). Attempts to characterize these nematodes reliably have involved a range of techniques, including allozyme electrophoresis for analyzing DNA. The family Heterorhabditidae is monotypic, represented by the genus *Heterorhabditis*. The systematic problems encountered in this group when applying the phonetic approach arise because the adult nematodes feed and reproduce in the protected environment of the insect hemocoel (Liu et al. 1999). Such specialized, but essentially similar modi operandi imply a considerable degree of similarity in morphological features expressed in combination with excessive morphometric variability attributable to density-dependent nutritional factors (Liu et al. 1999). Classical techniques have therefore concentrated on the free-living infective stage, which, although lacking considerable gross morphological variation as a result of being a nonfeeding stage, does show enhanced morphometric consistency. These problems have resulted in considerable confusion as to the status of the nominal species, a confusion that has enormous practical importance now that the nematodes have attracted commercial interest as potential biological control agents. In addition, accurate identification is often demanded by quarantine regulations stipulating that only indigenous species/isolates can be released as part of a biological control program (Hunt 1997). Taxonomic relationships of both heterorhabditid and steinernematid nematodes are usually based on morphological characters; sometimes crossbreeding is used with members of the genus *Steinernema*. Morphological characters cannot be used unambiguously to place new isolates into a particular species. Hashmi et al. (1996) reported that the feasibility of using heterorhabditid and steinernematid nematodes as biological control agents depends on the resources required for a rapid and accurate means to determine the genetic diversity among existing populations of EPN species. These methods can also be used for the development of identification tools.

### 5.2.2 Life Cycle and Host Relationship

The general life cycle of heterorhabditid and steinernematid nematodes involves a free-living infective third-stage juvenile or dauer stage that carries species-specific bacterial symbionts, *Xenorhabdus* or *Photorhabdus*, along its gut or in a pouch off the gut depending on the nematode family (Akhurst 1986). The only life cycle difference between *Heterorhabditis* and *Steinernema* is in the first generation. *Steinernema* species are amphimictic; this means that for successful reproduction, male and female infective juveniles must

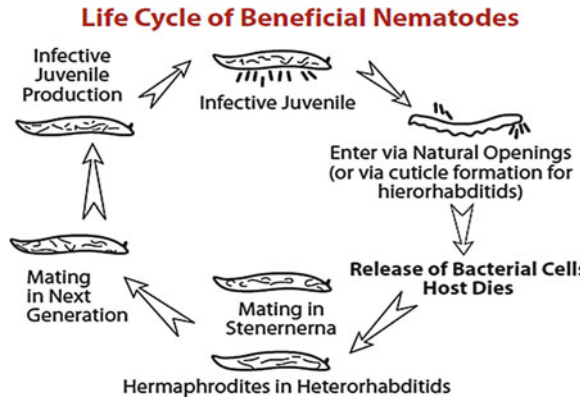


**Fig. 5.1** The generalized life cycle of *Steinernema* and *Heterorhabditis* nematodes. (After Grewal 1999)

enter the host, whereas *Heterorhabditis* species are hermaphroditic, and only one infective juvenile in the host is sufficient for successful reproduction. In the second generation of both nematode genera, reproduction is amphimictic (Poinar 1990). The infective juveniles of both nematodes commonly seek out and enter a suitable insect host through a natural opening such as the spiracles, mouth, and anus, or in the case of *Heterorhabditids*, additionally by penetration of the cuticle by use of a tooth. Once the infective juveniles have penetrated into the host's hemocoel, the nematode releases the bacterial symbiont, which propagates and causes a rapid and fatal septicemia. The bacteria digest the contents of the cadaver, and the nematode feeds on the bacterial culture. The bacteria turn the freshly killed insect larvae a reddish color, and the tissue takes on a characteristic gummy consistency. Undoubtedly, the host is killed by multiplication of the bacteria associated with the nematodes (Poinar 1990). The heterorhabditid infective juvenile grows to become a self-fertile adult inside the invaded insect and reproduces hermaphroditically, whereas the steinernematid infective juvenile becomes either a male or a female and reproduces amphimictically (Fig. 5.1). Interestingly, Grewal et al. (1993) reported that the male infective juveniles of *Steinernema* spp. migrate and penetrate hosts earlier than do females. The females then seek out and penetrate the male-occupied insects. Later, each of the two nematodes passes through two or three dioecious generations before they produce new dauer larvae (infective juveniles), which emerge from the depleted host cadaver (Fig. 5.1) into the soil within 2–3 weeks depending on the conditions (Grewal 1999).

### 5.2.3 Detection of EPNs in Soil

To detect the presence of EPNs in soil, a search for infected insect cadavers should be conducted. Since infected cadavers disintegrate within about 2 weeks, finding these is at best haphazard. Also, this method is unsatisfactory when host insect or nematode densities in nature are low or at low soil temperatures. Bedding and Akhurst (1975) found that the last instar larvae of the greater wax moth, *Galleria mellonella* L., when buried in soil are more susceptible to parasitism by EPNs than are the usual hosts. Normally, *Galleria* larvae live in beehives and are not exposed to nematodes, whereas soil-inhabiting insects have been exposed to EPNs for millions of generations and are expected to have evolved some immune protection. Thus, the *Galleria*-bait method of extracting EPNs from soil samples has become the standard in soil surveys (Fig. 5.2). In addition, laboratory cultures of nematodes can be initiated by force-feeding the infective stages, as they emerge from field-infected insects, to the last instar larvae of the greater wax moth. Until now, larvae of *G. mellonella* have been used as universal hosts for all species and strains of EPNs. However, *G. mellonella* is neither a good host for *S. scapterisci* nor does it reproduce in it (Nguyen and Smart 1990).

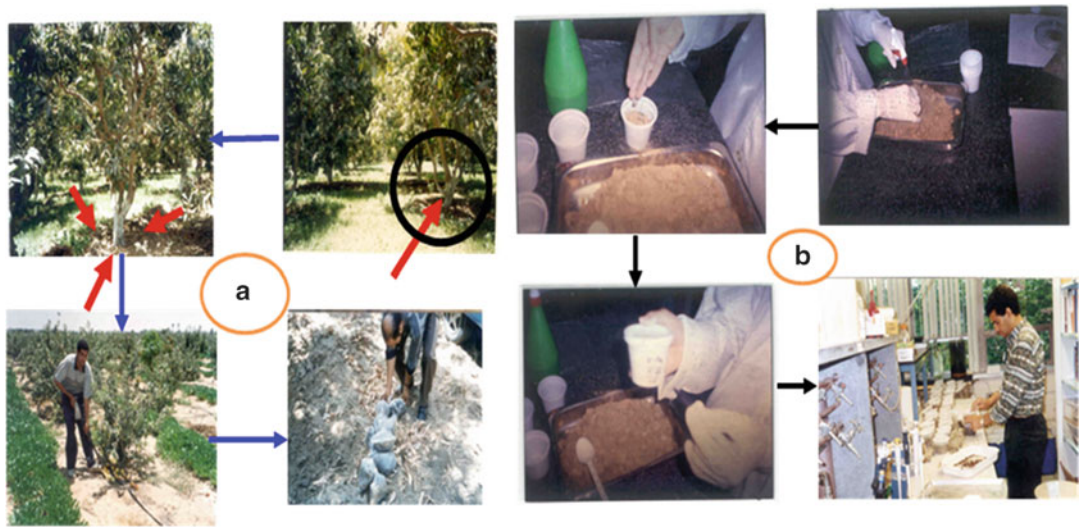


**Fig. 5.2** Isolation of entomopathogenic nematodes from soil. **a** Sequence and locations of soil sampling in the field; **b** the preparation of soil samples in the laboratory to isolate the nematodes. (After Atwa 2002)

### 5.2.4 Factors Affecting the Distribution of the Surveyed Nematodes

Great understanding of the abiotic and biotic factors governing the natural occurrence and abundance of EPNs is of importance in determining the distribution of these species in any survey.

#### 5.2.4.1 Soil Type

Many EPNs have been isolated from different soil types. These nematodes have been associated with humus and organomineral soil layers in Czechoslovakia (Mrácek 1982), humus and sandy soils in Sweden (Burman et al. 1986), sandy loam and loam soils in Ireland (Blackshaw 1988), calcareous soils in England (Hominick and Briscoe 1990), a coral sand in Hawaii (Lindegren et al. 1990), sandy soil restricted to ocean beach areas in the Hawaiian Islands (Hara et al. 1991), sandy and loamy soils in Egypt (Shamseldean and Abd-Elgawad 1994), and sandy soils rather than clay soil in Pakistan (Shahina et al. 1998). Apparently, EPNs travel less well through soils with a small pore space (Molyneux and Bedding 1984). In contrast, the occurrence of EPNs was not influenced by soil or vegetation type in Italy (Deseö et al. 1988). Therefore, Akhurst and Brooks (1984) speculated that the difference in the distribution of nematodes in various countries may reflect the availability of suitable host insects, although environmental influences such as soil type may also determine their distribution. Shapiro-Ilan et al. (2012) indicated that *Steinernema carpocapsae*'s response to electrical fields diminishes with infective juvenile age. Conceivably, the importance of a directional response in foraging strategies may be most important early in the nematode's life cycle. Alternatively, sensitivity to electrical fields may simply degenerate with age. Additionally, in a broader sense, differing substrates may affect EPN response in different soil types.

### 5.2.5 Moisture Content

The infective juvenile or dauer stage carries, initially at least, the unshed second-stage cuticle as a sheath (Nguyen 1993). These infective juveniles can survive the stress of desiccation, particularly if dehydration occurs very slowly (Womersley 1990). This indicates that, under natural conditions, infective juveniles can

survive slow drying, perhaps by aggregating alone or in association with soil colloids, plant root gels, or cadavers (Downes and Griffin 1996). In contrast, Hominick and Briscoe (1990) indicated that the temperate and moist climate of Britain provides conditions suitable for the year-round presence of steinernematids. Also, Garcia Del Pino and Palomo (1996) stated that the greater frequency of occurrence of EPNs in surveyed areas in Spain was associated with medium temperatures and higher rainfalls. They suggest that these climatic conditions are more favorable to nematode survival in the western Mediterranean area. EPNs are frequently found in sites adjacent to the sea (Griffin et al. 1994; Hara et al. 1991) in associations that are intriguing and unexplained. However, it has been shown that infective juveniles are capable of surviving in seawater for several weeks. They suggested that postglacial recolonization by EPNs may have been aided by the migration of coastal sand dune systems under the influence of a rising sea.

#### 5.2.5.1 Temperature

Steinernematids are widely distributed in temperate and cool areas, for example, Czechoslovakia (Mrácek 1980), Sweden (Burman et al. 1986), Britain (Hominick and Briscoe 1990), Germany (Ehlers et al. 1991), Ireland (Griffin et al. 1991; Downes and Griffin 1991), Scotland (Boag et al. 1992), and Norway (Haukeland 1993). These observations seem to suggest that steinernematids prevail in cool and temperate climates because they are better adapted to low temperature (Hominick et al. 1995). Similarly, steinernematids are prevalent, but there is a greater or lesser presence of heterorhabditids, in temperate areas of North America (Akhurst and Booker 1984), Australia (Akhurst and Bedding 1986), and Canada (Mrácek and Webster 1993).

*Heterorhabditis* seems to be commoner in tropical and subtropical climates such as those of Puerto Rico (Roman and Beavers 1982), Hawaii (Hara et al. 1991), Israel (Glazer et al. 1996), Egypt (Shamseldean and Abd-Elgawad 1994), and Pakistan (Shahina et al.1998). These findings may indicate that heterorhabditids are better adapted to warm and hot weather since they need higher temperatures than steinernematids (Molyneux 1986). Recent publications have reported the occurrence of steinernematids in warm and tropical countries, for example, Puerto Rico (Roman and Figueroa 1994), Spain (Garcia Del Pino and Palomo 1996), Portugal (Rosa et al. 1994), Argentina (Stock 1994), Korea (Stock et al. 1997), and Kenya (Waturu et al. 1997). These findings support the view that the broad generalization which holds that steinernematids are temperate species whereas heterorhabditids are tropical species must be questioned (Garcia Del Pino and Palomo 1996).

#### 5.2.5.2 UV Light

EPNs have very poor UV tolerance, indicating that the UV hazard is rarely encountered by natural EPN populations. However, the superior tolerance of *S. carpocapsae* over *Heterorhabditis bacteriophora* may be related to the tendency of that species to nictate at the soil surface (Gaugler et al. 1992).

#### 5.2.5.3 Seasonal Variation

There is some evidence of seasonality in the occurrence of the EPNs in different surveys (Griffin et al. 1991). This may presumably be due to the different climates and/or localities (Akhurst and Bedding 1986) where nematode infectivity is affected by many environmental conditions, including temperature (Grewal et al. 1994) and moisture (Kung et al. 1991), or both factors (Shahina et al. 1998). In contrast, there was no apparent seasonality to the EPN population densities throughout many surveys (Campbell et al. 1995). This indicates that EPNs are present during periods when pest insects are also present and/or suitable climatic conditions for nematode infection and reproduction prevail throughout the year (Hominick and Briscoe 1990). Kanga et al. (2012) illustrated that the diversity of the EPNs found in Cameroonian soils was low, with only three species detected, viz., *Heterorhabditis baujardi*, *Steinernema* sp. A, and *Steinernema* sp. B. *H. baujardi* was much more frequently isolated than the other

species. This suggests a wide range of susceptible hosts for the species.

#### 5.2.5.4 Dominating Vegetation

The literature on the habitat preference of EPNs is contradictory. In Tasmania, Akhurst and Bedding (1986) stated that there were no differences between forests and pasture regarding the presence of EPNs. In Britain, Hominick and Briscoe (1990) pointed out that vegetation had little effect on nematode persistence, similar to results of surveys in Ireland (Griffin et al. 1991) and Spain (Garcia Del Pino and Palomo 1996). Other surveys assessed habitat preferences of these nematodes. In Czechoslovakia, nematodes were commoner in forest than in cultivated fields and were not found in meadows (Mrácek 1980). In North Carolina, woodlands were less suitable than cultivated soils or pastures (Akhurst and Brooks 1984). Nematodes were commoner in Scottish pastures than in forests or croplands (Boag et al. 1992). In New Jersey, nematodes were more abundant in a weedy area than in nearby turf, but across some sites, nematodes appeared to be equally abundant in turf and weedy habitats (Stuart and Gaugler 1994). In this respect, Akhurst and Bedding (1986) suggested that these differences in nematode distribution are related to differences in the distribution of suitable insect hosts and to differences in the species or nematode involved.

#### 5.2.5.5 Host-Finding Ability

Different EPN species and strains exhibit differences in searching behavior which make them more or less suitable for insect pest infectivity; for example, *Steinernema glaseri* dispersed up to 90 cm in a sandy soil (Kaya 1990), whereas some species of *Heterorhabditis* migrate very actively through the soil (Smits et al. 1991), and other species such as *S. carpocapsae* migrate less and may nictate on a solid surface when relative humidities are high (Ishibashi et al. 1994) but become inactive in soil in the absence of hosts (Ishibashi and Kondo 1986). Generally, heterorhabditid infective juveniles are more migratory than those of steinernematids (Downes and Griffin 1996).

In seeking new hosts, EPNs that search by moving throughout their environment to find hosts are termed "cruisers," whereas those that wait for hosts to come to them are termed "ambushers" (Lewis et al. 1992). *S. glaseri* is a cruiser that actively moves in the soil (Schroeder and Beavers 1987), responds strongly to host cues, and is adapted to infect sedentary hosts (Campbell and Gaugler 1993). In contrast, *S. carpocapsae* is an ambusher that stays near the soil surface and does not disperse into the soil, is unresponsive to host cues, and is adapted to infect mobile hosts on the soil surface (Moyle and Kaya 1981). However, cruiser and ambusher behaviors reflect different balances of advantage for the species that display them. Movement increases the probability of encounter with a stationary host, but also with the nematode's natural enemies (Downes and Griffin 1996). Furthermore, an active nematode undoubtedly uses up its limited reserves more quickly. Regarding the attraction of nematodes to insect hosts, EPNs have been shown to respond positively to a chemical gradient around the host (Schmidt and All 1979), carbon dioxide and thermal gradients (Burman and Pye 1980), and materials from hosts or their feces (Kondo and Ishibashi 1986). Further, they can be activated by thermal or mechanical shock, and by certain chemicals (Gaugler and Campbell 1991).

#### 5.2.5.6 Dispersal Agents

Since the infective juveniles are adversely affected by desiccation and UV light, aerial dispersal over great distances is not likely (Downes and Griffin 1996). On the other hand, many adult insect hosts are capable of flying after infection over a period of at least 1 or 2 days after inoculation, and for longer if survival factors are suboptimal for the development of the infection. For example, the infected adults of coleopteran (Glaser and Farrell 1935) and lepidopteran (Timper et al. 1988) species serve as dispersal agents for EPNs. Although such internal infection or external phoresis may be a common method of dispersal in EPNs over a relatively short distance, wind-transported insects are capable of traveling up to 2,000 miles.

Humans are the most effective dispersal agents for nematodes (Ferris et al. 1976). Akhurst and Bedding (1986) speculated that nematodes were introduced into Australia during the immigration of Europeans, probably in soil introduced with exotic plants or ship ballast, or both. Also, EPNs are more frequently found in areas such as parks, lawns, seashores, and nurseries, where human impact has been substantial, rather than in natural habitats (Mrácek and Webster 1993). In addition, EPNs may be imported by researchers for laboratory and limited field testing (Hara et al. 1989). Reasonably, many countries have quarantine laws concerning importation of exotic organisms to protect the natural fauna and flora and local agriculture.

#### 5.2.5.7 Nematode Antagonists

Potential interactions between EPNs and predatory mites, nematodes, and pathogenic fungi in soil fields might have been at least partially responsible for an extended period of infectivity, lack of infectivity, and discontinuities in the temporal pattern of infectivity (Kaya 1990). Accordingly, the persistence of EPNs in sterilized soil was greater than that in unsterilized soil (Curran and Heng 1992). In addition, Fan and Hominick (1991) reported that only 30–40 % of EPNs present in the soil are capable of establishing themselves in *G. mellonella* larvae although all environmental conditions are optimal.

Abiotic stress factors negatively influence the persistence of EPNs. Mrácek and Webster (1993) reported that the absence of EPNs from forest nursery tree beds in Canada may be due to the use of chemical insecticides against root weevils in those tree beds. Similarly, the absence of nematodes from a British Columbian forest, where western spruce budworm larvae and pupae were present, may be due to the unsuitability of the forest litter for nematode survival (Mrácek and Webster 1993).

## 5.3 Techniques for Application of EPNs as Biopesticides

EPNs have received increasing attention because of their potential as bioinsecticides aganist soil insect pests easily found in soil. Poinar and Lindhardt (1971) found that bibionid fly larvae and pupae (*Bibio hortulanus*) in Denmark are probably continuously associated with steinernematids; hence, they may reduce host numbers in barley fields. Poinar (1975) reported that *H. bacteriophora* appeared to be an important pathogen of *Heliothis punctigera* in alfalfa fields in South Australia. Cabanillas and Raulston (1994) stated that *Steinernema riobravis* appears to be endemic in Texas, where it was found parasitizing prepupae and pupae of both corn earworm (*Helicoverpa zea*) and fall armyworm (*Spodoptera frugiperda*).

EPNs possess many attributes of an ideal bioinsecticide: they have a wide host spectrum, are environmentally safe, can be produced in large-scale bioreactors, are easily applied, are compatible with most chemical pesticides, are applied in diverse climatic conditions, and are capable of finding hosts in soil (Garcia Del Pino and Palomo 1996). In addition, the use of naturally occurring nematodes in a particular area as biological control agents may also reduce the risk to nontarget organisms when compared with the use of exotic isolates (Blackshaw 1988).

Selection of appropriate EPNs as bioinsecticides includes bioassays in the laboratory to identify virulent strains and evaluating efficacy under simulated field conditions (Jansson et al. 1993). Gray and Webster (1986) demonstrated that differences in virulence among nematode strains were influenced by temperature. It affects their motility, infectivity, pathogenicity, survival, and reproduction (Glazer et al. 1996). For example, Grewal et al. (1993) stated that *H. bacteriophora* adapted to cold or warm temperature by improving reproduction, but not virulence, whereas *Steinernema anomali* improved virulence, but not reproduction. Additionally, co-inhabiting nematode species may reduce competition in their niche by having different thermal optima (Freckman and Caswell 1985). For the above-mentioned reasons, temperature may be one of the most important factors limiting the success of *Heterorhabditis* ssp. and *Steinernema* ssp. in biological control of insect pests as bioinsecticides.

Application techniques were summarized by Atwa (2011), who reported that the application of EPN studies indicated that *S. carpocapsae* applied to soil may survive relatively longer than when applied foliarly. Soil applications should include the insecticide acephate or permethrin to maintain nematode activity for a long time without having a detrimental effect on these nematodes. For controlling insect borers, the injection technique achieved better control than the spray technique in separate applications of either *Heterorhabditis* sp. or *Steinernema* sp. (1,000 nematodes per milliliter) or the chemical insecticides Cidial 50 % EC and Basudin 60 % EC (3,000 ppm). The best results were obtained by injecting Basudin at 750 ppm with 500 infective juveniles of *Heterorhabditis* sp. per milliliter (64.74 % mortality) or by injecting it at 1,500 ppm with 500 infective juveniles of *Heterorhabditis* sp. per milliliter (63.89 % mortality). Atwa and Shamseldean (2008) found that *Steinernema* sp. (EGB20) was superior to *H. bacteriophora* (EGB13) and *Heterorhabditis indica* (EBN16) when applied for control of *Zeuzera pyrina* with 1,000 infective juveniles of EPNs per milliliter.

The effects of different application technologies were evaluated on the concentration, viability, and efficacy of infective juveniles of *H. indica* and *Steinernema* sp. (IBCB-n6) to control *S. frugiperda* Smith on corn plants by Garcia et al. (2008). Two hundred eighty infective juveniles of *Steinernema* sp. were required to kill 100 % of third-instar fall armyworms in petri dishes, as compared with 400 infective juveniles of *H. indica* to acheive 75 % fall armyworm control. It is possible to spray EPNs without significant loss of their concentration and viability with equipment that produces electrically charges to the spraying mix, and with equipment using hydraulic and rotary nozzle tips. The concentrations of infective juveniles of *H. indica* and *Steinernema* sp. were reduced by 28 and 53 %, respectively, when hydraulic spraying nozzles that require 100-mesh filtrating elements were used (Garcia et al. 2008). Tensoactive agents of the organosilicone and ethoxylate groups did not affect the viability of infective juveniles of *Steinernema* sp. Spraying corn plants (V6 growth stage) with up to 288 million infective juveniles of *Steinernema* sp. per hectare, diluted in the spraying mix to 800 L $ha^{-1}$, with 0.01 % ethoxylate tensoactive agent, or at the same volume followed by artificial rain (6-mm water depth), was not sufficient to control *S. frugiperda* in a controlled environment (Garcia et al. 2008).

## 5.4 Field Application of EPNs

### 5.4.1 Efficacy of EPNs

The efficacy of biopesticides is determined by the biological characters of the agent and the intended target, the physical aspects of the site to which they are applied, and the interactions of the biopesticide and the environment. For biopesticides that are applied to manage soil pests, the opaque, patchy, and complex milieu of soil presents an especially challenging suite of environmental characteristics to consider when trying to predict efficacy. EPNs are used to control insect pests primarily in soil, and can serve as part of a model system to study the interaction of soil processes with soilborne biological control organisms. EPNs in the families Steinernematidae and Heterorhabditidae use symbiotic bacteria (in the genera *Xenorhabdus* and *Photorhabdus*, respectively) to kill and develop inside their hosts (Kaya and Gaugler 1993). On finding a host, infective juveniles penetrate the hemocoel, usually via natural openings, and release symbiotic bacteria which kill the host usually within 24–48 h and provide essential nutrients for nematode development (Fig. 5.3). The nematodes generally complete two to three generations within the host's cadaver and emerge as infective juveniles, which forage for new hosts (Poinar 1990).

Infective juveniles, the only stage existing outside the insect, locate their host by responding to cues such as $CO_2$, temperature, feces, cuticle, electromagnetic fields, and vibration. They can also find their host via indirect cues from plants damaged by insect feeding. The foraging strategy varies with the species; some cruise through the

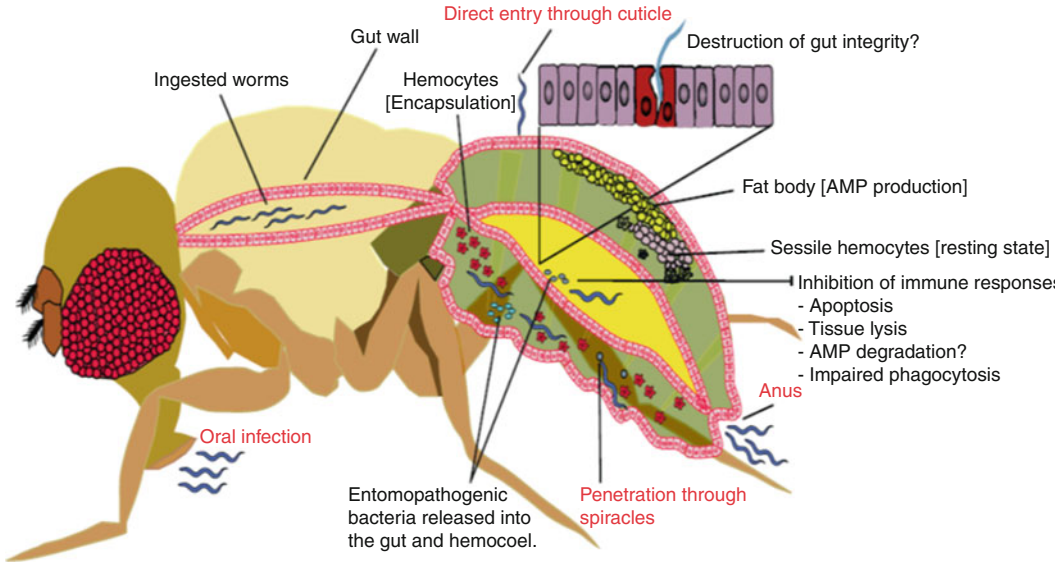


**Fig. 5.3** The routes most commonly used by entomopathogenic (or insect-pathogenic) nematodes to infect their insect hosts. Infective juveniles enter the insect body cavity through the mouth, anus, or spiracles. Once nematodes have gained access to the hemocoel (the insect open circulatory system), they may physically damage various insect tissues and organs, such as the gut and fat body. In the case of entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema*, the release of symbiotic bacteria (*Photorhabdus* and *Xenorhabdus*, respectively) into the insect host leads to suppression of the insect immune response as the bacteria are able to inhibit key cellular immune mechanisms (e.g., phagocytosis). In turn, this leads to a pathological state within the insect (septicemia) that results in rapid insect death. Nematodes are potentially able to cross the disrupted midgut epithelium. The nematodes and their symbiotic bacteria replicate within the insect, where they complete their life cycles before they emerge as a complex from the insect carcass in search of new suitable hosts. The main insect immune-related tissues (circulating, sessile hemocytes, and fat body) are shown. *AMP* antimicrobial peptide. (After Castillo et al. 2011)

soil following cues associated with hosts, others wait to ambush hosts near the soil surface, and many use intermediate foraging strategies (Atwa 2011). Compared with ambushers, cruisers spend more time moving and actively following host-associated cues in the soil, increasing the probability of locating sedentary and cryptic insect hosts.

The efficacy of aboveground applications of EPNs can be limited by the harmful effects of UV radiation and desiccation. Nonetheless, a number of studies indicate aboveground applications of EPNs can result in high levels of control for a variety of pests, including several *Synanthedon* spp. In the case of *Synanthedon pictipes*, however, our initial studies indicated that aboveground field applications with *S. carpocapsae* failed to cause significant *S. pictipes* mortality. Conceivably, improved formulations or application techniques may improve the efficacy of aboveground applications of EPNs. For example, addition of antidesiccants or other adjuvants has been reported to provide improved aboveground control of various foliar pests, including the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), the sweet potato whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), and the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae). Compared with foliar applications, relatively little attention has been devoted to improvement of EPN formulations for application to borer pests.

### 5.4.2 Virulence of EPNs

Selection of appropriate EPNs as biological control agents includes bioassays in the laboratory to identify virulent strains and evaluating efficacy under simulated field conditions

(Jansson et al. 1993). Gray and Webster (1986) demonstrated that differences in virulence among nematode strains were influenced by temperature. It affects their motility, infectivity, pathogenicity, survival, and reproduction (Selvan et al. 1992). For example, Grewal et al. (1993) stated that *H. bacteriophora* adapted to cold or warm temperature by improving reproduction, but not virulence, whereas *S. anomali* improved virulence, but not reproduction. Additionally, co-inhabiting nematode species may reduce competition in their niche by having different thermal optima. For the above-mentioned reasons, temperature may be one of the most important factors limiting the success of *Heterorhabditis* ssp. and *Steinernema* ssp. in biological control of insect pests.

#### 5.4.2.1 Temperature and Infectivity

Soil temperature may be a limiting factor in the ability of nematodes to attack a host. For example, *S. carpocapsae* and *Heterorhabditis* spp. are less adapted to controlling pests at 6 °C (Steiner 1996). Grewal et al. (1994) revealed that differences in thermal adaptation may result in host specialization among EPN species that are adapted to cool-temperature reproduction; for example, *Steinernema feltiae* would be effective against insects that are more active during winter seasons, whereas species that are adapted to warm-temperature reproduction, for example, *S. riobravis* and *Steinernema scapteriscae*, would parasitize insects that are more prevalent during summer. However, Molyneux (1986) and Wright (1992) reported that the Australian and New Zealand strains of *S. feltiae* were virulent at low temperatures, even though they were isolated from warmer climates. Also, Jaworaska (1992) reported that the Polish local strain of *H. bacteriophora* was virulent at a lower temperature of 10 °C although heterorhabditids are endemic to warmer climates. In addition, Grewal et al. (1994) found that the strains of *S. feltiae* isolated from France and Argentina had cool-temperature activities as they infected insects between 8 and 30 °C and reproduced between 10 and 25 °C. They propose that nematodes may have colonized diverse climatic regions without alterations in thermal niche breadth. In this context, the relationship between insect mortality and the number of infective juveniles seems to be density-dependent under certain temperature. Koppenhöfer and Kaya (1997) stated that increasing densities of *S. glaseri* infective juveniles in soil affected the penetration efficiency and reproduction of the nematodes in larvae of *G. mellonella*.

#### 5.4.2.2 Temperature and Activity

Heat may affect "short-range" attraction of nematodes over a few millimeters, as well as host arrest (Burman and Pye 1980). Byers and Poinar (1981) indicated that EPNs aggregate in response to temperature gradients even less than 0.3 °C above ambient temperature, which was the temperature of *G. mellonella* larvae. Apparently, insects in the soil lose very little heat by evaporation. Therefore, their body temperature may rise a "few degrees above ambient" owing to metabolic processes. However, EPNs are attracted not only to the insect body temperature, but also to various stimuli; for example, aqueous surface washes of *G. mellonella* larvae (Schmidt and All 1978), $CO_2$ (Gaugler et al. 1980), the symbiotic bacterium *Xenorhabdus nematophilus* (Ishibashi and Kondo 1990), and the components of insect feces (Schmidt and All 1979). Heat may also stimulate nematode entry through insect orifices (Byers and Poinar 1981).

Khlibsuwan et al. (1992) indicated that nematode migration toward the source of attraction was impaired at 35 and 37 °C. In addition, Steiner (1996) stated that failure of *S. feltiae* nematodes to parasitize *G. mellonella* larvae depends on their poor ability to move at low temperatures (6 °C). These examples illustrate that the nematode's searching strategy depends on temperature, which has important consequences for biological control under field conditions. Nevertheless, host-finding ability appears to be related also to nematode body length, or more likely to the amount of food reserves. Steiner (1996) found that *Steinernema kraussei* traveled a significantly longer distance than *S. feltiae*, and the smallest species of *Heterorhabditis* ssp. and *S. carpocapsae* dispersed only a short distance.

#### 5.4.2.3 Temperature and Reproduction

The influence of ambient temperatures on the development, maturation, and reproduction of EPNs is well documented in the literature. Dutky et al. (1964) and Kaya (1977) confirmed that the most favorable temperature for the growth and reproduction of the DD-136 strain of *Steinernema (=Neoaplectana) carpocapsae* is between 23 and 28 °C, whereas no development was observed at 10 and 33 °C. However, this nematode developed to the adult stage at 30 °C but did not reproduce. Members of the genus *Heterorhabditis* generally have a wider host range than most steinernematid species, but their activity and reproduction are restricted by cool temperatures (Wright et al. 1989). Molyneux (1983) found that *H. bacteriophora* strain V16 and *Heterorhabditis zealandica* strain HNZ were unable to reproduce at 10 °C. In contrast, Wright (1992) stated that the two nematode strains CA and AKLD of *S. feltiae* could produce infective juveniles within *G. mellonella* larvae at 10 °C and the rate of reproduction was directly correlated to the rate of growth of their associated *Xenorhabdus* clones at 10 °C. Grewal et al. (1994) indicated that the thermal niche breadth for reproduction was wide for *S. glaseri* (12–32 °C) and *Steinernema* sp. (20–32 °C). They were more adapted to warm temperatures, whereas *S. feltiae* was more adapted to cooler temperatures (10–25 °C). The inability of the other steinernematid and heterorhabditid species to reproduce at 10 °C may result from the lack of viable sperms or ova or from the mating behavior of the nematodes (Kaya 1977) or may be correlated with the lack of a hot-temperature-active *Xenorhabdus* clone (Wright 1992). In addition, Zervos et al. (1991) observed that the reproduction rate of *S. glaseri* in wax moth larvae was affected by inoculum levels as well as ambient temperatures.

#### 5.4.2.4 Temperature and Survival

Infective juveniles of EPNs may have mechanisms to survive under adverse thermal environments. The nematodes may survive in soil in a quiescent state (Ishibashi and Kondo 1986; Womersley 1990), migrating downward to avoid adverse conditions (Kaya 1990), remaining in the host cadaver for extended periods, lowering the nematode and bacterial metabolic rates and oxygen demands (Brown and Gaugler 1997), or synthesizing trehalose, which prevents freezing, in response to cold environmental stresses. Also, survival may be partly density dependent; hence, no surviving infective juveniles of *S. carpocapsae* were found in heavily infected cadavers (Brown and Gaugler 1997). The relationship between temperature and survival has been studied in many nematode species. Infective juveniles of steinernematids such as *S. carpocapsae*, *S. feltia*e, and *S. glaseri* can survive prolonged storage at 1–5 °C (Bedding 1984), and an Arkansas isolate of *S. carpocapsae* survived for 2 weeks in soil at 40 °C (Gray and Johnson 1983). The contrast among these results could be due to differences in heat tolerance of the strains used. Nevertheless, heat shock treatment for 2 h at 37 °C before exposure to 40 °C enhanced the survival of *Heterorhabditis* sp. IS-5 juveniles to 43 % as compared with a non-heat-shocked control (Glazer et al. 1996). Ogura and Nakashima (1997) indicated that storage of *Steinernema kushidai* at 5 °C caused 90 % mortality within 10 days, but when these nematodes were preconditioned at 10 °C for more than 8 days, a survival rate exceeding 50 % was recorded 100 days after storage at 5 °C.

#### 5.4.2.5 Temperature and Pathogenicity

Pathogenicity of the EPN–bacterium associations of *Steinernema* and *Xenorhabdus* and *Heterorhabditis* and *Photorhabdus* was investigated as a promising means of biological control, including broad host range, high virulence, and host-seeking capability (Poinar 1990). The bacteria converted the insect into a suitable environment for development and reproduction of the nematode's feeding stages (Poinar 1990).

Temperature may be directly related to the growth rate of bacteria, and nematode biology and virulence. Milstead (1981) indicated that development of *H. bacteriophora* was inhibited at 12 and 30 °C, whereas *Xenorhabdus* bacteria

can grow and cause mortality at 12–33 °C, and the length of the incubation period depended on the bacterial growth rate. Furthermore, bacterial dose–mortality responses in *G. mellonella* were similar for all temperatures (15, 20, 25, 28.5, 30 °C), except at 12 °C, where a larger dose was required to kill that host. Wright (1992) stated that the reproduction capacity of different *S. feltiae* strains was related to the growth rate of their associated *Xenorhabdus* clone. Grewal et al. (1993) demonstrated that improvement in nematode virulence and establishment and extension of the thermal infection niche breadths may be fully or partially due to improvements in the growth rate of symbiotic bacteria, *Xenorhabdus* sp.

### 5.4.3 Field Trials

EPNs have been field-tested against numerous agricultural insect pests; forest, vegetable, corn, and turf insect pests (soil, cryptic habitat, or foliar insects) are the targets of EPNs to be controlled. Comprehensive reviews have recently been published on the efficacy of EPNs against insects inhabiting soil and other habitats. Field application showed that EPNs of the genera *Steinernema* and *Heterorhabditis* are effective biopesticides against a wide variety of soil insect pests and for various cropping systems (Atwa 2011), such as the black vine weevil, *Otiorhynchus sulcatus* (F.), the citrus weevil, *Diaprepes abbreviatus* (L.), fungus gnats (Diptera; Sciaridae), various white grubs (Coleoptera; Scarabaeidae) (Atwa 2003), and some lepidopterous insects—the leopard moth, *Z. pyrina*, the Egyptian cotton leafworm, *S. littoralis*, and the cabbage looper, *Pieris brassica* (Atwa 1999). The inoculate release of nematode-based biopesticides is thought to succeed when (1) the pest is present throughout most of the year, (2) the pest has a high economic threshold, and (3) soil conditions are favorable to nematode survival (Atwa 2009). All these criteria can be met in a turf system in which the scarab's larvae are present in the soil for most of the year and the turf is irrigated during dry conditions favorable to nematodes (Atwa 2009). In this section, we will focus on some models of EPNs used under field conditions.

A promising and highly successful use of EPNs as bioinsecticides has been achieved against the soil stage of the fruit borer *Carposina nipponensis* in apple orchards in China and the strawberry scarab *Temnorhynchus baal* in Egypt (Atwa 2003). *Carposina* larvae overwinter in the soil at the base of the trees and emerge in the spring when the temperature reaches 19 °C. Invectives of *S. carpocapsae* are applied to the soil at the time of emergence. In trials performed for 4 years in succession, *Carposina* larval mortality was more than 90 % and fruit damage was below 3 %, values superior to those achieved with chemical insecticides. Inoculate release of *S. glaseri* is applied annually to achieved more than 95 % reduction of the scarab population. Such dramatic success resulted from an extensive systematic effort by Chinese and Australian scientists, and depended on detailed knowledge of the biology of the insect collected over many years by the Chinese. EPN species were screened for effectiveness in the laboratory and in small-scale trials. Extensive field trials with the most appropriate nematodes were then performed. Currently, trials are being conducted over hundreds of hectares of apple orchard. This effort will stand as an exemplary model for the development of an insect control strategy using EPNs.

In Europe, Australia, and North America, the most successful use of nematodes has been against several species of weevils (Fig. 5.4). Applications of *Heterorhahditis* sp. against *O. sulcatus*, the black vine weevil, in containerized soil repeatedly reduced insect densities by 90 %. Other weevils successfully controlled by nematodes include *D. abbreviatus*, the citrus weevil (Schroeder 1990; Tomalak 2005) ,and *Hyalohius ahiefis*, the large pine weevil. The excellent control of weevils that is usually achieved is probably due to a combination of their susceptibility to EPNs and favorable conditions for EPN survival and infection (Fig. 5.4).

In the USA, extensive efforts have been made to control *Popillia japonica*, the Japanese beetle, a major pest of turfgrass. Beetle larvae emerge to feed on grass roots in the spring and autumn.

**Fig. 5.4** Efficacy of the entomopathogenic nematode *Heterorhabditis megidis* on the pupal stage of weevil. Healthy pupae (*right*) and infected pupa (*left*). (After Tomalak 2005)

EPNs are applied in the autumn because temperatures in the spring are usually too low for the EPNs to be effective. *S. carpocapsae* and *Heterorhabditis* sp. have been field-tested the most, simply because of availability. Heterorhabditids have been generally more effective, although their performance has not been consistent. Although approximately 100 field trials against *P. japonica* have been performed, some notable gaps in the knowledge of the interactions among EPNs, insects, and the environment remain. Published data from laboratory screening of different EPN species and strains are scanty, little is known about the ability of different nematode species and strains to pass through the thatch layer (a dense layer of dead roots and organic matter that accumulates above the living root zone) to the root zone where the insects occur, few experiments have been performed to identify the physical factors which limit nematode effectiveness in turf, and the effect of biotic factors is unknown. Consequently, low efficacy in field trials often goes unexplained. Improvements in efficacy may come from subsurface injection of EPNs (Berg et al. 1987), which delivers them directly to the zone of insect activity, and spring applications of strains that are infective at low temperatures, for example, *S. feltiae*. However, what is most required is a redirection of effort from repetitive field trials to the acquisition of more knowledge of the interactions between different nematode species and strains with the target insect and the turf environment. The results of attempts to control the corn rootworm, *Diabrotica* sp., a major pest in the USA, have also been variable. Results of field tests with various strains of *S. carpocapsae* have ranged from no control to control superior to that achieved with chemicals. Once again, the factors contributing to success and failure were not always identified, and the use of nematodes in this application remains unpredictable.

Cryptic habitats within plants, although not the natural habitat of EPNs, provide ideal conditions for their survival and infectivity. Indeed, some of the most reliable results have been achieved against plant-boring insect pests. The blackcurrant borer, *Synanthedon tipuliformis*, was successfully controlled by applying *S. feltiae* to blackcurrant cuttings. In China, the tree-boring cossid moth, *Holcocercus insularis*, has been successfully controlled by manual application of EPNs to the uppermost entry and exit holes on the tree. This species of borer produces interconnecting galleries,

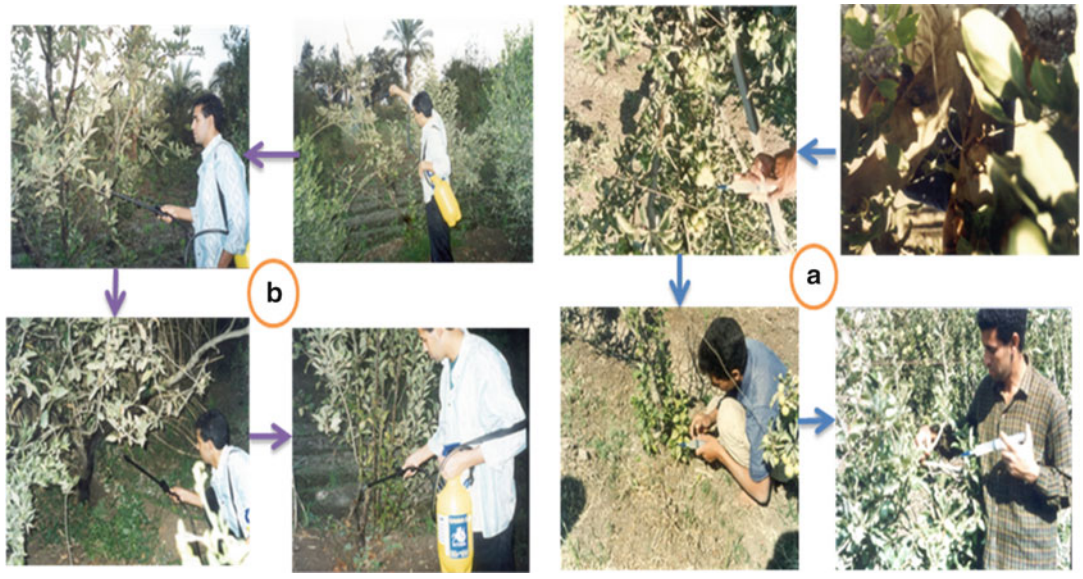


**Fig. 5.5** Control of the leopard moth, *Zeuzera pyrina*, with entomopathogenic nematodes in Egypt. **a** Injection technique for controlling larvae in infected tunnels; **b** direct spraying technique. (After Atwa 1999)

which facilitate EPN recycling; insect mortalities in excess of 90 % are common. In developed countries, lack of a cost-effective method of delivery to gallery openings, which are often difficult to find, is a major limitation to the use of EPNs against boring insects. The injection technique achieved better control than the spray technique (Atwa 1999) in separate applications of either *Heterorhabditis* sp. or *Steinernema* sp. (1,000 nematodes per milliliter). Atwa and Shamseldean (2008) found that *Steinernema* sp. (EGB20) was superior to *H. bacteriophora* (EGB13) and *H. indica* (EBN16) when applied for control of *Z. pyrina* with 1,000 infective juveniles of EPNs per milliliter (Atwa 1999). Injection of the tested nematode suspension into the insect galleries of *Z. pyrina* was more effective than the spray technique (Fig. 5.5). The addition of an evaporation retardant and sticker agent was associated with efficient insect control. Moreover, *S. glaseri* (NJ strains) was tested in the field against *T. baal* infestation on strawberry plants, with the population reduction ranging from 89.2 to 96.8 % after four field applications. The overall population reduction after eight field applications was 96.3–99.1 % (Atwa 2009). The results also showed that both *H. bacteriophora* (EGB13) and *Steinernema* sp. (EGB20) nematode isolates were more effective in reducing the larval population of *S. littoralis* and *P. brassica* on cabbage plants than *H. indica* (EBN16) (Atwa and Shamseldean 2008). Application of *S. carpocapsae* to artichoke plume moth larvae infesting artichoke leaf stalks has been successful. This part of the plant provides conditions suited to EPN survival, as does the cool foggy climate of the artichoke growing area. In contrast to the use of EPNs in cryptic habitats, attempts to use EPNs for insect control in foliar, manure, and aquatic habitats have met with little success, largely because the environmental conditions are not suitable for EPN survival and/or infectivity.

### 5.4.4 Ecological Considerations

In more than three decades there has an explosion of activity in the use of EPNs for insect control, yet, with a few exceptions, their efficacy has generally been lower than that of chemicals, and the effects of nematode application have been less predictable. In this section, a number of important principles to be followed to obtain the best possible field results are described, and some areas for research which could lead to

better exploitation of the nematodes are recommended. Although EPNs are not host-specific, each nematode species and strain has a number of preferred hosts rather than being equally efficient at infecting all insects. There are significant differences in pathogenicity toward sheep blowfly (*Lucilia cuprina*) larvae between *Heterorhabditis* sp. (median lethal dose 18 IJs) and *S. feltiae* (median lethal dose 53,490 IJs). Differences in median lethal time as great as 50-fold were also observed between strains of the same species (Grewal et al. 1993). It is now generally accepted that a number of nematode species and strains should be tested against a particular insect prior to field testing. The median lethal time should preliminarily be determined for individual insects in sand. Two or three EPN species which are the most effective should then be evaluated in pot tests using appropriate soil and plants, followed by small-scale field trials. Although this is possible in theory, in practice few EPN species and strains are available in large enough numbers for field trials, making it impossible to field-test some EPN strains which show most promise in laboratory tests. For example, *S. glaseri* and *Heterorhabditi megidis* were the most effective species against *P. japonica* larvae in laboratory tests (Klein and Georgis 1992), but they have yet to be produced in sufficient numbers for field testing. Thus, for many insect pests, acceptable control with nematodes will not be achieved until an appropriate production method has been developed.

Although strain variability of EPNs is a recognized phenomenon, the possibility of strain variability of the hosts has been neglected. This is no doubt a complicating factor, which will play a part in affecting the efficacy of nematodes. It is necessary to time EPN applications to coincide with or slightly precede the peak occurrence of the most susceptible stage of the insect's life cycle. This is especially critical where the life span or accessibility of the target stage is short, for example, in root maggots. More than one application may be required when insects feed on plants for longer than 2 months, for example, root weevils and mole crickets, or in cases in which there is more than one generation of insects per year, for example, Japanese beetle. Applications are best done at dusk to allow the EPNs time to disperse to cryptic habitats and avoid the lethal effects of UV light and desiccation. For turf and soil applications, irrigation before and after application is recommended for EPN movement and persistence. However, in soils close to their saturation points, EPNs are less effective (Molyneux and Bedding 1984), so moisture levels are critical. In general, temperatures above 30 °C and below 18 °C are held to be outside the optimum for EPN effectiveness. However, temperatures in this range are rare in the UK, and the EPNs are widely distributed (Hominick and Briscoe 1990), so temperature optima should be investigated for species and strains. Applications of at least one billion nematodes per acre are recommended for adequate control, but spot application in containers and greenhouses can lower this density.

Even when all of the above-mentioned factors are considered, unsuccessful field trials are often unexplained. Hundreds of field trials have been performed, yet few have included investigations of the dispersal and persistence of the EPNs or the environmental barriers to infection using appropriate controls. This huge information void is discussed at length by Gaugler (1988), who suggests that further knowledge of nematode soil ecology could be gained from a critical analysis of the differences between successful and unsuccessful trials. Indeed, researchers are increasingly reporting a list of field test parameters, which include the method, the time of application, air and soil temperatures, cloud cover, soil type, soil moisture, stage of pest and pest density, irrigation, and rainfall, all of which help interpretation of field trials. Clearly, the analysis of the results of multiple field trials is valuable. However, when trials are unsuccessful or the results are variable, this approach is a poor second best to performing ecological experiments designed to evaluate the important variables. More emphasis must be placed on this experimental approach for better understanding of the ecological issues of each pest problem. It is often claimed that infective juveniles actively seek out their hosts, but there is little evidence to support this. In a laboratory

assessment of the host-finding capability of S. *curpocupsue*, although a small proportion of infective juveniles moved toward the host, most remained inactive. The available data indicate that EPNs tend to remain at the point of application (Moyle and Kaya 1981). Information on nematode movement in the soil is important because dispersal ability may affect interactions with soil antagonists and strategies with respect to the most advantageous placement of infective juveniles during application. A major focus of research should be on application techniques to determine how best to obtain the optimum distribution of EPNs for a given pest. For example, an approach advocated by Ishibashi et al. (1987) is to use chemicals to activate nematodes to overcome poor nematode mobility in soil. Various agents, including dilute oxamyl (an insecticide/nematicide) and kale and aloe extracts, were shown to stimulate EPN activity. When these agents were applied with EPNs in field trials, higher insect mortalities were achieved.

## 5.5 Mass Culture of EPNs

Many insect antagonists are found within the phylum Nematoda, but only members of the genera *Steinernema* and *Heterorhabditis* have gained major importance as bioinsecticides. These genera are closely related to *Caenorhabditis* (Ehlers 2001). Furthermore, *Caenorhabditis elegans*, the genome sequence of which has been obtained, is the current model organism for studying animal development and genetics. *Steinernema* and *Heterorhabditis* have a symbiotic relationship with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively, and the nematode–bacteria complexes are used in the biological control of insects. In the field, EPNs are mobile and persistent in soil; furthermore, they are highly effective as bioinsecticides and often render better results than those obtained by means of chemical compounds used also for control.

Since EPNs are safe for humans and are environmentally friendly, the commercialization of these nematodes and their associated bacteria is, in many cases, exempt from legislative hurdles and requirements in many countries. Nowadays, EPNs are mainly used in environments in which chemical compounds fail (soil, galleries of boring insects, etc.), or in cases where resistance to insecticides has developed (Ehlers 2001); however, they are used primarily against insects that occur in high-value crops. The main commercial production of EPNs occurs in Asia, Europe, and the USA, but only very few companies produce them in liquid culture using bioreactors. The price of EPNs is still too high to permit their application on low-priced crops. Therefore, the successful commercialization of nematode products depends on the ability to produce sufficient quantities of the product containing infective juvenile forms with the symbiotic bacteria at convenient prices for a full pest-control program. Major problems related to mass production of nematodes in submerged culture remain unsolved. In vivo mass culture can be used for small-scale production of EPNs used with soil insects or cryptic habitat insects, and in vitro mass culture can be used for large-scale production of EPNs for insect control.

### 5.5.1 In Vivo Mass Culture of EPNs

In vivo production methods have been used in the past to produce relatively large numbers of EPNs. Bedding (1981) developed a solid culture technology using flask cultures involving coating crumbed polyurethane foam sponge with poultry offal homogenate. The porous foam afforded a very high surface area to volume ratio for growth while providing adequate gas exchange. The next advance was the adoption of large autoclavable plastic bags to replace flasks as rearing vessels. Medium and shredded foam were hand-blended and sealed in bags before sterilization by an autoclave. Bacterial inoculum was injected into the bags, the contents of the bags were mixed manually, and the bags were placed on racks in an incubation room. After 24 h of bacterial growth, the bags were inoculated with nematode infective juveniles. The bags were kept on racks equipped with a small air compressor and able to deliver air

to each bag over the 2-week incubation period. A conventional medium based on an animal protein and lipid was used. A scaled-up version of the Bedding process would require expensive automated equipment, would have difficulty to maintain an aseptic state, and would present difficulties in medium preparation and nematode harvesting (Gaugler et al. 2002).

Since the early 1980s, EPN liquid culture has been actively researched. One of the great difficulties in optimizing a liquid monoxenic culture is to provide sufficient aeration for both the bacteria and the EPNs without exposing the nematodes to excessive shear forces. Oxygen transfer is not a limiting factor for cultures in shaker flasks, but it is the main problem for the bacterial symbiont growth in commercial bioreactors; in contrast, nematodes have a comparatively low oxygen demand. Further, it has long been recognized that intense agitation can inhibit nematode reproduction. One of the approaches to overcome these problems was to use a bubble column bioreactor for commercial production. These reactors use only air injected at the base for mixing. This bioreactor type proved satisfactory until product demand increased and the need for a more scalable, widely available, conventional stirred tank reactor became evident. Others used low-shear paddle impellers to gently mix the medium and a downward-pointing air sparger. Another bioreactor type for nematode mass production was a stirred-tank bioreactor with an internal draft tube or central cylinder, using a marina impeller that improves circulation and oxygen transfer, reducing shear forces.

In vivo culture is a two-dimensional system that relies on production in trays and shelves (Friedman 1990; Gaugler et al. 2002). Production methods for culturing EPNs in insect hosts have been reported by various authors (Poinar 1990; Woodring and Kaya 1988). All of these references describe (with some variation) a system based on the White trap (White 1927), which takes advantage of the infective juvenile's natural migration away from the host cadaver on emergence.

For commercial purposes, harvested nematodes have to be concentrated prior to formulation. This can be accomplished by gravity settling (Dutky et al. 1964), but prolonged periods of settling may be detrimental to the nematodes because of oxygen deprivation (Burman and Pye 1980). The process can be accelerated by vacuum filtration (Lindegren et al. 1993). Centrifugation is also feasible, but, for commercial in vivo operations, the capital outlay for a centrifuge of sufficient capacity may be excessive. Prior to formulation, EPNs (produced in vivo or in vitro) can be stored in aerated holding tanks for up to 3 months (Georgis et al. 1995). In the White trap method, contamination is minimized because infective juveniles migrate away from the cadaver, leaving most potential contaminants behind. However, some host material or microbial contamination is possible and can be reduced by repeatedly washing the harvested nematodes using the concentration methods described previously. Additionally, decontamination can be accomplished by use of antimicrobial compounds (Dutky et al. 1964; Woodring and Kaya 1988) such as streptomycin sulfate, Hyamine® (methylbenzethonium chloride), merthiolate, NaOCl, and $HgCl_2$ (Lunau et al. 1993), but the effects of these compounds on nematodes for commercial application have not been reported.

### 5.5.2 Factors Affecting In Vivo Yield of EPNs

In vivo production yields differ greatly among different insect hosts and nematode species. The insect host most commonly used for laboratory and commercial EPN culture is the last instar of the larvae of the greater wax moth, *G. mellonella*, because of its high susceptibility to most nematodes, wide availability, ease of rearing, and ability to produce high yields (Woodring and Kaya 1988). There are only a couple of EPNs not amenable to culture in *G. mellonella* (due to extremes in host specificity): *S. kushidai* is most amenable to culture in scarab beetle larvae (Coleoptera: Scarabaeidae), and *S. scapterisci* is most amenable to culture in mole crickets (*Scapteriscus* spp.) (Nguyen and Smart 1990). Other hosts in which in vivo production has been studied include the navel orangeworm (*Amyelois transitella*), tobacco budworm (*Heliothis virescens*), cabbage looper (*Trichoplusia ni*), pink bollworm (*Pectinophora*

*gossypiella*), beet armyworm (*Spodoptera exigua*), corn earworm (*H. zea*), gypsy moth (*Lymantria dispar*), house cricket (*Acheta domesticus*), and various beetles (Coleoptera), including the yellow mealworm (*Tenebrio molitor*) (Lindegren et al. 1979). Other than *G. mellonella*, the host most commonly used for in vivo culture is *T. molitor*, but little research has been reported for production in this host. In response, Gaugler et al. (2002) compared relative yields in *T. molitor* for a number of EPNs. Clearly, nematode yield in *T. molitor* differs among nematode strains and species; for example, *H. bacteriophora* (TF strain) produced approximately twice the progeny of *H. indica* (Hom1 strain) and *Heterorhabditis marelatus* (Point Reyes strain). Higher reproductive potential of one nematode relative to another (e.g., as observed in the TF strain) may result from a closer natural association with the host or its relatives (Shapiro et al. 1999).

In general, nematode yield is proportional to host size (Flanders et al. 1996), yet yield per milligram of insect (within the host species) and susceptibility to infection are often inversely proportional to host size or age (Shapiro et al. 1999). Ease of culture and ease of infection are important factors when choosing a host; for example, the long-horned beetle (Cerambycidae) can produce more than twice the number of nematodes as *G. mellonella*, but (as with many of the insects listed above) difficulty or cost of rearing, and inconsistency of infection, precludes these insects from being suitable hosts. Among nematode species, yield is generally inversely proportional to size (Grewal et al. 1994; Hominick et al. 1997).

The choice of the host species and the nematode for in vivo production should ultimately rest on nematode yield per cost of insect and the suitability of the nematode for the pest target. Cost analysis among different host species has rarely been addressed. In a crude approach to the problem (i.e., without statistical analysis), Blinova and Ivanova (1987) reported *T. molitor* to be more cost-efficient than *G. mellonella* and *T. ni* for producing *S. carpocapsae*. A hastened life cycle within the host might affect the cost by allowing faster production cycles; recently, *Steinernema abbasi* was reported to produce a roughly equivalent number of progeny in half the time of other EPNs (first emergence beginning after only 3.5 days) (Atwa 1999; Grewal et al. 1994). Another issue that has rarely been addressed in the choice of nematode and host is the resulting quality of the product. Nematode quality appears to be greater when the nematode is cultured in hosts that are within the nematode's natural host range (Abu Hatab and Gaugler 2001). Furthermore, nematodes can adapt to the host on which they are reared (Stuart and Gaugler 1996), which could reduce field efficacy if that host is not related to the target. Therefore, although *G. mellonella* may often be the most efficient host to use, it may not be the most appropriate "medium" for maximizing efficacy with regard to a particular target pest.

In vivo production yields are dependent on nematode dose (Boff et al. 2000). A dose that is too low results in low host mortality, and a dose that is too high often results in a high level of failed infections owing to competition with secondary invaders (Woodring and Kaya 1988). These outcomes reduce production efficiency owing to the need to remove live or poorly infected insects. The number of nematodes that invade a host is proportional to the exposure concentration. Selvan et al. (1993) found that optimization of the initial nematode density within the host (e.g., at 100 *H. bacteriophora* and *S. carpocapsae* nematodes per *G. mellonella* moth) maximizes nematode survival and fecundity. Thus, intermediate doses maximize yield (Boff et al. 2000). Similarly, host density per unit area affects nematode invasion and thus may affect yield.

Environmental factors such as temperature, aeration, and moisture can affect the yield of infective juveniles produced. The rearing temperature affects both the yield and the life-cycle duration (time to emergence) (Grewal et al. 1994). Generally, the optimum culture temperature is related to the nematode's climate of origin (Grewal et al. 1994; Molyneux 1986). Grewal et al. (1994) determined the optimum rearing temperature and time to emergence in *G. mellonella* for 12 species and strains of EPNs; the optimum temperatures ranged from 18 to 28 °C. Adequate aeration is necessary for nematode development (Friedman 1990). The moisture level is another

essential component for in vivo culture. High levels of humidity must be maintained throughout the production cycle (Woodring and Kaya 1988). In the White trap method, the substrate must remain moist to prevent cadaver desiccation and allow emerging infective juveniles to migrate, but too much water will prevent movement and interfere with oxygen exchange.

The inoculation method can affect infection efficiency and thus yield potential. Inoculation for in vivo production can be accomplished by pipetting or spraying nematodes onto a substrate, immersion of hosts in a nematode suspension, or (for some hosts) applying the nematodes to the insect's food. Comparison of methods has rarely been addressed. Immersion of hosts is more time-efficient but requires more nematodes than other procedures. Additionally, some host–nematode combinations may not be suitable for the immersion method; for example, it appears *H. bacteriophora* cannot infect *T. molitor* at levels required for mass production (90 % or higher) using the immersion method, but can do so when applied by feeding or pipette. Blinova and Ivanova (1987) reported that infectivity of *S. carpocapsae* in *T. molitor* was increased using the feeding method relative to other methods. Feeding, however, would require an additional step of removing infected cadavers from food remnants (which may cause contamination); thus, the inoculation procedure must be included in a cost-efficiency analysis before a method is decided on.

A concern for both in vivo and in vitro production is strain deterioration. When a biological control agent is isolated from nature and reared in the laboratory, or mass-produced for commercial purposes, it may lose beneficial traits because of genetic processes, including drift, inbreeding, and inadvertent selection (Hopper et al. 1993). Thus, repeated culturing of nematodes can result in reduction of quality and fitness characters such as virulence, environmental tolerance, and reproductive capacity (Stuart and Gaugler 1996). Therefore, precautions against strain deterioration should be taken; for example, cryopreservation of stock cultures, minimization of serial passages, and introduction of fresh genetic material (Gaugler et al. 2000).

### 5.5.3 In Vitro Mass Culture of EPNs

In vitro technology requires substantial capital investment in sterilization equipment, as well as considerable technical expertise. However, these disadvantages are offset by production costs as low as US$12 for *S. carpocapsae* (Gaugler and Han 2002). In contrast, in vivo production has low requirements for capital or expertise, but is difficult to scale up and hence it is difficult to achieve economies of scale. Lindegren et al. (1993) estimated in vivo production costs of US$150 per billion EPNs. Consequently, nematode producers reliant on in vivo methods form a cottage industry of low-volume producers (Gaugler et al. 2000). In vivo production is based on the adaptation of the White trap (White 1927) by Dutky et al. (1964), albeit with some modifications (e.g., Lindegren et al. 1993), in which nematode-killed hosts are placed above a water reservoir. The method exploits the tendency of infective nematodes to migrate from depleted host cadavers into the reservoir, which is decanted to collect infective juveniles. This system is appropriate for laboratory bench-scale production of inoculum for experiments, but its labor-intensive nature makes it inefficient for large-scale production.

Scale-up of in vivo production has consisted of providing larger White traps, reducing the extraction efficiency by increasing the migration distance to the reservoir. Apart from Carne and Reed (1964),who described a harvest system that was conceptually similar to the Baermann funnel, no further ideas for mechanizing in vivo production surfaced over the intervening more than 50 years until now. We report the first scalable in vivo system for mass production of EPNs.

### 5.5.4 Overview of Mass Production of EPNs

Mass production on artificial media was realized 30 years before Dutky et al. (1964) established effective in vivo methods. Solid culture was pioneered by Rudolf Glaser, who was the first to artificially culture a parasitic nematode. Glaser and his coworkers, in one of the most ambitious

and least known experiments in biological control, produced and released billions of *S. glaseri* throughout New Jersey to attack Japanese beetles from 1939 to 1942 (Fleming 1968). Regrettably, early workers were unaware of the nematode's bacterial partner. Nematode mass production was conducted in shallow trays of veal-pulp medium with salicylic acid and formaldehyde to repress contaminating microbes (McCoy and Girth 1938), including apparently the natural symbiont, *Xenorhabdus poinarii*. Today, the need for monoxenicity is universally recognized as one of the cornerstones of nematode in vitro culture.

Others extended Glaser's accomplishment by developing other media as alternatives to costly animal tissue homogenates, such as the dog food medium of House et al. (1965). Regardless of the growth medium, cultures were produced on the substrate surface because of the need for adequate gas exchange. That is, cultures were two-dimensional, perfectly suited for laboratory cultures, but a limitation that precluded commercial-scale production. The development by Bedding (1981, 1984) of practical solid culture technology was a seminal step in nematode production because it made the leap from two-to three-dimensional substrates. Bedding flask cultures involved thinly coating crumbed polyurethane foam sponge with poultry offal homogenate. The porous foam afforded an outstanding surface area to volume ratio for growth while providing adequate gas exchange. A primer for preparing Bedding flasks is found in Woodring and Kaya (1988). The next advance was the adoption of large autoclavable plastic bags to replace flasks as rearing vessels (Bedding 1984). Medium and shredded foam were hand-blended and sealed in bags before sterilization by an autoclave. Bacterial inoculum was injected into the bags, the contents of the bags were mixed manually, and the bags were placed on racks in an incubation room. After 24 h of bacterial growth, nematode inoculum was injected into the bags (e.g., *S. scapterisci* was introduced at 2,000 infective juveniles per gram of medium), and the contents were mixed again. Holding racks were equipped with a small air compressor and gang valve leading to a network of hoses delivering air to each bag over the 2-week incubation period.

A conventional medium was also developed because poultry entrails cannot be standardized and so provide unreliable results. Spurred by the development by Wouts (1981) of a practical yeast extract, corn oil, and soy flour medium for the Bedding flask, a new medium based on yeast extract, corn oil, corn starch, and dried egg solids was developed. Scaling up Bedding's advances to commercial production was undertaken in collaboration with Biotech Australia, which licensed the technology. The use of bags and an improved medium permitted commercial-scale nematode production, but shortcomings were encountered that reduced the effectiveness. Most troublesome was that each bag required a laborious custom fitting of costly inlet and outlet microbial filters. The compressors increased the air-conditioning load in the incubation room, which became problematic for *S. scapterisci* as this species produced metabolic heat within the bag to the point that growth could be retarded. Condensation (metabolic water) sometimes saturated bag edges and was associated with poor growth. These limitations contributed to inconsistent yield.

Bedding et al. (1996) addressed the gas-exchange issue in developing a stainless steel box system. The key innovation was a foam sponge gasket lining the inside edge of the box lid that provided passive ventilation. Nevertheless, Biotech Australia judged there was insufficient improvement to justify the expense of constructing the boxes, and the new system was not implemented. Nematode extraction from the foam medium was accomplished using active migration and sedimentation as in a Baermann funnel apparatus. Harvest trays were constructed, several square meters in size, with a bottom support screen of aluminum. The water level in the trays was adjusted to the same height as the screen, and a cloth fabric was placed over the screen. The bags were emptied onto the cloth, and nematodes migrated through the cloth and into the water reservoir. Trays were hinged so

they could be decanted after migration to remove bacteria and medium residue. The collected nematodes were then pumped to a chilled holding tank with a bacteriostat to await formulation. This manufacturing process worked moderately well for highly mobile species such as *H. bacteriophora*. By contrast, migration rates for sedentary nematodes such as *S. scapterisci* ranged from 50 to 75 %, often with excessive numbers of noninfective stages that stimulated microbial activity and reduced shelf life.

## 5.6 Safety of EPNs and Quality Control of EPN Production

### 5.6.1 Safety of EPNs

EPNs are exceptionally safe biological control agents; they are certainly more specific and are less of a threat to the environment than chemical insecticides. Since the first use of the EPN *S. glaseri* against the white grub *P. japonica* in New Jersey (USA) (Glaser and Farrell 1935), not even inferior damage or hazards caused by the use of EPNs to the environment have been recorded. The use of EPNs is safe for the user. EPNs and their associated bacteria have detrimental effect on mammals or plants (Akhurst and Smith 2002). A joint workshop supported by EU COST Action 819 "Entomopathogenic Nematodes" and the OECD research program "Biological Resource Management for Sustainable Agriculture Systems," which met in 1995 to discuss potential risks related to the use of EPNs in biological control, concluded that EPNs are safe for production and application personnel and consumers of agriculture products treated with EPNs. The expert group could not identify any risk to the general public related to the use of EPNs.

No reports exist documenting any effect on humans caused by the symbiotic bacteria. A related nonsymbiotic species, *Photorhabdus asymbiotica*, was reported five times from humans in the USA (Farmer et al. 1989). Another group of nonsymbiotic *Photorhabdus* was reported from five patients in Australia (Peel et al. 1999). From most of the patients, other human-pathogenic bacteria were also recorded; for example, *Photorhabdus* spp. were opportunistic. The route of the infections was not established. Three infections might have been related to spider bites. Both clinical groups lack symbiotic relations with nematodes, and strains within each group have a high level of within-group relatedness but do not cluster in groups containing the nematode symbionts (Akhurst and Smith 2002). The existence of bacterial species with and without pathogenic effects on humans within one genus is common (e.g., *Bacillus*). No action is therefore required and no conclusions should be drawn from the reports of pathogenic effects on humans caused by nonsymbiotic *Photorhabdus* spp. or potential risks related to the use of EPNs and their symbiotic bacteria.

Naturally occurring nematode populations cause sustainable effects on pest populations. These effects have not been very well exploited because we understand little of EPN population dynamics and the possibilities to enhance EPN populations by culture methods (Fischer and Führer 1990). At present, we cannot evaluate the economic benefits of sustainable effects. The economic effect of introducing an exotic species is easier to assess. In the case of a pest population surpassing the economic threshold, the use of an exotic nematode might be economically reasonable. It is often argued that prior to the release of exotic species, it should be tested whether an endemic population might also be the solution to a problem. However, the naturally occurring species, even if superior in its control potential, might not be commercially available. Waiting until the endemic population has increased and reached an even distribution to significantly reduce the pest population will result in economic losses. The benefit from introducing the exotic species will overwhelm the damage caused by a reduction of the population of the endemic EPN species. Should the exotic species persist, we will have a case of "biological pollution." However, is this

damage or a benefit for the farmer? As exotic species have not been recorded to eliminate the endemic EPN species, no real hazard has yet been identified with the introduction of the exotic species and the "biological pollution."

### 5.6.2 Quality Control of EPN Production

The quality of EPNs, when applied to steinernematids and heterorhabditids and their associated bacteria from a technical perspective, is usually defined as a set of linked parameters to be monitored and evaluated, such as nematode viability or percent viable, total viable nematodes per unit of product, nematode virulence (as indicated via bioassay), nematode age (after harvest, formulation, shelf life, etc.), and morphological measurements, and demonstrated performance of all these parameters should be available on the product label. Until now and according to different points of view for most farmers and end users of chemical products, they believe that the liquid formulation is the best among all types of formulations. Concurrently, most nematologists believe that the liquid formulation of nematodes is the best one to be used in the field. However, to obtain a promising EPN as a bioinsecticide, some points should be taken into consideration, such as storage, packaging, transportation, and field application methods (injection and/or spraying). The quality of the nematode products was determined under field conditions and after field transportation. The quality test was accomplished on nematodes before field trips, in the laboratory, and after transportation to the field. Viability (total viable nematodes), nematode virulence, storage ability of nematodes, morphological examination, and field efficacy were recorded. The data showed that, with optimal conditions, the nematode quality did not change (Atwa 2003). All tested parameters have indicated that, despite the low nematode numbers obtained from the in vivo methods, the nematodes have better quality, virulence, and better performance in the field compared with the nematodes produced by in vitro mass culture methods. The low numbers of nematodes produced through in vivo mass culture methods is not a great problem yet with the use of automated in vivo mass culture such as the LOTEK system (Gaugler et al. 2002).

## 5.7 Future Focus of EPNs as Bioinsecticides

This chapter has provided information on the applied aspects of EPNs as biopesticides against insect pests. These EPNs are already applied on ornamentals and vegetables in greenhouses and container crops in tree nurseries in Europe and the USA. EPNs as biopesticides can make an important contribution to the development of sustainable agriculture, but relatively few EPN biopesticides have been commercialized. EPNs as biopesticides can make important contributions to integrated control management and help reduce reliance on chemical pesticides. Hence, they have a major role to play in the development of sustainable farming. There are a range of definitions of what constitutes EPNs as biopesticides, and the terms used can be confusing at times. Essentially, we are dealing with a broad group of agents. We have defined and illustrated EPNs as biopesticides as mass-produced, biologically based agents used for the control of plant insect pests. This definition encompasses not only the active ingredient of a biopesticide, but also how it is used. Nowadays, the only bioinsecticide manufactured on an industrial scale and available on the market at prices which farmers can afford is the bacterium *Bacillus thuringiensis*. However, *B. thuringiensis* is not effective with the soil insect while EPNs are ideal to control soil insect. The great potential of EPNs for effective insect pest control, the possibility to apply them with conventional sprayers, and the possibility to produce them in liquid culture bioreactors make them a good candidate for large-scale manufacturing processes at reasonable cost. Two research and development strategies will provide the necessary progress to achieve this goal; further formulation improvement to stabilize the quality of nematode-based products and reduction of nematode application concentration. The information in this chapter on isolation of

EPNs to mass culture and product safety and quality control will allow the necessary progress to be made to achieve the main goals of this chapter to scale up the use of EPNs as bioinsecticides. In general, this chapter will have a significant economic and technical impact on the overall progress in agriculture practice and will stimulate further development and application of biotechnology and bioengineering within India and some countries in Asia and the Middle East.

## 5.8 Summary

Data were collected from field studies of EPNs to discuss isolation techniques and nematode distribution in different soil types all over the world. Environmental factors affecting nematode distribution were also discussed. EPNs have received increasing attention because of their potential as bioinsecticides against soil insect pests easily found in soil. Selection of appropriate EPNs as bioinsecticides includes bioassays in the laboratory to identify virulent strains and evaluating efficacy under simulated field conditions. EPN studies indicated the differences between EPN species, isolates, and/or strains applied to soil or applied foliarly. The effects of different application technologies were evaluated with regard to the concentration, viability, and efficacy of infective juveniles of the nematode species, isolates, and/or strains, and factors affecting field efficacy and reproduction were discussed. The efficacy of biopesticides is determined by biological characters of the agent and the intended target, the physical aspects of the site to which they are applied, and the interactions of the biopesticide and the environment. EPNs are used to control insect pests primarily in soil, and can serve as part of a model system to study the interaction of soil processes with soilborne biological control organisms. The effects of temperature and sunlight on EPN virulence were discussed. The mass production of EPNs depends on in vivo production in the greater wax moth, *G. mellonella*, and/or the beetle *T. molitor*. Novel devices (LOTEK) have been developed for in vivo mass production. The number of infective juveniles of EPNs produced from one unit has reached $50 \times 10^7$–$75 \times 10^7$. One unit consists of ten racks with 500 cadavers of insects per rack, with a total of 5,000 insect larvae per unit, with a total weight ranging from 750 to 900 g, which may be enough for a small-scale experiment. This chapter closed with discussion of the safety of EPNs and quality control. EPNs are exceptionally safe biological control agents: they are certainly more specific and are less of a threat to the environment than chemical insecticides. Since the first use of the EPN *S. glaseri* against the white grub *P. japonica* in New Jersey (USA), not even inferior damage or hazards caused by the use of EPNs to the environment or humans have been recorded. The use of EPNs is safe for the user. Quality control of EPN production is very important. Nematode viability or percent viable, total viable nematodes per unit of product, nematode virulence (as indicated via bioassay), nematode age (after harvest, formulation, shelf life, etc.), morphological measurements, and demonstrated performance are all parameters that should be available on the product label. Finally, we conclude that EPNs are very promising for future use as bioinsecticides, mass production of EPNs can be conducted, and the nematodes produced can be easily applied in the field by any farmer. Field releases of EPNs by farmers can be applied through the spread of insect cadavers infected with the nematodes in fields infested with insect pests, where emerged nematode infective juveniles will infect other insect pests. EPNs can be used exclusively to control insect pests which live in cryptic habitats such as tree borers and soil insects, for example, the red palm weevil and white grubs of scarab pests of strawberries.

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# Microbial Elicitors to Induce Immunity for Plant Disease Control in Chilli and Tomato

6

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**Abstract**

The induced immunity in long cayenne pepper in terms of phytoalexin production using microbial elicitors as chaetoglobosin C from *Chaetomium globosum*, chaetomanone A from *Chaetomium lucknowense*, and trichotoxin A50 from *Trichoderma harzianum* PC01 was investigated in pot experiments. Stem inoculation of chilli plants with *Colletotrichum capsici* isolate C208 resulted in necrosis and accumulation of capsidiol, a phytoalexin. Stem inoculation of chilli plants resulted in different degrees of sensitivity to *C. capsici*, expressed as necrosis along the stems. The microbial elicitors chaetoglobosin C, chaetomanone A, and trichotoxin A50 induced an immunity response against *C. capsici*. Qualitative analysis of capsidiol accumulation in the upper part of the chilli plant was detected by thin-layer chromatography and showed that capsidiol had accumulated in the chilli plant at 5 and 10 days after treatment with the microbial elicitors, whereas no capsidiol was detected in the noninoculated and untreated plants. Chilli cultivation of long cayenne pepper in vivo was conducted by spraying chaetoglobosin C, chaetomanone A, and trichotoxin A50, and the findings were compared with cultivation using a chemical fungicide

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_6, 

(difenoconazole). The results showed that application of each microbial elicitor reduced disease incidence by 42–55 % when compared with an inoculated control, whereas difenoconazole reduced disease incidence by 33 %. Additionally, the yield increased by 47–55 % when compared with the yield for the inoculated control. Chilli cultivation of long cayenne pepper in vivo using the biological fungicides Bio-CG from *C. globosum* N0802, Bio-CLT from *C. lucknowense* CLT, and Bio-T from *T. harzianum* PC01 resulted in reduction of disease incidence by 39–55 % when compared with the inoculated control, whereas difenoconazole reduced disease incidence by 33 %. Moreover, treatment with Bio-CG, Bio-CLT, and Bio-T increased the yield by 39–53 % when compared with yield for the inoculated control.

Moreover, the bioactive compounds chaetoglobosin C, chaetomanone A, and trichotoxin A50 were used as microbial elicitors to elicit α-tomatine in tomato. α-Tomatine was detected by high-performance liquid chromatography after treatment with the bioactive compounds. Chaetoglobosin C, chaetomanone A, and trichotoxin A50 were sprayed onto inoculated tomato seedling variety Sida inoculated with *Fusarium oxysporum* f. sp. *lycopersici* NKSC01, and plant disease immunity and quantity of α-tomatine were assessed. The results revealed that plant disease immunity of tomato seedlings treated with chaetoglobosin C, chaetomanone A, and trichotoxin A50 at a concentration of 50 μg/ml after 10 days was 44.97, 35.18, and 39.43 %, respectively, whereas tomato seedlings treated with prochloraz that showed plant disease immunity of 29.95 %. The stems and leaves of tomato were extracted and spotted on thin-layer chromatography paper and produced a green spot with a retention factor of 0.23, the same as for a spot of standard α-tomatine. Tomato extracts were analyzed for α-tomatine by high-performance liquid chromatography. The α-tomatine quantification data were analyzed using a linear regression curve. Tomato treated with chaetoglobosin C, chaetomanone A, and trichotoxin A50 at 15 days expressed α-tomatine at levels of 207.87, 254.25, and 205.04 μg/g, levels which are significantly higher than those resulting from prochloraz and inoculation treatments, for which the quantities of α-tomatine were 131.56 and 77.46 μg/g, respectively. It is shown that the induction of α-tomatine by chaetoglobosin C, chaetomanone A, and trichotoxin A50 in tomato plants implies disease immunity against fusarium wilt of tomato variety Sida through phytoalexin production. The bioactive compounds were tested for their efficacies to control tomato wilt in vivo. The results revealed that all the bioactive compounds at concentrations of 10, 50, and 100 μg/ml could induce plant disease immunity in tomato between 53.80 and 65.15 %, which is significantly higher than the plant disease immunity induced by prochloraz, which was 26.73 %.

**Keywords**
Phytoalexin • Plant immunity • Elicitor

## 6.1 Microbial Elicitors Inducing Plant Disease Immunity in Chilli

The prospect of broad-spectrum disease control using the plant's own resistance mechanisms has increased interest in the development of agents which can mimic natural inducers of resistance (Walters et al. 2005). Research on resistance mechanisms has shown that plant defenses are activated after infection. Many biochemical changes occur in plants after infection, and some of these have been associated with the expression of defense because they have activities against pathogens in vitro (Hammerschmidt 1999). One type of biochemical response that is strongly associated with defense is the accumulation of phytoalexins, which are low molecular weight antimicrobial compounds that are produced after infection. Their production is triggered either by pathogen attack or by biotic and abiotic elicitors such as synthetic chemicals, substances from microorganisms, polysaccharides, proteins, and cellulose (Chávez-Moctezuma and Lozoya-Gloria 1996; Walters et al. 2005). Phytoalexins are produced by plants not only in response to interactions with fungi or other living organisms, but also following treatment with many chemicals, irradiation by ultraviolet light, and exposure to the products of microbial metabolites (Zbell and Walter-Back 1989). In addition, phytoalexin production can also be induced by fungicides. Hwang and Sung (1989) demonstrated that metalaxyl induced the phytoalexin capsidiol in the stem of chilli plants infected with *Phytophthora capsici*.

Capsidiol is the major phytoalexin that accumulates in tobacco (*Nicotiana tabacum*) and chilli (*Capsicum annuum*) in response to fungal infection as reported by Hoshino et al. (1995). Capsidiol is biosynthetically derived from the mevalonate pathway via farnesyl pyrophosphate. Farnesyl pyrophosphate is a key intermediate in the biosynthesis of terpenes. Specific cyclization reactions of farnesyl pyrophosphate are catalyzed by particular sesquiterpene cyclases. The enzyme 5-*epi*-aristolochene synthase from tobacco and chilli produces 5-*epi*-aristolochene, which is the immediate precursor of the bicyclic phytoalexin capsidiol (Zook et al. 1996; Back et al. 1998; Zavala-Páramo et al. 2000). The sesquiterpenoid phytoalexin capsidiol, the principal phytoalexin of *C. annuum*, has been isolated from fruits, leaves, and stems of chilli. Watson and Brooks (1984) reported pectinase (from *Aspergillus niger*) and cellulase (from *Trichoderma viride*) caused rapid accumulation of capsidiol in the diffusates and flesh of green chillies, and apparently that was the first reported example of the potency of these two agents in eliciting sesquiterpenoid phytoalexins. Capsidiol production was observed in vitro root cultures of chilli after treatment with cellulase. The maximal secretion of capsidiol to the medium was achieved at 24 h after treatment (Chávez-Moctezuma and Lozoya-Gloria 1996). Ma (2008) reported that the production of a sesquiterpenoid from a *C. annuum* suspension culture was induced by the elicitor cellulase, and the induced compound, capsidiol, mainly accumulated in the culture medium. Moreover, the elicitation may be mediated by a second messenger, jasmonic acid, linked by preceding phospholipase $A_2$ activation. Bhandal and Paxton (1991) suggested that Polytran L, a commercially produced glucan of fungal origin, can be used as an elicitor at 0.25 % (w/v) for inducing capsidiol accumulation in unripe chilli fruits. The resistance of *C. annuum* to chilli blight causd by *P. capsici* was observed after inoculating the chilli stems with the fungal pathogen. Capsidiol accumulated in the area of necrosis and appeared to be involved in this resistance (Egea et al. 1996a, b; García-Pérez et al. 1998). Ahmed et al. (2000) suggested that *Trichoderma harzianum* probably uses a combination of biocontrol mechanisms against *P. capsici* to induce resistance in chilli.

As microbial elicitors, we evaluated pure compounds from fungi—namely, chaetoglobosin C from *Chaetomium globosum*, chaetomanone A from *Chaetomium lucknowense*, and trichotoxin A50 from *T. harzianum* (Fig. 6.1). Stock solutions (50 ml) of each microbial elicitor at a concentration of 1,000 µg/ml were prepared by dissolving 0.05 g of the elicitor in dimethyl sulfoxide. Then, the stock solutions were diluted with distilled

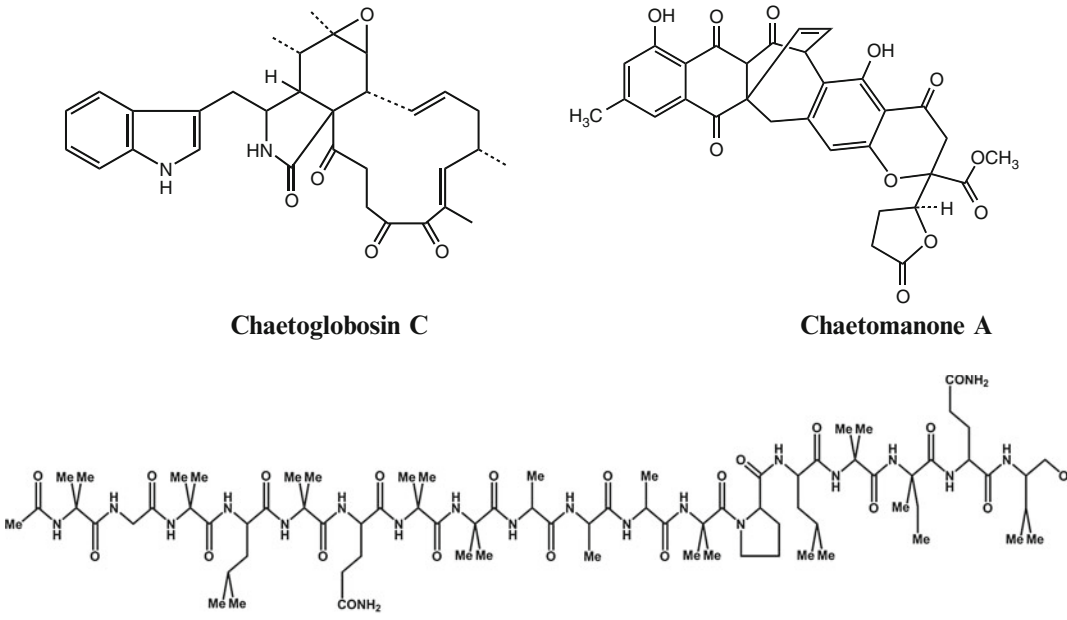


**Fig. 6.1** Chemical structure of microbial elicitors (Kanokmedhakul et al. 2002; 2006; Suwan et al. 2000)

water to elicitor concentrations of 10, 50, and 100 μg/ml.

The immunity induced in long cayenne pepper plants as shown by phytoalexin production was achieved by inoculation with a highly virulent *Colletotrichum* isolate, using the plug inoculation method over the wounded stem (5 mm long). Five-millimeter-diameter plugs of the *Colletotrichum* isolate were cut from the edge of the colony, the wounded sites were inoculated with the *Colletotrichum* isolate, and were then covered with aluminum foil containing moist cotton wool (Fig. 6.2a). Noninoculated chilli plants were also wounded on their stems, and only a potato dextrose agar plug was placed over the wound; they were not inoculated with the pathogen and were not treated with the microbial elicitors. Then, chilli plants were separately treated by spraying the microbial elicitors—chaetoglobosin C, chaetomanone A, and trichotoxin A50—over the plant canopy. The experiments were performed as three factor factorial (2 × 3 × 4) experiments in a randomized complete block design with three replications. Factor A was the inoculation conditions, where A1 represented no inoculation and A2 represented inoculation, factor B was the microbial elicitors, where B1 represented chaetoglobosin C, B2 represented chaetomanone A, and B3 represented trichotoxin A50, and factor C was the concentrations, where C1 represented 0 μg/ml, C2 represented 10 μg/ml, C3 represented 50 μg/ml, and C4 represented 100 μg/ml.

The length of the necrosis lesion on the stems was measured at 5, 10, and 20 days after treatment, and the accumulation of capsidiol was also measuered at 5, 10, and 20 days after treatment. To detect capsidiol, the upper parts of the chilli plant (Fig. 6.2b) were cut off and ground to a fine powder with a mortar and pestle using liquid nitrogen. Capsidiol was extracted from the resulting powder by adding a mixture of dichloromethane and methanol (2:1, v/v) following a modified protocol of Egea et al. (1996a). Plant debris was removed by filtration, and then the extract was washed with water and with dichloromethane. Finally, the organic phase was evaporated to dryness, redissolved in dichloromethane, and subjected to thin-layer chromatography (TLC) and developed with a mixture of hexane and ethyl acetate (1:1, v/v). The capsidiol spot was detected after spraying

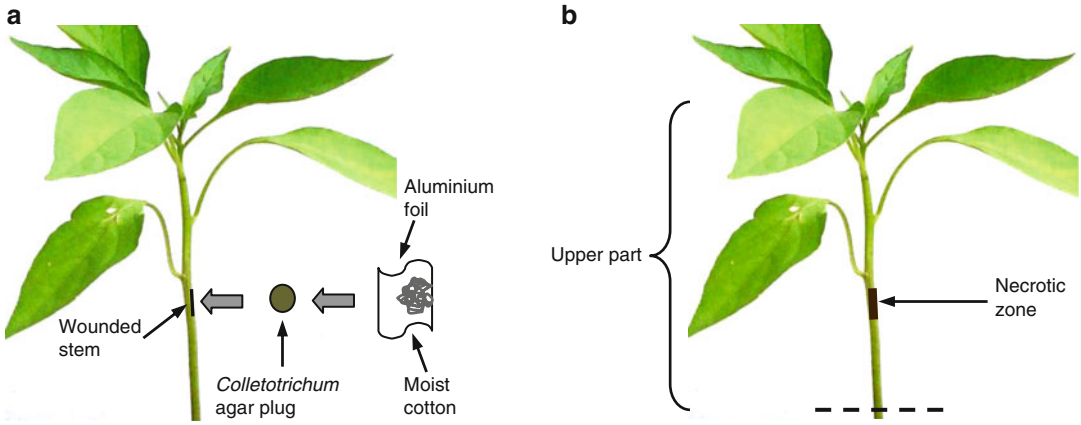


**Fig. 6.2** Stem inoculation (**a**) and upper part of chilli plant for capsidiol detection (**b**)

the TLC plate with vanillin reagent (1 g of vanillin in 30 ml of methanol, to which 0.2 ml of concentrated sulfuric acid was added). A characteristic blue spot with a retention factor (Rf) of 0.2 developed after the TLC plate had been heated at 80 °C by 3–4 min.

Long cayenne pepper seedlings were transplanted into 11-in.-diameter plastic pots which contained sterilized soil mixture (soil–sand–compost, 4:1:1). Thirty-day-old chilli plants, except for the noninoculated control, were inoculated by spraying the spore suspension ($1 \times 10^6$ conidia per milliliter) over the plant canopy (2 ml per plant). The experiment was performed by using a randomized complete block design with four replications (five plants per replication). The treatments were designed as follows: T1 represented the untreated control, T2 represented the inoculated control, T3 represented treatment with chaetoglobosin C at a concentration of 50 μg/ml every 15 days, T4 represented treatment with chaetomanone A at a concentration of 50 μg/ml every 15 days, T5 represented treatment with trichotoxin A50 at a concentration of 50 μg/ml every 15 days, and T6 represented treatment with a chemical fungicide (difenoconazole) at the recommended rate (10 ml per 20 l of water) every 15 days. The number of fruits which showed anthracnose symptoms per plant was used as a measure of disease incidence. Disease incidence was assessed at 90 days after treatment and was based on a disease rating scale (modified from Gopinath et al. 2006) as follows:1 for uninfected fruit, 2 for 1–25 % of fruits infected per plant, 3 for 26–50 % of fruits infected per plant, 4 for 51–75 % of fruits infected per plant, and 5 for 76–100 % of fruits infected per plant. The percent disease reduction (PDR) was calculated as follows:

$$\mathrm{PDR} = \frac{\text{Disease rating in inoculated control} - \text{Disease rating in treatment}}{\text{Disease rating in inoculated control}} \times 100.$$

Plant height (cm) was recorded every 15 days. Moreover, plant fresh weight (g), root fresh weight (g), plant dry weight (g), and root dry weight (g) were recorded at harvesting (90 days after treatment). Fruit diameter (cm), fruit length (cm), and fruit weight per plant (g) were also recorded. The percent increase in yield (PIY) was calculated as follows:

$$\mathrm{PIY} = \frac{\text{Fruit weight per plant in treatment} - \text{Fruit weight per plant in inoculated control}}{\text{Fruit weight per plant in treatment}} \times 100.$$

Data were analyzed by analysis of variance. Treatment means were statistically compared by Duncan's multiple range test at $P = 0.05$ and $P = 0.01$.

Biological fungicides were separately formulated from *C. globosum* KMITL-N0802, *C. lucknowense* CLT, and *T. harzianum* PC01 as oil formulations according to the work of Soytong et al. (2001)—namely, Bio-CG, Bio-CLT, and Bio-T, and were separately tested for their abilities to induce immunity in chilli against anthracnose. Long cayenne pepper seedlings were transplanted into 11-in.-diameter plastic pots which contained sterilized soil mixture (soil–sand–compost, 4 1 1). Thirty-day-old chilli plants, except for the noninoculated control, were inoculated by spraying the spore suspension ($1 \times 10^6$ conidia per milliliter) over the plant canopy (2 ml per plant). The experiment was peformed by using a randomized complete block design with four replications (five plants per replication). Treatments were designed as follows:T1 represented the untreated control, T2 represented the inoculated control, T3 represented treatment with Bio-CG at a rate of 10 ml per 20 l of water every 15 days, T4 represented treatment with Bio-CLT at a rate of 10 ml per 20 l of water every 15 days, T5 represented treatment with Bio-T at a rate of 10 ml per 20 l of water every 15 days, and T6 represented the treatment with a chemical fungicide (difenoconazole) at the recommendation rate (10 ml per 20 l of water) every 15 days. The number of fruits which showed anthracnose symptoms per plant was used as a measure of disease incidence. Disease incidence was assessed at 90 days after treatment and was based on a disease rating scale (modified from Gopinath et al. 2006) as follows: 1 for uninfected fruit, 2 for 1–25 % of fruits infected per plant, 3 for 26–50 % of fruits infected per plant, 4 for 51–75 % of fruits infected per plant, and 5 for 76–100 % of fruits infected per plant. The PDR was calculated as follows:

$$\text{PDR} = \frac{\text{Disease rating in inoculated control} - \text{Disease rating in treatment}}{\text{Disease rating in inoculated control}} \times 100.$$

Plant height (cm) was recorded every 15 days. Moreover, plant fresh weight (g), root fresh weight (g), plant dry weight (g), and root dry weight (g) were recorded at harvesting (90 days after treatment). Fruit diameter (cm), fruit length (cm), and fruit weight per plant (g) were also recorded. The PIY was calculated as follows:

$$\text{PIY} = \frac{\text{Fruit weight per plant in treatment} - \text{Fruit weight per plant in inoculated control}}{\text{Fruit weight per plant in treatment}} \times 100.$$

Data were analyzed by analysis of variance. Treatment means were statistically compared by Duncan's multiple range test at $P = 0.05$ and $P = 0.01$.

The immunity induced in long cayenne pepper plants as shown by phytoalexin production was assessed by inoculation with the highly virulent *C. capsici* isolate C208, using the plug inoculation method over the wounded stem and treatment by spraying the microbial elicitors—chaetoglobosin C, chaetomanone A, and trichotoxin A50—thoroughly over the plant canopy. The experiments were performed as three factor factorial ($2 \times 3 \times 4$) experiments in a randomized complete block design with three replications. Factor A was the inoculation conditions, where A1 represented no inoculation and A2 represented inoculation, factor B was the microbial elicitors, where B1 represented chaetoglobosin C, B2 represented chaetomanone A, and B3 represented trichotoxin A50, and factor C was the concentrations, where C1

represented 0 μg/ml, C2 represented 10 μg/ml, C3 represented 50 μg/ml, and C4 represented 100 μg/ml. The results showed that stem inoculation of chilli plants with *C. capsici* isolate C208 resulted in necrosis and accumulation of capsidiol. Stem inoculation of chilli plants showed different degrees of sensitivity to the fungal pathogen *C. capsici*, expressed as necrosis along the stems. Treatment with all three microbial elicitors induced an immunity response against *C. capsici* in the upper part of the chilli plant. The length of the necrosis was significantly different at 5 days after treatment with the microbial elicitors. For both no inoculation and inoculation followed by treatment with chaetoglobosin C, chaetomanone A, and trichotoxin A50 at concentrations of 10, 50, and 100 μg/ml, there was no significant difference in the length of the necrosis, but the difference was significant when compared with the lengths of the necrosis of plants inoculated with the pathogen and not treated with chaetoglobosin C, chaetomanone A, and trichotoxin A50, for which the lengths of the necrosis were 8.0, 8.3, and 8.3 mm, respectively. The length of the necrosis on the plants not inoculated with the pathogen and treated with chaetoglobosin C, chaetomanone A, and trichotoxin A50 at concentrations of 10, 50, and 100 μg/ml was the same—5.0 mm. The lengths of the necrosis on the plants inoculated with the pathogen and treated with chaetoglobosin C at concentrations of 10, 50, and 100 μg/ml were 5.3, 5.3, and 5.2 mm, respectively, lengths which are shorter than the length of the necrosis on the plants inoculated with the pathogen and treated with chaetomanone A and trichotoxin A50. The lengths of the necrosis on the plants inoculated with the pathogen and treated with chaetomanone A at concentrations of 10, 50, and 100 μg/ml were 5.3, 5.5, and 5.4 mm, respectively, whereas the lengths of the necrosis on the plants inoculated with the pathogen and treated with trichotoxin A50 at concentrations of 10, 50, and 100 μg/ml were 6.1, 5.8, and 5.9 mm, respectively.

The lengths of the necrosis on the plants not inoculated with the pathogen and treated with chaetoglobosin C, chaetomanone A, and trichotoxin A50 at concentrations of 10, 50, and 100 μg/ml and on the plants inoculated with the pathogen and treated with chaetoglobosin C, chaetomanone A, and trichotoxin A50 at concentrations of 10, 50, and 100 μg/ml were not increased at 10 and 20 days after treatment with the microbial elicitors, whereas at 10 days the lengths of the necrosis on the plants inoculated with the pathogen and not treated with chaetoglobosin C, chaetomanone A, and trichotoxin A50 were 11.0, 11.3, and 11.7 mm, respectively, which are significantly different from those resulting from the other treatments. At 20 days the lengths of the necrosis on the plants inoculated with the pathogen and not treated with chaetoglobosin C, chaetomanone A, and trichotoxin A50 were 14.0, 14.3, and 14.3 mm, respectively, which are significantly different from those resulting from the other treatments (Table 6.1 and Fig. 6.3).

Qualitative analysis of capsidiol accumulation in the upper part of the chilli plant was detected by TLC according to the establishment a blue spot and the respective Rf (0.2). Capsidiol was detected in chilli plants at 5 and 10 days after treatment with chaetoglobosin C, chaetomanone A, and trichotoxin A50 at concentrations of 10, 50, and 100 μg/ml in both no-inoculation and inoculation treatments, whereas capsidiol was not detected in plants that were not inoculated and not treated with chaetoglobosin C, chaetomanone A, and trichotoxin A50. After 20 days, for all the treatments, capsidiol was not detected in the plants (Fig. 6.4).

Moreover, microbial elicitors and a chemical fungicide (difenoconazole) were applied to the pathogen-inoculated chilli plants. Disease incidence after treatment with chaetoglobosin C was 2.00 (18.68 % of fruits infected per plant), which was significantly different when compared with disease incidence after difenoconazole treatment (3.00; 31.06 % of fruits infected per plant) ($P = 0.05$). Fo treatment with chaetomanone A and trichotoxin A50, disease incidence was 2.25 (24.96 %of fruits infected per plant) and 2.50 (26.36 % of fruits infected per plant), respectively. The plants inoculated with the pathogen exhibited the highest disease incidence, which was 4.50 (74.65 % of fruits infected per plant), whereas te untreated control

**Table 6.1** Length of necrosis in the stem of long cayenne pepper plants infected with *Colletotrichum capsici* isolate C208

| Inoculation conditions | Elicitor | Concentration (μg/ml) | Length of necrosis (mm) | | |
|---|---|---|---|---|---|
| | | | 5 days | 10 days | 20 days |
| No inoculation | Chaetoglobosin C | 0 | 0.0d | 0.0e | 0.0e |
| | | 10 | 5.0c | 5.0d | 5.0d |
| | | 50 | 5.0c | 5.0d | 5.0d |
| | | 100 | 5.0c | 5.0d | 5.0d |
| | Chaetomanone A | 0 | 0.0d | 0.0e | 0.0e |
| | | 10 | 5.0c | 5.0d | 5.0d |
| | | 50 | 5.0c | 5.0d | 5.0d |
| | | 100 | 5.0c | 5.0d | 5.0d |
| | Trichotoxin A50 | 0 | 0.0d | 0.0e | 0.0e |
| | | 10 | 5.0c | 5.0d | 5.0d |
| | | 50 | 5.0c | 5.0d | 5.0d |
| | | 100 | 5.0c | 5.0d | 5.0d |
| Inoculation | Chaetoglobosin C | 0 | 8.0a | 11.0a | 14.0a |
| | | 10 | 5.3bc | 5.3bcd | 5.3bcd |
| | | 50 | 5.3bc | 5.3bcd | 5.3bcd |
| | | 100 | 5.2bc | 5.2cd | 5.2cd |
| | Chaetomanone A | 0 | 8.3a | 11.3a | 14.3a |
| | | 10 | 5.3bc | 5.3bcd | 5.3bcd |
| | | 50 | 5.5bc | 5.5bcd | 5.5bcd |
| | | 100 | 5.4bc | 5.4bcd | 5.4bcd |
| | Trichotoxin A50 | 0 | 8.3a | 11.7a | 14.3a |
| | | 10 | 6.1b | 6.1b | 6.1b |
| | | 50 | 5.8bc | 5.8bcd | 5.8bcd |
| | | 100 | 5.9bc | 5.9bc | 5.9bc |

Means are based on data from three replications. Means followed by a common letter in each column are not significantly different by Duncan's multiple range test at $P = 0.01$

showed no anthracnose symptoms on chilli fruit. Chaetoglobosin C, chaetomanone A, and trichotoxin A50 induced immunity in chilli against anthracnose which significantly reduced disease incidence by 55.0, 50.0, and 42.5 %, respectively, when compared with difenoconazole, which reduced disease incidence by only 32.5 % (Table 6.2). All microbial elicitors tested resulted in significantly higher plant growth parameters (plant height, root length, plant fresh and dry weight, and root fresh and dry weight) than for the inoculated control at 90 days. The plant height at 15 days after treatment with chaetoglobosin C was 12.88 cm, which was the highest and was significantly different from the heights resulting from the other treatments ($P = 0.05$),. For treatment with chaetomanone A, the plant height was 11.28 cm. For plants treated with trichotoxin A50 and difenoconazole and the untreated control, the plants heights were not significantly different—8.33, 8.53, and 7.85 cm, respectively. The plant height of the inoculated control was 5.95 cm, which was the lowest height and significantly differed from the heights resulting from the other treatments (Table 6.3). The plant height at 30 days after treatment with chaetoglobosin C was 29.85 cm, which was the highest and significantly differed from the heights resulting from the other treatments ($P = 0.05$). For plants treated with chaetomanone A and trichotoxin A50 and the untreated control, the plant heights were not significantly different (18.28, 18.23, and 19.63 cm, respectively), in contrast to the plant height resulting from treatment with difenoconazole (15.10 cm). The plant height of

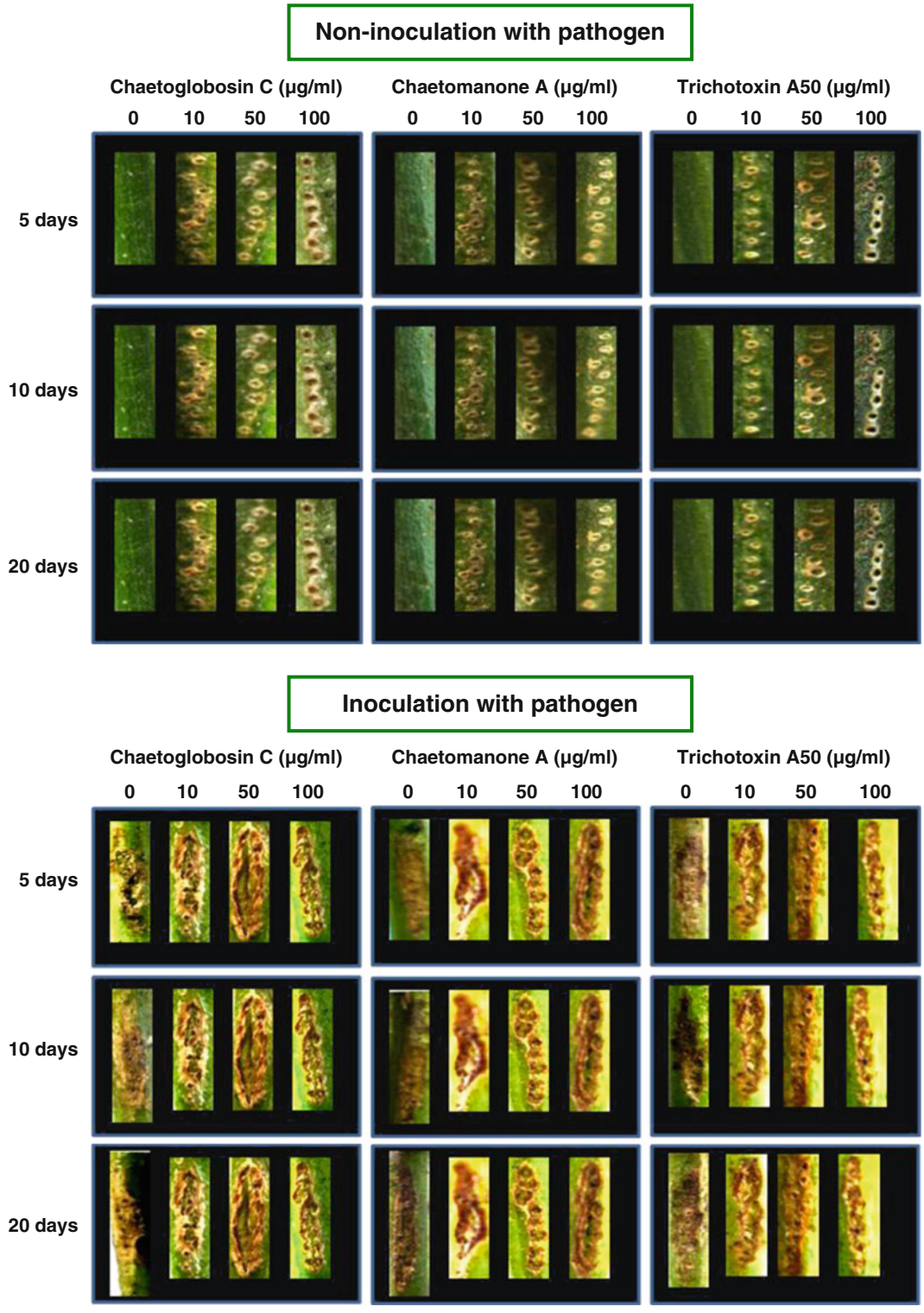


**Fig. 6.3** Necrosis on the chilli stem after treatment with microbial elicitors

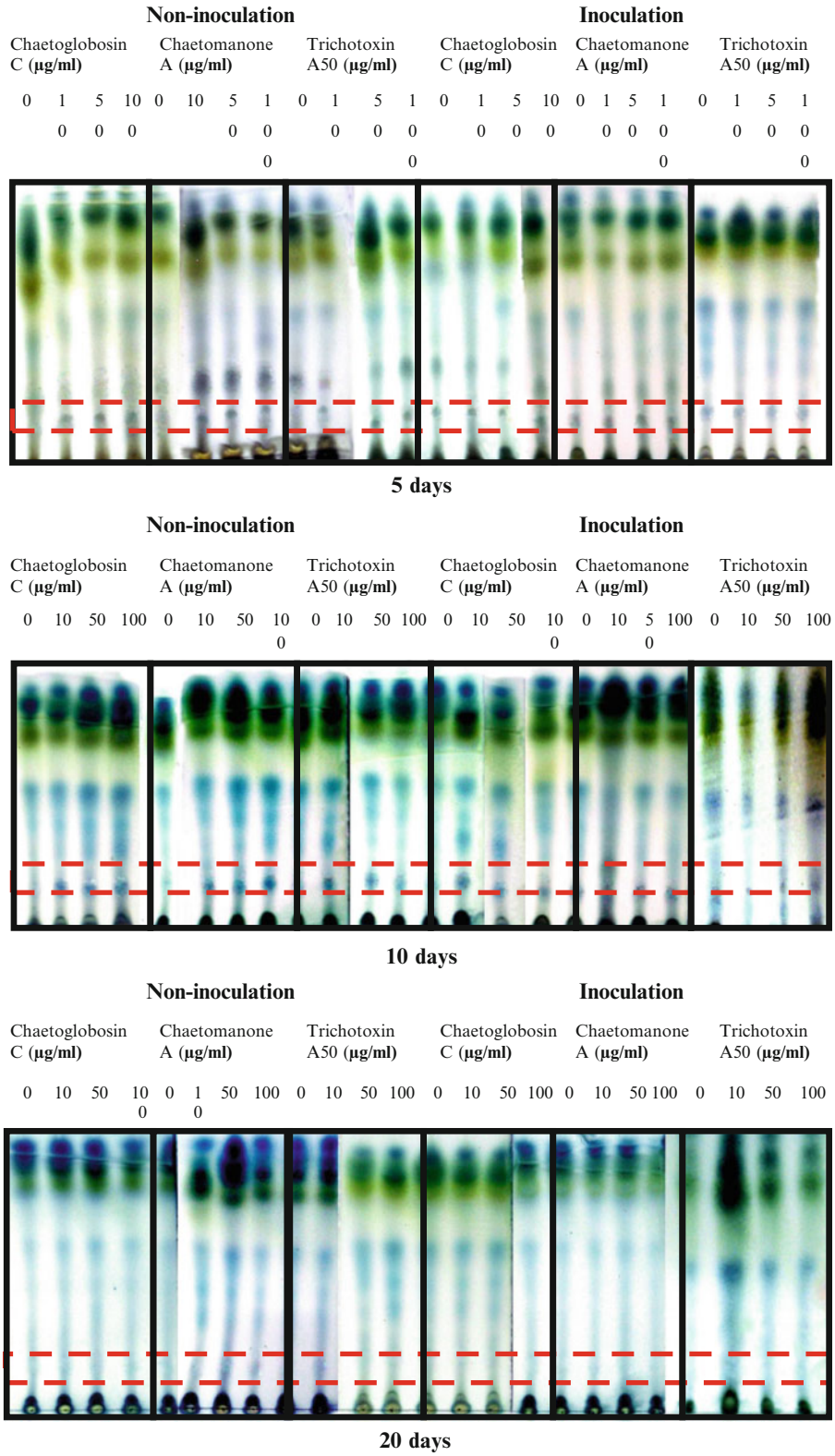


**Fig. 6.4** Capsidiol accumulation after treatment with microbial elicitors detected by using thin-layer chromatography

**Table 6.2** Efficacy of microbial elicitors to control chilli anthracnose

| Treatment | Fruit (fruits/plant) | Infected fruit (fruits/plant) | Infected fruit (%) | Disease incidences[a] | Disease reduction[b] (%) | Fruit weight (g/plant) | Fruit length (cm) | Fruit diameter (cm) | Increase in yield[c] (%) |
|---|---|---|---|---|---|---|---|---|---|
| Untreated control | 10.15bc | – | – | – | – | 68.57b | 8.38c | 1.36cd | 18.40b |
| Inoculated control | 8.50c | 6.30a | 74.65a | 4.50a | – | 55.89b | 7.68c | 1.30d | – |
| Chaetoglobosin C | 13.10a | 2.45c | 18.68c | 2.00c | 55.00a | 124.64a | 10.78a | 1.64a | 55.17a |
| Chaetomanone A | 12.40ab | 3.05bc | 24.96bc | 2.25bc | 50.00a | 113.58a | 10.36a | 1.57ab | 48.49a |
| Trichotoxin A50 | 12.25ab | 3.18b | 26.36bc | 2.50bc | 42.50ab | 106.43a | 9.37b | 1.50b | 46.53a |
| Difenoconazole | 10.85ab | 3.35b | 31.06b | 3.00b | 32.50b | 76.11b | 8.13c | 1.39c | 25.31b |

Means are based on data from four replications. Means followed by common letter in each column are not significantly different by Duncan's multiple range test at $P = 0.05$

[a]Disease incidence based on a disease rating scale: 1 for uninfected fruit, 2 for 1–25 % of fruits infected per plant, 3 for 26–50 % of fruits infected per plant, 4 for 51–75 % of fruits infected per plant, and 5 for 76–100 % of fruits infected per plant

[b]Percent disease reduction = (Disease rating of inoculated control – Disease rating of treatment)/Disease rating of inoculated control × 100

[c]Percent increase in yield = (Fruit weight per plant of treatment – Fruit weight per plant of inoculated control)/Fruit weight per plant of treatment × 100

**Table 6.3** Plant height of long cayenne pepper after treatment with microbial elicitors

| | Plant height (cm) | | | | |
|---|---|---|---|---|---|
| Treatment | 15 days | 30 days | 45 days | 60 days | 90 days |
| Untreated control | 7.85d | 19.63b | 25.75c | 26.05d | 40.60d |
| Inoculated control | 5.95e | 11.03d | 21.04d | 23.90e | 34.35e |
| Chaetoglobosin C | 12.88a | 29.85a | 36.48a | 38.65a | 53.15a |
| Chaetomanone A | 11.28b | 18.28b | 31.33b | 34.15b | 51.08b |
| Trichotoxin A50 | 8.33cd | 18.23b | 25.40c | 32.05c | 48.90c |
| Difenoconazole | 8.53c | 15.10c | 25.05c | 27.35d | 41.83d |

Means are based on data from four replications. Means followed by a common letter in each column are not significantly different by Duncan's multiple range test at $P = 0.05$

the inoculated control was 11.03 cm, which was the lowest plant height and significantly differed from the heights resulting from the other treatments.

Chaetoglobosin C promoted plant growth significantly differently from the other treatments at 45 days after treatment ($P = 0.05$). For treatment with chaetoglobosin C, the plant height was 36.48 cm, whereas for treatment with chaetomanone A, the plant height was 31.33 cm. For plants treated with trichotoxin A50 and difenoconazole and the untreated control, there was no significant difference in the plant heights, which were 25.40, 25.05, and 25.75 cm, respectively. The plant height of the inoculated control was 21.04 cm, which was the lowest plant height and significantly differed from the heights resulting from the other treatments (Table 6.3, Fig. 6.5). The plant height at 60 days after treatment showed that all the microbial elicitors tested resulted in significantly different plant heights when compared with the chemical fungicide ($P = 0.05$). Chaetoglobosin C promoted plant growth significantly differently


**Fig. 6.5** Plant height of long cayenne pepper 45 days after treatment with microbial elicitors. *T1* untreated control, *T2* inoculated control, *T3* treatment with chaetoglobosin C, *T4* treatment with chaetomanone A, *T5* treatment with trichotoxin A50, *T6* treatment with difenoconazole


**Fig. 6.6** Plant height of long cayenne pepper 60 days after treatment with microbial elicitors. *T1* untreated control, *T2* inoculated control, *T3* treatment with chaetoglobosin C, *T4* treatment with chaetomanone A, *T5* treatment with trichotoxin A50, *T6* treatment with difenoconazole

from the other treatments. For treatment with chaetoglobosin C, the plant height was 38.65 cm, and for treatment with chaetomanone A and trichotoxin A50, the plant heights were 34.15 and 32.05 cm, respectively. For the plants treated with difenoconazole and the untreated control, there was no significant difference in the plant heights, which were 27.35 and 26.05 cm, respectively, whereas the plant height of the inoculated control was 23.90 cm, which was the lowest plant height and significantly differed from the heights resulting from the other treatments (Fig. 6.6). It was clearly demonstrated that at 90 days after treatment, treatment with all the microbial elicitors tested also resulted in significantly different plant heights when compared with the chemical fungicide ($P = 0.05$). Chaetoglobosin C promoted plant growth significantly differently from the other treatments. For treatment with chaetoglobosin C, the plant height was 53.15 cm, whereas for treatment with chaetomanone A and trichotoxin A50, the plant heights were 51.08 and 48.90 cm, respectively.


**Fig. 6.7** Plant height of long cayenne pepper 90 days after treatment with microbial elicitors. *T1* untreated control, *T2* inoculated control, *T3* treatment with chaetoglobosin C, *T4* treatment with chaetomanone A, *T5* treatment with trichotoxin A50, *T6* treatment with difenoconazole

For the plants treated with difenoconazole and the untreated control, there was no significant difference in the plant heights, which were 41.83 and 40.60 cm, respectively, whereas the plant height of the inoculated control was 34.35 cm, which was the lowest plant height and significantly differed from the heights resulting from the other treatments (Fig. 6.7). There was a significant difference in the root length at 90 days after treatment. Treatment with all the microbial elicitors tested resulted in a significant difference in the root length when compared with the chemical fungicide ($P = 0.05$). Treatment with chaetoglobosin C resulted in a significantly different root length of 40.50 cm, whereas treatment with chaetomanone A and trichotoxin A50 resulted in root lengths of 32.55 and 32.13 cm, respectively. For the plants treated with difenoconazole and the untreated control, there was no significant difference in the root lengths, which were 29.13 and 28.03 cm, respectively, whereas the root length of the inoculated control was 22.53 cm, which was the shortest root length and significantly differed from the root lengths resulting from the other treatments (Fig. 6.8). The plant fresh and dry weights showed that treatment with all the microbial elicitors tested resulted in significantly different plant fresh and dry weights when compared with the chemical fungicide ($P = 0.05$). Treatment with chaetoglobosin C resulted in a significantly different plant fresh weight, which was 54.81 g, whereas treatment with chaetomanone A and trichotoxin A50 resulted in plant fresh weights of 46.06 and 44.69 g, respectively. For the plants treated with difenoconazole and the untreated control, there was no significant difference in the plant fresh weights, which were 35.67 and 35.16 g, respectively, whereas the plant fresh weight of the inoculated control was 25.79 g, which was the lowest plant fresh weight and significantly differed from the fresh weights resulting from the other treatments. Likewise, the plant dry weight after treatment with chaetoglobosin C was 17.29 g, which significantly differed from the dry weights resulting from the other treatments, For treatment with chaetomanone A and trichotoxin A50, the plant dry weights were 13.96 and 13.23 g, respectively. For the plants treated with difenoconazole and the untreated control, there was no significant difference in the plant dry weights, which were 10.07 and 9.77 g, respectively, whereas the plant dry weight of the inoculated control was 6.82 g, which was the lowest plant dry weight and significantly differed from the dry weights resulting from the other treatments. The root fresh weight showed that for all the microbial elicitors tested, there was a significant difference in the root fresh weight when compared with the root fresh weight of the inoculated control ($P = 0.05$). The root fresh weight after treatment with chaetoglobosin C was 11.06 g, which was


**Fig. 6.8** Root length of long cayenne pepper 90 days after treatment with microbial elicitors. *T1* untreated control, *T2* inoculated control, *T3* treatment with chaetoglobosin C, *T4* treatment with chaetomanone A, *T5* treatment with trichotoxin A50, *T6* treatment with difenoconazole

**Table 6.4** Efficacy of microbial elicitors for plant growth at 90 days

| | | Fresh weight (g) | | Dry weight (g) | |
|---|---|---|---|---|---|
| Treatment | Root length (cm) | Plant | Root | Plant | Root |
| Untreated control | 28.03c | 35.16c | 8.41bc | 9.77c | 2.01c |
| Inoculated control | 22.53d | 25.79d | 7.54c | 6.82d | 1.63d |
| Chaetoglobosin C | 40.50a | 54.81a | 11.06a | 17.29a | 2.75a |
| Chaetomanone A | 32.55b | 46.06b | 9.70ab | 13.96b | 2.53b |
| Trichotoxin A50 | 32.13b | 44.69b | 9.19b | 13.23b | 2.34b |
| Difenoconazole | 29.13c | 35.67c | 8.60bc | 10.07c | 2.06c |

Means are based on data from four replications. Means followed by a common letter in each column are not significantly different by Duncan's multiple range test at $P = 0.05$

significantly different from the root fresh weight resulting from the other treatments. For plants treated with chaetomanone A, trichotoxin A50, and difenoconazole and the untreated control, the root fresh weights were 9.70, 9.19, 8.60, and 8.41 g, respectively. The root fresh weight of the inoculated control was 7.54 g, which was the lowest root fresh weight and significantly differed from the root fresh weights resulting from microbial elicitor treatments. However, the root dry weight showed that for all the elicitors tested there was a significant difference in the root dry weight when compared with the root dry weight resulting from treatment with the the chemical fungicide ($P = 0.05$). The root dry weight after treatment with chaetoglobosin C was 2.75 g, which significantly differed from the root dry weight after the other treatments. For treatment with chaetomanone A and trichotoxin A50, the root dry weights were 2.53 and 2.34 g, respectively. For the plants treated with difenoconazole and the untreated control, there was no significant difference in the root dry weights, which were 2.06 and 2.01 g, respectively, whereas the root dry weight of the inoculated control was 1.63 g, which was the lowest root dry weight and significantly differed from the root dry weights resulting from the other treatments (Table 6.4).

In addition to reducing disease incidence, application of microbial elicitors also increased fruit yield and the overall quality of the fruits.


**Fig. 6.9** Fruit number of long cayenne pepper after treatment with microbial elicitors. *T1* untreated control, *T2* inoculated control, *T3* treatment with chaetoglobosin C, *T4* treatment with chaetomanone A, *T5* treatment with trichotoxin A50, *T6* treatment with difenoconazole

For all the microbial elicitors tested, the number of fruits per plant was significantly different when compared with the number of fruits per plant for the inoculated control ($P = 0.05$). Treatment with Chaetoglobosin C resulted in the highest fruit number, which was 13.10 fruits per plant, followed by treatment with chaetomanone A, trichotoxin A50, and difenoconazole, for which the fruit numbers were 12.40, 12.25, and 10.85 fruits per plant. The lowest fruit numbers were obtained for the untreated control and the inoculated control, and were 10.15 and 8.50 fruits per plant, respectively (Table 6.2 and Fig. 6.9).

For all the microbial elicitors tested, the overall quality of the fruit was significantly different when compared with that resulting from treatment with the chemical fungicide ($P = 0.05$). The fruit lengths after treatment with chaetoglobosin C and chaetomanone A were 10.78 and 10.36 cm, respectively, which were the longest and significantly differed from the lengths resulting from the other treatments. For treatment with trichotoxin A50, the fruit length was 9.37 cm. For the plants treated with difenoconazole, the untreated control, and the inoculated control, there was no significant difference in the fruit lengths, which were 8.13, 8.38, and 7.68 cm, respectively. The fruit diameter after treatment with the microbial elicitors was significantly different when compared with the fruit diameter after the other treatments. The fruit diameter of chaetoglobosin C was 1.64 cm, which was the largest diameter. For treatment with chaetomanone A and trichotoxin A50, the fruit diameters were 1.57 and 1.50, respectively. The smallest fruit diameters were obtained for the plants treated with difenoconazole, the untreated control, and the inoculated control, and were 1.39, 1.36, and 1.30 cm, respectively (Table 6.2).

Moreover, the results showed that for all the microbial elicitors tested, the yield was significantly different from that resulting from treatment with the chemical fungicide ($P = 0.05$). Treatment with chaetoglobosin C resulted in the highest yield, which was 124.64 g per plant, followed by treatment with chaetomanone A and trichotoxin A50, which resulted in yields of 113.58 and 106.43 g per plant, respectively. For the plants treated with difenoconazole, the untreated control, and the inoculated control, there was no significant difference in the yields, which were 76.11, 68.57,and 55.89 g per plant, respectively. Lastly, the increases in yield after treatment with chaetoglobosin C, chaetomanone A, and trichotoxin A50 were 55.17, 48.49, and 46.53 %, respectively, which significantly differed from the increase in yield after treatment with difenoconazole (25.31 %).

Biological fungicides formulated in an oil form—namely, Bio-CG, Bio-CLT, and Bio-T—were tested in comparison with a chemical fungicide (difenoconazole) in pot experiments for 90 days. The disease incidence after treatment with Bio-CG was 2.00 (19.41 % of fruits infected per plant), which was significantly different when compared with the disease incidence after treatment with difenoconazole (3.00; 29.16 % of fruits infected per plant) ($P = 0.05$). For treatment with Bio-CLT and Bio-T, the disease incidences were 2.50 (26.04 % of fruits infected

**Table 6.5** Efficacy of biological fungicides to control chilli anthracnose

| Treatment | Fruit (fruits/plant) | Infected fruit (fruits/plant) | Infected fruit (%) | Disease incidence[a] | Disease reduction[b] (%) | Fruit weight (g/plant) | Fruit length (cm) | Fruit diameter (cm) | Increase in yield[c] (%) |
|---|---|---|---|---|---|---|---|---|---|
| Untreated control | 11.38ab | – | – | – | – | 91.51bc | 8.92b | 1.40bc | 22.89b |
| Inoculated control | 9.85b | 7.38a | 75.28a | 4.50a | – | 71.20c | 8.72b | 1.36c | – |
| Bio-CG | 13.50a | 2.63b | 19.41c | 2.00c | 55.00a | 152.77a | 10.89a | 1.66a | 52.54a |
| Bio-CLT | 11.95a | 3.13b | 26.04bc | 2.50bc | 42.50ab | 119.17b | 10.77a | 1.64a | 39.65ab |
| Bio-T | 11.95a | 3.30b | 27.64b | 2.75bc | 38.75b | 118.20b | 10.52a | 1.64a | 38.95ab |
| Difenoconazole | 11.45ab | 3.35b | 29.16b | 3.00b | 32.50b | 99.13bc | 9.36b | 1.48b | 28.04b |

Means are based on data from four replications. Means followed by a common letter in each column are not significantly different by Duncan's multiple range test at $P = 0.05$

[a]Disease incidence based on a disease rating scale: 1 for uninfected fruit, 2 for 1–25 % of fruits infected per plant, 3 for 26–50 % of fruits infected per plant, 4 for 51–75 % of fruits infected per plant, and 5 for 76–100 % of fruits infected per plant

[b]Percent disease reduction = (Disease rating of inoculated control – Disease rating of treatment)/Disease rating of inoculated control × 100

[c]Percent increased in yield = (Fruit weight per plant of treatment – Fruit weight per plant of inoculated control)/Fruit weight per plant of treatment × 100

per plant) and 2.75 (27.64 % of fruits infected per plant), respectively. The highest disease incidence of 4.50 (75.28 % of fruits infected plant) occurred with the inoculated control, and significantly differed from the disease incidences resulting from the other treatments, whereas the untreated control showed no anthracnose symptoms on chilli fruit. For treatment with Bio-CG and Bio-CLT, the reduction of disease incidence on chilli fruit was significantly different, for which the PDR was 55.00 and 42.50 %, respectively, when compared with diseases incidence following treatment with difenoconazole ($P = 0.05$). The PDR of the Bio-T and difenoconazole treatments was 38.75 and 32.50 %, respectively (Table 6.5).

Treatment with all the biological fungicides resulted in significantly higher plant growth parameters (plant height, root length, plant fresh and dry weights, and root fresh and dry weights) than for the inoculated control. The plant height at 15 days after treatment with Bio-CG was 12.13 cm, which was the highest and significantly differed from the plant heights resulting from the other treatments ($P = 0.05$). For treatment with Bio-CLT, the plant height was 10.93 cm. For the plants treated with Bio-T and difenoconazole and the untreated control, there was no significant difference in the plant heights, which were 8.36, 8.29, and 8.33 cm, respectively. The lowest plant height occurred for the inoculated control (7.03 cm), and was significantly different from the plant heights resulting from the other treatments (Fig. 6.10). Bio-CG also promoted plant growth significantly differently (the plant height was 28.10 cm) when compared with the other treatments for 30 days ($P = 0.05$). For the plants treated with Bio-CLT, Bio-T, and difenoconazole and the untreated control, the plant heights were 20.19, 18.09, 16.30, and 19.95 cm, respectively (Fig. 6.11). The plant height at 45 days after treatment showed that treatment wit Bio-CG and Bio-CLT resulted in significantly different plant heights, which were 36.15 and 35.05 cm, respectively, when compared with the other treatments ($P = 0.05$). For the plants treated with Bio-T and difenoconazole and the untreated control, the plant heights were 27.23, 25.80, and 23.85 cm, respectively. The plant height of the inoculated control was 20.78 cm, which was the lowest plant height and significantly differed from the plant heights resulting from the other treatments (Fig. 6.12). The plant height at 60 days after treatment showed that treatment with all the biological fungicides tested resulted


**Fig. 6.10** Plant height of long cayenne pepper 45 days after treatment with biological fungicides. *T1* untreated control, *T2* inoculated control, *T3* treatment with Bio-CG, *T4* treatment with Bio-CLT, *T5* treatment with Bio-T, *T6* treatment with difenoconazole


**Fig. 6.11** Plant height of long cayenne pepper 60 days after treatment with biological fungicides. *T1* untreated control, *T2* inoculated control, *T3* treatment with Bio-CG, *T4* treatment with Bio-CLT, *T5* treatment with Bio-T, *T6* treatment with difenoconazole


**Fig. 6.12** Plant height of long cayenne pepper 90 days after treatment with biological fungicides. *T1* untreated control, *T2* inoculated control, *T3* treatment with Bio-CG, *T4* treatment with Bio-CLT, *T5* treatment with Bio-T, *T6* treatment with difenoconazole

**Table 6.6** Plant height of long cayenne pepper after treatment with biological fungicides

| | Plant height (cm) | | | | |
|---|---|---|---|---|---|
| Treatment | 15 days | 30 days | 45 days | 60 days | 90 days |
| Untreated control | 8.33c | 19.95b | 23.85d | 27.85d | 44.93d |
| Inoculated control | 7.03d | 13.55e | 20.78e | 24.35e | 35.65e |
| Bio-CG | 12.13a | 28.10a | 36.15a | 40.45a | 60.25a |
| Bio-CLT | 10.93b | 20.19b | 35.05a | 36.05b | 51.55b |
| Bio-T | 8.36c | 18.09c | 27.23b | 32.80c | 49.20c |
| Difenoconazole | 8.29c | 16.30d | 25.80c | 28.35d | 45.60d |

Means are based on data from four replications. Means followed by a common letter in each column are not significantly different by Duncan's multiple range test at $P = 0.05$

in significantly different plant heights when compared with the plant height resulting from treatment with the chemical fungicide ($P = 0.05$). Bio-CG promoted plant growth significantly differently, and the resulting plant height was 40.45 cm. For treatment with Bio-CLT and Bio-T, the resulting plant heights were 36.05 and 32.80 cm, respectively. For the plants treated with difenoconazole and the untreated control, there was no significant difference in the plant heights, which were 28.35 and 27.85 cm, respectively, whereas the plant height of the inoculated control was 24.35 cm, which was the lowest plant height and significantly differed from the plant heights resulting from the other treatments. It was clearly demonstrated that treatment with all the biological fungicides tested also resulted in significantly different plant heights at 90 days after treatment, when compared with the plant height resulting from treatment with the chemical fungicide ($P = 0.05$). Bio-CG promoted plant growth significantly differently, and the resulting plant height was 60.52 cm. For treatment with Bio-CLT and Bio-T, the resulting plant heights were 51.55 and 49.20 cm, respectively. For the plants treated with difenoconazole and the untreated control, there was no significant difference in the plant heights, which were 45.60 and 44.93 cm, respectively, whereas the plant height of the inoculated control was 35.65 cm, which was the lowest plant height and significantly differed from the plant heights resulting from the other treatments (Table 6.6).

The results showed that for treatment with all the biological fungicides tested, the root length was significantly different when compared with the root length resulting from treatment with the chemical fungicide ($P = 0.05$). Bio-CG promoted plant growth significantly differently, and the resulting root length was 37.55 cm. For treatment with Bio-CLT and Bio-T, the resulting root lengths were 32.73 and 31.48 cm, respectively. For the plants treated with difenoconazole and the untreated control, there was no significant difference in the root lengths, which were 29.18 and 30.53 cm, respectively, whereas the root length of the inoculated control was 23.60 cm, which was the shortest root length and significantly differed from the root lengths resulting from the other treatments (Fig. 6.14). For all the biological fungicides tested, the plant fresh and dry weights were significantly different when compared with those of the inoculated control ($P = 0.05$). Treatment with Bio-CG resulted in a significantly different plant fresh weight of 51.55 g, whereas for treatment with Bio-CLT and Bio-T, the plant fresh weights were 43.61 and 41.75 g, respectively. For the plants treated with difenoconazole and the untreated control, the plant fresh weights were 33.43 and 34.92 g, respectively, and were not significantly different, whereas the plant fresh weight of the inoculated control was 22.63 g, which was the lowest plant fresh weight and significantly differed from the plant fresh weights resulting from the other treatments. Similarly, the plant dry weight after treatment with Bio-CG was 16.79 g, which significantly differed from the plant dry weights after the other treatments. For treatment with Bio-CLT and Bio-T, the plant dry weights were 14.44 and 12.40 g, respectively. For the plants

**Table 6.7** Efficacy of biological fungicides for plant growth at 90 days

| Treatment | Root length (cm) | Fresh weight (g) | | Dry weight (g) | |
|---|---|---|---|---|---|
| | | Plant | Root | Plant | Root |
| Untreated control | 30.53bc | 34.92c | 7.17c | 10.20c | 1.91d |
| Inoculated control | 23.60d | 22.63d | 6.44c | 6.49d | 1.35e |
| Bio-CG | 37.55a | 51.55a | 10.97a | 16.79a | 2.73a |
| Bio-CLT | 32.73b | 43.61b | 9.08b | 14.44ab | 2.43b |
| Bio-T | 31.48b | 41.75b | 7.94bc | 12.40bc | 2.21bc |
| Difenoconazole | 29.18c | 33.43c | 7.45c | 11.82c | 1.98cd |

Means are based on data from four replications. Means followed by a common letter in each column are not significantly different by Duncan's multiple range test at $P = 0.05$

treated with difenoconazole and the untreated control, there was no significant difference in the plant dry weights, which were 11.82 and 10.20 g, respectively, whereas the plant dry weight of the inoculated control was 6.49 g, which was the lowest plant dry weight and significantly differed from the plant dry weights resulting from the other treatments. For treatment with all the biological fungicides tested, the plant dry weight was significantly different when compared with that of the inoculated control ($P = 0.05$). The root fresh weight after treatment with Bio-CG was 10.97 g, which was significantly different from that after the other treatments. For treatment with Bio-CLT and Bio-T, the root fresh weights were 9.08 and 7.94 g, respectively. For the plants treated with difenoconazole and the untreated control, there was no significant difference in the root fresh weights, which were 7.45 and 7.17 g, respectively. The lowest root fresh weight was obtained for the inoculated control, and was 6.44 g and significantly differed from the root fresh weights resulting from the other treatments. Likewise, the root dry weight after treatment with Bio-CG was 2.73 g, which was the highest and significantly differed from the root dry weights resulting from the other treatments. For the plants treated with Bio-CLT, Bio-T, and difenoconazole and the untreated control, the root dry weights were 2.43, 2.21, 1.98, and 1.91 g, respectively. The lowest root dry weight was obtained for the inoculated control; it was 6.44 g and significantly differed from the root dry weights resulting from the other treatments (Table 6.7).

In addition to reducing disease incidence, biological fungicides increase fruit yield and the overall quality of the fruits. For all the biological fungicides tested, the number of fruits per plant was significantly different when compared with the number of fruits per plant for the inoculated control ($P = 0.05$). Treatment with Bio-CG, Bio-CLT, and Bio-T resulted in the highest fruit numbers, which were 13.50, 11.95, and 11.95 fruits per plant, respectively. For the plants treated with difenoconazole and the untreated control, there was no significant difference in the fruit numbers, which were 11.45 and 11.38 fruits per plant, respectively, whereas for the inoculated control, there were 9.85 fruits per plant, which was the lowest fruit number and significantly differed from the fruit numbers resulting from the other treatments (Fig. 6.14). For treatment with all the biological fungicides, the overall quality of the fruit was significantly different when compared with the overall quality resulting from treatment with the chemical fungicide ($P = 0.05$). The fruit lengths after treatment with Bio-CG, Bio-CLT, and Bio-T were 10.89, 10.77 and 10.52 cm, respectively, which significantly differed from the fruit lengths resulting from the other treatments. For the plants treated with difenoconazole, the untreated control, and the inoculated control, there was no significant difference in the fruit lengths, which were 9.36, 8.92, and 8.72 cm, respectively. Similarly, treatment with Bio-CG, Bio-CLT, and Bio-T also resulted in fruit diameters significantly different from those resulting from the other treatments; they were 1.66, 1.64, and 1.64 cm, respectively. For the plants treated with difenoconazole and the untreated control, the fruit diameters were 1.48 and 1.40 cm, respectively. The smallest fruit diameter was obtained for the

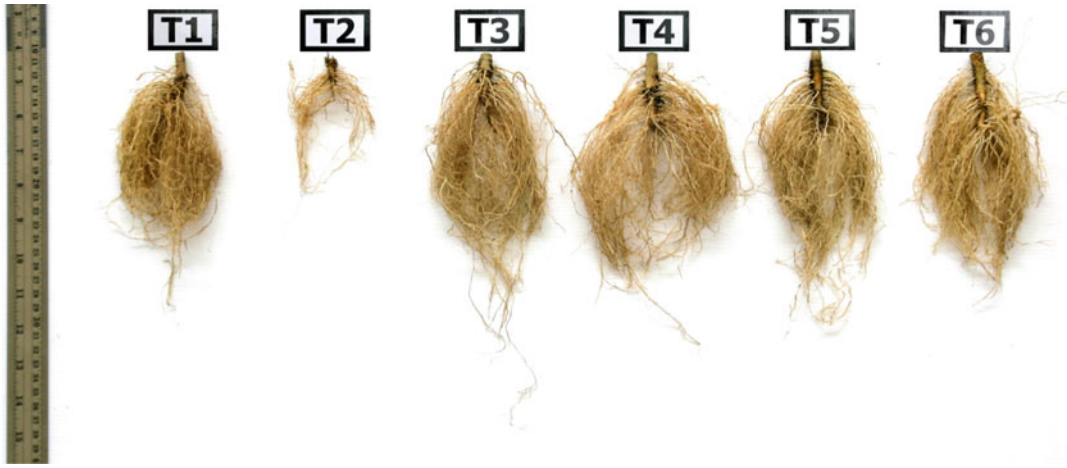


**Fig. 6.13** Root length of long cayenne pepper 90 days after treatment with biological fungicides. *T1* untreated control, *T2* inoculated control, *T3* treatment with Bio-CG, *T4* treatment with Bio-CLT, *T5* treatment with Bio-T, *T6* treatment with difenoconazole


**Fig. 6.14** Fruit number of long cayenne pepper 90 days after treatment with biological fungicides. *T1* untreated control, *T2* inoculated control, *T3* treatment with Bio-CG, *T4* treatment with Bio-CLT, *T5* treatment with Bio-T, *T6* treatment with difenoconazole

inoculated control, and was 1.36 cm and significantly differed from the fruit diameters resulting from the other treatments. Moreover, treatment with all the biological fungicides resulted in significantly different yields when compared with the yield for the inoculated control ($P = 0.05$). Treatment with Bio-CG gave the highest yield, which was 152.77 g per plant, and this significantly differed from the yields resulting from the other treatments. Treatment with Bio-CLT and Bio-T gave yields of 119.17 and 118.20 g per plant, respectively. For the plants treated with difenoconazole and the untreated control, there was no significant difference in the yields, which were 99.13 and 91.51 g per plant. The lowest yield was obtained for the inoculated control, which was 71.20 g per plant (Fig. 6.11). It is clearly demonstrated that Bio-CG treatment increased the yield by 52.54 % which was significantly higher than that obtained by difenoconazole treatment ($P = 0.05$). Treatment with Bio-CLT and Bio-T increased the yield by 39.65 and 38.95 %, respectively. Application of difenoconazole gave the lowest yield of 28.04 %. (Figs. 6.12, 6.13, and 6.14).

The stem of long cayenne pepper exhibited a reaction when inoculated with *C. capsici* and treated with chaetoglobosin C from *C. globosum*, chaetomanone A from *C. lucknowense*, and trichotoxin A50 from *T. harzianum* PC01 at different concentrations. The reactions resulted in the induction of immunity response against *C. capsici* by a necrosis being expressed in the upper part of the plant. The length of the necrosis was

significantly different at 5, 10, and 20 days after treatment with the microbial elicitors. The inoculated plants treated with chaetoglobosin C at concentrations of 10, 50, and 100 μg/ml showed shorter necrosis than the inoculated plants treated with chaetomanone A and trichotoxin A50. The length of the necrosis at each concentration did not differ significantly. This suggested that long cayenne pepper has a mechanism for recognizing this pathogen for the defense response. This finding is supported by Egea et al. (1996a), who reported that the stems of three cultivars of *C. annuum* exhibited a classic hypersensitive reaction when inoculated with *P. capsici*, and found that a resistant cultivar developed a defense response that resulted in a rapid and lasting restriction of the pathogen. Qualitative analysis of capsidiol accumulation was detected by TLC in this study. The results showed that capsidiol had accumulated in chilli plants 5 and 10 days after treatment with microbial elicitors, whereas no capsidiol was detected in noninoculated plants and the untreated control. After 20 days, no capsidiol was detected in all treatments, a finding supported by the previously mentioned report. Egea et al. (1996b) stated that the maximum level of capsidiol detected was in the necrotic zone 6 days after inoculation, whereas after 9 days, capsidiol levels fell in all chilli cultivars. Moreover, no capsidiol was induced in the healthy control. Ahmed et al. (2000) also found a susceptible variety of chilli which was treated with *T. harzianum* and inoculated with *P. capsici*, which causes root rot in chilli, stimulated the production of capsidiol, the concentration of which was highest after 6 days and fell after 9 days. In this study, capsidiol accumulation was also detected in noninoculated plants treated with chaetoglobosin C, chaetomanone A, and trichotoxin A50 at concentrations of 10, 50, and 100 μg/ml. This suggests that capsidiol, a phytoalexin, is produced by chilli plants not only in response to interaction with the fungal pathogen but also following treatment with microbial elicitors, in the same way as in report by Back et al. (1998), which suggested that phytoalexins are produced by plants following treatment with many chemicals, irradiation by ultraviolet light, and exposure to the products of microbial metabolism. Hwang and Sung (1989) demonstrated that the chemical fungicide metalaxyl also induced capsidiol in the stem of chilli plants infected with *P. capsici*. Bhapdal and Paxton (1991) state that phytoalexin biosynthesis are induced by the fungal glucan Polytran in sweet pepper tissues.

It is clearly demonstrated that the microbial elicitors chaetoglobosin C, chaetomanone A, and trichotoxin A50 can induce immunity against chilli anthracnose in pot experiments. This experiment showed that the PDR of chaetoglobosin C was 55 %, which was the highest reduction. This demonstrates that chaetoglobosin C is potent in inducin immunity in chilli plants. Similarly, induced immunity in citrus seedlings was reported by Treetong et al. (2000), who stated that chaetoglobosin C could be the induced immunizing agent against *Phytophthora* root and stem rot of Shogun citrus seedlings that could reduce the pathogen inoculum and incidence of root and stem rot. Moreover, Soytong et al. (2005) also stated that chaetoglobosin C could inhibit the growth of *Colletotrichum gloeosporioides* stain WMF01, which causes grape anthracnose, with a median effective dose between 1 and 50 ppm. Likewise, this study showed that antagonistic fungi, *C. globosum* KMITL-N0802 and *C. lucknowense* CLT, significantly reduced disease incidence by 55 and 42.5 %. This demonstrates that *Chaetomium* spp. are potent in reducing chilli anthracnose. This finding is also similar to that of Shternshis et al. (2005), who suggested that the mycofungicide Ketomium, which was developed from *Chaetomium* species, shows promise as a new means of biological control of plant diseases in Siberian conditions such as raspberry spur blight, which is caused by *Didymella applanata*, and potato disease caused by *Rhizoctonia solani*. Moreover, Soytong et al. (2001) reported that biological control of plant disease has been successfully reported by using a new broad-spectrum biological fungicide from *Chaetomium* and this has successfully been applied to infested field soils and integrated with cultural control measures and organic amendments for the long-term protection against durian and black pepper disease caused by *Phytophthora palmivora*, tangerine disease caused by

*Phytophthora parasitica*, strawberry disease caused by *Phytophthora cactorum*, wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici*, and basal rot of corn caused by *Sclerotium rolfsii*. In this study, *T. harzianum* PC01 and trichotoxin A50 reduced disease by 38.75 and 42.5 %, respectively, whereas difenoconazole reduced disease by only 32.5 %. This demonstrates the potency of *Trichoderma* spp. and the active substance trichotoxin A50. A similar result was reported by Jebessa and Ranamukhaarachchi (2006), who stated that *T. harzianum* collected in the same regions as the chilli samples had some effect in controlling *C. gloeosporioides*, which causes chilli anthracnose in southern, central, and western chilli-growing regions of Ethiopia. In addition, Soytong et al. (2005) reported that crude extracts from *T. harzianum* PC01, *T. harzianum* PC02, and its antibiotic substance, namely, trichotoxin A50, could inhibit the growth of *C. gloeosporioides* strain WMF01, which causes grape anthracnose, with median effective doses between 1 and 50 ppm, and bioproducts of *Trichoderma* significantly reduced the incidence of disease on leaves, twigs, and fruits of grape as compared with the chemical control. Moreover, there is a report indicating that trichotoxin A50 from *T. harzianum* PC01 has significant immunizing ability against *Phytophthora* root rot of citrus seedling when compared with the chemical fungicide metalaxyl (Treetong et al. 2000). In this experiment, all the microbial elicitors and antagonistic fungi as biological fungicides were significantly different in their effectiveness in disease reduction when compared with a chemical fungicide (difenoconazole). This finding is also similar to that of Sariah (1989), who suggested that using a chemical fungicide resulted in little or no control of anthracnose in some major chilli-growing areas of Selangor, Malaysia. However, Gopinath et al. (2006) studied the efficacy of the chemical fungicide difenoconazole for chilli anthracnose management through extensive greenhouse and field trials and found that application of difenoconazole at 0.05 % reduced disease incidence by 58 %, whereas this study showed that difenoconazole reduced disease incidence by 32.5 %. The use of antagonistic fungi as biological fungicides and microbial elicitors from these antagonistic fungi not only reduced disease incidence but also increased the yield. This may be interpreted as being due to some antagonistic fungi not only controlling disease but also promoting growth (Jebessa and Ranamukhaarachchi 2006).

## 6.2 Microbial Elicitors Inducing Plant Disease Immunity in Tomato

The α-tomatine quantification of the extracts was performed by using high-performance liquid chromatography (HPLC) with a modified method of Friedman et al. (1994). The crude extracts (1 mg) from the tested plants were dissolved in 1 ml of mixed solvent comprising 50 % methanol and 0.1 % acetic acid and were filtered through a0.45-μm nylon membrane. The filtrate (50 μl) was subjected to HPLC (instrument from Agilent). The HPLC eluent for α-tomatine analysis was a combined solvent system comprising 80 % water, 15 % acetonitrile, and 5 % methanol (all HPLC grade). The flow rate was set to 1.0 ml/min. The $C_8$ chromatography column was eluted with the HPLC solvent system before injection of the filtrates. The α-tomatine quantification was performed using a linear regression curve. Data were statistically analyzed by analysis of variance. Treatment means were compared by Duncan's multiple range test at $P = 0.05$ and $P = 0.01$.

The experiment was performed to detect α-tomatine in the laboratory. The trial was conducted with the modified method of Melton et al. (1998) and designed in a completely randomized design with six treatments and three replications. Twenty-day-old seedlings of variety Sida were inoculated with a conidial suspension of *F. oxysporum* f. sp. *lycopersici* at a concentration of $2 \times 10^6$ conidia per milliliter and planted in 4-in.-diameter plastic pots containing a sterilized soil mixture as in the previous experiments, followed by spraying each bioactive compound or chemical fungicide on seedling leaves. The treatments were as follows: in treatment 1, the seedling was inoculated with the pathogen and sprayed with chaetoglobosin C at 50 μg/ml; in treatment 2, the seedling was inoculated with the

pathogen and sprayed with chaetomanone A at 50 μg/ml; in treatment 3, the seedling was inoculated with the pathogen and sprayed with trichotoxin A50 at 50 μg/ml; in treatment 4, the seedling was inoculated with the pathogen and sprayed with prochloraz at 20 g/20 l water; in treatment 5, the seedling was inoculated with the pathogen. A noninoculated control was also studied. The disease severity scale of Silva and Bettiol (2005) as follows:- 1= no symptom; 2= plant showed yellowing leaves and wilting 1-20%, 3= plant showed yellowing leaves and wilting 21-40%, 4= plant showed yellowing leaves and wilting 41-60%, 5= plant showed yellowing leaves and wilting 61-80%, and 6= plant showed yellowing leaves and wilting or die 81-100%. The percentage of plant disease immunity (PDI) of the treated tomato seedlings was calculated using the following formula: PDI = DSI of inoculated control – DSI of each treatment/DSI of inoculated control × 100. The α-tomatine was extracted from 6 g of stems and leaves of tomato plants which were harvested at 5, 10, and 15 days after treatment. Then, plant tissues were ground in 95 % methanol with a mortar and pestle. The extracts was evaporated in a vacuum evaporator, and the volume was adjusted with methanol, and then they were subjected to TLC with standard α-tomatine for comparison. The spot of the extracts from the tested tomato which showed Rf = 0.23 was expected to be α-tomatine in each treatment.

The phytoanticipin α-tomatine was induced by the bioactive compounds chaetoglobosin C, chaetomanone A, and trichotoxin A50. The DSI was determined before the seedlings were harvested and analyzed for PDI. The PDI of tomato seedlings treated with chaetoglobosin C, chaetomanone A, and trichotoxin A50 after 10 days was 44.97, 35.18, and 39.43 %, respectively, whereas tomato seedlings treated with prochloraz showed PDI of 29.95 %. The extracts from tested tomato were spotted on TLC paper with standard α-tomatine for comparison. The spots of extracts on TLC paper were green in all treatments with Rf = 0.23, the as same for standard α-tomatine (Fig. 6.15). The quantity of α-tomatine at 5 days was analyzed, and the data revealed that inoculated seedlings treated with chaetoglobosin C contained the highest quantity of α-tomatine (746.67 μg/g sample), whereas inoculated seedlings treated with chaetomanone A and trichotoxin A50 contained significantly lower quantities of α-tomatine (535.01 and 599.79 μg/g sample, respectively). For inoculated seedlings treated with prochloraz, tomato seedlings inoculated with the pathogen only, and the noninoculated control, there was no significant difference in the quantity of α-tomatine—368.68, 361.51, and 294.38 μg/g sample, respectively. The seedlings treated with trichotoxin A50 contained 492.22 μg of α-tomatine per gram of sample, which was significantly higher than for seedlings treated with chaetoglobosin C and chaetomanone A and for the noninoculated control, which contained 348.72, 322.98, and 321.19 μg of α-tomatine per gram of sample after 10 days (Table 6.8). In addition, inoculated seedlings treated with prochloraz and tomato seedlings inoculated with the pathogen contained the lowest quantities of α-tomatine—190.82 and 179.87 μg/g sample. The quantity of α-tomatine was lower for all treatments at 15 days, except for the noninoculated control, which contained 365.91 μg of α-tomatine per gram of sample. The quantities of α-tomatine for seedlings treated with chaetoglobosin C, chaetomanone A, and trichotoxin A50 were not significantly different (207.87, 254.25, and 205.04 μg/g sample, respectively), whereas for seedlings treated with prochloraz and the inoculated control, the quantities were 131.56 and 77.46 μg/g sample, respectively.

In line with these findings, Soytong et al. (2001), Kanokmedhakul et al. (1993) mentioned that chaetoglobosin C extracted from *C. globosum* acts as an inducer of PDI for disease resistance by inducing a localized and subsystemic oxidative burst in tomato, tobacco, potato, and carrot. This ability of chaetoglobosin C is also supported by the work of Soytong et al. (2005). Chaetoglobosin C, chaetomanone A, and trichotoxin A50 exhibited strong inhibitory activity in vivo against tomato fusarium wilt. Inoculated tomato plants to which bioactive

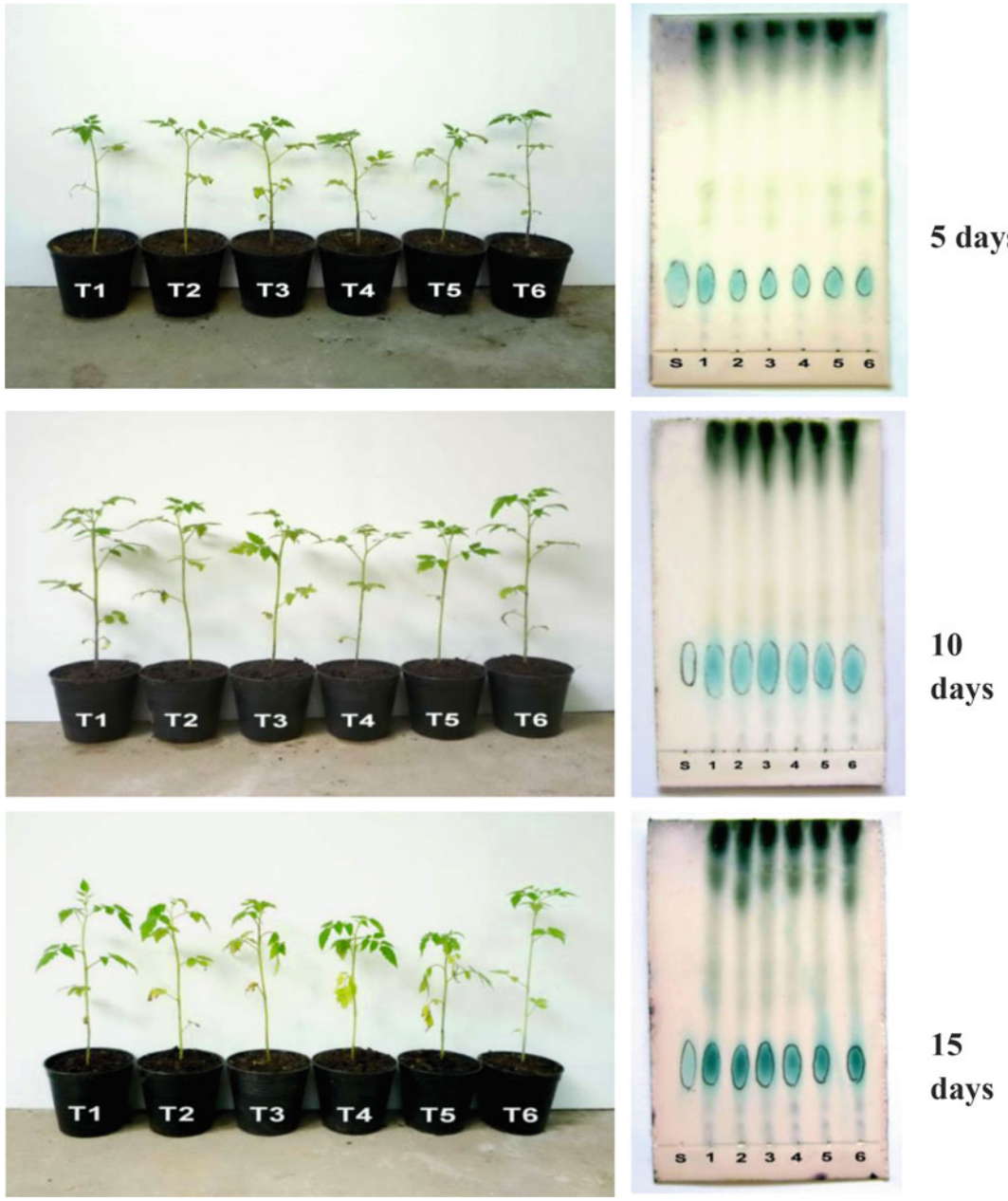


**Fig. 6.15** Detection of α-tomatine 5, 10, and 15 days. after inoculation with *Fusarium oxysporum* f. sp. *lycopersici* and treatment with bioactive compounds. *T1* treatment with chaetoglobosin C, *T2* treatment with chaetomanone A, *T3* treatment with trichotoxin A50, *T4* treatment with prochloraz, *T5* inoculation with the pathogen only, *T6* noninoculated control. For thin-layer chromatography, *S* standard α- tomatine, *1* treatment with chaetoglobosin C, *2* treatment with chaetomanone A, *3* treatment with trichotoxin A50, *4* treatment with prochloraz, *5* inoculated control, *6* noninoculated control

compounds were applied at 10–100 μg/ml showed higher PDI (53.80–65.15 %) than those to which prochloraz was applied (26.73 %). This result is supported by reports of Soytong et al. (2001) and Suwan et al. (2000), who stated that chaetoglobosin C and trichotoxin A50 can elicit resistance or immunity in tomato and other plants, inhibit the pathogen, and stimulate plant

**Table 6.8** Disease severity index (*DSI*) and α-tomatine quantification of tomato after inoculation with a pathogen and spraying with bioactive compounds

| | 5 days | | 10 days | | 15 days | | |
|---|---|---|---|---|---|---|---|
| Treatment | DSI[a] | Quantity of α-tomatine (ug/g) | DSI | Quantity of α-tomatine (ug/g) | DSI | PDI[b] (%) | Quantity of α-tomatine (ug/g) |
| Chaetoglobosin C | 1.0a | 746.67a | 1.0a | 348.72b | 2.43b | 44.97a | 207.87b |
| Chaetomanone A | 1.0a | 535.01b | 1.0a | 322.98b | 2.87b | 35.18a | 254.25b |
| Trichotoxin A50 | 1.0a | 599.70b | 1.0a | 492.22a | 2.67b | 39.43a | 205.04b |
| Prochloraz | 1.0a | 368.68c | 1.0a | 190.82c | 3.43ab | 26.95a | 131.56c |
| Inoculated control | 1.0a | 361.51c | 1.0a | 179.87c | 4.37a | – | 77.46c |
| Noninoculated control | 1.0a | 294.38c | 1.0a | 321.19b | 1.0c | – | 365.91a |
| CV (%) | NS | 13.71 | NS | 13.74 | 20.8 | 25.85 | 14.62 |

Average of three replications. Means with the same common letter in each column are not significantly different according to Duncan's multiple range test at $P = 0.01$

*CV* coefficient of variation, *NS* not significant

[a]Disease severity idex(DSI): 1 = no symptom; 2 = plant showed yellowing leaves and wilting 1–20%, 3 = plant showed yellowing leaves and wilting 21–40%, 4 = plant showed yellowing leaves and wilting 41–60%, 5 = plant showed yellowing leaves and wilting 61–80%, and 6 = plant showed yellowing leaves and wilting or die 81–100%

[b]Plant disease immunity (*PDI*) = (DSI of inoculated control – DSI of each treatment)/DSI of inoculated control × 100

growth. From the results, tomato plants treated with bioactive compounds showed significant differences in plant height, plant fresh weight, and plant dry weight in comparison with tomato plants tread with prochoraz and the inoculated control. This finding is similar to that of Soytong et al. (2007), who reported that trichotoxin A50 exhibited potential as a plant growth regulator in Chinese cabbage, kale, and mungbean. Moreover, Phuwiwat and Soytong (1999) stated that the same strain of *T. harzianum* PC01 could also promote the growth of Chinese radish and improve yield.

## 6.3 Concluding Remarks

Long cayenne pepper clearly has a mechanism for recognizing the fungal pathogen *C. capsici* C208 for the defense response by necrosis when inoculated with *C. capsici* and treated with chaetoglobosin C, chaetomanone A, and trichotoxin A50 at different concentrations. Qualitative capsidiol accumulation was found in chilli plants at 5 and 10 days after treatment with all three microbial elicitors at all concentrations, whereas no capsidiol was detected in the noninoculated and untreated control. Moreover, capsidiol was not detected in plants subjected to all treatments after 20 days.

The long cayenne pepper production in pot experiments using microbial elicitors, that is, chaetoglobosin C, chaetomanone A, and trichotoxin A50, and using biological fungicides as Bio-CG (*C. globosum* KMITL-N0802), Bio-CLT (*C. lucknowense* CLT), and Bio-T (*T. harzianum* PC01) compared with a chemical fungicide (difenoconazole) clearly demonstrated that these microbial elicitors and biological fungicides can reduce disease incidence. Chaetoglobosin C, chaetomanone A, and trichotoxin A50 significantly reduced disease incidence by 55.0, 50.0, and 42.5 %, respectively, when compared with difenoconazole, which reduced disease incidence by only 32.5 %. Additionally, treatment with chaetoglobosin C, chaetomanone A, and trichotoxin A50 significantly increased the yield by 55.17, 48.49, and 46.53 %, respectively, when compared with treatment with difenoconazole, which increased the yield by 25.31 %. Likewise, treatment with Bio-CG, Bio-CLT, and Bio-T significantly reduced disease incidence by 55.0, 42.5, and 38.75 %, respectively, when compared with treatment with difenoconazole, which reduced disease incidence by only 32.5 %. Finally, treatment with Bio-CG,

Bio-CLT, and Bio-T also significantly increased the yield by 52.54, 39.65, and 38.95 %, respectively, when compared with treatment with difenoconazole, which increased the yield by 28.04 %. These results suggest a new means of inducing immunity against chilli anthracnose in chilli production. Moreover, it was clearly shown that these antagonistic fungi and their active substances not only control disease but also promote the growth of chilli.

Chaetoglobosin C, chaetomanone A, and trichotoxin A50 also acted as microbial elicitors to induce immunity in tomato plant variety Sida by eliciting α-tomatine. Tomato plants treated with the bioactive compounds had PDI between 35.18 and 44.97 %. The bioactive compounds elicit α-tomatine between 205.04 and 254.25 μg/g, which is higher than the concentration elicited by the chemical fungicide (prochloraz) and in the inoculated control (131.56 and 77.46 μg/g) at 15 days after treatment with the pathogen. Further, the bioactive compounds were tested for their abilities as microbial elicitors to induce PDI to control tomato wilt in vivo. Chaetoglobosin C, chaetomanone A, and trichotoxin A50 at concentrations of 10, 50 and 100 μg/ml induced immunity in tomato plants with PDI between 53.80 and 65.15 %, which is higher than that for prochloraz, which resulted in PDI of only 26.73 %.

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# Part II

# Botanicals

# Plant Essential Oils and Pest Management

7

Saeid Moharramipour and Maryam Negahban

**Abstract**

Insect pest management in agriculture is facing challenge in several problems of using synthetic pesticides and toxic fumigants including environmental contamination, pesticide resistance, and destruction of nontarget organisms. So, public and environmental pressure can support environmentally safe pesticide alternatives to the use of synthetic pesticides. In recent years, a new field is developing on the use of botanical pesticide origin in the pest management practices. Botanicals have been considered as potential pest management agents, because they demonstrate to have a wide range of bioactivity and possess contact and fumigant toxicity and repellent, oviposition, and feeding deterrence. In addition, the main advantages of many plant-based pesticides lie in their low mammalian toxicity and rapid degradation with broad-spectrum activity. Botanical insecticides composed of essential oils may prove to be a reasonable alternative to the more persistent synthetic pesticides. The essential oils obtained by the distillation of aromatic plants can be utilized to protect agricultural product pests. Recently, the essential oils and their constituents have received a great deal of attention as pest control agents.

They are volatile and can function as fumigants and, in some instances, are comparable to methyl bromide in laboratory tests with insects. Their action against stored product insects has been extensively studied. Moreover, these natural oil and new formulations are considered to be an alternative means of controlling harmful larvae of field crop insects. Recent research has demonstrated their larvicidal and antifeeding effects, their capacity to delay

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_7, 

development and adult emergence and cause egg mortality, their deterrent effects on oviposition, and their arrestant and repellent action. Also the combined effects of gamma radiation or diatome with essential oil on some stored product insect have been reported. Despite these most promising properties, problems related to their volatility, poor water solubility, aptitude for oxidation, and high sorption are the important limiting factors for the application of natural compounds in large-scale commodity fumigations, and it might lead to more residue-treated commodities. In view of the problem, it is necessary to do a kind of research such as work on new formulations of the oil components and their effects on sorption, tainting, and residues in food commodities. Nowadays, using new technologies such as nanoencapsulated formulation can overcome the constraints of plant essential oils. It seems that the findings of research could be promising to make practical use of plant essential oils. As the new technology in nanoencapsuled essential oil through the control release of active ingredients overcome the restrictions of plant essential oils usage in storage and farms. Finally, most of the natural pest control measures using botanicals are becoming important tools by the development of their use in pest management, because they could be economical and eco-friendly for both the public health and the environment.

**Keywords**
Botanicals • Biopesticides • Nanocapsule • Plant essential oil • Stored-product pests • Field crop insects

## 7.1 Introduction

Widespread insecticide resistance has been a major problem in a sustainable and cost-effective biointensive integrated pest management (BIPM) strategy. In addition, the increasing public concern over pesticide safety and possible damage to the environment has resulted in increasing attention being given to natural products for the control of agricultural pests (Rajendran and Sriranjini 2008; Zapata and Smagghe 2010). The coevolution of plants along with insects has compelled the use of natural chemical defenses for the management of insect pests. Several studies have focused on the potential use of botanical applications in biological control of different insect pests, since some are selective, biodegrade to nontoxic products, and have few effects on nontarget organisms and the environment (Singh and Upadhyay 1993; Isman 2000; Kim et al. 2010). Apart from all the advantages and safety aspects of botanicals as compared to synthetics, it will also give farmers the necessary psychological satisfaction. At the same time, it will be helpful in reducing the present excessive use of synthetic insecticides, which are not compatible with many biological and microbial components of an IPM package (Rattan 2010). In the past few decades, several studies have focused on the potential use of the essential oil applications in biological control of different economically important insect pests.

The essential oils may be more rapidly degraded in the environment than synthetic compounds, and some have increased specificity that favors beneficial insects, and the pesticides of plant origin are gaining increased attention and interest among those concerned with environment-friendly, safe, and integrated crop management approaches. In addition, they are playing a vital role in organic food production globally (Pillmoor et al. 1993; Rattan 2010). Their action against stored product insects has been extensively studied (Negahban et al. 2007a; Sahaf et al. 2008a, b, c; Rastegar

et al. 2008; Saeidi and Moharramipour 2008; Sahaf and Moharramipour 2007; Arabi et al. 2008a, b; Ghasemi et al. 2009). Moreover, these natural derivatives are considered to be an alternative means of controlling pestiferous larvae of Lepidoptera (Jamal et al. 2011; Hasheminia et al. 2011; Yi et al. 2007; Vanichpakorn et al. 2010; Lee et al. 2001a, b). That is to say that recent research has demonstrated their larvicidal and antifeeding effects (Negahban and Moharramipour 2007a; Negahban and Moharramipour 2008; Sahaf and Moharramipour 2008), their capacity to delay development and adult emergence and cause egg mortality, their deterrent effects on oviposition (Negahban and Moharramipour 2007b; Shakarami et al. 2004; Sahaf et al. 2008b), and their arrestant and repellent action (Negahban et al. 2007b; Moharramipour et al. 2008; Sahaf et al. 2008a). In view of the various activities of the essential oils against agriculture product pests as reported by various workers, plant extracts contain compounds that show ovicidal, repellent, and antifeedant properties and can combine with other methods such as gamma radiation (Ahmadi et al. 2008a,b).

The insecticidal constituents of many plant extracts and the essential oils are monoterpenoids. Camphor, camphene, 1,8-cineol and α-pinene, linalool, methyl acetate, limonene, menthone, geraniol, citral, citronellal, thymol, carvacrol, eugenol, geraniol, and *trans*-anethole are well-known examples of pesticide compounds (Phillips et al. 2010; Negahban et al. 2007a; Isman and Machial 2006; Isman 2000). Additionally, monoterpenoid compounds have been considered as potential pest control agents because they are acutely toxic to insects and possess repellent (Mediouni-Ben Jemaa and Tersim 2011; Kim et al. 2010) and antifeedant properties (Sbeghen-Loss et al. 2011; Shukla et al. 2012) and ovicidal, larvicidal, pupicidal, and adulticidal activities (Yang et al. 2004; Waliwitiya et al. 2009; Murugan et al. 2012).

Finally, mostly the work has been carried on studying the effects of the essential oils, their lethal doses, and the time to achieve lethal effects, but their formulations especially as a form of micro- or nanoencapsules are in general not fully elucidated. Nanotechnology of the essential oils may act through the control release of active ingredients and overcome the restrictions of plant essential oils usage in storage and farms. Nanoencapsulation is a process in which tiny particles or droplets are surrounded by a coating to give small capsules many useful properties. In a relatively simplistic form, a nanocapsule is a small sphere with a uniform wall around it. The material inside the nanocapsule is referred to as the core, internal phase, or fill, whereas the wall is sometimes called a shell, coating, or membrane. Most nanocapsules have dimensions measured in nanometers. Nanocapsules have a polymeric shell; the active substances are usually dissolved in the inner core but may also be adsorbed at their surface (Khoee and Yaghoobian 2009).

Nanoencapsulation technique can serve as new model formulations for the development of the essential oils and their compound derivatives with enhanced activity or environmental friendliness. However, their functions and clarity on the specificity of the metabolites responsible for proclaimed insecticidal activity is lacking. Worthwhile for us, the plant origin nanoinsecticides could be exploited for the development of novel formulations with highly precise target for sustainable insect pest management in agriculture. In this chapter, we review the essential oil sources of insecticidal activity, their constituents, and mode of action and discuss the few botanical materials with potential action and their development of formulation with encapsulation for producing nanoinsecticidal products.

## 7.2 Potential and Improvement of Plant Essential Oils as New Insecticides

The use of the essential oils extracted from aromatic plants to control pests has been investigated and is well documented (Isman 2006). As a result, most essential oils come from highly aromatic species such as those in the Asteraceae, Myrtaceae, Apiaceae, Verbenaceae, and Lamiaceae plant families. Many aromatic plant species are indigenous to Iran (Naghibi et al. 2005). Iran is situated in arid and semiarid areas and has many endemic

aromatic plants from different families. It therefore seems very worthwhile to mount a comprehensive screening program to determine the insecticidal efficacy of such plants. Additional research from our laboratory shows that several essential oils possess insecticidal activity effects (Table 7.1). The genus *Artemisia* is a member of the large and evolutionary advanced plant family (Asteraceae). More than 300 different species comprise this diverse genus which is mainly found in arid and semiarid areas of Europe, America, and North Africa as well as in Asia (Heywood and Humphries 1977). Many *Artemisia* species are used medicinally and hence to be of more commercial values. It has been reported that *Artemisia herba-alba* Asso. (Asteraceae) inhibits the asexual reproduction of *Aspergillus niger* Tiegh (Eurotiales: Trichocomaceae), *Penicillium italicum* Wehmer (Eurotiales: Trichocomaceae), and *Zygorhychus* sp. Vuillemin (Tantaoui-Elaraki et al. 1993). *Artemisia scoparia* Waldst et Kit (Asteraceae) is used as a choleretic, anti-inflammatory, and diuretic agent in the treatment of hepatitis (Hikino 1985).

**Table 7.1** Examples of essential oils and insects which are known to be affected by these oils

| Plant source | Insect species[a] | Action[b] | Selected references |
|---|---|---|---|
| *Perovskia atriplicifolia* | *T. castaneum* | F, SG, MFO | Ahmadi et al. (2008a, b, c, d, e), (2009a, b), (2011a, b) |
| | *C. maculatus* | F, SG, MFO | |
| *Rosmarinus officinalis* | *T. castaneum* | F, SG, MFO | |
| | *C. maculatus* | F, SG, MFO | |
| *Artemisia sieberi* | *C. maculatus* | F, R, OD, O | Negahban et al. (2006a, b), Negahban and Moharramipour (2008), (2007a, b) |
| | *S. oryzae* | F, R | |
| | *T. castaneum* | | |
| *Artemisia scoparia* | *C. maculatus* | F, R, FD | Negahban et al. (2006a), Negahban and Moharramipour (2007a, b) |
| | *S. oryzae* | F, R, OD, O | |
| | *T. castaneum* | F, R | |
| | | F, R, FD | |
| *Thymus kotschyanus* | *C. maculatus* | F, R, OD, O | Akrami et al. (2011) |
| *Mentha longifolia* | *C. maculatus* | F, R, OD, O | |
| *Perovskia abrotanoides* | *T. castaneum* | F, R, FD | Arabi et al. (2008a, b) |
| | *S. oryzae* | F, R | |
| *Tanacetum polycephalum* | *T. castaneum* | F | |
| *Thymus kotschyanus* | *V. destructor* | F | Ghasemi et al. (2009, 2011) |
| *Ferula assa-foetida* | *V. destructor* | F | |
| *Mentha longifolia* | *V. destructor* | F | |
| *Artemisia annua* | *P$_i$. rapae* | FD | Hasheminia et al. (2011) |
| *Achillea millefolium* | *P$_i$. rapae* | FD | |
| *Ruta graveolens* | *C. maculatus* | F | Hosseinpour et al. (2011) |
| *Ferula gummosa* | *C. maculatus* | F | |
| *Carum copticum* | *P$_I$. xylostella* | F, R, FD | Jamal et al. (2012) |
| *Eucalyptus leucoxylon* | *C. maculatus* | F | Kambouzia et al. (2009) |
| | *S. oryzae* | F | |
| | *T. castaneum* | F | |
| *Vitex pseudo-negundo* | *B. brassicae* | F | Moharramipour and Sahaf (2006) |
| *Salvia mirzayanii* | *C. maculatus* | F | Nikooei et al. (2011) |
| | *T. confusum* | F | |
| *Zhumeria majdae* | *T. confusum* | F, R, FD | |

(continued)

**Table 7.1** (continued)

| Plant source | Insect species[a] | Action[b] | Selected references |
|---|---|---|---|
| *Achillea wilhelmsii* | *P. interpunctella* | F, R, FD, O | Rafiei Karahroodi et al. (2008, 2009, 2011) |
| *Achillea millefolium* | *P. interpunctella* | F, R, FD, O | |
| *Artemisia dracunculus* | *P. interpunctella* | F, R, FD, O | |
| *Salvia multicaulis* | *P. interpunctella* | F, R, FD, O | |
| *Thymus vulgaris* | *P. interpunctella* | F, R, FD, O | |
| *Ziziphora clinopodioides* | *P. interpunctella* | F, R, FD, O | |
| *Rosmarinus officinalis* | *P. interpunctella* | F, R, FD, O | |
| *Lavandula angustifolia* | *P. interpunctella* | F, R, FD, O | |
| *Mentha piperita* | *P. interpunctella* | F, R, FD, O | |
| *Hyssopus officinalis* | *P. interpunctella* | F, R, FD, O | |
| *Salvia officinalis* | *P. interpunctella* | | |
| *Anethum graveolens* | *P. interpunctella* | | |
| *Foeniculum vulgare* | *P. interpunctella* | | |
| *Carum carvi* | *P. interpunctella* | | |
| *Petroselinum sativum* | *P. interpunctella* | | |
| *Artemisia absinthium* | *P. interpunctella* | | |
| *Santolina chamaecyparissus* | *C. maculatus* | F | Saeidi et al. (2011), Saeidi and Moharramipour (2008) |
| *Citrus reticulata* | *C. maculatus* | F, R, OD, O | |
| *Citrus limon* | *C. maculatus* | F, R, OD, O | |
| *Citrus aurantium* | *C. maculates* | F, R, OD, O | |
| | *C. maculatus* | | |
| *Carum copticum* | *C. maculatus* | F, R, OD, O | Sahaf and Moharramipour (2007, 2008, 2009), Sahaf et al. (2007, 2008a, b, c) |
| *Vitex pseudo-negundo* | *S. oryzae* | F, FD | |
| | *T. castaneum* | F, R, FD | |
| *Salvia bracteata* | *C. maculatus* | F, R, OD, O | Shakarami et al. (2004, 2005) |
| | *S. oryzae* | F, R | |
| | *T. castaneum* | F, R, FD | |
| | *S. granarius* | F, R | |
| *Thymus persicus* | *C. maculatus* | F, R, OD, O | Taghizadeh Saroukolai (2008a, b), (2009), (2010), Taghizadeh Saroukolai and Moharramipour (2011) |
| *Prangos acaulis* | *S. oryzae* | F, R | |
| | *T. castaneum* | F, R, FD | |
| | *C. maculatus* | F, R, OD, O | |
| | *S. oryzae* | F, R | |
| | *T. castaneum* | F, R, FD | |

[a]Genus; *B*, *Brevicoryne; C*, *Callosobruchus; P*, *Plodia; $P_i$*, *Pieris; $P_l$*, *Plutella; S*, *Sitophilus*; *T*, *Tribolium*
[b]Action, *F* fumigant, *R* repellent, *FD* feeding deterrence, *OD* oviposition deterrence, *O* ovicide, *SG* synergism with gamma radiation, *MFO* micronuclei formation in ovaries induced by gamma radiation and oil

*Artemisia* is a genus that grows in many areas of Iran. There are several reports showing that species of this genus are highly toxic to stored product insects such as *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae), *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) (Negahban et al. 2006b, 2007a). The effects of *Artemisia annua* L. (Asteraceae) crude leaf extracts on the toxicity, development, feeding efficiency, and chemical activities of small cabbage *Pieris rapae* L. (Lepidoptera: Pieridae) have been evaluated (Hasheminia et al. 2011). *Artemisia vulgaris* L. (Asteraceae) has been reported to be repellent and toxic to *T. castaneum* (Wang et al. 2006). The fumigant toxicity of the essential oils

extracted from two plants of the Iran flora, *Ruta graveolens* L. (Rutaceae) belonging to Rutaceae and *Ferula gummosa* Boiss from Apiaceae, was investigated on *C. maculatus* (Hosseinpour et al. 2011). Also the toxicity of 42 essential oils extracted from Myrtaceae has been tested against *S. oryzae* and *T. castaneum* (Lee et al. 2004). Negahban and Moharramipour (2007c) reported the fumigant toxicity of three Myrtaceae species oil, *Eucalyptus intertexta* R.T. Baker (Myrtaceae), *Eucalyptus sargentii* Maiden (Myrtaceae), and *Eucalyptus camaldulensis* Dehnh (Myrtaceae), against three major stored-product beetles, *C. maculatus*, *S. oryzae*, and *T. castaneum*.

Lamiaceae family is best known for their essential oils common to many members of the family. These plants have been used by human since prehistoric times and are one of the major sources of culinary, vegetable, and medicinal plants all over the world (Naghibi et al. 2005). From this family, the genus *Thymus* (Lamiaceae) consists of 14 species in Iranian flora, four of which including *Thymus persicus* (Ronniger ex Rech. f.) are indigenous to Iran (Rechinger 1982; Nickavar et al. 2005). *Thymus* species have strong antifungal, antibacterial, and insecticidal activity (Stahl-Biskup and Saez 2002; Rasooli et al. 2006). These plant species and their extracts are known to have various effects on insect pests, including stored-product insects. Several studies have assessed the ability of the *Thymus* essential oils and their constituents as fumigants and repellents against a number of insect pests. The insecticidal and repellent activities of *Thymus vulgaris* L. have been reported against *T. castaneum* (Clemente et al. 2003), *S. oryzae* (Lee et al. 2001a), *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) (Passino et al. 2004), *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae) (Hori 2003), *Spodoptera litura* (F.) (Lepidoptera: Noctuidae) (Hummelbrunner and Isman 2001) and mushroom sciarids (*Lycoriella mali* Fitch, Sciaridae) (Choi et al. 2006). Other plant essential oils with biological activity include *Thymus mandschuricus* Ronniger against *S. oryzae* (Kim et al. 2003) and *Thymus mastichina* (L.) against *T. castaneum* (Pascual-Villalobos and Robledo 1998). Moreover, *Thymus serpyllum* L. which is rich in the phenols thymol and carvacrol has fumigant effect against the bean weevil *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae) (Regnault-Roger et al. 1993; Regnault-Roger and Hamraoui 1995). The fumigant effect of the essential oil extracted from aerial part of the *T. persicus* has been investigated against the red flour beetle, *T. castaneum,* and rice weevil *S. oryzae* (Taghizadeh Saroukolai et al. 2010). *Salvia mirzayanii* (Rech. F. and Esfand) (Lamiaceae) is a wild-growing flowering plant belonging to the family Lamiaceae and is found in the southern area of Iran (Yamini et al. 2008). Many studies indicated antioxidant, antimicrobial, and antiviral activities of some *Salvia* species (Sivropoulou et al. 1997; Javidnia et al. 2002). Soleimannejad et al. (2011) has reported the high-toxicity effects of *S. mirzayanii* essential oil on nutritional indices of the *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) adults.

*Perovskia abrotanoides* Karel (Lamiaceae), the Caspian Russian sage, is a perennial silvery white spicy foliage plant with long-lasting dark blue flowers. Despite its common name, *P. abrotanoides* is native to Iran, Afghanistan, Pakistan, Tajikistan, Turkmenistan, and Tibet China province (Xizang region) (Rechinger 1982; USDA 2007). The efficiency of the essential oil from *P. abrotanoides* has been reported as a fumigant in the management of *S. oryzae* and *T. castaneum* (Arabi et al. 2008a). Also, the oviposition deterrence and repellent activity of *Thymus kotschyanus* Boiss and Hohen and *Mentha longifolia* L. (Lamiaceae) was reported on *C. maculatus* (Akrami et al. 2011). Ajwain, *Carum copticum* C. B. Clarke (Apiaceae), is a medicinal plant, and its oil is used as pharmaceutical and in flavoring. It is an annual plant which grows in the Iran, Pakistan, and Egypt with white flowers and small brownish fruits (Zargari 1991). Chaste tree, *Vitex pseudo-negundo* Hand I. MZT (Verbenaceae), naturally grows around seasonal rivers in Iran. Medicinal properties of this species caused to introduce *V. pseudo-negundo* as a familiar drug (Filekesh et al. 2005). The insecticidal activity of *C. copticum* (Sahaf et al. 2007) and *V. pseudo-negundo* (Sahaf et al. 2008a, b) has been determined against *T. castaneum*, *S. oryzae*, and *C. maculatus* (eggs, larvae and adults), as

an important storage pest of legume seeds in Iran (Sahaf and Moharramipour 2008).

Repellency effect, oviposition deterrency, and ovicidal activity of several essential oils of medicinal plant species growing in Iran have been investigated by Rafiei Karahroodi et al. (2008; 2009; 2011). In recent years, some commercial plant extracts have been introduced to varroa control. Acaricidal activity of the essential oils of *T. kotschyanus*, *Ferula assa-foetida* L. (Apiaceae), and *E. camaldulensis* has been demonstrated against *Varroa destructor* Anderson (Mesostigmata: Varroidae) (Ghasemi et al. 2011). In addition, combined application of the essential oils and gamma radiation is an ecologically safe method which could be used in the management of stored-product pests (Ahmadi et al. 2008a, b). Under in vitro experimental conditions, the essential oil had no significant direct effect on frequency of micronucleus induced in *T. castaneum* ovaries. However, using gamma radiation alone and in combination with *Rosmarinus officinalis* L. (Lamiaceae) has significantly increased the induced micronucleus. They indicated that gamma radiation can induce significant cytogenetic effects and in combination with *R. officinalis* could show synergistic effect (Ahmadi et al. 2009b).

In spite of these hopeful properties, problems related to their volatility, poor water solubility, and oxidation potentiality have to be resolved before use as an alternative pest control system, in the hope that using encapsulated formulations of the essential oil components seems to be the best choice (Clancy et al. 1992; Moretti et al. 2002). Controlled release by nanoencapsulated formulations allows the essential oil to be used more effectively over a given time interval, suitability to mode of application, and minimization of environmental damage. Our studies indicated a postingestive toxicity of the essential oil from *Artemisia sieberi* Besser (Asteraceae) using the nanoencapsulated formulation as potential insecticide for the control of *Plutella xylostella* (L.), (Lepidoptera: Plutellidae), and *T. castaneum* (Negahban et al. 2011b; 2013a). Also, ovicidal activity of nanoencapsulated essential oil of *C. copticum* on diamondback moth *P. xylostella* was evaluated by Jamal et al. (2012). Consequently, our results showed higher repellency rates in nanocapsule than in pure essential oil due to controlled-release formulations allowing smaller quantities of the essential oil to be used more effectively over a given time interval. The reasons for nanocapsulating the essential oil have been to improve its stability to reduce side effects or to reduce dosing frequency and total dosing amount, to obtain better repellent activity, and for sustained (long-lasting) release. Therefore, the nanocapsulation of the essential oil might provide a new method for the management of pests (Negahban et al. 2013a, b). Also, persistence or half-life time of the nanoencapsulated essential oil of *A. sieberi* was significantly longer than *A. sieberi* oil against stored product insect, such as *T. castaneum*. These results elucidate the suitability of nanoencapsulated essential oil as an insect control agent in organic food protection (Negahban et al. 2010). Despite fundamental differences in biology, physiology, and feeding behavior of tested insects, the general response of insect's toxicity, repellency, and antifeedant activities of nanoencapsuled essential oil was similarly effective in many aspects compared to pure essential oil. Overall, it seems that the findings of research could be promising to make practical use of plant essential oils, as the new technology in nanoencapsuled essential oil through the control release of active ingredients overcome the restrictions of plant essential oils usage in storage and farms. Nevertheless, more studies are necessary to economize these novel technologies in natural environments such as warehouses, greenhouses, and farms.

## 7.3 Essential Oils: Extraction Methods, Constituents, and Their Efficacy

Methods used for extracting the essential oil include traditional press extraction (Chen et al. 2008), steam distillation extraction (Mostafa et al. 2010; Xavier et al. 2011), organic solvent extraction (Sarikurkcu et al. 2009), subsequent

ultrasound-assisted solvent extraction (Sereshti et al. 2011), supercritical CO2 extraction (Akgun et al. 2009; Zizovic et al. 2007), microwave-assisted steam distillation (Chemat et al. 2006; Sahraoui et al. 2008), and so on. On the other hand, some known disadvantage factors, such as lower extraction rate, loss of active composition, residual of organic solvents, and higher production cost, limit their application in industrial process. Compared with others, hydrodistillation is the most commonly used method due to its advantages: easy to operate, lower cost, and good quality. However, because of involvement of higher temperatures and long time in the extraction process, some thermal-sensitive compounds in the essential oil are destroyed (Liu et al. 2009). Thus, reduction of distillation time in hydrodistillation process will provide a promising extraction method for the essential oil. If the distillation time could be reduced significantly under the premise of full extraction, the advantages of hydrodistillation are retained, while the quality of the resulting oil can be improved. Extracting the essential oil by hydrodistillation can be described as several steps. First, the essential oil molecules exist initially in plant cells in contact with water molecules, then the total pressure of mixture will reach the amount of partial pressure of each component at same temperature, finally the essential oil is brought out by water vapor. Ultrasonic processing technology is the most effective method for mixing liquid material currently. It is reasonable to believe that ultrasonic processing has positive effects on reducing distillation time through promoting contact of the two molecules. Additionally, it has been reported that the extraction rate of the essential oil can be enhanced by adding an appropriate amount of inorganic salts during the extraction process (Ji et al. 2008).

The essential oils are complex mixtures of 20–60 organic compounds and hundreds of different constituents (Bakkali et al. 2008; Rajendran and Sriranjini 2008). Depending on its chemistry, an essential oil can have widely different therapeutic and insecticidal actions. The ingredients found in the essential oils are organic due to their molecular structure which is based on carbon atoms held together by hydrogen atoms. Oxygen atoms and sometimes nitrogen and sulfur atoms are also present. Chemical structures of some of the essential oil constituents possess potent biological activity (Fig. 7.1) and are responsible for the bitter taste and toxic properties. Essential oil constituents are primarily lipophilic compounds that act as toxins, feeding deterrents, and oviposition deterrents to a wide variety of insect pests. Insecticidal properties of several monoterpenoids have been reported on housefly, red flour beetle, and southern corn rootworm (Rice and Coats 1994; Lee et al. 2004; Wang et al. 2006, Kim et al. 2010). As mentioned above, the essential oils are complex mixtures of natural organic compounds which are predominantly composed of terpenes (hydrocarbons), such as myrcene, pinene, terpinene, limonene, phellandrene, etc., and terpenoids (oxygen containing hydrocarbons), such as acyclic monoterpene alcohols (geraniol, linalool), monocyclic alcohols (menthol, 4-carvomenthenol, terpineol, carveol, borneol), aliphatic aldehydes (citral, citronellal, perillaldehyde), aromatic phenols (carvacrol, thymol, safrole, eugenol), bicyclic alcohol (verbenol), monocyclic ketones (menthone, pulegone, carvone), bicyclic monoterpenic ketones (thujone, verbenone, fenchone), acids (citronellic acid, cinnamic acid), and esters (linalyl acetate). Some essential oils may also contain oxides (1,8- cineole), sulfur-containing constituents, methyl anthranilate, and coumarins. Zingiberene, curcumene, farnesol, sesquiphellandrene, termerone, and nerolidol are examples of sesquiterpenes (C15) isolated from essential oils. Mono and sesquiterpenoidal essential oil constituents are formed by the condensation of isopentenyl pyrophosphate units. Diterpenes usually do not occur in the essential oils but are sometimes encountered as by-products (Koul et al. 2008). The eight main chemical components found in the essential oils are as follows.

## 7.4 Monoterpene Alcohols

They contain 10 carbon atoms often arranged in a ring or in acyclic form. They are colorless and highly volatile. They can deteriorate very quickly

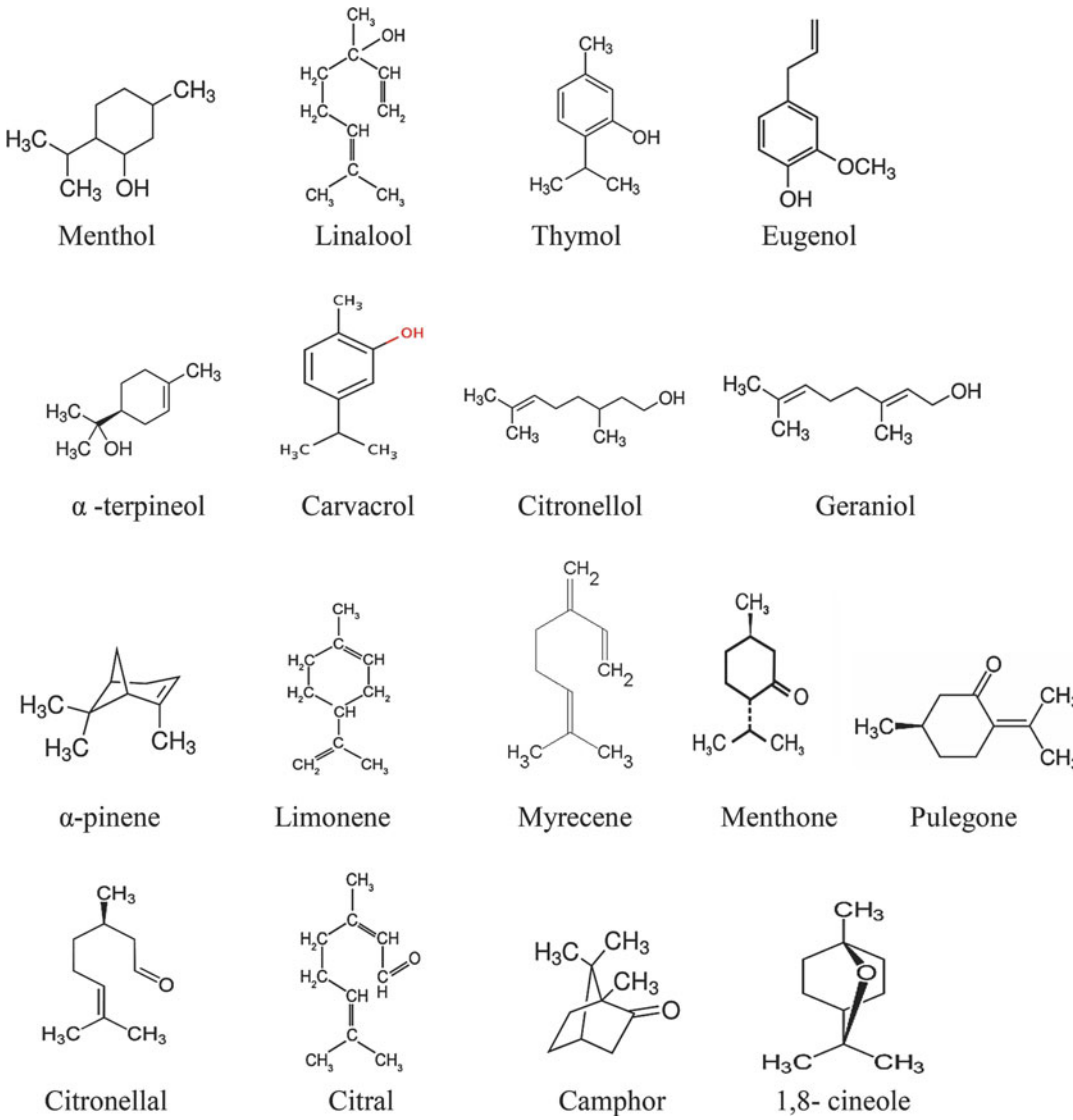


**Fig. 7.1** Chemical structure of some plant essential oil components with insecticidal activities

and therefore need to be kept at cool temperatures and to be antiviral, antibacterial, and antifungal. Being typically volatile and rather lipophilic compounds, they can rapidly penetrate into insects and interfere with their physiological functions (Lee et al. 2002) and have insecticidal properties. Eugenol is reported as toxic to *S. litura*, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae), *Musca domestica* L. (Diptera: Muscidae), and *Diabrotica virgifera* Lee Conte (Coleoptera: Chrysomelidae) (Hummelbrunner and Isman 2001; Obeng-Ofori et al. 1997; Lee et al. 1997). Eugenol is also active against *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), *Aedes aegypti* (L.) (Diptera: Culicidae), and American cockroach, *Periplaneta americana* (L.) (Blattodea: Blattidae) (Bhatnagar et al. 1993, Ngoh et al. 1998). Cornelius et al. (1997) evaluated toxicity of monoterpenoids against *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae) (a subterranean termite) of which eugenol was found most effective as termiticide. Moreover,

Meepagala et al. (2006) found that apiol isolated from *Ligusticum hultenii* Fernald (Apiaceae) exhibited high termiticidal activity and similar effect was shown by vulgarone B, isolated from *Artemisia douglasiana* Besser (Asteraceae). According to Raina et al. (2007) and Chauhan and Raina (2006) *d*-limonene, *E*, *Z*- nepetalactone, and *Z*, *E*-nepetalactone caused mortality to formosan subterranean termite, *C. formosanus*, and there was significant reduction in feeding. It was also effective as a fumigant and as feeding deterrent. Similarly, thymol has shown to have direct contact toxicity to larvae of *Agriotes obscurus* (L.) (Coleoptera: Elateridae) (Waliwitiya et al. 2000). Varying concentrations of the essential oil of red thyme having thymol are toxic to *A. aegypti* and *Aedes albimanus* Wiedemann (Barnard 1999). Thymol was also found to repel mosquitoes (Kalemba et al. 1991, Chokechaijaroenporn et al. 1994). According to Taghizadeh Saroukolai et al. (2010), the insecticidal activity of *T. persicus* could be related to these constituents.

Citronellal is toxic to *S. litura*, *M. domestica* (Hummelbrunner and Isman 2001; Lee et al. 1997), and *C. maculatus* (Don-Pedro 1996). There are several reports on insecticidal activity of camphor (Negahban et al. 2007a) in some species of *Artemisia*, and 1,8-cineole was the most toxic fumigant found in eucalyptus, rosemary, and *Perovskia* essential oils (Lee et al. 2003; Negahban and Moharramipour 2007c, Lee et al. 2001a; Negahban et al. 2007a; Arabi et al. 2008a). Coats et al. (1991) found the fumigant toxicity effect of myrcene and a-terpineol against *S. oryzae* after 24 h. α-Pinene is reported to be toxic to *T. confusum* (Ojimelukwe and Alder 1999). Hierro et al. (2004) have reported the toxicity activity of geraniol, citronellol, citral, carvacrol, and cuminaldehyde against *Anisakis simplex* Rudolphi (Ascaridida: Anisakidae). Menthone, trans-anethole, and cinnamaldehyde are well-known anti-insect compounds (Marcus and Lichtenstein 1979, Harwood et al. 1990, Lee et al. 1997, Franzios et al. 1997, Huang and Ho 1998; Hummelbrunner and Isman 2001; Chang and Ahn 2001; Chang and Cheng 2002). *d*- Limonene, linalool, myrcene, and terpineol significantly increased the nymphal duration in German cockroach, *Blattella germanica* (L.) (Blattodea: Blattellidae), when fed through artificial diet (Karr and Coats 1992). Also, *d*-limonene is toxic to *M. domestica*, *D. virgifera*, *S. litura* (Lee et al. 1997; Hummelbrunner and Isman 2001), and some stored grain pests and cockroaches (Don-Pedro 1996, Coats et al. 1991). Similarly, limonene found in the essential oil of various citrus leaves and fruit peels has exhibited significant insect control properties (Karr and Coats 1988). Acaricidal activity of thymol and 1,8-cineole from the essential oils of *T. kotschyanus*, *F. assa-foetida*, and *E. camaldulensis* against *V. destructor* was evaluated (Ghasemi et al. 2011). Essential oils rich in 1,8-cineole are also effective against house dust mites (Miresmailli et al. 2006). Among pure constituents, citronellal, eugenol, menthol, pulegone, and thymol are moderately active against various mites (Calderone and Spivak 1995; Perrucci 1995; Ellis and Baxendale 1997).

## 7.5 Sesquiterpene Alcohols

Which hydrocarbons comprise of 15 carbon atoms. These terpenes are not as volatile as monoterpenes and having a calming effect as well as being anti-inflammatory and anti-infectious. Varying properties include anti-inflammatory, antiviral, and anticarcinogenic. New insecticidal sesquiterpene extracts of the root bark of *Celastrus angulatus* M. have been reported (Wei et al. 2011).

## 7.6 Other Compounds

*Aldehydes*: antimicrobial, anti-inflammatory, disinfectant, and sedative

*Esters*: antispasmodic, anti-inflammatory, antifungal, calming, and sedative

*Ethers*: antispasmodic and analgesic

*Ketones*: anticatarrhal, regenerative, and analgesic

*Oxides*: expectorant and stimulant

*Phenols*: Strongly antimicrobial, stimulants to the immune and nervous system, and irritating to mucous membranes.

The essential oils work as the chemical defense mechanism of the plant. They ward off insects and play a role in initiating the regeneration process for the plant. They have a chemical structure that is similar to that found in human and animal cells and tissues. This makes the essential oils compatible with human and animal protein and enables them to be readily identified and accepted by the body. Diffused essential oils can increase atmospheric oxygen and provide negative ions that inhibit bacterial growth and can break down and render potentially harmful chemicals nontoxic. Some benefits of pure therapeutic grade essential oils are as follows: regenerating, oxygenating, and immune defense properties of plants, also containing oxygen molecules, which help to transport nutrients to the starving human cells. Because a nutritional deficiency is an oxygen deficiency, disease begins when the cells lack the oxygen for proper nutrient assimilation. By providing the needed oxygen, the essential oils also work to stimulate the immune system, very powerful. Antioxidants create an unfriendly environment for free radicals. They prevent all mutations, work as free radical scavengers, prevent fungus, and prevent oxidation in the cells, removing metallic particles and toxins from the air.

Plant essential oils are produced commercially from several botanical sources. Examples include 1,8-cineole, eugenol, thymol, menthol, asarones, carvacrol, and linalool from many plant species. A number of source plants have been traditionally used for protection of stored commodities (Koul et al. 2008), especially in the Mediterranean region and in Southern Asia, but interest in the oils was renewed with emerging demonstration of their fumigant and contact insecticidal activities to a wide range of pests in the 1990s (Isman 2000). The rapid action against some pests is indicative of a neurotoxic mode of action, and there is evidence for interference with the neuromodulator octopamine (Kostyukovsky et al. 2002) by some oils and with GABA-gated chloride channels by others (Priestley et al. 2003). Recent evidence for an octopaminergic mode of action for certain monoterpenoids (Bischof and Enan 2004; Kostyukovsky et al. 2002), combined with their relative chemical simplicity, may yet find these natural products useful as lead structures for the discovery of new neurotoxic insecticides with good mammalian selectivity. The knowledge on the mode of action of available insecticides will help to determine the chemical properties of novel compounds that may be ideally suited for modification of insect behavior at doses that can be used safely and economically in agriculture (Haynes 1988). The plant origin insecticides could be exploited for the development of novel molecules with highly precise target for sustainable insect pest management in agriculture (Rattan 2010). Given these encouraging results, further experiments are in progress to assess the suitability of natural active principle formulations for application as a new tool in integrated control of different pest.

## 7.7 Fumigant Properties of Essential Oils

More current research shows that the essential oils and their constituents may have the potential as alternative compounds to currently used fumigants (Nattudurai et al. 2012; Kim et al. 2010; Suthisut et al. 2011). Major constituents from aromatic plants, mainly monoterpenes, are of special interest to industrial markets because of other potent biological activities in addition to their toxicity to insects (Kubo et al. 1994; Isman 2000; Weinzierl 2000). Several studies have been undertaken in our laboratory to explore the potential of the essential oils and their constituents as insect fumigants. Table 7.2 provides examples of bioassay test to determine the $LC_{50}$ values of insecticidal activity of a few of the better-known essential oils. Their action against stored product insects has been extensively studied. Moreover, these natural derivatives are considered to be an alternative means of controlling harmful larvae of field crop insect pests. The optimum use of the essential oils with maintaining their active ingredients by developing applicable methods is the essential way to control insects. For taking into account the limitations and the physicochemical characteristics of the essential oils, nanoencapsulated formulations of the essential oil components seem to be the best choice. To get the best out of our

**Table 7.2** Fumigant toxicity of some selected plant essential oils on insects and mites

| Plant source | Insect species[a] | Stage | $LC_{50}$ (μL/L air) (95 % fiducial limits) | Selected references |
|---|---|---|---|---|
| *Artemisia scoparia* | *C. maculatus* | Adult | 1.46 (1.29–1.68) | Negahban et al. (2006a) |
| | *S. oryzae* | Adult | 1.87 (1.70–2.11) | |
| | *T. castaneum* | Adult | 2.05 (1.72–2.37) | |
| *Artemisia sieberi* | *C. maculatus* | Adult | 1.45 (1.23–1.66) | Negahban et al. (2007a) |
| | *S. oryzae* | Adult | 3.86 (3.49–4.28) | |
| | *T. castaneum* | Adult | 16.76 (14.64–18.61) | |
| *Eucalyptus intertexta* | *C. maculatus* | Adult | 2.55 (2.24–2.84) | Negahban and Moharramipour (2007c) |
| | *S. oryzae* | Adult | 6.93 (6.45–7.34) | |
| | *T. castaneum* | Adult | 11.59 (11.28–11.90) | |
| *Eucalyptus camaldulensis* | *C. maculatus* | Adult | 3.97 (3.64–4.31) | |
| | *S. oryzae* | Adult | 12.06 (11.63–12.43) | |
| | *T. castaneum* | Adult | 33.50 (30.47–36.51) | |
| *Eucalyptus sargentii* | *C. maculatus* | Adult | 3.87 (3.56–4.19) | |
| | *S. oryzae* | Adult | 12.91 (12.47–13.33) | |
| | *T. castaneum* | Adult | 18.38 (17.65–19.07) | |
| *Carum copticum* | *S. oryzae* | Adult | 12.30 (6.42–42.74) | Sahaf et al. (2007) |
| | *T. castaneum* | Adult | 150.36 (102.69–284.81) | |
| *Vitex pseudo-negundo* | *C. maculatus* | Egg | 2.20 (1.81–2.94) | Sahaf and Moharramipour (2008) |
| | | Larvae | 8.42 (6.87–10.06) | |
| | | Adult | 9.39 (6.63–14.22) | |
| *Vitex pseudo-negundo* | *S. oryzae* | Adult | 31.96 (26.60–39.25) | Sahaf et al. (2008a) |
| | *T. castaneum* | Adult | 47.27 (42.31–52.70) | |
| *Carum copticum* | *C. maculatus* | Egg | 1.01 (0.88–1.12) | Sahaf and Moharramipour (2008) |
| | | Larvae | 2.50 (1.95–3.17) | |
| | | Adult | 0.90 (0.08–1.03) | |
| *Perovskia abrotanoides* | *S. oryzae* | Adult | 18.75 (16.59–21.26) | Arabi et al. (2008a) |
| | *T. castaneum* | Adult | 11.39 (9.35–13.56) | |
| *Thymus vulgaris* | *C. maculatus* | Egg | 1.99 (1.40–2.95) | Dezfouli et al. (2010) |
| | | Larvae | 6.14 (5.47–6.88) | |
| *Eucalyptus leucoxylon* | *C. maculatus* | Adult | 2.76 (2.29–3.18) | Kambouzia et al. (2009) |
| | *S. oryzae* | Adult | 8.48 (8.02–8.88) | |
| | *T. castaneum* | Adult | 13.15 (12.83–14.09) | |
| *Thymus persicus* | *S. oryzae* | Adult | 3.34 (2.62–4.28) | Taghizadeh Saroukolai et al. (2009) |
| | *T. castaneum* | Adult | 236.9 (186.27–292.81) | |
| *Salvia mirzayanii* | *C. maculatus* | Adult | 2.58 (2.00–3.20) | Nikooei and Moharramipour (2010) |
| *Citrus reticulata* | *C. maculatus* | Adult | 8.70 (8.30–9.15) | Saeidi et al. (2011) |
| *Citrus limon* | *C. maculatus* | Adult | 7.21 (6.79–7.71) | |
| *Citrus aurantium* | *C. maculatus* | Adult | 6.33 (5.88–6.88) | |
| *Thymus kotschyanus* | *V. destructor* | Adult | 1.07 (0.87–1.26) | Ghasemi et al. (2011) |
| *Ferula assa-foetida* | *V. destructor* | Adult | 2.46 (2.10–2.86) | |
| *Eucalyptus camaldulensis* | *V. destructor* | Adult | 1.74 (0.96–2.50) | |
| *Artemisia annua* | *P. rapae* | Larvae | 9.38 (6.84–14.49) | Hasheminia et al. (2011) |
| *Achillea millefolium* | *P. rapae* | Larvae | 4.19 (3.1–6.18) | |
| *Ruta graveolens* | *C. maculatus* | Adult | 14.7 (12.3–17) | Hosseinpour et al. (2011) |
| *Ferula gummosa* | *C. maculatus* | Adult | 29.2 (25.9–32.7) | |
| *Carum copticum* | *P. xylostella* | Larvae | 3.50 (3.32–3.71) | Jamal et al. (2012) |

[a]Genus; *C*, *Callosobruchus; P*, *Pieris; P*, *Plutella; S*, *Sitophilus*; *T*, *Tribolium*; *V*, *Varroa*

data, high fumigant toxicity and persistence of nanoencapsulated *A. sieberi* essential oil have been demonstrated as a new formulation against *C. maculatus* and *T. castaneum* (Negahban et al. 2011a; 2010). Also, preparation and characterization of nanoparticles containing *Cuminum cyminum* L. (Apiaceae) oil as a potential agriculture insecticidal application have been evaluated against insect pests (Zandi et al. 2010).

## 7.8 Antifeedant Properties of the Essential Oils

Antifeedant chemicals may be defined as being either repellent without making direct contact to insects or deterrent from feeding once contact has been made with insects (Koul et al. 2008).

### 7.8.1 Repellent Properties

One other great thing about the essential oils is that they have been tested as potential sources of insect repellents. Similarly, our laboratory bioassays have been conducted to determine the activity of some natural essential oils against some stored product and field crop pests. Repellency of *M. longifolia* and *T. kotschyanus* on *C. maculatus* was recorded at 90 % and 73.33 % At 800 ppm, respectively (Akrami et al. 2011). Also, at 1.5 ppm, the essential oil of *A. sieberi* was significantly more repellent to *T. castaneum* (65.90 %) than *S. oryzae* (59.70 %) and *C. maculatus* (55.80 %) (Negahban et al. 2007b), while *A. scoparia* strongly repelled *T. castaneum* (63.80 %) and *S. oryzae* (62.01 %) than *C. maculatus* (48.57 %) (Negahban et al. 2006a). Equally at 3 ppm, *S. mirzayanii* was significantly more repellent to *T. confusum* (86.66 %) than *C. maculatus* (70 %) (Nikooei and Moharramipour 2010). The strongest repellency has been shown in *Anethum graveolens* L. (Apiaceae) (100 %), *T. vulgaris* (100 %), and *R. officinalis* (93.33 %) and the weakest repellency in *Hyssopus officinalis* L. (Lamiaceae) (7.69 %) and *Petroselinum sativum* Hoffm. ex Gaudin (Apiaceae) (9.48 %) against *P. interpunctella* (Rafiei Karahroodi et al. 2009). Repellency of *C. reticulata* was significantly higher than *Citrus limon* (L.) Burm.f. (Rutaceae) and *Citrus aurantium* L. (Rutaceae). The adult insects were exposed to the concentration of 1, 3, 5, and 7 ppm of citrus peel essential oils to estimate repellent activities. Repellent values for *Citrus reticulata at the abovementioned concentrations* were estimated to be 26.66, 33.33, 33.66, and 40 %, respectively. *C. reticulata* essential oil was significantly more repellent to *C. maculatus* at 7 ppm (Saeidi et al. 2011).

Analysis of the data by Taghizadeh Saroukolai et al. (2009) has shown that the essential oil of *P. acaulis* strongly repelled adult insects and was significantly differed between insect species. The repellency of oil tested at the highest concentration (2 µl/ml acetone) was 83.6, 71.6, and 63.6 % on *S. oryzae*, *C. maculatus*, and *T. castaneum*, respectively. However, reverse observations have been made by several other studies concerning the strong repellent effects on *T. castaneum* rather than *S. oryzae* and *C. maculatus*. Therefore, further study is necessary to elucidate the mode of action of these essential oils. To the best knowledge of Negahban et al. (2013b), at 1.9 ppm, the nanocapsule of *Artemisia* oil was shown here to possess more repellent activity (80 %) to *P. xylostella* compared to *Artemisia* pure oil (62 %). The results showed higher repellent rates in nanocapsule than in the essential oil. The reasons for nanocapsulating the essential oil have been to improve its stability to reduce side effects or to reduce dosing frequency and total dosing amount, to obtain better repellent activity, and for sustained (long-lasting) release. These results showed that medicinal plants could be used as repellent of insects. These essential oils can be used for protecting agricultural products from pest injury.

Recent research has focused on insecticidal property of essential oil plants in biological control of insects. Nerio et al. (2010) reported that the increase in repellence activity for the essential oils is highly dependent on the product composition. Formulations based on creams, polymer mixtures, or microcapsules for controlled release resulted in an increase of repellency duration (Nentwig 2003;

Chang et al. 2006). For example, *Zanthoxylum limonella* oil was successfully microencapsulated in glutaraldehyde cross-linked gelatin (a polymer), in order to improve mosquito-repellent properties (Maji et al. 2007). Consequently, controlled release by nanoencapsulated essential oil seems to get the best out of the formulation for increasing the efficiency and providing a new method for the pest management.

### 7.8.2 Deterrent Properties

One of the significant aspects to the application of plant essential oils is that they have been considered as an important feeding deterrence in integrated pest management (IPM) programs. Needless to say, these are environmentally less harmful than synthetic pesticides and act in many insects in different ways. Several experiments have been designed to measure the nutritional indices such as relative growth rate (RGR), relative consumption rate (RCR), efficiency of conversion of ingested food (ECI), and feeding deterrence index (FDI). Bioefficacy of *A. sieberi* and *A. scoparia* exhibited antifeeding activity against *T. castaneum*, while *A. sieberi* oil was highly effective compared to *A. scoparia* and significantly decreased the RGR and RCR. Moreover, *A. sieberi* oil was more effective on FDI than *A. scoparia* (Negahban and Moharramipour 2007b). In another study (Sahaf and Moharramipour 2009), the efficacy of *C. copticum* and *V. pseudo-negundo* essential oils has also been determined against *T. castaneum* related to increase FDI.

Generally, antifeedant activity of *C. copticum* was more effective than *V. pseudo-negundo*. Soleimannejad et al. (2011) studied the effects of *Salvia mirzayanii* essential oil on nutritional indices of the *T. confusum* adults. *S. mirzayanii* showed a strong feeding deterrence action against adults of *T. confusum*. As there was an increase in the concentration of the essential oil, the growth rate (RGR), relative consumption rate (RCR), and efficiency of conversion of ingested food (ECI) were reduced significantly. However, there was a significant increase in feeding deterrence (FDI) action as increase in concentration of the essential oil. Therefore, the essential oil by fumigation could affect nutritional indices of the food by pre-ingestive and postingestive behavior. Reduction in growth and feeding rate may affect ECI and FDI, respectively. ECI indicates a sign of postingestive physiological effect. As in our experiments, food did not contaminated with the essential oil directly; therefore, it seems that indirect toxic properties of fumigation may have reduced growth and consequently ECI. Nevertheless, FDI shows pre-ingestive behavior of the insect. Consequently, the reduction in growth rate could be due to the feeding behavior of insect. It is possible that the reduction in feeding and growth rate of insects was mainly due to the feeding deterrent action. As in this study, strong feeding deterrent properties of the essential oils were observed. So, in addition to toxic effect of the essential oils, they have potential to affect growth and consumption rate of the insect.

## 7.9 Mechanism of Action of Essential Oils

As a matter of fact, the mode of action and site of effect for insecticidal activities from the essential oil is worthwhile for various authors (Enan 2001; Kostyukovsky et al. 2002; Priestley et al. 2003). Mostly the work has been carried on studying the effects of the essential oils, their lethal doses, and time to achieve lethal effects, but mode of action is in general not fully elucidated (Rattan 2010). Since large numbers of chemical defenses are present in the nature, very little is known about their mode of action at molecular level. The most striking thing to mention here is that the essential oils and their constituents disrupt the endocrinologic balance of insects (Rattan 2010). Toxicity from the essential oils in insects and other arthropods points to a neurotoxic mode of action; most prominent symptoms are hyperactivity followed by hyperexcitation leading to rapid knockdown and immobilization (Enan 2001).

Several essential oils from aromatic plants, monoterpenes, and natural products have been shown as inhibitors of acetylcholinesterase (AChE) against different insect species

(Kostyukovsky et al. 2002; Shaaya and Rafaeli 2007; Rajendran and Sriranjini 2008). Recently, it was found that azadirachtin (a tetraterpenoid) significantly inhibits the activity of AChE in *Nilaparvata lugens* Stal (Nathan et al. 2004). Furthermore, octopamine is a target for the essential oils activity in insects. The acute and sublethal behavioral effects of the essential oil compounds on insects are consistent with an octopaminergic target site in insects, which acts by blocking octopamine receptors (Enan 2001, 2005). Another possible target suggested for the essential oils is the interference with GABA-gated chloride channels in insects (Priestley et al. 2003). A case in point is that the thujone has been classified as a neurotoxic insecticide, which acts on GABA receptors (Hold et al. 2000; Ratra and Casida 2001). Koul et al. (2008) reported that the rapid action against some pests is indicative of a neurotoxic mode of action, and there is evidence for interference with the neuromodulator octopamine (Kostyukovsky et al. 2002) by some oils and with GABA-gated chloride channels by others (Priestley et al. 2003). On the whole, however, their functions on the specificity of the metabolites responsible for proclaimed insecticidal activity are lacking. Nevertheless the plant-based insecticides could be exploited for the development of novel molecules with highly precise target. For moving on the way to green chemistry processes and continuing need for developing new crop protection tools with novel modes of action makes discovery and commercialization of natural products as green pesticides with more empirical evaluation of active components for sustainable insect pest management in agriculture

## 7.10 Problems and Prospects

As a matter of fact, the essential oils and their components have certain advantages since they have been used in traditional medicine, in pharmaceutical preparations, and as natural flavorings. In addition to insecticidal activity, some of the essential oils have the advantage of showing fumigant, repellent, and contact and antifeedant action against agricultural product pests. Nonetheless, fumigants from plant sources lack the most important property of an ideal fumigant, i.e., sufficient vapor pressure for diffusion and penetration into commodities to kill the pests. Evidently, compounds of plant origin can be used only for small-scale applications or for space treatments. They require some carrier gases (e.g., $CO_2$) for even distribution and penetration into the commodities or they can act as adjuvant for conventional fumigants. Sometimes it has been claimed that monoterpenoids have comparable fumigant action to that of methyl bromide (Rajendran and Sriranjini 2008). However, this has not been established in large-scale trials with mixed-age cultures of target insects. The stability of the essential oils and their components is very important for toxic action. Negahban et al. (2006a) noted that the fumigant action of *A. sieberi* leaves decreased after processing over a period.

Very few studies have been conducted on the stability of promising the essential oils and their constituents. High sorption is one of the important limiting factors for the application of natural compounds in large-scale commodity fumigations, and it might lead to more residues; treated commodities will require longer aeration period. The most striking thing to mention here is that the nanoencapsulation process is a really helpful method for entrapping the essential oils of a very different chemical composition. In order to take full advantage of the essential oils, nanoencapsulated formulation reduces loss of the active principles, leading to high-loaded nanoparticles that offer protection against environmental agents; it also offers the possibility of controlled oil release. These effects appear maximized by nanocapsule adhesion to the hair structures typically present in some of the main defoliator families. Negahban et al. (2011a, b) reported that the fumigant toxicity of nanoencapsules was significantly higher than non-formulated oil at sublethal doses after 7 days exposure. When the nanoencapsulated oil is sprayed on the insect body, because of suspension capability in water, it completely wets all organs of the insect which is covered with fuzz.

Low viscosity of suspension resulted in uniform coatings, and the presence of surfactant in nanoformulation suspension causes a decrease in surface tension which increases stability of minute droplets of the essential oil in the suspension. Lai et al. (2006) have investigated the formulation of emulsion of *Artemisia arborescens* L. (Asteraceae) essential oil with solid lipid and its toxic effect on *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae). They showed that the type and amount of the essential oil did not alter fast during the period after being sprayed. The evaporation rate of the essential oil in the formulation is extremely low in comparison with control samples. Nanoformulation can be an up-to-date asset for pest control in agriculture. Moretti et al. (2002) reported the highest larval mortality occurred by microparticle adhesion to the hair structures of insects. Besides, the mortality rate was significantly high in experiment in closed condition in all fumigant, contact, and feeding nanoencapsulated treatments. This could be as a result of high volatility of the toxic mono- and sesquiterpene compounds which likely delivered fumigant toxicity by vapor action via the respiratory system, leading to more influence of complex and higher amount of active principles (Phillips et al. 2010, Kim et al. 2010). It can be concluded that mortality caused by nanoformulations is mainly wrought by contaminating the diet with the active principles released from the capsules and is a consequence of ingestion (Passino et al. 2004).

In general, the results show that the toxicity of various formulations besides chemical components depends on different factors such as application method as fumigant or contact toxicity or feeding repellent effects. Nanoencapsulation protects the quality and durability of active ingredients and thus increases the contact toxicity of the essential oil (Ziaee et al. 2014a, b). Moretti et al. (2002) and Passino et al. (2004) reported that insects' mortality due to nanoencapsulation is higher than non-formulated oil as the release rate of active ingredients can be controlled by loading them into nanocapsules over time. Therefore, according to researches, the appropriate application of nanoformulations can increase the insecticidal potential of the essential oils. Nevertheless, more studies are necessary to economize these novel technologies in the IPM.

The results of repellency tests indicate that although the non-formulated oil was more effective than nanoformulation in short time period (maximum 6 h after exposure), it is a strong repellent after 24-h exposure. In addition to feeding deterrency, the possibility of postingestive toxicity of nanoformulations is clearly increased.

## 7.11 Conclusion

Plant essential oils and their individual metabolites have demonstrated optimal potential for insecticidal activity against several species, not only insects, but other kinds of arthropods. However, its high volatility decreases the times of protection. What it does is that the major inconvenience of the use of this oil and, in general, of the essential oils is their chemical instability in the presence of air, light, moisture, and high temperatures that can determine the rapid evaporation and degradation of some active components. It was concluded that essential oils could be encapsulated successfully. All studied formulations demonstrated a high physical stability and a good capability to reduce the essential oil evaporation. The best results were obtained with nanoencapsulated formulation, which did not vary in size even after the spraying procedure. A central feature is that nanocapsules can be generally applied over large areas with conventional spray equipment, and numerous variables can be manipulated to control the release characteristics, e.g., capsule wall thickness, capsule size, capsule wall composition, and internal composition. Given these encouraging results, further experiments are in progress to assess the suitability of natural active principle formulations for application as a new tool in integrated control of different pests. Therefore, encapsulating the essential oils has a considerable potential as commercial insecticide products. Incorporation of the essential oils in controlled release with nanocapsule formulations could solve their problems and offer several advantages. Following

an extensive literature review of the existing nanocapsulation technology, we concluded that several nanocapsulation applications would bloom if a low-cost continuous polymerization process for high-performance nanocapsules became available.

The essential oils have the ability to control a wide range of insects. Unfortunately, this broad-spectrum nature can adversely affect many nontarget beneficial species such as bees and parasitoid wasps along with the pest. Effectiveness of the essential oils can vary depending on where the chemicals are being applied. For instance, they may kill a large percentage of the target pest because it is a controlled environment, but in a real-life situation, the number may be much smaller. Evaluating uses of new formulations in similar situations as that of natural conditions may help in estimating the kind of effect it will have.

The essential oils are considered as a shorter-lived, fast-acting, and more acutely toxic material. But encapsulation technologies may modify them to longer-lasting, slow-acting, and less toxic materials that may be better for chronic pest problems. As essential oils are reported to be often the most phytotoxic, this property requires serious attention when formulating products. Other constraints such as lack of data for the essential oils on sorption, tainting, and residues in food commodities are included. Therefore, implementation of essential oil-based control systems will require more knowledge about their side effects in pest management systems. Because the knowledge of essential oils in this area is very limited, considerably more research will be required to develop and implement these new growing technologies. Acquiring this knowledge base will necessitate coordinated efforts among many scientific disciplines.

The main objective in future work is to achieve reactive polymerization of nanocapsule compositions to be used in controlled-release applications. These new nanocapsule formulations will achieve a high selectivity, specificity, and accuracy in delivering the optimum dose of the active ingredient to the desired site at the appropriate time. It will also seek to obtain a maximum activity on the target while producing minimal effect on the nontarget materials. A secondary goal to the main objective is to extend this research by improving the understating of the released mechanisms. Therefore, it is time to focus the attention of the researchers toward the development and application of known essential oils and their constituents by advanced formulation technologies.

Plant essential oils and their individual metabolites have demonstrated optimal potential for insecticidal activity against several species, not only insects, but other kinds of arthropods. However, its high volatility decreases the times of protection. There are likely several reasons for encapsulating method that are good potential carrier to bring into being the biopesticides of the essential oil in agriculture:

1. What it does is that the major inconvenience of the use of this oil and, in general, of the essential oils is their chemical instability in the presence of air, light, moisture, and high temperatures that can determine the rapid evaporation and degradation of some active components. It was concluded that the essential oils could be encapsulated successfully. All studied formulations demonstrated a high physical stability and a good capability to reduce the essential oil evaporation. The best results were obtained with nanoencapsulated formulation, which did not vary in size even after the spraying procedure. A central feature is that nanocapsules can be generally applied over large areas with conventional spray equipment, and numerous variables can be manipulated to control the release characteristics, e.g., capsule wall thickness, capsule size, capsule wall composition, and internal composition. Given these encouraging results, further experiments are in progress to assess the suitability of natural active principle formulations for application as a new tool in integrated control of different pests. Therefore, encapsulating the essential oils has a considerable potential as commercial insecticide products. Incorporation of the essential oils in controlled release with nanocapsule formulations could solve their problems and offer several advantages. Following an extensive literature review of existing nanocapsulation technology, we concluded

that several nanocapsulation applications would bloom if a low-cost continuous polymerization process for high-performance nanocapsules became available.

2. The essential oils have ability to control a wide range of insects. Unfortunately, this broad-spectrum nature can adversely affect many nontarget beneficial species such as bees and parasitoid wasps along with the pest. Effectiveness of the essential oils can vary depending on where the chemicals are being applied. For instance, they may kill a large percentage of the target pest because it is a controlled environment, but in a real-life situation, the number may be much smaller. Evaluating uses of new formulations in similar situations as that of natural conditions may help in estimating the kind of effect it will have.
3. The essential oils are considered as a shorter-lived, fast-acting, and more acutely toxic material. But encapsulation technologies may modify them to longer-lasting, slow-acting, and less toxic materials that may be better for chronic pest problems.
4. As essential oils are reported to be often the most phytotoxic, this property requires serious attention when formulating products.
5. Other constraints such as lack of data for the essential oils on sorption, tainting, and residues in food commodities are included. Therefore, implementation of the essential oil-based control systems will require more knowledge about their side effects in pest management systems. Because the knowledge of essential oils in this area is very limited, considerably more research will be required to develop and implement these new growing technologies. Acquiring this knowledge base will necessitate coordinated efforts among many scientific disciplines.

## 7.12 Future Focus of the Work

The main objective in future work is to achieve reactive polymerization of nanocapsule compositions to be used in controlled-release applications. These new nanocapsule formulations will achieve a high selectivity, specificity, and accuracy in delivering the optimum dose of the active ingredient to the desired site at the appropriate time. It will also seek to obtain a maximum activity on the target while producing minimal effect on the nontarget materials. A secondary goal to the main objective is to extend this research by improving the understating of the released mechanisms. Therefore, it is time to focus the attention of the researchers toward the development and application of known essential oils and their constituents by advanced formulation technologies.

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# Relevance of Botanicals for the Management of Forest Insect Pests of India

8

R. Sundararaj

**Abstract**

Insect pests are major biological determinants of forest productivity, and integrated pest management is not new to Indian forestry. In this chapter, insect pests of economic importance in Indian forest scenario are summarized, and the possibility of managing them with botanical pesticides is discussed. Proper understanding of the biology and ecology of the tree species and their associated fauna and flora plays an important role in the pest management. In general, nurseries and monoculture plantations are much more susceptible to insect epidemics as compared to mixed plantations and natural forests. Besides, the number of insect species acquiring pest status is increasing day by day possibly due to environmental imbalances, climate change and bioinvasion. Large-scale spraying of chemicals, microbes or botanicals cannot be feasible in large-scale plantations and natural forest areas, but it is feasible to use maximum quantity of neem cake and other neem products as an integral component for pest management. Besides, it is essential to promote the use of plant products in the forest insect pest management programmes which will encourage the users to grow more trees in their homesteads and boost the greening India programme.

**Keywords**

Intergrated forest pest management • Botanicals • India

## 8.1 Introduction

Forests are vital for life on earth; without healthy, thriving forests, earth cannot sustain life. They have many functions integral for our survival and sustenance. They offer watershed protection, timber and non-timber products and various recreational options. They prevent soil erosion, help in maintaining the water cycle and check global warming by using carbon dioxide in photosynthesis. About 30 % of the world is forested today, but the ratio between forest and population varies immensely. The United States and Canada share 16 % of the world's forests; the former Soviet Union contains 21 %, Africa has

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_8, 

**Fig. 8.1** Raising of nurseries, an important activity for forest plantation

20 % and Latin America has 24 %. As per the estimate of FAO (2001), about 47 % of the world's total forests lie in the tropics and 8 % in the subtropics, together making up 55 % of the total. Of the tropical forests, the largest portion is in Latin America (52 %), centred on the Amazon river basin; followed by Africa (28 %), centred on the Congo river basin; and the rest in the Asia-Pacific (19 %), where it is more scattered. Forests provide habitat for many living organisms. The forest ecosystems deliver a vast array of products and services to the society. The most famous product from the forest is wood, which has an enormous diversity of applications and purposes. However, apart from that, there are a large number of non-timber forest products, like medicinal plants, honey, fruits, etc. The availability, the use and the importance of these products vary per region and per culture. Most often, the poorest and vulnerable part of society depends very much on the forest. Over the years, the area under forest cover has decreased steadily, as forests have been cleared for agriculture, industry, housing and other development activities like the construction of roads, railways and hydro-electric plants. Today tropical rainforests are disappearing from the face of the globe. Despite growing international concern, rainforests continue to be destroyed at a pace exceeding 80,000 acres (32,000 ha) per day, a concern for climate change. It is a global environmental problem and can manifest itself in gradual shifts in temperature, precipitation and a rise in sea level (Dubey 2009). New challenges due to climate change require increasing the forest cover and protecting environmentally important forests in the world. Reforestation is a way to help restore the balance. Planting trees, especially quick-growing native trees that will not be logged or burned, remove carbon from the cycle and sequester, or 'fix' it, within the wood itself. For these, raising forest nurseries (Fig. 8.1) which can supply plants for planting to many geographical areas is vital in which keeping pests out of nurseries is especially important. Forest nurseries use intensive management practices which, if not properly done, may promote pest buildup. The artificial environment of the nursery, such as planting density, species or clone choice and monoculture, can be favourable to pest development. To minimize damage, detecting and treating pests before they spread are essential. Some of the nursery IPM practices are also useful in managing planted forests.

## 8.2 Status of Indian Forest

Forest is the second largest land use in India next to agriculture. India's forest cover accounts for 23.84 % of the total geographical area of the country with very dense forest (more than 70 %) 2.54 %, moderately dense forest (between 40 % and 70 %) 9.71 % and open forest (between 10 % and 40 %) 8.77 %. In addition, tree cover accounts for 2.82 % of India's geographical area (Table 8.1). It constitutes one of the principal natural resource and occupies a permanent

**Table 8.1** Forest and tree cover of India in 2007 (FSI 2009)

| Class based on canopy density (%) | Area (million ha) | Geographical area (%) |
|---|---|---|
| Very dense forest (more than 70) | 8.35 | 2.54 |
| Moderately dense forest (between 40 and 70) | 31.90 | 9.71 |
| Open forest (between 10 and 40) | 28.84 | 8.77 |
| Total forest cover | 69.09 | 21.02 |
| Tree cover | 9.28 | 2.82 |
| Total forest and tree cover | 78.37 | 23.84 |

place in enhancement of national economy, poverty alleviation and tribal development. Forests of India form a unique national treasure and are responsible for India's rich biodiversity making the country as one of the 12 'megadiverse' countries in the world. They play a vital role in harbouring more than 45,000 floral and 81,000 faunal species of which 5,150 floral and 1,837 faunal species are endemic. The panorama of Indian forests ranges from evergreen tropical rain forests in Andaman and Nicobar Islands, the Western Ghats and northeastern states to dry alpine areas in the Himalayas in the north, and between these two extremes, the country has semievergreen, deciduous, subtropical and thorn forests. These forests hold within them a unique wildlife, flora and fauna, and are also a source of sustainable livelihoods to over 200 million people in the country. With 17 % of the world's population and 18 % livestock population over 2.4 % of the world's total geographical area, India's forests are facing severe biotic pressures as nearly 40 % of domestic fuelwood needs of the people and 30 % of fodder needs of the cattle population in the country are met from forests. The demand and supply gap of timber, fuelwood and fodder are widening. Shifting cultivation (slash and burn cultivation) practised over about 1.2 m ha., though associated with sociocultural, legal and biophysical characteristics, is also a cause of degradation of forests predominately in eastern and northeastern India. Dealing with the stupendous task to overcome the problems forests are facing, the National Forest Commission has recommended an allocation of a minimum of 2.5 % of the national budget to the forestry sector. Besides, progressive national forestry legislations and policies in India aimed at conservation and sustainable management of forests have reversed deforestation. One of the primary objectives as laid in the National Forest Policy is to increase forest productivity per unit area and time. Accordingly, in the forestry research, the topmost priority has been enhancement of productivity. The average annual productivity of wood per ha. in India has been worked out at 0.7 cubic metres which is much less than the world average of 2.1 cubic metre (Gairola and Aggarwal 2005), whereas as per the reports of Sachin Kumar and Thakur (2011), the average potential productivity of Indian forests has been estimated at 6 cubic metres per ha. per annum. Concerted efforts are therefore required to bridge this large gap between the potential and realized productivity. Efforts were therefore directed to raise large-scale plantations under various forestation programmes. Insect pests and diseases are major biological determinants which limit forest productivity. Rising of large-scale plantations are bound to be fraught with alarming pest problems (Sachin Kumar and Thakur 2011). Many such a forestation programmes, at times, suffer total failures due to insect problems. Pests and diseases cause catastrophic damage that result in loss of the planting stock and failure of plantations and the associated economic losses. Hence, it is vital to maintain the health and vitality of forests, forest ecosystems particularly nurseries and trees outside forests with reference to pests and diseases.

## 8.3 Forest Insect Pests

Forest vegetation provides food and breeding sites for many insect species. A great diversity of forest insects adapted morphologically, physiologically and behaviourally to feed on almost all

**Fig. 8.2** Termite infestation on sandal (*left*) and *Terminalia* sp. (*right*)

forest vegetation and organic matter derived from it. In general, insects are more numerous in the tropics than temperate forests. Insects, as a group, are capable of feeding on almost all parts of a tree – the leaves, flowers, fruit, seed, shoot, bark, sapwood, heartwood and the roots. All insect orders are present in the forest ecosystems. Almost all organic matter in the forest is eaten by one or other insect species. These insects play key roles in ecosystem processes at two trophic levels – as primary consumers and as decomposers. They also play minor roles as secondary and tertiary consumers. In addition, they interact with many other life forms in innumerable ways. These direct and indirect effects of insects on trees, other organisms and the physical environment can influence primary production, succession and evolution of plant communities. The diversity of forest insects is also reflected in their feeding habits. Among them, leaf feeders constitute a large proportion of forest insects. Members of the orders Lepidoptera, Coleoptera and Orthoptera are the common leaf-feeding insects. During outbreaks, they consume most or all the foliage and if it occurs over several successive years, causes reduced seed yields, growth loss, dieback and sometimes death of the tree itself. Sap feeders constitute a comparatively small proportion of species, but some are of economic significance because of population outbreaks and many act as vectors of disease. Most sap feeders belong to the order Hemiptera and a small scale of Diptera and Thysanoptera. They suck the plant juices, and heavy infestations can kill stems and premature defoliations, and they act as vectors. Stem feeders include shoot borers, bark borers, sapwood borers and sapwood cum heartwood borers. Shoot borers are mostly lepidopteran larvae of the families Pyralidae, Oecophoridae and Cossidae. Bark borers include the bark surface-feeding caterpillar *Indarbela quadrinotata* as well as the more economically important 'bark beetles' of the family Curculionidae (Scolytinae). Sapwood cum heartwood borers are mainly the coleopteran family Cerambycidae bore deep into the tree trunk and cause more serious damage. These wood-boring insects reduce the structural integrity of trees and structural damage to poles, fences and wooden buildings. Shoot- and stem-infesting insects kill growing portion of trees and cause growth reduction and tree deformity. Other economically important groups are flower, nectar, pollen and seed feeders which can reduce the seed yields. Gall inducers often kill branches and reduce seed production. Dead wood feeders are mainly termites (Fig. 8.2) and to some extent cerambycid beetles. Termites cause considerable destruction to plants from nurseries to trees in plantations and natural forests, and they probably do more damage to dry wood or seasoned timber

than any other insects in the tropics (Harries 1965). If the damage they cause is significant steps to be taken to reduce those losses so that human's dependent on these resources may continue to exist in comfort. Fortunately, only a small group of the insect species found in the world forests competes with humans for forest resources. However, that relatively small number can have a wide range of ecological, social, or economic impacts. Their damage can be significant, sometimes accounting for millions of US$ in resource losses and pest management costs (Ciesla 2011).

## 8.4 Forest Insect Pests of India and Their Impacts

Indian forests are mainly tropical, which are characterized by high species diversity and generally are free of pest outbreaks, although the trees may support small populations of phytophagous insects (Nair 2007). In tropical evergreen forests with their numerous species of trees and still more numerous hordes of insect species, the absence of epidemics is not surprising (Beeson 1941). Mixed stands are much safer from insect injury than are pure stands. This is true because the forests have a high degree of species diversity, and most insects have a narrow host range. When, however, single-species plantations are established, the probability of an outbreak of a defoliating insect increases substantially (Wagner et al. 1991). A large number of insects and diseases are known to damage both naturally regenerating forests and plantations in India, although little statistics are available on the area affected by these insects (FAO 2007).

### 8.4.1 Insect Pests in Natural Habitats

Nair et al. (1986a) reported that all the observed 20 tree species in moist deciduous forests and 18 tree species in evergreen forests in Kerala suffered different types of damage by insects like defoliation, sap sucking, gall inducing and wood boring. The annual defoliation percentage ranged from 0.1 to 6.7 for the different tree species. The mean monthly defoliation was 21 % for moist deciduous species and 17 % for evergreen species. In general, evergreen tree species suffered less damage than moist deciduous species. *Tectona grandis* (teak), one of the important tree species in the moist deciduous forest, showed more 50 % defoliation due to its defoliator *Hyblaea puera*. *H. puera* is also known to cause heavy defoliation in Karnataka, Andhra Pradesh and Madhya Pradesh. Teak is also affected periodically by teak skeletonizer *Paliga machaeralis* in India. Its outbreaks in natural teak areas in central India were reported as early as 1892–1898 (Thompson 1897; Fernandez 1898). *Pteroma plagiophleps*, a polyphagous bagworm, was known to outbreak in plantations of pine *Falcataria moluccana* in India (Nair and Mathew 1992) and also infest many other trees such as *Tamarindus indica*, *Delonix regia*, *Emblica officinalis*, *Syzygium cumini*, *Populus deltoides*, *Tectona grandis* and *Trema orientalis* (Mathew and Nair 1986).

In India, the incidence of cerambycid borer *Hoplocerambyx spinicornis* on Indian sal *Shorea robusta* is in epidemics in many times throughout the range of central and northern India. This is the most economically important pest of Indian forest. In 1997–1998, it has become an endemic pest of sal in Madhya Pradesh and has affected one-sixth of the total sal forests that cover an area of 300,000 ha inflicting a loss of Rs. 250 crores. The beetle, which has an annual life cycle, lays eggs under the bark of the trunk. The grubs bore into the sapwood and heartwood and remain active for 6–7 months, creating extensive galleries and causing partial or complete girdling, eventually killing the tree. In an epidemic in 1923–1928, about seven million sal trees were killed in Madla Forest Division in Madhya Pradesh (Roonwal 1978). Another epidemic in the same state from 1994 to 1998 which covered half a million ha of sal forests resulted in death of about three million trees, before it subsided naturally in 1999 (Dey 2001). The lesser leaf roller of bamboos *Pyrausta bambucivora* is injurious in bamboo forests of northwest Himalaya particularly in moist nullahs. Two bamboo weevils, viz.,

**Fig. 8.3** Infestation of *Aristobia octofasciculata* (*left*) and *Zeuzera coffeae* (*right*) on sandalwood

*Cyrtotrachelus dux* and C. *longimanus* and the bamboo hispine beetle *Estigmena chinensis* are common shot and cum borers of bamboos in natural forests and young plantations. *Dinoderus ocellaris*, *D. minutus* and *D. brevis* are important borers of felled bamboos, and they cause immense damage, and their attack occurs when the bamboos are in the process of drying. Singh et al. (2001) reported heavy mortality of chir pine (*Pinus roxburghii*) in India which occurs in the subtropical mixed forest at Morni Hills in Haryana, patches comprising young and middle-aged trees, heavy mortality of pine trees. Four species of beetles, viz., *Sphenoptera aterrima* (Buprestidae), *Cryptorhynchus rufescens* (Curculionidae), *Platypus biformis* (Curculionidae, Platypodidae) and *Polygraphus longifolia* (Curculionidae: Scolytinae), were considered as causative organisms for the death of trees.

Sandalwood is infested by six species of stem borers, viz., *Aristobia octofasciculata* (Fig. 8.3), *Aeolesthes holosericea*, *Purpuricenus sanguinolentus*, *Capnolymma cingalensis*, *Zeuzera coffeae* (Fig. 8.3) and *Indarbela quadrinotata*. All these borers infest and damage standing sandalwood trees and often lead to mortality in young trees. The trees infested by these borers show poor growth and poor quality of wood. Sandalwood kotis in south India showed sandalwood logs with hollowed heartwood and an average loss of 198.6 kg of heartwood to every ton of wood produced by these trees (Remadevi et al. 1998). There are several important insect species causing qualitative as well as quantitative losses by making galleries in the bark and wood of trees. The affected tress show less resistance to diseases and natural calamities. Breaking of shoots by winds often facilitates entry of pathogens/microorganisms. Trees in a pool physiological state and stress become more liable to attack by xylophagous insects. *Hypsipyla robusta* is an important pest of Meliaceae and causes considerable losses to seeds, shoots and stem of *Toona ciliata*, *Swietenia macrophylla* and *S. mahagoni*. *Tonica niviferana* is a major shoot borer of semul. The beetle *Estigmena chinensis* (Chrysomelidae) is a serious pest of bamboo forests. The pentatomid bug, *Udonga montana*, feeds on the developing seeds of bamboos, and a very heavy build up of this bug was noticed periodically in bamboo forests coincident with gregarious flowering of bamboos.

### 8.4.2 Insect Pests in Plantations

About 170 species have been tried in plantations forestry which is a major activity in forestry

**Fig. 8.4** Infestation of gall wasp *Leptocybe invasa* in *Eucalyptus* sp. (*left*) and flower gall inducer *Asphondylia pongamiae* on *Pongamia pinnata* (*right*)

**Fig. 8.5** Infestation of *Icerya* sp. on *Tectona grandis* (*left*) and *Rastrococcus iceryoides* on *Pongamia pinnata* (*right*)

sector in India (Ghosh 1977). It includes species of Acacias, Ailanthus, Bamboo, Casuarina, Dalbergia, Eucalyptus (Fig. 8.4), Pinus, Poplar, Pongam, Shorea, Swietenia and *Gmelina arborea*, *Leucaena leucocephala* and *Tectona grandis* (Fig. 8.5). These tree species are selected irrespective of the extent of the area planted and whether they suffer from serious pest problems or not. The pests associated with these plantation tree species fall under three major categories – nursery pests, sapling pests and pests of older, established plantations.

### 8.4.3 Nursery Pests

Forest nurseries in India are grown and maintained to overcome the problems of deforestation. Government of India is spending large amount of money to bring about afforestation and reforestation programmes, but the programmes are facing a lot of problems due to nursery pests. Generally, forest tree seedlings are raised in nursery beds and planted out in the field when they are 6–12 months old. Nursery pests include root-feeding white grubs and termites; shoot-cutting caterpillars, crickets and grasshoppers (Fig. 8.6); leaf-feeding caterpillars and beetles; sap-sucking bugs (Fig. 8.7); and shoot-boring scolytine beetles. White grubs are the immature stages of some beetles of the family Scarabaeidae which live in soil and feed underground on the roots of seedlings, and their adult beetles feed on the foliage of trees. White grubs of *Holotrichia consanguinea* and *H. serrata* are

**Fig. 8.6** Grasshoppers affecting sandal nurseries

**Fig. 8.7** *Pseudoregma bambusicola* infestation on *Bamboo* sp. and *Acaudaleyrodes rachipora* on *Prosopis cineraria*

serious pests of teak nurseries in some localities. Some species of termites attack the roots of seedlings, killing the plants. In well-managed nurseries, the problem of termite damage to young seedling, sampling transplants, or cuttings is almost insignificant. However, under unfavourable conditions, termite menace assumes alarming proportions, often resulting in the total loss and abandonment of nurseries (Thakur 1983, 1988). Caterpillars of some noctuid moths which are commonly known as 'cutworms' characteristically cut off the shoots of small seedlings at ground level. Crickets and mole crickets cause damage in forest nurseries which nest in tunnels made in the ground, come out at night and feed on seedlings, cutting them and dragging pieces to their tunnels. Seedlings of *Casuarina equisetifolia*, *Tectona grandis*, *Dalbergia sissoo*, eucalypts, etc., are damaged by these insects. Several species of grasshoppers and leaf-feeding caterpillars feed on the foliage of seedlings and saplings. Several species of leaf-

feeding caterpillars damage seedlings in forest nurseries. *Diacrisia obliqua* and *Spodoptera litura* are polyphagous. *Eutectona machaeralis* and *Hyblaea puera* attack teak (Ambika-Varma et al. 1996), and *Eligma narcissus* attacks *Ailanthus* spp. (Sivaramakrishnan and Remadevi 1996). *Strepsicrates rhothia* is a cosmopolitan pest which attacks seedlings of many species of eucalypts. Many species of chrysomelid and curculionid beetles also cause damage to forest nursery seedlings. The chrysomelids *Chrysomela populi* and *Nodostoma waterhousie* in poplar nurseries in Kashmir and Himachal Pradesh, respectively, are examples from India (Khan and Ahmad 1991; Singh and Singh 1995). Many species of psyllids cause serious damage to nursery seedlings. *Arytaina* sp. causes serious damage to seedlings of *Albizia lebbeck* in Karnataka (Sivaramakrishnan and Remadevi 1996), and an unidentified species attacks seedlings of *A. odoratissima* and *Pterocarpus marsupium* in Kerala (Mathew 1993).

The babul whitefly *Acaudaleyrodes rachipora* is known to attack the seedlings of species of *Prosopis*, *Acacia* and many other plants of Indian arid zone (Sundararaj and Murugesan 1996). In exceptional cases, losses up to 20–30 % of seedlings of teak due to white grubs, 80 % of eucalypts due to termites, 10–20 % of Albizia lebbeck or eucalypts due to cutworms, 40 % of eucalypts due to crickets, 40 % of *Acacia mangium* due to scolytines, 30–40 % of *Pericopsis elata* due to a pyralid, etc., have sometimes been reported (Nair 2007). The invasive gall-inducing *Leptocybe invasa* (Plate 4) was reported to take heavy toll on eucalyptus nurseries and plantations. Seedlings in nurseries and 6–8-month-old saplings are susceptible to *L. invasa*, which produces galls in young shoot terminals, petioles and midribs, while in mature trees the galls occur only on midribs. A heavy infestation of the wasp results in loss vigour and growth retardation. The spread of gall wasp is a huge concern to the country as eucalypts occupy 25 % of the plantation estate of the country (Jacob et al. 2007). Generally, serious damage in nurseries can usually be prevented by application of prophylactic or curative control measures.

### 8.4.4 Sapling Pests

The insects, which attack the sapling stage of trees, include root, stem, or terminal shoot borers, leaf-feeding caterpillars and sap-sucking bugs. Termites usually attack saplings of eucalypts, pines, casuarina, etc., during their establishment stage after transplanting into the field. The grub of the cerambycid beetle *Celosterna scabrator* bore into the root-shoot portion of the saplings of *Prosopis* spp., *Acacia* spp., *Eucalyptus* spp., etc., often causes death of host trees. Sandal plants of girth class 5–12 cm were found heavily infested by *Purpuricenus sanguinolentus*. The nature of attack was from the stem towards the basal portion of sandal causing death to the sandal saplings and posing threat to young sandal plantations (Raja Muthukrishnan et al. 2009). Larvae of the cossid moth *Zeuzera coffeae* bore into the stem of saplings of teak, sandal, eucalypts, etc., and some bostrichid beetles bore into the stem of saplings of *Acacia mangium*. Larvae of the moths *Hypsipyla robusta* bore into the terminal shoot of saplings of mahogany and other meliaceous trees, causing severe growth retardation. Leaf-feeding caterpillars of the moth *Eligma narcissus* cause defoliation of saplings of *Ailanthus* species. Kadam, *Anthocephalus cadamba*, commonly raised in large scale under agroforestry conditions suffers severe defoliation due to *Arthroschista hilaralis*. The defoliation retards growth of young trees in 2–5-year-old plantations result in stunned growth. *Pteroma plagiophleps* often defoliates albizias which are raised in large scale in various states. Hypocala rostrata causes large-scale defoliation in tendu (*Diospyros melanoxylon*) and makes the leaves unfit for making bidi wrapper (Khan and Bhandari 2001).

Sandal saplings were infested by 73 species of hemipteran- and 2 species of thysanopteran-sucking pests. The hemipteran-sucking pests include 21 species of Cicadellidae followed by 7 species of Pentatomidae; 6 species each of Coccidae, Margarodidae, Membracidae and Pseudococcidae;

**Fig. 8.8** Infestation of *Megapulvinaria maxima* on sandal (left) and *Pyrausta coclesalis* on *Bamboo* sp.

4 species of Aleyrodidae; 2 species each of Alydidae, Coreidae, Delphacidae, Diaspididae, Gerridae, Pyrrhocoridae and Scutelleridae; and 1 species each of Cercopidae, Eurybrachidae and Ortheziidae (Sundararaj and Raja Muthukrishnan 2008a). The sap-feeding bug *Tingis beesoni* attacks saplings of *Gmelina arborea* and causes dieback of shoots. The mealy scale *Megapulvinaria maxima* (Plate 8) and tea mosquito bug *Helopeltis antonii* are common sucking pests on Neem. Its infestation on sandal often leads to death of young plants. Various insect pests belonging to the orders Coleoptera, Hemiptera, Orthoptera and Lepidoptera attack bamboo in nurseries and plantations, and they are grouped as defoliators, borers and sap. Aphids were major pests on bamboo species damaging the sap from the lower surface of leaves. The major sap-sucking aphid pests are *Astegopteryx bambusae*, *Astegopteryx formosana*, *Hysteroneura setariae*, *Pseudoregma bambusicola* and *Melanaphis bambusae*. The bamboo bug, *Notobitus meleagris*, is a pest of bamboo, and the injection of its toxic saliva into bamboo shoot at feeding causes the death of plant cells and necrosis. *Estigmena chinensis* is the most important pests of standing bamboos in natural forests and plantations. Sometimes, 100 % culms in clump are attacked. *Pyrausta coclesalis* (Fig. 8.8) is a common defoliator of bamboo in Indian subcontinent. Depending on the tree species, sapling pests can cause serious economic loss, particularly where no effective control methods is available.

### 8.4.5 Pests of Older Plantations

Generally, a large number of insect species are associated with each tree species. The number of phytophagous insect species associated with a tree species ranges from 10 to 200 in general, with a mean of 65 with an exceptional 920 species associated with Eucalyptus (Nair 2007). The number of species of insects associated with a plantation tree species will be influenced by several factors – the chemical profile of the species, the extent and climatic diversity of the geographical area covered, the period over which the species has been cultivated on a large scale, etc. In most of the cases, although fairly large numbers of insects are associated with all tree species, many of them are casual or minor pests, and only a few species are major pests on a tree species. Major pests include defoliators, sapsuckers and stem borers. Leaf-feeding insects occur on all tree species, with serious pests occurring on *Ailanthus*, *Dalbergia sissoo*, eucalypts, *Gmelina arborea*, teak, oak sal, pine deodar and poplars. The insect *Atteva fabriciella* commonly known as Ailanthus webworm causes defoliation in plantations of *Ailanthus* species. *D. sissoo* suffers extensive damage due to attack of key pests known as shisham defoliator, *Plecoptera reflexa*, and shisham leaf roller, *Dichomeris eridantis*.

The epidemic by *Ascotis selenaria imparata* in sal forests often causes complete defoliation of

**Fig. 8.9** Defoliated teak plantation

sal trees. Poplar is defoliated by two major defoliators, viz., *Clostera fulgurita* and *C. cupreata*. Large-scale defoliation due to these insects has been reported in poplar plantations in Uttar Pradesh. Khasi pine is defoliated by *Eterusia pulchella* and *Metanastria ampla*. *Lymantria obfuscate* commonly known as India gypsy moth or Kashmir willow defoliator attacks willow, poplars and oak (Khan and Bhandari 2001). Sap-sucking insects are major pests in *Leucaena leucocephala*. A sap-sucking bug, *Rederator bimaculatus*, is responsible for transmitting spike disease, caused by a mycoplasma-like organism in the sandal tree, *Santalum album*. Sundararaj and Raja Muthukrishnan (2008b) reported the presence of six species of stem borers in sandalwood trees. Remadevi and Raja Muthukrishnan (2008) observed significant difference in the distribution of *A. octofasciculata* and *I. quadrinotata* on different girth classes of sandal. Ghate et al. (2011) reported the incidence of *Capnolymma cingalensis* from sandal forest areas of Karnataka. Stem borers are major pests of *Shorea robusta*. *Celosterna scabrator* is a major pest of babul (*Acacia nilotica*), khair (*Acacia catechu*) and Eucalyptus plantations in Uttar Pradesh, Madhya Pradesh and Haryana. Combined defoliation of teak by *Paliga machaeralis* and *Hyblaea puera* is a major problem in India (Fig. 8.9). *Alcterogystia cadambae* is a major pest in teak plantations, and *Batocera rufomaculata* is a polyphagous pest species affecting the timber of many tree species.

### 8.4.6 Pest of Timber

Various groups of insects, belonging to several orders, commonly known as wood borers, confine their attack to various timber species under different conditions. Among these, the most important order is Coleoptera (or beetles). Wood wasps and ants belonging to Hymenoptera and termites (Isoptera) are the other major wood invaders. Timber in structures/houses is mainly attacked by insects belonging to the Coleopteran families, Platypodidae and Scolytidae (ambrosia beetles), Lyctidae, Bostrichidae, Cerambycidae and Anobiidae. Ghoon beetles/borers or bostrichids or shot hole borers particularly *Heterobostrychus aequalis* and *Sinoxylon* spp. also cause extensive damage to timber in service and storage. Termites are one of the

principle destroyers of wood in buildings in the tropics and the subtropics. The extent of financial loss due to termite damage has not been computed precisely due to the difficulties associated with such computations. Edwards and Mill (1986) estimated that US$ 1,920,000 was spent worldwide per annum by the pest control operations for the treatment of buildings against termites. In India itself, the cost of treatments as preventive and remedial measures comes to about Rs 28 million annually (Rawat 2004). Damage to constructional timbers and timber products in buildings can be caused either by dry wood termites which do not maintain soil connection for moisture requirements or by the soil-dwelling termites that obligatorily maintain soil connection for moisture requirements. However, *Coptotermes* spp. are known to survive without soil connection provided that moisture source is available near their colony sites. The soil-dwelling termites cause the maximum infestation and damage in India and other tropical countries. Timbers put in use in marine environment are commonly affected by marine wood borers which fall under two main classes, viz., Bivalvia of Mollusca and Crustacea of Arthropoda.

## 8.5 Insect Pest Management

In natural tropical forests where serious pest attack is exceptional, most tree species raised in plantations are attacked by one or more serious pests. For some tree species, pests have a devastating impact in plantations, much more serious than in the mixed species natural stands. Integrated pest management practices are well recognized in the management of forest insect pests. It largely depends upon the sound knowledge of forest nature and the natural regulating forces operating in it. Strategy of wood protection is by avoiding all chemical and adopting natural ways to prevent the attack of insect pests by adopting physical and cultural methods or by employing the biocontrol methods. Naturally, many timbers are durable timbers but which account for only 10 % of the total volume of wood used in the industrial sector (Purushotham 1975). The presence of various chemical compounds and lignin are the factors which account for the natural resistance of timbers (Walcott 1946, 1947; Abushama and Abdel Nur 1973; Behr et al. 1972). The cultural methods include avoiding sapwood, following safe-felling period, debarking, starch depletion and following proper storage methods. Surface protection like polishing, lamination, as well as sterilization is very effective natural methods. The concept of integrated pest management (IPM) exploits all the available options so that the insecticide load to the environment can be minimized. The IPM practices are adopted from raising nurseries, seed sowing, bed preparation, etc., but most of the control operation in forestry is limited to the nursery stage in the form of chemical inputs. However, the use of synthetic pesticides during the last half century has often been careless and indiscriminate which resulted in malicious effects on the environment and leads to 'ecological backlash' (Sundararaj 1997). Concern about this has led to a surge of research into alternative pest control technologies. One of the efforts is the development of botanical insecticides as a novel and safer alternative strategy. It is this place where these botanical insecticides could be of great use along with other options to minimize the use of chemicals. Botanical insecticides, which contain plant extracts as active components, are safer as well as environmentally friendlier than synthetic insecticides. The use of these chemicals of plant origin, commonly called 'botanicals' or 'phytochemicals', has attracted particular attention because of their specificity to insect pests, their biodegradable nature and their potential for commercial application (Bishop and Thornton 1997; Shukla et al. 2000). These materials have been, since time immemorial, reported to be devoid of the various disadvantages, which are associated with the use of synthetics. Bioactivity of plant-based compounds is well documented in literature and is a subject of increasing importance. Knowledge of the toxic plants, their toxic principles and their biological activity is of paramount importance not only to enable them to be utilized as natural pest control

agents and replace the commercial synthetic pesticides but also to enable us to understand the nature of their toxicity to nontargeted animals (Shukla et al. 2001). The efficient use of such renewable natural resources is becoming increasingly important worldwide. There is no doubt that many plant secondary metabolites affect insect behaviour, development and reproduction. Characterization and identification of these substances is an important first step in understanding the effect of plants on insect life. The botanicals thus obtained offer better compatibility with other biological pest control agents than that of the synthetics, and this has brought them to a sudden prominence in pest management programme.

### 8.5.1 Neem Products for the Management of Forest Insect Pests

Neem (*Azadirachta indica*) products are known in use in India from time immemorial against noxious insects. Because of its legendary insect repellent and medicinal properties, it is being identified as 'the most promising of all plants', and at the present moment, it is the source of the most promising pesticides. More than 100 protolimonoids, limonoids, or tetranortriterpenoids and some nonterpenoid constituents have been isolated from various parts of neem (Koul et al. 1990; Lim and Dale 1994). From the neem seed extract alone, over 57 components (Table 8.2) have been isolated and identified (Jacobson 1988). It is now well established that azadirachtin, the most important phagorepellent of neem kernels, protects plants against insect attack. Bernays and Chapman (1977) indicated azadirachtin as the most potent antifeedant against insects like *Locusta migratatoria migratorioides* and *Schistocerca gregaria*. It exhibits strong antifeedant activity against locusts as well as growth-inhibiting properties (Rembold et al. 1980). Neem kernel extracts or their oil repel insects act as antifeedant, cause growth disruption, deformities or mortality and impair egg production (Sieber and Rembold 1983).

**Table 8.2** Some of the important biologically active chemicals from *A. indica*

| Compound name | Molecular formula | Activity |
|---|---|---|
| Azadirachtin | $C_{35}H_{44}O_{16}$ | AF, GR, OR |
| Azadiradion | $C_{28}H_{43}O_5$ | AF |
| Azadiron | $C_{28}H_{36}O_4$ | AF |
| Deacetyl azadirachtinol | $C_{32}H_{42}O_{15}$ | AF, GR |
| 6-Deacetylnimbinen | $C_{26}H_{32}O_6$ | AF |
| Nimbinen | $C_{28}H_{34}O_7$ | AF |
| 3-Deacetylsalannin | $C_{32}H_{42}O_8$ | AF, GR |
| Salannin | $C_{34}H_{44}O_9$ | AF |
| 1, 3-Diacetyl vilasmin | $C_{30}H_{40}O_7$ | AF, GR |
| Eposyazadiradion | $C_{28}H_{34}O_6$ | AF |
| Gedunin | $C_{28}H_{34}O_7$ | AF |
| Meliantriol | $C_{30}H_{50}O_5$ | AF |
| Nimbandiol | $C_{26}H_{32}O_7$ | AF |

*AF* Antifeedant activity, *GR* Growth and development regulation, *OR* Ovipositional repellent

This chapter offers further evidence for the impact of neem products against the major forest insect pests of India. The control of forest pests like poplar defoliator, *Pygaera cupreata* (Bhandari et al. 1988); babul defoliator, *Taragama siva* (Sundararaj et al. 1995); the rohida defoliator, *Patialus tecomella* (Sundararaj and Murugesan 1995); the babul whitefly, *Acaudaleyrodes rachipora* (Sundararaj et al. 1995, 1996; Sundararaj 1999a, b); and the teak defoliators, *Eutectona machaeralis* and *Hyblaea puera* (Kulkarni et al. 1996; Remadevi and Raja Muthukrishnan 1998; Murugan et al. 1999; Sree et al. 2008) using different neem products have been tested and found useful. Dubey and Sundararaj (2004) demonstrated neem oil as effective like that of commercial neem formulations and Chlorpyrifos in containing the nymphal populations of *A. disperses*-infesting trees of *Michelia champaca* and *B. variegata*. Neem seed kernel suspension as effective repellent against the polyphagous desert locust *Schistocerca gregaria* was demonstrated (Pradhan and Jotwani 1971; Singh 1985; Sundararaj et al. 1995). Ramarethinam et al. (2002a, b) reported insecticidal property of azadirachtin against *Eurema hecabe* on *Cassia fistula* Ambika et al. (2007). The application of

neem cake alone or in combination with other seed cakes and VAM was recommended to control whiteflies in nurseries (Sundararaj 2010). As the neem products proved its practical utility, they are recommended for large-scale application in forestry. The types of neem products and their effect on the insect pest of forest importance and their host spectrum are given in the Table 8.3. The reports confirm that the neem constituents are effective against forest insect pests. Neem products particularly neem cake form an integral component of potting mixture in raising seedlings. It is mixed with other potting mixtures along with other biofertilizers. The number of neem trees in India is estimated to be around 18 million with potential of 54,000 t of seeds/year, and only 25 % of the seeds are used (Gahukar 2010) and hence its full potential to be exploited. Besides, neem-based pesticides can further be fortified against dynamic pests by optimizing their use with microbial agents. From the standpoint of safety, neem-based pesticides, with their low mammalian toxicities, offer attractive alternatives to many hard conventional pesticides in use today. However, as per the report of the Directorate of Nonedible Oils and Soap Industry, Khadi and Village Industries Commission, Bombay, 1976 (Rajasekaran 1991), less than one-fourth of the neem seeds produced in this country is collected for utilizations purpose (Table 8.4). Hence, it is the right time to make more awareness to use neem products more as well as the users should be encouraged to grow more neem trees on their domestic homesteads and educated about its value in pest control.

### 8.5.2 Other Plant Products for the Management

Considerable amount of research is carried out in India with emphasis on screening and development of plant products, phytochemicals and natural products. Over 2,000 plant species have been reported to possess pesticidal activity (Crosby 1971; Chakraboraty and Basu 1997) out of about 2,500,000 angiosperms so far documented. A perusal review indicated that other than neem, about 64 plant species were reported to have pest management properties on forest insect pests (Table 8.5). In these plants, mostly crude extracts were found to have different type of pest management properties in laboratory condition against defoliating pests of teak, poplar, subabul, bamboo, etc., without identifying the active principles in the plant products. Shukla et al. (2001) commented that only a fraction of pesticidal plants have been analysed for active principles. Defoliating insects were mostly used as test insects except for the report of Sharma et al. (1992) who reported insecticidal properties of 15 plant oils against the sap-sucking psyllid *Heteropsylla cubana*. Cashew nut shell liquid has been found effective in protecting the wood for shorter durations against termites (Remadevi et al. 2002). These plant products are probable sources of some biologically active agents for pest management for the future. However, since almost all plant products other than neem and cashew nut shell liquid were evaluated in the laboratory condition as of now, they were not practically used in pest control.

## 8.6 Conclusion

The forest is a dynamic ecosystem constituting of principal natural renewable resources of multifarious uses which fulfill the requirement of the society and sustainability of the earth. Insect pests are major biological determinants of forest productivity, and integrated pest management is not new to Indian forestry. Adoption of wood protection technologies alone has the potential to save approximately 2 $m^3$ of wood for every 3 $m^3$ and because of increase in average life of timber from 5 to 15 years would save at least 5.6 m ha of forests raising the forest cover by 2 % in India. A good and ideal silviculture system means healthy, resistant, tolerant and vigorous tree. Proper understanding of the biology and ecology of the tree species and their associated fauna and flora plays an important role in the pest management. The nurseries and monoculture plantations are much more susceptible to insect

**Table 8.3** Effect of neem products on insect pests having the host spectrum of forest trees

| Pest species | Neem products | Host plant | Effects | References | Known host spectrum of forest trees |
|---|---|---|---|---|---|
| *Achaea janata* | NSKE | Castor | Antifeedant | Chari and Muralidharan 1985; Ramarethinam et al. 2002a | *Acacia arabica*, *Albizia amara*, *Anogeissus latifolia*, *Bauhinia vahlii*, *Grewia microcos*, *Phyllanthus emblica*, *Ziziphus jujuba* |
| | NSO and neem formulations | | Insecticidal | | |
| *Agelastica alni* | NSKE | Alder | Fecundity reduction | Speckbacher 1977 | – |
| *Aphis gossypii* | NSO | Brinjal | Insecticidal | Cherian and Gopal 1944 | *Tecomella undulata* |
| *Bemisia tabaci* | NSKE | Black gram | Insecticidal | Mariappan et al. 1987; Natarajan and Sundaramurthy 1990 | *Clerodendron infortunatum* |
| | NSO | Cotton | Growth suppression | | |
| *Bupalus piniarius* | NSKE | *Pinus sylvestris* | Insecticidal | Speckbacher 1977 | – |
| *Callosobruchus* spp. | NKP | Pulses | Grain protection | Jotwani and Sircar 1967 | Many tree species |
| *Caryedon serratus* | Nimbicidin | Seeds | Antifeedancy and growth regulation | Murugesan et al. 2008 | *Acacia nilotica*, *Pongamia pinnata* |
| | | | | | *Tamarindus indica* |
| *Choristoneura fumiferana* | Margosan-O | Spruce | Antifeedant, growth regulation, insecticidal | Thomas et al. 1992 | – |
| *Diprion pini* | NSKE | Forest trees | Insecticidal | Speckbacher 1977 | – |
| *Drosicha mangiferae* | NC | Mango | Insecticidal | Tandon and Lal 1980 | *Artocarpus integrifolia*, *Dalbergia sissoo*, *Ficus benghalensis*, *F. religiosa*, *F. glomerata* |
| *Dysdercus cingulatus* | NSKE | Cotton | Insecticidal and juvenile hormone mimic activity | Abraham and Ambika 1979 | *Bombax malabaricum*, *Thespesia populnea* |
| *Dysdercus koenigii* | NSKE | – | Growth inhibitor | Jaipal et al. 1983 | *Grewia tiliifolia*, *Sida rhombifolia* |
| | NSO | – | Antifeedant, growth inhibitor | Gujar and Mehrotra 1990 | – |
| *Euproctis lunata* | NSKE | Castor | Repellant | Babu and Beri 1969 | *Acacia nilotica*, *Terminalia tomentosa*, *Ziziphus jujuba* |
| *Euproctis fraterna* | NSKE | Castor | Antifeedant | Kareem et al. 1988 | *Terminalia tomentosa*, *Tectona grandis*, *Shorea robusta*, *Ziziphus jujuba*, *Ougeinia dalbergioides* |
| *Euproctis chrysorrhoea* | NSKE | Oak | Antifeedant | Speckbacher 1977 | – |
| *Eurema hecabe* | Azadirachtin | *Cassia fistula* | Insecticide | Ramarethinam et al. 2002b | – |
| *Eutectona machaeralis* | NSE | Teak | Antifeedant | Kulkarni et al. 1996 | *Tectona grandis*, *Callicarpa* spp., *Tectona hamiltoniana* |
| | NSO | Teak | Antifeedant | Remadevi and Rajamuthukrishnan 1998 | |
| *Fenusa pusilla* | NSKE | Birch trees | Insecticidal | Larew et al. 1987 | – |
| *Fenusa pusilla* | Margosan-O | *Betula papyrifera* | Insecticidal | Marion et al. 1990. | – |
| *Helicoverpa armigera* | NSKE | Bengal gram and red gram | Insecticidal | Srivastava et al. 1984 | *Albizia* sp., *Pinus* sp. |
| | NSO | Chickpea | Crop protectant | Sinha 1993 | |
| *Heteronygmia dissimilis* | NSKE | Khayanyasica | Insecticidal | Rwamputa and Schabel 1989 | – |

(continued)

**Table 8.3** (continued)

| Pest species | Neem products | Host plant | Effects | References | Known host spectrum of forest trees |
|---|---|---|---|---|---|
| *Hieroglyphus banian* | NSKE | Rice | Crop protection | Dhaliwal et al. 1993; Mohan et al. 1991 | *Dendrocalamus strictus* |
| | NSO, NC | | Crop protection | | |
| *Holotrichia consanguinea* | Neem cake | Groundnut | Crop protection | Rao and Bajaj 1984 | Forest nurseries |
| *Holotrichia insularis* | NC | Chilli | Insecticidal | Sachan and Pal 1976 | Nursery pest of many species |
| *Hyblaea puera* | NSKE | Teak | Antifeedant ovipositional deterrent | Murugan et al. 1999 | *Tectona grandis*, *Tecomella undulata*, *Millingtonia hortensis*, *Callicarpa* spp., *Vitex* spp. |
| | NSO | Teak | Antifeedant | Remadevi and Rajamuthukrishnan 1998 | |
| *Hylobius abietis* | Azadirachtin-enriched product | Spruce | Repellent | Beitzen-Heineke and Hofmann 1992 | – |
| *Hyloicus pinastri* | NSKE | *Pinus sylvestris* | Insecticidal | Speckbacher 1977 | – |
| *Lipaphis erysimi* | NSKE | Mustard | Insecticidal | Sharma et al. 1984 | *Tecomella undulata* |
| *Locusta migratoria* | NSKE | – | Protected maize, cabbage and sorghum plants | Pradhan and Jotwani 1971 | Many tree species |
| *Lymantria dispar* | NSKE | Oak | Insecticidal | Skatulla and Meisner 1975 | – |
| *L. monacha* | Azadirachtin-enriched product | – | Insecticidal | Beitzen-Heineke and Hofmann 1992 | – |
| *Melolontha hippocastani* | Azadirachtin-enriched product and NSO | Oak | Fecundity reduction | Schmutterer and Kaethner 1988 | Many tree species |
| *M. melolontha* | Azadirachtin-enriched product and NSO | Oak | Fecundity reduction | Kaethner 1991 | – |
| *Nephotettix virescens* | NSKE | Rice | Antifeedant | Krishnaiah and Kalode 1991 | *Santalum album* |
| | NSO | Rice | Insect growth disruptant | Kareem et al. 1988 | |
| | NKP | Rice | Insecticidal | | |
| *Paliga machoeralis* | LE | Teak | Insecticidal | Sree et al. 2008 | – |
| *Panolis flammea* | NSKE | *Pinus sylvestris* | Insecticidal | Speckbacher 1977 | – |
| *Pericallia ricini* | NSO | – | Antifeedant | Mala 1987 | *Cassia tora*, *Santalum album*, *Bombax ceiba* |
| | NOE | | Antifeedant and growth regulation | Mala and Muthalagi 2008 | |
| | | | | | – |
| *Phyllocnistis citrella* | NSO | Citrus | Insecticidal | Dhara Jothi et al. 1990 | *Aegle marmelos*, *Murraya koenigii* |
| | NC | | | | |
| *Pityogenes chalcographus* | Neem oil, NSKE | Spruce | Sterilizing effects | Wulf and Scheidemann 1990 | – |
| *Pristiphora abietina* | Azadirachtin-enriched product | Spruce | Insecticidal | Schmutterer 1995 | – |

(continued)

**Table 8.3** (continued)

| Pest species | Neem products | Host plant | Effects | References | Known host spectrum of forest trees |
|---|---|---|---|---|---|
| *Pygaera cupreata* | NSKE | Poplar | Antifeedant | Bhandari et al. 1988 | *Populus* sp., *Salix* spp. |
| *Schistocerca gregaria* | NSKP | Fed with diet | Repellant toxicity | Singh 1985 | Many tree species of Indian arid zone |
| *Spodoptera litura* | NSKE | Tobacco | Antifeedant | Joshi et al. 1984 | *Cassia tora*, *Diospyros montana*, *Tectona grandis* |
| | NSO | – | Antifeedant and gustatory repellant | Koul 1987 | |
| | NC | Tobacco | Insecticidal | Murthy et al. 1990 | |
| | Azadirachtin | Sugar beet | Larvicidal | Shivankar et al. 2008 | |
| *Stilpnotia salicis* | Neem leaf | Willow | Antifeedant | Speckbacher 1977 | – |
| *Thaumetopoea pityocampa* | NSKE | *Pinus sylvestris* | Insecticidal | Speckbacher 1977 | – |
| *White grub* | NKP | Groundnut | Insecticidal | Raodev 1973 | *Shorea robusta*, *Tectona grandis*, *Cassia* sp., *Lagerstroemia sp.* |
| *Yponomeuta padellus* | NSKE | *Prunus spinosa* | Antifeedant | Speckbacher 1977 | – |

*NSKE* Neem seed/neem seed kernel extract, *NSO* Neem seed oil, *NKP* Neem kernel powder, *NOE* Neem oil extractive, *NC* Neem cake, *LE* Leaf extract

**Table 8.4** Distribution of neem trees in India and the potential collection

| State | No. of trees ('000) | Total seed potential ('000 t) | Total oil potential ('000 t) | Actual collection (%) |
|---|---|---|---|---|
| Andhra Pradesh | 653.9 | 12.2 | 2.5 | 27 |
| Gujarat | 636.2 | 21.0 | 4.2 | 1 |
| Madhya Pradesh | 735.6 | 18.2 | 3.6 | 2 |
| Tamil Nadu | 2,544.1 | 57.1 | 11.4 | 29 |
| Maharashtra | 710.1 | 28.2 | 5.6 | 1 |
| Karnataka | 790.6 | 20.1 | 4.0 | 20 |
| Orissa | 48.7 | 1.2 | 0.2 | -NA- |
| Punjab | 391.3 | 12.0 | 2.4 | -NA- |
| Rajasthan | 183.8 | 3.9 | 0.8 | -NA- |
| Uttar Pradesh | 7972.6 | 265.9 | 53.2 | -NA- |
| West Bengal | 273.0 | 2.5 | 0.5 | 27 |
| Total | 14,939.90 | 442.3 | 88.5 | 24 |

Source: Directorate of Nonedible Oils and Soap Industry, Khadi and Village Industries Commission, Bombay, 1976; Rajasekaran 1991
*NA* Not available

epidemics as compared to mixed plantations and natural forests. Besides, the number of insect species acquiring pest status is increasing day by day possibly due to environmental imbalances. Climate change and bioinvasion are expected to bring extension in the host range of many pests and diseases. Shift in population growth rate among insect species due to global warming will have profound ecological effect by altering species composition and disrupting food webs. Forest managers realize that large-scale spraying of chemicals, microbes, or botanicals cannot be feasible in large-scale plantations and natural forest areas. However, they find it is feasible to use a maximum quantity of neem cake as an integral component of potting mixture for raising nurseries. Also, the herbal product cashew nut shell liquid is commonly used for short-term wood protection. Most of the control operation in forestry is limited to the nursery

**Table 8.5** Effect of plant products other than neem against forest insect pests of India

| Plant species | Product | Pest species | Host plant | Effects | References |
|---|---|---|---|---|---|
| *Acacia mangium* | Leaf extract | *Hyblaea puera* | *Tectona grandis* | Antifeedant | Ramanna and Bhat 2006 |
| *Acacia auriculiformis* | Leaf extract | *Hyblaea puera* | *Tectona grandis* | Antifeedant | Ramanna and Bhat 2006 |
| *Acorus calamus* | Plant oil | *Heteropsylla cubana* | *Leucaena leucocephala* | Insecticidal | Sharma et al. 1992 |
| *Aegle marmelos* | Seed oil | *Hyblaea puera* | *Tectona grandis* | Insecticidal | Krishnakumar et al. 2011 |
| *Adina cordifolia* | Leaf extract | *Clostera cupreata* | Poplar | Antifeedant | Ahmad et al. 1997 |
| *Adhatoda vasica* | Leaf extract | *Atteva fabriciella* | *Ailanthus* sp. | Antifeedant | Ahmad et al. 1991 |
| *Aloe vera* | Leaf extract | *Paliga machaeralis* | *Tectona grandis* | Antifeedant | Kulkarni et al. 1997a |
| *Amarphophallus componata* | Tuber extract | *Hyblaea puera* | *Tectona grandis* | Larvicidal | Javaregowda and Naik 2006 |
| *Anacardium occidentale* | Cashew nut shell liquid | Termites | *Hevea brasiliensis* | Wood protection | Remadevi et al. 2002 |
| *Angelica glauca* | Plant oil | *Heteropsylla cubana* | *Leucaena leucocephala* | Insecticidal | Sharma et al. 1992 |
| *Annona squamosa* | Leaf extract | *Crypsiptya coclesalis* | *Bamboo* spp. | Antifeedant | Kulkarni et al. 2003 |
| *Apium graveolens* | Plant oil | *Heteropsylla cubana* | *Leucaena leucocephala* | Insecticidal | Sharma et al. 1992 |
| *Bassia latifolia* | Plant oil | *Heteropsylla cubana* | *Leucaena leucocephala* | Insecticidal | Sharma et al. 1992 |
| *Bixa orellana* | Leaf extract | *Paliga machaeralis* | *Tectona grandis* | Growth inhibition | Sree et al. 2008 |
| *Butea frondosa* | Plant oil | *Heteropsylla cubana* | *Leucaena leucocephala* | Insecticidal | Sharma et al. 1992 |
| *Calophyllum inophyllum* | Plant oil | *Heteropsylla cubana* | *Leucaena leucocephala* | Insecticidal | Sharma et al. 1992 |
| *Calotropis procera* | Leaf extract | *Paliga machaeralis* | *Tectona grandis* | Antifeedant | Meshram 1995 |
| *Carum capticum* | Essential oil | *Odontotermes obesus* | '*no choice bioassay*' | Mortality | Gupta et al. 2011 |
| *Cassis fistula* | Leaf and bark extract | *Hyblaea puera* | *Tectona grandis* | Larvicidal | Javaregowda and Naik 2006 |
| *Cassia siamea* | Leaf extract | *Hyblaea puera* | *Tectona grandis* | Larvicidal | Javaregowda and Naik 2006 |
| *Casuarina equisetifolia* | Leaf extract | *Hyblaea puera* | *Tectona grandis* | Antifeedant | Ramana and Bhat 2006 |
| *Catharanthus roseus* | Leaf extract | *Paliga machaeralis* | *Tectona grandis* | Growth inhibition | Sree et al. 2008 |
| *Cedrus deodara* | Plant oil | *Heteropsylla cubana* | *Leucaena leucocephala* | Insecticidal | Sharma et al. 1992 |
| | Essential oil | *Odontotermes obesus* | '*no choice bioassay*' | Mortality | Gupta et al. 2011 |
| *Chromolaena odorata* | Leaf extract | *Termites* | *Bambusa balcooa* | Resistant to degradation | Borthakur and Gogoi, 2009 |
| *Cinnamomum camphora* | Camphor oil | *Calopepla leayana* | *Gmelina arborea* | Antifeedant | Singh and Sushilkumar, 1998 |
| | | *Eupterote geminate* | | | |

(continued)

**Table 8.5** (continued)

| Plant species | Product | Pest species | Host plant | Effects | References |
|---|---|---|---|---|---|
| *Clerodendrum inerme* | Leaf extract | *Hyblaea puera* and *Paliga machaeralis* | *Tectona grandis* | Antifeedant | Sundararaj et al. 2004 |
| | Leaf extract | *Hyblaea puera* | *Tectona grandis* | Larvicidal | Javaregowda and Naik 2006 |
| | Leaf extract | *Hyblaea puera* | *Tectona grandis* | Ovicidal | Ramana 2005 |
| *Cymbopogon citratus* | Essential oil | *Odontotermes obesus* | '*no choice bioassay*' | Mortality | Gupta et al. 2011 |
| *Eucalyptus hybrid* | Leaf extract | *Clostera cupreata* | Poplar | Antifeedant | Ahmad et al. 1997 |
| *Eucalyptus globulus* | Essential oil | *Odontotermes obesus* | '*no choice bioassay*' | Mortality | Gupta et al. 2011 |
| *Eugenia caryophyllata* | Essential oil | *Odontotermes obesus* | '*no choice bioassay*' | Mortality | Gupta et al. 2011 |
| *Daucus carota* | Plant oil | *Heteropsylla cubana* | *Leucaena leucocephala* | Insecticidal | Sharma et al. 1992 |
| *Dalbergia stipulacea* | Bark and Root extract | *Clostera cupreata* | Poplar | Antifeedant | Ahmad et al. 1997 |
| *Derris indica* | Leaf extract | *Hyblaea puera* | *Tectona grandis* | Larvicidal | Javaregowda and Naik 2006 |
| *Datura metel* | Leaf extract | *Paliga machaeralis* | *Tectona grandis* | Antifeedant | Meshram 1995 |
| | Leaf extract | *Plecoptera reflexa* | *Dalbergia sissoo* | Antifeedant | Kulkarni et al. 1997b |
| | Leaf extract | *Paliga machaeralis* | *Tectona grandis* | Growth inhibition | Sree et al. 2008 |
| *Dirca palustris* | Seed extract | *Paliga machaeralis* | *Tectona grandis* | Antifeedant | Murugesan et al. 2003 |
| *Dodonaea viscosa* | Leaf extract | *Lamprosema niphaelis* | *Pongamia pinnata* | Growth inhibition | Deepa and Remadevi 2007b |
| | | *Hyblaea puera* | *Tectona grandis* | | Deepa and Remadevi 2008 |
| *Dryopteris* sp. | Leaf extract | *Termites* | *Bambusa balcooa* | Resistant to degradation | Borthakur and Gogoi 2009 |
| *Eucalyptus hybrid* | Leaf extract | *Plecoptera reflexa* | *Dalbergia sissoo* | Antifeedant | Meshram 2000 |
| *Gnidia glauca* | Leaf and bark extract | *Hyblaea puera* | *Tectona grandis* | Larvicidal | Javaregowda and Naik 2006 |
| *Hevea brasiliensis* | Plant oil | *Heteropsylla cubana* | *Leucaena leucocephala* | Insecticidal | Sharma et al. 1992 |
| *Holigarna arnottiana* | Leaf and bark extract | *Hyblaea puera* | *Tectona grandis* | Antifeedant | Ramana et al. 2004 |
| *Ipomea carnea* | Leaf and flower extract | *Crypsiptya coclesalis* | *Bamboo* spp. | Antifeedant | Kulkarni and Joshi 1998 |
| *Jatropha curcas* | Plant oil | *Heteropsylla cubana* | *Leucaena leucocephala* | Insecticidal | Sharma et al. 1992 |
| | Leaf extract | *Papilio demoleus* | *Feronia elephantum* | Antifeedant | Meshram et al. 1996 |
| | Leaf and seed extract | *Hyblaea puera* | *Tectona grandis* | Larvicidal | Javaregowda and Naik 2006 |
| *Juniper communis* | Plant oil | *Heteropsylla cubana* | *Leucaena leucocephala* | Insecticidal | Sharma et al. 1992 |
| *Lantana caftera* | Leaf extract | *Hyblaea puera* | *Tectona grandis* | Larvicidal | Javaregowda and Naik 2006 |

(continued)

**Table 8.5** (continued)

| Plant species | Product | Pest species | Host plant | Effects | References |
|---|---|---|---|---|---|
| *Lantana camara* | Leaf extract | *Atteva fabriciella* | *Ailanthus* sp. | Antifeedant | Ahmad et al. 1991 |
| | | *Plecoptera reflexa* | *Dalbergia sissoo* | | Kulkarni et al. 1997b |
| | Leaf extract | *Paliga machaeralis* | *Tectona grandis* | Antifeedant | Kulkarni et al. 1997a |
| | Leaf and flower extract | *Crypsiptya coclesalis* | *Bamboo* spp. | Antifeedant | Kulkarni et al. 1999 |
| *Lobelia nicotianaefolia* | Leaf extract | *Hyblaea puera* | *Tectona grandis* | Larvicidal | Javaregowda and Naik 2006 |
| | Leaf extract | *Lamprosema niphaelis* | *Pongamia pinnata* | Growth inhibition | Deepa and Remadevi 2007a |
| *Persea macrantha* | Solid colouring matter from the bark | *Hyblaea puera* and *Paliga machaeralis* | *Tectona grandis* | Antifeedant | Sundararaj et al. 2004 |
| *Melia azedarach* | Leaf extract | *Atteva fabriciella* | *Ailanthus* sp. | Antifeedant | Ahmad et al. 1991 |
| | Leaf extract | *Plecoptera reflexa* | *Dalbergia sissoo* | Antifeedant | Meshram 2000 |
| | Leaf and seed extract | *Hyblaea puera* | *Tectona grandis* | Antifeedant | Senthilnathan and Sehoon 2006 |
| *Mentha arvensis* | Essential oil | *Odontotermes obesus* | '*no choice bioassay*' | Mortality | Gupta et al. 2011 |
| *Nerium oleander* | Leaf extract | *Paliga machaeralis* | *Tectona grandis* | Growth inhibition | Sree et al. 2008 |
| *Parthenium hysterophorus* | Leaf extract | *Paliga machaeralis* | *Tectona grandis* | Antifeedant | Durairaj 2009 |
| *Polygonum glabrum* | Leaf extract | *Termites* | *Bambusa balcooa* | Resistant to degradation | Borthakur and Gogoi 2009 |
| *Pongamia pinnata* | Leaf extract | *Plecoptera reflexa* | *Dalbergia sissoo* | Antifeedant | Meshram 2000 |
| *Pterocarpus marsupium* | Wood Extract | *Hyblaea puera* | *Tectona grandis* | Antifeedant | Deepa and Remadevi 2006 |
| *Ricinus communis* | Leaf extract | *Hyblaea puera* | *Tectona grandis* | Larvicidal | Javaregowda and Naik 2006 |
| *Saussurea lappa* | Plant oil | *Heteropsylla cubana* | *Leucaena leucocephala* | Insecticidal | Sharma et al. 1992 |
| *Semecarpus kathalekanensis* | Leaf extract | *Hyblaea puera* | *Tectona grandis* | Ovicidal | Ramana 2006 |
| | | | | Antifeedant | Ramana et al. 2007 |
| *Sesamum indicum* | Plant oil | *Heteropsylla cubana* | *Leucaena leucocephala* | Insecticidal | Sharma et al. 1992 |
| *Strychnos nux-vomica* | Leaf, bark and seed extract | *Hyblaea puera* | *Tectona grandis* | Larvicidal | Javaregowda and Naik 2006 |
| | Leaf extract | *Paliga machaeralis* | *Tectona grandis* | Growth inhibition | Sree et al. 2008 |
| *Trachyspermum ammi* | Plant oil | *Heteropsylla cubana* | *Leucaena leucocephala* | Insecticidal | Sharma et al. 1992 |
| *Valeriana wallichii* | Plant oil | *Heteropsylla cubana* | *Leucaena leucocephala* | Insecticidal | Sharma et al. 1992 |
| *Vitex negundo* | Leaf extract | *Hyblaea puera* | *Tectona grandis* | Larvicidal | Javaregowda and Naik 2006 |
| | Leaf powder | *Bruchidius sp.* and *Caryedon serratus* | *Tamarindus indica* and *Acacia nilotica* | Insecticidal | Murugesan et al. 2008 |

stage mainly in the form of chemical inputs. However, the consequent pollution jeopardizes the agricultural as well as forestry business. So usage of botanical insecticides could be of great use along with other options to minimize the use of chemicals in the future. Botanicals used as insecticides presently constitute 1 % of the world insecticide market, while currently Indian market for plant product is less than 1 %. Therefore, it is essential to promote the use of plant products in the insect pest management programmes for the benefit of users who are mainly the plantations growers, environmentalists, State Forest Departments, industries, farmers, etc. It will encourage the users to grow more trees in their homesteads and boost the greening India programme.

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# Use of Plant Volatile for Plant Pathogens and Pests Management

9

Hiromi Ikeura

**Abstract**

Antifungal activity of tomato leaf volatiles (TLV) against three types of plant pathogens of *Botryotinia fuckeliana*, *Glomerella cingulata*, and *Fusarium oxysporum* was investigated. Growths of *B. fuckeliana* and *G. cingulata* were completely inhibited by 700 and 1,000 μL of TLV extract, respectively. These suggest that TLV are proven to be efficacious as a biological control agent and that there is the defense response against plant pathogens in tomato plant. In another study, repellent and insecticidal effects of eight (peppermint, cherry sage, dokudami, sweet pepper, eucalyptus, lavender, chives, and tansy) and seven (rosemary, spearmint, eucalyptus, chives, sage, tansy, and sweet pepper) extracts were against *Myzus persicae* and *Pieris rapae crucivora* Boisduval, respectively, in laboratory and field conditions. In the laboratory, sweet pepper and tansy extracts proved to be a potent insecticide against *M. persicae*. In the field test, dokudami extract was a highly effective repellent against *M. persicae*. These findings suggest that dokudami extract can be used to control wingless green peach aphids in the field. Moreover, neem cake showed a strong repellent effect against green peach aphid. Among the seven plants, the tested herbs, rosemary and spearmint volatile extracts have a notable feeding repellent effect against *P. rapae* larvae.

**Keywords**

Tomato leaf volatiles • Herb • Volatile • Neem • *Myzus persicae* Sulzer • *Pieris rapae crucivora* Boisduval

## 9.1 Introduction

Recently, there are growing concerns on the use of chemical pesticides and fertilizers due to environmental pollution, adverse effects on human health, disruption of natural biological control,

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_9, 

and evolution of pesticide-resistant pest populations (Flint 2012). As consumer interest in the safety of food and agricultural products has risen in recent years, producers are increasingly focused on pesticide-free and organic farming that take human health and the environment into account. Without the use of chemicals, however, farmers risk disease and insect damage to crops (Perry et al. 1998; Isman 2006; Mulumba et al. 2012).

Many plant species are known to possess insecticidal properties, and the compounds from these plants have a number of useful activities such as toxicity, repellency, feeding, oviposition deterrence, and insect growth regulation properties (Sharma and Gupta 2009; Mann and Kaufman 2012). In mixed vegetation, odors released by some plants can mask the effect of odors released by other plants, thereby disorientating insects such that they spend less time on plants suitable for egg laying and feeding. Consequently, plant and herb extracts and neem materials are attracting increased attention as a basis for botanical insecticides (Isman 2006; Tripathi et al. 2009; Schrader et al. 2010). Botanical insecticides are an environmentally friendly alternative to hazardous chemicals as they either are plant-derived insecticides which occur naturally or are extracts of such plants (Gupta et al. 2005).

In Japan, the amount of tomato (*Solanum lycopersicum* L.) fruit is produced at about 760,000 t year$^{-1}$, although the same amount of the leaf and stem is wasted. However, the leaf and stem may become valuable organic resources. The future challenge is to make use of them, since social structure of resources recycling is currently desired. There are food preservative and biological control agents as the usage of functional constituents in plant. In addition, it was reported that herbal aroma was different by picking season and by site and that the antibacterial activity varied greatly (Chorianopoulos et al. 2006; Kiralan et al. 2012). Thus, it can be expected that functional constituents in tomato leaf and stem are used as antibacterial agents. It is well known that there is characteristic odor in tomato leaves (Buttery et al. 1987).

The neem tree (*Azadirachta indica* L.) is well known in India and neighboring countries where it has been one of the most versatile medicinal plants for 2,000 years with a wide spectrum of biological activity (Alves et al. 2009; Atawodi and Atawodi 2009; Deng et al. 2012; Gangwar 2012). There are two types of harvestable neem materials. The first is neem oil which is compressed from neem seeds, concentrated, and purified. Neem oil functions as a pest repellent and is diluted with water and sprayed onto leaves. The second material is neem seed kernel oil cake, simply called neem cake, which is divided into fruitcake, seed cake, and neem seed kernel oil cake and is used primarily as an amendment and growth-promoting agent.

The neem tree synthesizes compounds for chemical defense to protect against herbivorous insects (Atawodi and Atawodi 2009). These compounds function by interfering with insect hormones (Mordue and Blackwell 1993; Anibal and Condor 2007). Nine limonoid compounds with pest control properties have been extracted from neem seeds and shown to inhibited pest growth, and the most effective of the main compounds is azadirachtin (Schmutterer 1990; Alves et al. 2009). Azadirachtin induces a physiological effect on insects by interfering with the synthesis and release of ecdysteroids which disrupts larval molting in hemimetabolous and holometabolous insects and interferes with pupation and/or eclosion of adults and with reproduction (Mordue and Blackwell 1993). Neem-based pesticides are known for their pesticidal activity against more than 400 species of insects (Siddiqui et al. 2003). However, they are not toxic to humans and many beneficial arthropods and target pests which are unlikely to develop resistance. Therefore, these pesticides have been advocated to replace synthetic pesticides as it is regarded as more sensible to use natural pesticides in most pest management programs (Schmutterer 1990; Mordue and Blackwell 1993; Isman 2006; Irigaray et al. 2010; Mulumba et al. 2012). Thus, neem oil-based pesticides are available for use against many pests and have been evaluated as an alternative to synthetic pesticides (Anibal and Condor 2007).

On the other hand, the wingless green peach aphid (*Myzus persicae* Sulzer, Hemiptera: Aphididae) is a major serious polyphagous pest carrying a multitude of viral diseases and infecting about 100 plant species worldwide, including those of Solanaceae, Fabaceae, and Brassicaceae (Feng and Isman 1995; Hori 1998). The aphid is difficult to eradicate as it multiplies in greenhouses and fields (Hori and Harada 1995). To date, azadirachtin and neem-based pesticides have been shown to be effective in controlling *M. persicae* and other aphid species (Lowery and Isman 1993; Lowery et al. 1993). However, no studies have been done on the potential of neem seed cake to repel *M. persicae*. In addition, there are only a few studies on the repellent and insecticidal activity of herb extracts on aphids (Hori and Harada 1995; Hori 1999a, b; Salari et al. 2012).

The cabbage white butterfly, *Pieris rapae crucivora* Boisduval, is a serious pest of Brassicaceae plants such as cabbage, cauliflower, and broccoli, and its larvae damage the host plant leaves when feeding (Ikeura et al. 2012). Gao et al. (2004) showed that deoxypodophyllotoxin, identified in *Juniperus sabina* L., has insecticidal activity against *P. rapae* larvae. In addition, Zhong et al. (2006) showed that rhodojaponin-III isolated from *Rhododendron molle* G. Don flowers is an antifeedant, stomach poison, contact toxicant, and growth inhibitor against *P. rapae*. However, the compounds identified in their study were nonvolatile. Only a few reports have been published on the feeding repellent effect of volatile extracts against *P. rapae* larvae.

In this chapter, we aimed to develop a biological pesticide alternative to chemical insecticide for future use. Therefore, we investigated as follows: I. antifungal activity of tomato leaf volatiles against *Botryotinia fuckeliana*, *Glomerella cingulata*, and *Fusarium oxysporum*, which were plant pathogens, II. the repellent effects of eight kinds of herb extracts from companion plants against wingless green peach aphids, III. repellent effect of neem materials against the green peach aphid and the effects of different concentrations of neem and repellent tests with the volatile extracts of neem materials, and IV. the feeding repellent effect of seven kinds of herb extracts to develop a botanical insecticide against *P. rapae* larvae. In this chapter, we introduce that plant volatile extracts have repellent effects and antifungal activity against insects and fungi.

## 9.2 Methodology Followed

### 9.2.1 Plants

Tomato cultivar 'Reiyo'(*S. lycopersicum* L.), from Daiichi Engei Co., Ltd., Tokyo, Japan, was cultivated in a greenhouse at Meiji University from August to December 2007. The leaves from fourth to seventh flower cluster were sampled at the anthesis stage of seventh flower cluster, frozen with liquid nitrogen, crushed, and stored at −40 °C until the time of extraction of volatile compounds.

Radish 'Comet' (*Raphanus sativus* var. *sativus*) was purchased from Takii Seed Co. (Kyoto, Japan), seeded in black polyethylene pods, and grown for 15 days. Peppermint (*Mentha piperita* L.), cherry sage (*Salvia microphylla* L.), dokudami (*Houttuynia cordata* L.), sweet pepper (*Capsicum annuum* L.), eucalyptus (*Eucalyptus globulus* L.), lavender (*Lavandula intermedia* L.), chives (*Allium schoenoprasum* L.), and tansy (*Tanacetum vulgare* L.) were used.

Raw neem leaves were obtained from Mizusaki Farm (Fukuoka, Japan). Neem oil ('AZ green N'; 1.2 % azadirachtin and 2.8 % neem extract) and neem seed kernel oil cake ('Rikunomegumi'; 0.3–0.5 % azadirachtin) were purchased from OM Science (Osaka, Japan). The following seven plants, well known for agriculturists and horticulturists and common in the literature, were selected as the basis for herbal extracts: rosemary (*Rosmarinus officinalis* L.), spearmint (*M. spicata* L.), eucalyptus (*E. globulus* L.), chives (*A. schoenoprasum* L.), sage (*Salvia officinalis* L.), tansy (*T. vulgare* L.), and sweet pepper (*C. annuum* L.).

### 9.2.2 Pests and Diseases

*Botryotinia fuckeliana* NBRC 9760, *G. cingulata* NBRC 5257, and *F. oxysporum* NBRC 6385 were purchased from the National Institute of Technology and Evaluation (Kisarazu, Japan). Wingless green peach aphids virginoparae (*M. persicae* Sulzer) were collected around the Meiji University and were reared on radish plants at 25 °C under a L16:D8 photoperiod in an incubator until reaching 1.8–2.0 mm in length after which they were used in the experiments.

Gravid female adults of *P. rapae crucivora* Boisduval were collected from cabbage patches in Kawasaki City. They were released in a cage (1.6 m × 1.6 m × 1.8 m) made from white shading net in a greenhouse of Meiji University, fed an approximately 10 % sucrose solution, and free to oviposit on potted cabbages. When eggs were laid, the cabbages were placed under a natural condition in the greenhouse until hatching. The hatched larvae, which were fed cabbage in a plastic case (10 cm × 20 cm × 7 cm), were reared at 25 °C under a L16: D8 regime until being at the fourth instar.

### 9.2.3 Plant Extracts

Volatile components were extracted from tomato leaves using the Porapak Q method (PQM) as described by Hayata et al. (2003). The eluate containing the volatile compounds was dried over hydrous sodium sulfate (Kanto Chemical Co., Ltd., Tokyo, Japan) overnight at room temperature and concentrated to 6 mL under a nitrogen stream.

Fresh herb leaves (10 g) were homogenized with 40 mL of 50 % EtOH for 24 h and filtered to obtain an herb extract. The control was 25 % EtOH solution. Each herb extract was added to a spreading agent (Dain®, Sumitomo Chemical Co. Ltd., Osaka, Japan). Neem seed cake (50 g) was added to 1,000 mL of diethyl ether and mixed for 4 h. It was then filtrated, and the filtrate was concentrated to 10 mL under nitrogen gas flow and used as a bioassay sample. Ten grams of fresh leaves were homogenized and mixed with 40 mL of diethyl ether at 4 °C for 4 h with a stirrer. Next, the sample was centrifuged at 12,000 rpm at 4 °C for 20 min and filtered, and the filtrate was used as the herb extract.

### 9.2.4 Antifungal Activity: Laboratory Conditions

One hundred microliter of each plant pathogens solution ($10^6$sporem$L^{-1}$) was plated on the potato dextrose agar plate (Difco, USA). Paper disks containing ~1,000 μL of extract were put on the lid of the plates turned upside down and the extract was dried. The plates were incubated at 25 °C for 7 days after sealed with parafilms. Antifungal activity of extract was shown as inhibition ratio calculated by the following equation: inhibition ratio (%) = d/D × 100, where, d is diameter of inhibition circle (mm) and D is diameter of plate (85 mm). All experiments were performed in triplicate. The data presented are the means with standard errors.

### 9.2.5 Antifungal Activity: Field Test

Radishes for the field test were seeded in 12-cm diameter pots and were harvested. The dorsal and ventral leaf surfaces of radish seedlings (four leaves) were coated with 2 mL of herb extract diluted twofold with water and control solution (25 % EtOH) once every 3 days. Aphid source plants were propagated in other plant pots. Four pots of each herb extract were placed around each radish pot. The total number of aphids on each radish plant was calculated from day 1 to day 21. Experiments for each extract were performed in triplicate.

### 9.2.6 Feeding Repellent Assay

*Y-Tube Olfactometer Bioassay:* An olfactometer made of a silicon tube and glass rods was used. A Y-junction mixed treatment and control airflows. Aphids moved into one of the Y-tube branches down an apparent chemotaxis gradient.

Air was drawn through the apparatus at a rate of 0.2 L $min^{-1}$. Wingless virginoparae were placed at the base of the central tube. Tests were carried out in a darkroom at 22–24 °C to eliminate the potential influence of light. The number of aphids in each of the Y-tube branches was counted 5 min after the start of the test. Each experiment used 30 aphids and was replicated three times. In tests with liquid samples, a 2-mL sample on filter paper (No. 5B, 90 mm; Advantec, Tokyo, Japan) was placed in the treatment flask with radish as the host plant, while filter paper without radish was placed in the control flask. Aliquots of 2-mL diethyl ether solution of each sample were applied to filter paper, and their effects on the aphids were compared against a diethyl ether control. In tests with solid samples, 1, 10, 20, and 100 g of raw leaves or neem seed cake were placed in the treatment flask with radish, while radish only was placed in the control flask. Both treatment and control filter papers were allowed to dry to remove diethyl ether before being placed in the flask.

*Leaf Disk Bioassay:* A feeding repellent assay was conducted on two cabbage leaf disks (3 cm × 3 cm) on filter paper (5 cm × 5 cm) treated with herb extract or diethyl ether in a box with dimensions of 20 cm × 15 cm × 8 cm. A larva was placed between the disks, and the filter paper chosen by the larva was noted. Thirty larvae were used for each treatment. The repellent rate is expressed as follows: number of larvae choosing treated paper/(number of larvae choosing treated paper + number of larvae choosing control paper) × 100.

## 9.3 Observation and Discussion

### 9.3.1 Antifungal Activity of Volatiles from Tomato

Antifungal activity against all plant pathogens tested increased linearly with the increase of TLV extract volume (Fig. 9.1). Growths of *B. fuckeliana* and *G. cingulata* were completely inhibited by 700 (equivalent to about 12 g of tomato leaves) and 1,000 μL (equivalent to about 17 g of tomato leaves) of TLV extract, respectively. On the other hand, against *F. oxysporum*, inhibition ratio by 1,000 μL of TLV extract was about 55 %. Accordingly, antifungal effect of TLV varies with the type of plant pathogens. It was, also, recognized that there was no antifungal activity in diethyl ether of extract solvent (data not shown). It was reported that volatiles from thyme (*Thymus vulgaris* L.) showed strong antifungal activities against *Aspergillus parasiticus* and *Cryptococcus neoformans* while not against *Candida albicans* (Martos et al. 2007). Furthermore, in the present study, TLV exhibited strong antifungal activities, even though *B. fuckeliana* infects easily into tomato leaves and causes illness. The reason was probably due to the concentration of the TLV.

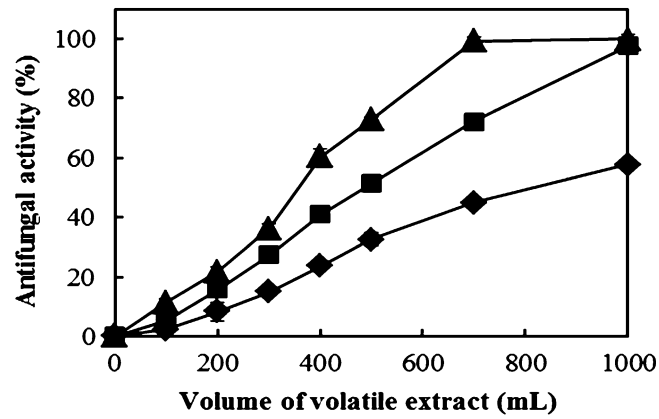


**Fig. 9.1** Antifungal activity of volatile extract from tomato leaves against *B. fuckeliana* (▲), *G. cingulata* (■), and *F. oxysporum* (◆)

$C_6$-alcohols and $C_6$-aldehydes described as green leaf volatiles (GLV) contained fresh green flavor and were reported to be rapidly produced within the plant when the plant incurred insect damages and pathogens infected to the plant (Nakamura and Hatanaka 2002). Furthermore, it is noted that GLV shows antibacterial activities against many types of microorganisms (Virginie et al. 2009). In tomato plant, GLV was synthesized due to infect damage by *Helicoverpa armigera* larva and released from the leaves (Rose et al. 1996). In cotton plant, GLV production increased at the upper leaves when the feeding damages to the lower leaves by *Spodoptera exigua* larva occurred (Mann et al. 2000). Therefore, it is presumed that there is the resistant response system due to insect damage or infection of plant pathogens in tomato plant. In addition, it was reported that essential oil showed antifungal activity at three steps of conidial germination, growth of vegetative hyphae and conidial forming (Inouye et al. 1998, 2001). However, it is not clear whether TLV acted as antifungal activity at any life cycle stage of plant pathogens.

### 9.3.2 Herb Extracts on *Myzus persicae* Sulzer

The number of aphids on leaves treated with all herb extracts and the control was equal until day 13 but changed after day 16 (Table 9.1). Dokudami extract prevented an increase in aphids and completely repelled them by day 21. Cherry sage extract repelled aphids from day 16. Peppermint extract increased the number of aphids at day 16, but decreased the number from day 19. Lavender extract significantly prevented an increase in aphids from day 16. These results show that dokudami extract had the highest repellency followed by sage and lavender extracts.

*Myzus persicae* aphids use visual cues such as plant size, shape, and color to search for host plants, with nonhost plant odor serving only as an avoidance measure (Hori 1999c). There was no significant difference between each herb extract and the control until day 13, because it was assumed that aphids entered the larval stage when they colonized the radish after moving from the source plant. From day 13, dokudami, cherry sage and lavender extracts continued to prevent an aphid increase, indicating their potential to protect against aphid damage. Peppermint extract had an immediate effect on reproductive inhibition of aphids, since although peppermint extract increased the number of aphids at day 16, it significantly decreased the number thereafter. Hori (1996) described that mint inhibited the sucking ability of aphids but did not repel them. The mint extract treatment inhibited the sucking activity of aphids causing them to move on to other host plants, likely because the host plant odor was masked by the mint extract.

The chives extract had a slow reproductive inhibiting effect since the number of aphids increased rapidly at day 16 and dramatically decreased from day 19 to day 21. Hori and Harada (1995) suggested that compounds in *Allium* genus plants play a role in repelling aphids. However, the reason that their data are not concomitant with ours can likely be attributed to the use of a solvent which could not extract the repellent from the chives in our study.

Of all the herbs, dokudami extract resulted in the lowest number of aphids throughout the field test period and proved to be a strong repellent against *M. persicae*. This result supports the finding that aphids did not settle on dokudami plants (Hori and Harada 1995). Harvey and Fortuna (2012) propose that components in essential oil have either a repellent or attractant effect on insects and that these compounds have various functions such as masking host plant odors.

### 9.3.3 Repellency Against *Myzus persicae* Sulzer

The average number of *M. persicae* for 10 and 20 g of raw leaves was 12.33 and 11.00, respectively, and the preference to 20 g of raw leaves was lower than that of the control (Fig. 9.2). This

**Table 9.1** Number of aphids on radish leaves sprayed with herb extracts in the field

| | The number of aphid on leaves (average ± standard deviation) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Plant materials | 1 day[a] | 4 days | 7 days | 10 days | 13 days | 16 days | 19 days | 21 days |
| Eucalyptus | 1.5 ± 2.1 a[b] | 1.0 ± 0.7 a | 1.5 ± 1.1 a | 2.8 ± 2.6 a | 1.8 ± 1.9 a | 34.5 ± 1.5 b | 139 ± 7.5 a | 163.0 ± 0.0 a |
| Dokudami | 3.8 ± 4.2 a | 2.5 ± 1.5 a | 0.5 ± 0.5 a | 1.0 ± 0.0 a | 9.7 ± 6.3 a | 3.5 ± 1.5 b | 9.0 ± 9.0 b | 0.0 ± 0.0 b |
| Cherry sage | 1.0 ± 1.0 a | 3.5 ± 3.5 a | 2.5 ± 2.1 a | 3.8 ± 2.9 a | 2.3 ± 2.8 a | 7.3 ± 8.2 b | 13.5 ± 8.3 b | 13.5 ± 7.2 b |
| Tansy | 1.5 ± 1.5 a | 1.0 ± 1.7 a | 2.5 ± 1.8 a | 4.0 ± 1.9 a | 2.0 ± 1.2 a | 133 ± 81.5 a | 69.0 ± 14.7 a | 36.0 ± 20.2 a |
| Sweet pepper | 0.5 ± 0.5 a | 1.5 ± 1.5 a | 1.3 ± 1.3 a | 11.0 ± 6.5 a | 11.7 ± 11.6 a | 58.0 ± 25.9 a | 127 ± 22.5 a | 7.0 ± 6.0 b |
| Chives | 2.8 ± 3.1 a | 3.5 ± 5.5 a | 4.0 ± 4.1 a | 6.3 ± 4.2 a | 15.8 ± 12.3 a | 127 ± 67.4 a | 120 ± 4.0 a | 24.0 ± 20.0 a |
| Peppermint | 1.3 ± 1.1 a | 2.5 ± 3.3 a | 2.0 ± 0.7 a | 5.8 ± 6.5 a | 4.3 ± 6.3 a | 56.8 ± 40.7 a | 14.5 ± 9.0 b | 15.8 ± 9.4 b |
| Lavender | 1.3 ± 1.1 a | 1.3 ± 0.8 a | 3.3 ± 3.0 a | 10.5 ± 13.1 a | 4.3 ± 5.7 a | 13.5 ± 9.5 b | 20.7 ± 12.9 b | 11.5 ± 11.9 b |
| Control | 0.5 ± 0.9 a | 2.5 ± 2.6 a | 1.5 ± 0.9 a | 3.5 ± 2.7 a | 8.0 ± 8.0 a | 124 ± 14.0 a | 156 ± 34.0 a | 89.5 ± 9.5 a |

[a]Days after placed with aphid source plant
[b]Different letters indicate a difference significant at the 5 % level by Tukey-Kramer test among plant materials ($n = 5$)

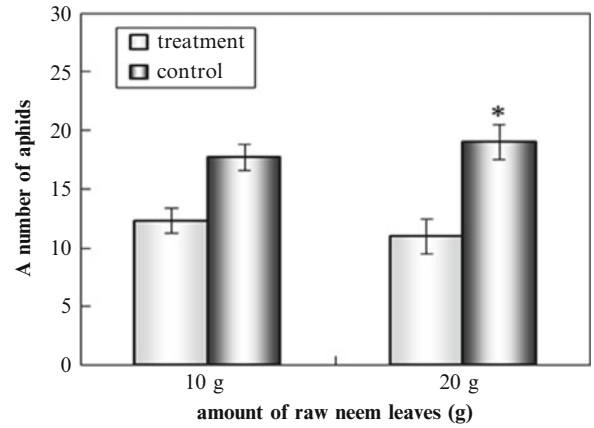


**Fig. 9.2** Repellent effect of raw neem leaves on *M. persicae*

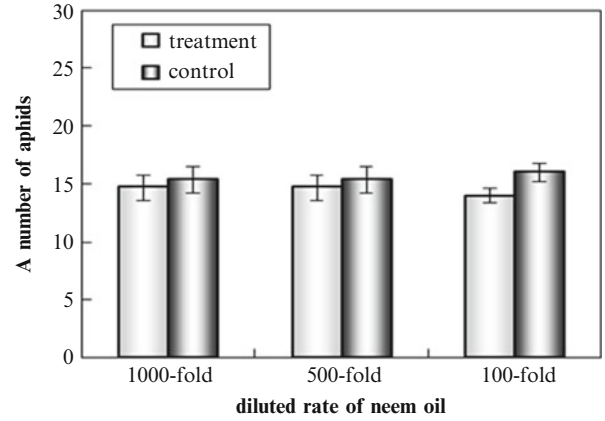


**Fig. 9.3** Repellent effect of neem oil on *M. persicae*

shows that raw neem leave have a slight repellent effect on *M. persicae*. The average number of *M. persicae* for neem oil diluted 100-fold, 500-fold, and 1,000-fold was 14.00, 14.67, and 14.67, respectively, indicating a nonsignificant effect (Fig. 9.3). The average number of *M. persicae* for 1, 10, and 100 g of neem seed cake was 15.00, 10.00, and 6.00, respectively; neem seed cake of $>10$ g had a significant repelling effect, which increased with treatment amount (Fig. 9.4). The average number of *M. persicae* for neem seed cake extract and control was 10.33 and 19.67, respectively (Fig. 9.5). Neem seed cake extract showed a repellent effect similar to that of 10 g of neem seed cake. This result revealed that neem seed cake extract contains repellent compounds.

In a previous study, Lowry et al. (1993) demonstrated that neem seed oil sprayed onto intact plants in the laboratory resulted in a significant reduction in the number of *M. persicae*; however, they also described that the effectiveness of neem appears to be influenced by the host plant, specific aphid species, and weather conditions. On the other hand, Hori (1996, 1999a) reported that mint, thyme, garlic, and onion oil strongly repel *M. persicae* and that the volatile constituents of essential oils of garlic and onion may inhibit *M. persicae* from settling on plants. In addition, rosemary and ginger oil repelled *M. persicae* in an olfactometer when linalool, camphor, and α-terpineol essential oils were used (Hori 1999b, c). Hori (1999c) suggests that polyphagous aphids such as *M. persicae* have a tendency to locate their host plants mainly by visual cue, avoiding the odors of some nonhost plants. Thus, the olfactory behaviors of aphids against host plant odors are related to host proximity. In the present study,

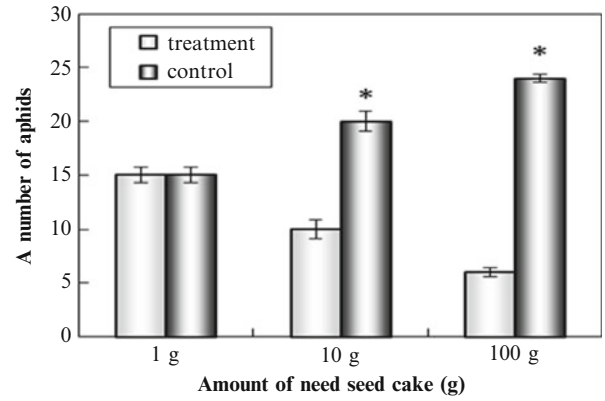


**Fig. 9.4** Repellent effect of neem seed cake on *M. persicae*

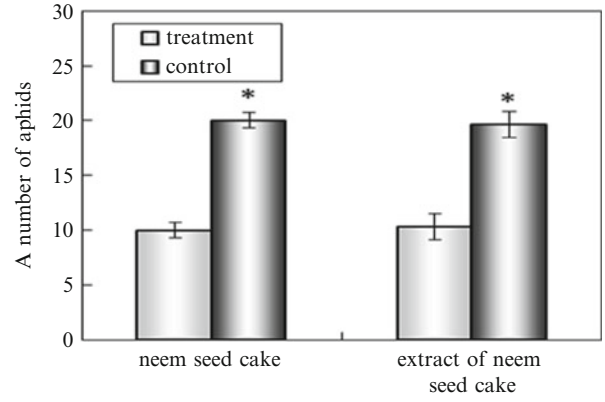


**Fig. 9.5** Repellent effect of neem seed cake extract and neem seed cake on *M. persicae* Extract was 10 g of neem seed cake

aphids detected volatile compounds more in the neem seed cake than in the host plant so preferred the host plant significantly more. There is no report on the repellency of neem seed cake on *M. persicae*. The present study is the first to show that neem seed cake has a repellent effect on these aphids. The present findings suggest that volatile compounds in neem seed cake have a repellent effect against *M. persicae*.

### 9.3.4 Volatile Extracts on *Pieris rapae crucivora*

Only rosemary (75 %; chi-test, $\chi^2 = 14.75$, $p < 0.001$) and spearmint (72 %; $\chi^2 = 11.36$, $p < 0.001$) extracts had a significant feeding repellent effect against the larvae (Fig. 9.6). Plants have wide action spectra, whereas a particular plant can affect insects though different mechanisms of action. In this study, rosemary and spearmint affected *P. rapae* larvae by inhibiting their feeding, suggesting the presence of feeding repellent compounds in the volatile extracts. To date, there have been many studies on repellency and the insecticidal effects of herbs against pests (Dover 1985; Sharma and Gupta 2009; Salari et al. 2012). Rosemary is known to have a repellent effect on various pests (Salari et al. 2012), and Sampson et al. (2005) reported that rosemary oil repelled *Lipaphis pseudobrassicae*. Waliwitiya et al.(2009) assessed the essential oil of rosemary against larval stages of *Aedes aegypti* and found that the larvicidal activity of 15 terpenoids, pulegone, thymol, eugenol, trans-anethole, and citronellal exhibited high larvicidal activity against *A. aegypti*. Dover (1985) reported that the alcohol extract of rosemary has a repellent effect on *P. brassicae*. Moreover, mint oil is known to repel various pests and insects (Sampson et al. 2005;

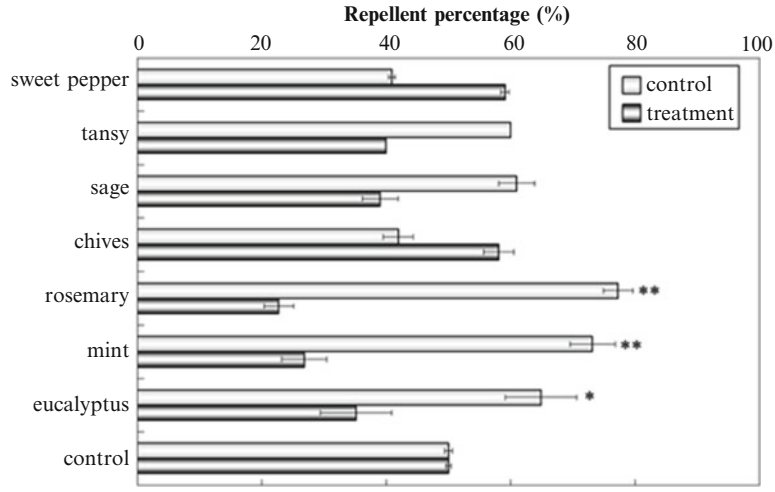


**Fig. 9.6** Repellent effect of rosemary, spearmint, eucalyptus, chives, sage, tansy, and sweet pepper herb extracts against *P. rapae* larvae

Abbaszadeh et al. 2009). However, there is no data on the antifeeding effects of rosemary on *P. rapae* larvae; nevertheless, rosemary and spearmint are known to repel various pests.

*Pieris brassicae* belongs to the same family as *P. rapae* and avoids feeding on leaves treated with hyssop, rosemary, sage, thyme, white clover, and eucalyptus (Sharma and Gupta 2009). On the other hand, *P. rapae* larvae avoid feeding on the wild mustard, *Erysimum cheiranthoides* (Sachdev-Gupta et al. 1993). Thus, *Pieris* spp. reject various plants, and these repellents are soluble. The findings of the present study suggest that compounds from rosemary may be volatile due to extract high volatile organic solvent. Furthermore, it seems desirable to use rosemary for companion plants.

## 9.4 Conclusions

In this chapter, we investigated that plant volatile extracts have repellent effects and antifungal activity against insects and fungi. First, it was clarified that the TLV showed strong antifungal activities against three types of plant pathogens of *B. fuckeliana*, *G. cingulata*, and *F. oxysporum*. Furthermore, it was presumed that there was the defense response against plant pathogens by TLV. Second, sweet pepper and tansy extracts proved to be a potent insecticide against aphids in the laboratory, and dokudami extract was a highly effective repellent against aphids in the field. Third, neem seed cake had the highest repellent effect on *M. persicae*, and effectiveness increased in line with increasing concentration. Finally, rosemary and spearmint volatile extracts have a notable feeding repellent effect against *P. rapae* larvae. Thus, we consider the repellent effect on insects and antifungal activity on fungi to be attributed to plant volatile compounds. In the future, we need to identify these repellent and antifungal compounds.

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# Part III

# Natural Enemies

# Reduviids and Their Merits in Biological Control

10

K. Sahayaraj

**Abstract**

Reduviids are the largest group of pest-specific, efficient hemipteran predators of economically important insect pests worldwide. They are found in the agroecosystems, semi-arid zones, scrub jungles and tropical rain forests. Reduviid predators have been distributed in various crops like soybean, groundnut, pigeon pea, cotton, castor, rice, cabbage, tobacco, pumpkin, bhindi, citrus, sugar cane, sesbania and apple and in secondary and tropical evergreen forests in many parts of the world. In natural and plantation forests, reduviids are living at various heights on vegetation but also in bird nests, caves and spider webs. Their success in every ecosystem/trophic niche is due to their morphological and physiological adaptations related to the predation. Reduviids are efficient predators on insect pests of crops, playing a significant role in keeping pest populations in check. Some important biological control agents are *Platymeris laevicollis* Distant, *Zelus renardii* Kolenati, *Rhynocoris marginatus* (Fab.), *Rhynocoris kumarii* Ambrose and Livingstone, *Rhynocoris fuscipes* (Fab.), *Pristhesancus plagipennis* Walker, *Blaptostethus pallescens* Poppius, *Acanthaspis pedestris* (Stål), *Catamiarus brevipennis* (Serville), *Ectomocoris tibialis* (Distant), etc.

Even though reduviids are polyphagous predators, many have prey specialisations. Its biological control potential has been evaluated on crops like cotton, soybean, groundnut, bhindi and coconut in field and laboratory experiments and is being commonly used in biological control programmes. Distribution pattern, morphological characters, prey specificity and biological control potential under laboratory, field cage and natural field conditions have been discussed in elaborate manner.

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_10, 

**Keywords**
Reduviid predators • Prey • Bioefficacy • Field evaluation • Mass production

## 10.1 Introduction

Reduviid (Hemiptera: Reduviidae) predators are the largest terrestrial bugs, consisting of 7,000 species (http://www.cirrusimage.com/bugs_assassin_zelus_luridus.htm) and subspecies, 913 genera and 25 subfamilies (Maldonado 1990). The subfamilies included Bactrodinae, Centrocnemidinae, Cetherinae (termites feeder), Chryxinae, Ectrichodiinae (millipede assassin bugs), Elasmodeminae, Emesinae, Hammacerinae, Harpactorinae, Holoptilinae (feather-legged assassin bugs), Manangocorinae, Peiratinae, Phimophorinae, Phymatinae (ambush bugs), Physoderinae, Pseudocetherinae (lobe-headed bugs), Reduviinae, Saicinae, Salyavatinae (termite-feeding assassin bugs), Sphaeridopinae, Stenopodainae, Triatominae (kissing bugs-blood-feeding insect), Tribelocephalinae, Vesciinae and Visayanocorinae. Few distinct features and characteristic features of reduviid subfamilies are as follows: Nothing is currently known about the life history of Bactrodinae; most of the Centrocnemidinae collected specimens have been located on tree trunks; nothing is currently known of Chryxinae life history; Ectrichodiinae species are most commonly found in leaf litter and are nocturnal; Elasmodeminae predators live under loose bark and appear to prey on insects; Harpactorinae is the largest reduviid subfamily, whereas subfamily Manangocorinae contains only one species described from Malaysia; Peiratinae species are primarily ground dwelling and feed on other arthropods and also fast runners; nothing is currently known of Phimophorinae life history; *Phymata pennsylvanica* Handlirsch (Phymatinae) has been observed to feed on nectar; Physoderinae mainly dwelled in caves, hollow trees, vegetable debris and at the bases of banana and *Pandanus* leaves; Reduviinae species live in animal burrows and also closely associated with humans; Saicinae species are commonly collected at lights; most species of Salyavatinae, Stenopodainae and Tribelocephalinae are nocturnal; most of the Stenopodainae species are found on soil, often covered with soil or sand; many Triatominae species are vectors of Chagas disease or trypanosomiasis; few species of Tribelocephalinae dwelled in litter; Vesciinae is found in secondary forests, tropical dry forests and on roadsides; and nothing else is known on Visayanocorinae natural history.

Concerning Indian reduviid fauna, there are about 464 species belonging to 144 genera and 14 subfamilies (Ambrose 2006). These bugs are particularly numerous and diverse on agricultural crops, but most species are specific on semi-arid zones and scrub jungles bordering agroecosystems. Reduviid predators are more resistant to chemical sprays than the coccinellids. Their adjustability to ecological factors and to the secondary effects of phytosanitary sprays makes heteropterans, especially reduviids, potentially good biological control agents. Reduviid predators have been recorded from many agroecosystems (see Table 10.1 for more details) such as sugar beet (Ehler et al. 1997), sesbania (Sileshi et al. 2001), oilseed brassica (Ruberson and Williams 2000), soybean (Grundy, and Maelzer 2000), orchard and field crops (Pyke and Brown 1996), cotton (Showler and Greenberg 2003; Grundy 2007), lady's finger, chilli, cinnamon, citrus, coconut, *Rhynocoris segmentarius* in cowpea (Suh Niba 2011), groundnut, maize, mango, mustard, pigeon pea (Ambrose and Claver 2001), potato, pumpkin, rice, sugar cane, sunflower, sweet potato, tobacco (Marques et al. 2006), teak (Das and Ambrose 2008), wheat (Sahayaraj 2007) and apple orchard (Sackett et al. 2007).

More importantly, *Apiomerus floridensis* Szerlip, *Arilus cristatus* (Linnaeus), *Sinea spinipes* (Herrich-Schaeffer) and *Zelus exsanguis*

**Table 10.1** Distribution of reduviids in agricultural ecosystems

| Crops | Reduviids | Location | Citation |
|---|---|---|---|
| Fruit crops and pecan | *Apiomerus floridensis* Szerlip, *Arilus cristatus* (Linnaeus), *Sinea spinipes* (Herrich-Schaeffer), *Zelus exsanguis* Stål | USA | Mizell and Tedders (1995) |
| Sugar cane | *Acanthaspis quinquespinosa* (Fabricius) | India | Butani (1985) |
| | *Pristhesancus plagipennis* (Walker) | Queensland | Illingworth (1921) |
| | *Rhynocoris marginatus* (Fabricius) | India | Sahayaraj (1999) |
| Pigeon pea | *Coranus* spp., *Rhynocoris marginatus, Rhynocoris fuscipes, Paralenaeus pyrrhomelas, Ectomocoris* | India | Claver (2011) |
| | *Rhynocoris marginatus* (Fabricius), *Irantha armipes* (Stål), *Sycanus pyrrhomelas* (Walker), *Rhynocoris longifrons* (Stål) | India | Ambrose and Claver (2001) |
| Cabbage | *Polybia* sp. | Sweden | Miranda Ortiz (2011) |
| | *Rhynocoris segmentarius* | South Africa | Suh Niba (2011) |
| Alfalfa | *Nagusta goedelii* (Kolenati) | Iran | Rakhshani et al. (2010) |
| Forest | *Heza* sp. and *Apiomerus* sp., *Arilus* sp., *Harpactor angulosus* | Brazil | Pereira et al. (2012) |
| | *Heza* sp., *Apiomerus* sp., *Harpactor angulosus, Arilus sp.* | Brazil | Pereira et al. (2012) |
| Cardamom ecosystem | *Sycanus indagator* Stål, *Rhynocoris longifrons* Stål, *Endochus migratorius* Distant, *Endochus atricapillus, Rihirbus trochantericus, Epidaus bicolor, Acanthaspis siva* Distant, *Ectomocoris tibialis* | India | Nagarajan and Varadarasan (2013) |
| Cotton | *Acanthaspis pedestris* (Stål) | India | Kalidas and Sahayaraj (2012) |
| | *Zelus longipes*, *Z. laticornis*, *Z. ruficeps*, *Z. armillatus*, *Atrachelus cinereus* ssp. *crassicornis* and *Apiomerus apicalis* | | Silvie et al. (2007) |
| | *Coranus aegyptiacus* (Fabricius) | India | Singh et al. (1987) |
| | *Coranus nodulosus* Ambrose and Sahayaraj | India | Sahayaraj (1991) |
| | *Phonoctonus nigrofasciatus* (Stål) | England | Evans (1962) |
| | *Phonoctonus fasciatus* (P. de B.) and *Phonoctonus subimpictus* (Stål) | Nigeria | Parker (1972) |
| | *Pisilus tipuliformis* (Fab.) | West Africa | Parker (1965) |
| | *Pristhesancus papuensis* (Stål) | Australia | Martin and Brown (1984) |
| | *Zelus renardii* Kolenati | California | Cisneros and Rosenheim (1997) |
| | *Zelus renardii* Kolenati | America | Bosch and Hagen (1966) |
| | *Zelus* sp. and *Sinea* sp. | Brazil | Gravena and Sterling (1983) |
| | *Zelus exsanguis* (Stål), *Zelus cervicalis* Stål, *Zelus socius* Uhler | North America | Ables (1978) |
| | *Pristhesancus plagipennis* (Walker) | Australia | Pyke and Brown (1996); Grundy and Maelzer (2000) |
| | *Rhynocoris fuscipes* (Fab.) | India | Singh and Sing (1987) |
| | *Rhynocoris marginatus* (Fab.) | India | Sahayaraj (1995) |
| | *Acanthaspis pedestris* (Stål), *Acanthaspis quinquespinosa* (Fab), *Acanthaspis subrufa* (Distant) | India | Rajagopal (1984) |

(continued)

**Table 10.1** (continued)

| Crops | Reduviids | Location | Citation |
|---|---|---|---|
| Cotton | *Oncocephalus fuscirostris* Stål | Australia | Miles and Bull (2000); Murray (1982) |
| Tobacco | *Agriocleptes bahianus* Wygodzinsky | Brazil | Marques et al. (2006) |
| | *Apiomerus lanipes* (Fabricius) | Brazil | Marques et al. (2006) |
| | *Coranus atricapillus* | India | Singh (1985) |
| | *Coranus spiniscutis* (Reuter) | India | Bose (1949) |
| | *Rhynocoris squaliua* (Distant) | India | Singh (1985); Rao et al. (1981) |
| Coconut | *Catamiarus brevipennis* (Serville) | India | Pawar et al. (1986) |
| | *Ectrychotes dispar* (Reuter) | | |
| | *Rhynocoris marginatus* (Fabricius) | | |
| | *Coranus atricapillus* | India | Singh (1985) |
| | *Coranus spiniscutis* (Reuter) | | |
| | *Cosmocolopius nigroannulatus* Stål | Brazil | Jahnke et al. (2002) |
| Oil palm | *Cosmelestes picticeps* | Malaysia | Cheong et al. (2010) |
| Coco | *Carcinoma astrologus* (Sign.) *Oncocephalus subspinosus* Aniyot | Ghana Rép. Congo | Babin (2009) |
| | *Phonoctonus* sp., *Polytoxus walbergi* St., *Rhynocoris bicolor* F., *R. loratus* St. | | |
| | *R. obtusus* (de Beauv.), *R. tristis* St. *Sastrapoda vicina* Schout. *Sphedanolestes picturellus* Schout. | | |
| | *Vestula lineaticeps* (Sign.) | | |
| Sweet potato | *Coranus spiniscutis* (Reuter) | India | Bose (1949) |
| Cowpea | *Coranus spiniscutis* (Reuter) | India | Bose (1949) |
| Mustard | *Coranus spiniscutis* (Reuter) | India | Bose (1949) |
| Lucerne | *Pirates ephippiger* White | Australia | Miles and Bull (2000); Murray (1982) |
| Maize | *Coranus spiniscutis* (Reuter) | India | Jalali and Singh (2002) |
| | *Ectomocoris cordiger* (Stål) | India | Misra (1975) |
| | *Cydnocoris gilvus* (Burmeister) | | |
| | *Oncocephalus impudicus* (Reuter) | | |
| | *Coranus spiniscutis* (Reuter) | India | Bose (1949) |
| Ailanthus | *Panthous bimaculatus* (Distant) | India | Varma (1989) |
| Rice | *Polytoxus fuscovittatus* (Stål) | India | Satpathy et al. (1975) |
| | *Coranus spiniscutis* (Reuter) | India | Bose (1949) |
| | *Rhynocoris fuscipes* (Fabricius) | India | Singh (1985) |
| Sunflower | *Pristhesancus plagipennis* | Australia | Grundy and Maelzer (2000) |
| Citrus | *Pristhesancus plagipennis* | Australia | Grundy and Maelzer (2000, 2002) |
| | *Rhynocoris albopunctatus* Stål | Uganda | Nyiira (1970) |
| | *Pristhesancus plagipennis* (Walker) | New South Wales | James, 1994 Grundy and |
| | *Pristhesancus plagipennis* | Australia | Grundy and Maelzer (2000, 2002) |
| Bhindi | *Rhynocoris fuscipes* (Fabricius) | India | Singh and Sing (1987) |
| Chillies | *Rhynocoris fuscipes* (Fabricius) | India | Singh and Sing (1987) |
| Pumpkin | *Rhynocoris fuscipes* (Fabricius) | India | Cherian and Brahmachari (1941) |
| | *Rhynocoris lapidicola* Samuel and Joseph | India | Joseph (1959) |
| | *Rhynocoris nysiiphagus* Samuel and Joseph | India | Joseph (1959) |

(continued)

**Table 10.1** (continued)

| Crops | Reduviids | Location | Citation |
|---|---|---|---|
| Groundnut | *Rhynocoris longifrons* (Stål) | India | Sahayaraj and Raju (2003) |
| | *Ectomocoris cordiger* (Stål) | | |
| | *Rhynocoris marginatus* (Fabricius) | India | Sahayaraj (1995) |
| | *Rhynocoris kumarii* (Ambrose and Livingstone) | India | Sahayaraj (1994) |
| | *Rhynocoris squaliua* (Distant) | India | Singh (1985) |
| | *Rhynocoris fuscipes* (Fabricius) | India | Singh and Sing (1987) |
| | *Oncocephalus annulipes* Stål | India | Sahayaraj and Raju (2003) |
| Low land Forest | *Salyavata variegata* Amyot | Costa Rica | McMahan (1983) |
| Oil palm | *Sycanus dichotomus* | Malaysia | Zulkefli et al. (2004) |
| *Acacia mangium* | *Sycanus leucomesus* Walker | Malaysia | Sajap et al. (1999) |
| Ornamental plant | *Zelus exsanguis* (Stål) | | Mizell (2007) |
| Soybean | *Coranus trabeatus* Horvath | Australia | Bishop and Blood (1977) |
| | *Gminatus wallengreni* Stål | | |
| | *Trachylestes aspericollis* (Stål) | | |
| | *Sastrapada australica* | | |
| | *Zelus longipes* (Lin.) | Colombia | Irwin and Shepard (1980) |
| | *Zelus socius* | USA | Irwin and Shepard (1980) |
| | *Sinea diadema* (Fabricius) | USA | Slater and Baranowsky (1978) |
| | *Sinea spinipes* (Herrich-Schaeffer) | USA | Irwin and Shepard (1980) |
| | *Sinea complexa* (Caudell) | Brazil | Irwin and Shepard (1980) |
| | *Sycanus indagator* (Stål) | India | Greene (1973) |
| | *Rhynocoris fuscipes* (Fabricius) | India | Singh and Singh (1987) |
| | *Rhynocoris fuscipes* (Fabricius) | India | Singh and Gangrade (1975) |
| | *Scadra annulipes* Reuter | | |
| | *Rhynocoris fuscipes* (Fabricius) | India | Singh and Singh (1987) |
| | *Pristhesancus papuensis* (Stål) | Australia | Shepard et al. (1983) |
| | *Pristhesancus plagipennis* (Walker) | Australia | Grundy and Maelzer (2000); Bishop and Blood (1977) |
| | *Coranus spiniscutis* (Reuter) | India | Bose (1949) |
| Potato | *Rhynocoris fuscipes* (F.) | India | Patalappa and Basavanna (1979); Schaefer (1983) |
| | | Pakistan | |
| Tea | *Acanthaspis quinquespinosa* (Fab.) | India | Das et al. (2010) |
| | *Rhynocoris marginatus* (Fab.) | | |
| | *Epidaus* sp. | | |
| *Coarse grain crops* | *Pristhesancus plagipennis* (Walker) | Queensland | Wade (1999) |

Stål were recorded from the fruit crops and pecan (Mizell and Tedders 1995), while *Zelurus angularis* (Stål) (Reduviinae), *Apiomerus mutabilis* (Costa Lima, Seabra and Hathaway) and *Apiomerus nigricollis* Stål (Apiomerinae) (Hemiptera, Reduviidae) from Brazilian forests (Grossi et al. 2012). A reduviid predator, *Sycanus pyrrhomelas* Walker was observed in parthenium preying on old-stage grubs or adults *Zygogramma bicolorata* (Gupta et al. 2004). Reduviids like *Pnirontis modesta* Banks and *Rhynocoris ventralis* (Say) were recorded in the USA east of the Rocky Mountains (Hagerty and McPherson 1999; Paiero et al. 2003). *Acanthaspis quinquespinosa* Fab., *Allaeocranum quadrisignatum* Reuter, *Rhynocoris marginatus* Fab., *Epidaus* sp., *Opistoplatys* sp. and *Sycanus croceovittatus* Dohrn were recorded from tea plantation in India (Das et al. 2010).

*Nagusta goedelii* (Kolenati) was collected in low densities and recorded as predator of pea aphid, *A. pisum*, from Alfalfa (*Medicago sativa* L.) (Rakhshani et al. 2010).

## 10.2 Why Reduviids Are Dominant Predator?

Generally, coccinellids, chrysopids, other hemipteran predators, ground-dwelling coleopteran predators and spiders are considered as common natural enemies present in any agroecosystems. At the same time, generalist reduviid predators are considered as dominant ones than coccinellids (Sahayaraj and Raju 2006), *Chrysoperla carnea* (Rosenheim et al. 1999) and spiders (Wignall and Taylor 2008; 2009). Furthermore, reduviid predators are resistant to major pesticides (James and Vogele 2001; Zulkefli et al. 2004; Abdul Hakeem 2008) and biopesticides (Jaronski et al. 1998; Fadare and Osisanya 1998; Fadare and Amusa 2003). This group of predators has been considered as an important biocontrol agent in the pest management programme. This chapter deals with the prey record, prey selection, bioefficacy, mass production and augmentative biological control of reduviid predators in a concise manner.

## 10.3 Plant-Associated Reduviids

According to Cohen (1996), in some heteropteran families (Reduviidae, Phymatinae and Nabidae), all members are obligate carnivores (including entomophages), whereas in other families (Lygaeidae and Pentatomidae), it appears that carnivory is secondary to herbivory and only a few species of a subfamily have taken up a predaceous mode of life. Considering the feeding adaptive features, reduviid predators have mandibular stylet tips with barbs or tooth or knifelike (Cohen 1996; Sahayaraj and Vinothkanna 2011; Kumar and Sahayaraj 2012), an organ usually used for predator. Though more than 96 % of reduviid predators are zoophagous, Harpactorinae species live in a specific relationship with certain plant species from which they obtain carbohydrates from food bodies, extrafloral nectar and hemipteran honeydew, or from sap by biting the plants (says Stoner et al. 1975; Bérenger and Pluot-Sigwalt 1997; Tallamy et al. 2004). In another study, Stoner et al. (1975) recorded *Sinea confuse* Caudell, *S. complexa*, *Zelus renardii, Zelus socius and Atrachelus cinereus* feed plant to obtain adequate food to live for a long time. It was also evident from the study of Guillermo-Ferreira and co-workers (2012) that *Atopozelus opsimus* feed on extrafloral nectaries of *Inga vera* (Fabaceae) in a Neotropical savanna area. Furthermore, indirect relation of reduviids with specific plant was also recorded; for instance, some reduviids prey on insects associated with a particular plant species (Miller 1953). However, no literature is available about the defoliation or plant sap sucking to sustain the life of the reduviids, clearly revealing reduviid predators are zoophagous animals rather than omnivorous.

## 10.4 Prey Record

### 10.4.1 Insect Pest Feeders

Predatory reduviids feed on a variety of food sources and encounter several preys with different nutritional value and defensive mechanisms, and the predator must develop several attack strategies to exploit a variety of preys. According to Begon et al. (1996), a predator is classified as truly generalist when its prey selection is proportional to the relative abundance of the prey species in its environment. However, some predators show some preference, that is, they preferentially select a prey over others, whatever the relative abundance of that prey is (Cock 1978; Hassell and Southwood 1978). Food preference was observed for several insect predators (Cock 1978) and has been identified as a species-specific characteristic (Hassell and Southwood 1978). Prey selection by a predator may be attributed to one of the two distinct mechanisms: active predator choice or passive selection. Active choice occurs when a predator actively chooses a prey according to its nutritional value,

while the prey physical and/or behavioural characteristics (mobility, size) do not influence the selection. On the other hand, passive selection is the result of predation opportunity based on prey physical and/or behavioural characteristics (vulnerability), rather than an active selection. For example, the mobility of different prey species may influence their encounter rates with a predator and thus influence their susceptibility to predation (Provost et al. 2006). Most reduviids are predators of insect pests of agricultural as well as forest vegetation. The prey record includes various families of Coleoptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera and Orthoptera. Prey record of reduviids is large and diverse, and conservation and augmentation of the reduviid predator and its utilisation in biological control of the insect pests has been gaining momentum in India and other countries in recent years (Ambrose 1995; Schaefer 1988; Sahayaraj 2007). Reduviid predators are often assumed to feed on a diverse diet of insects and other arthropods. For instance, hunter reduviids that feed on insects belong to Lepidoptera, Hemiptera, Orthoptera, Hymenoptera, Coleoptera, Isoptera, etc. Even though this seems to be true for many species of Harpactorinae (Louis 1974; Ables 1978; James 2000), prey specialisations are known for a number of Reduviidae. Whitefly *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) is an important pest of many agricultural crops, and reduviid predators have been used for its management (Williamson (1998).

### 10.4.2 Millipede Feeders

Many Ectrichodiinae (>600 spp.) are specialist feeds on Diplopoda (Livingstone and Ambrose 1984; Louis 1974; Cobben 1978; Miller 1956).

### 10.4.3 Termite Feeders

Salyavatinae (99 spp.) specialised on termites. Similarly, *Petalochirus brachialis* feeds only on termites (Sahayaraj 1991). *Ptilocerus ochraceus, Ptilocnemus femoralis* and *Ptilocnemus lemur* feed on ants (Jacobson 1911; McKeown 1944). Denlinger (1994) noted reduviid nymphs feeding on aggregating adults of *Stenotarsus subtilis* Arrow on Barro Colorado Island in Panama. *Vesbius sanguinosus* was found feeding on the termite *O. obesus* and the ant *Oecophylla* sp. (Das and Ambrose 2008).

### 10.4.4 Spider Feeders (Araneophagic)

Spiders are dangerous predators that can impose substantial pressure on natural populations as well as in agroecosystems. Emesine assassin bugs, or thread-legged bugs, are known for living in and around spider webs (Usinger 1941; Wygodzinsky 1966) and feeding on insects caught in spider webs (Villiers 1962), but other emesines, including those in the genus *Stenolemus*, appear to be araneophagic (i.e. predators that prey on spiders). *Stenolemus lanipes* feeds on spiderlings of *Achaearanea tepidariorum* (Hodge (1984) and *Stenolemus bituberus* on *Achaearanea* sp. and *Pholcus phalangioides* (Wignall and Taylor 2009). Then, Snoddy et al. (1976) similarly noted that *S. lanipes* did not feed on insects caught in the webs of *A. tepidariorum* (although young nymphs were observed feeding on psocids around the periphery of webs). However, an Australian species, *Stenolemus edwardsii*, preys on the young of the spider *Ixeuticus robustus* (*Badumna insignis*) and sometimes other small spiders but has not been observed feeding on insects or psocids (Hickman 1969). Recently, Wignall and Taylor (2008) reported that *Stenolemus bituberus* (Emesinae) typically found living in spider webs and feeding on them. East African assassin bugs, *Scipinnia repax* (Stål) and *Nagusta* sp. (Harpactorinae) were also reported as araneophagic (Jackson et al. 2010).

### 10.4.5 Ant Feeders

Some assassin bug groups specialise on certain prey groups, such as ants (feather-legged bugs – Holoptilinae) (78 spp.) (http://www.cirrusimage.com/bugs_assassin_zelus_luridus.htm; Hwang

and Weirauch 2012). In French Guiana, assassin bug *Zelus annulosus* (Harpactorinae) frequently observed on the leaves of *Hirtella physophora* (Chrysobalanaceae), for example, houses *Allomerus decemarticulatus* (Myrmicinae) that build gallery-shaped traps to catch large prey (Dejean et al. 2006; Revel et al. 2010).

## 10.5 Bee Feeders

Grossi et al. (2012) reported that *Apiomerus* spp. prey upon bees (Hymenoptera, Apidae), such as *Apis mellifera* Linnaeus (Apinae: Apini) (Costa Lima 1940; Marques *et al*. 2003) and in particular stingless bees (Apinae, Meliponini) (Silva and Gil-Santana 2004; Gil-Santana et al. 2006). Indeed, they are commonly known as bee killers or bee assassins, due to their recorded attraction to and predation on bees (Berniker et al. 2011). Species of *Apiomerus* have also been recognised as mimics of meliponine bees (Gil-Santana et al. 2003). In India, *Acanthaspis siva* D. adults and nymphs reported to feeding on Indian honey bee *Apis indica* F. (http://deriv.nls.uk/dcn6/7549/75493405.6.pdf). *Apiomerus crassipes* (F.) also reported to feeding on a species from Apis, namely, *Apis mellifera* (L.) (Swadner and Yonke 1973).

## 10.6 Vector Feeders

Along with the above mentioned arthropods belong to different insect orders, spiders and millipedes, reduviid predators have the tendency of feeding vectors like mosquitoes (Diptera) and Triatominae (Reduviidae). For instance, in Argentina, *A. nigricollis* was found in caves of armadillos (*Dasypus* sp.), together with *Triatoma rubrovaria* (Blanchard) (Carpintero 1981). Further, it was reported that *A. nigricollis* was considered to be a possible prey for this apiomerine. Later, it was also reported that some species of *Zelurus* have been observed preying on nymphs of some species of Triatominae, both under natural and under laboratory conditions (Coscarón et al. 1999).

## 10.7 Postharvest Pests Feeders

In addition to the agricultural pests, reduviids are also predating upon postharvest pests. Several species of reduviids have been studied as biological control agents. Specifically, *Xylocoris flavipes* (Reuter) is the most studied candidate biological control agent among predatory bugs. *X. flavipes* is advantageous because it has a high population increase capacity and wide distribution. *X. flavipes* has been reported to suppress populations of small insects, but it cannot predate large insects and internal grain-feeding insects. As *Amphibolus venator* (Klug), *Peregrinator biannulipes* (Montrouzier and Signoret) and *Joppeicus paradoxus* Puton can attack large insects, more research should be carried out on the suppression effects of these bugs (Imamura et al. 2008). Previously Awadallah et al. (1984, 1990) also reported that *Allaeocranum biannulipes* (Montr. et Sign.) has been considered as a stored-product pest predator. *Peregrinator biannulipes* Montrouzier et Signoret is a worldwide distributed bug (Ghauri 1962) and is known as a predator for stored-product pests including *Stegobium paniceum* (L.), *Lasioderma serricorne* (F.), *Anagasta kuhniella* (Zell), *Pyralis farinalis* (L.) and *Tribolium* spp. (Awadallah et al. 1990).

## 10.8 Prey Choice

Generalist insect predators are the most abundant natural enemies in annual agroecosystems. Prey-stage preference was evaluated in both choice and non-choice tests. Among the tested predator stages, third, fourth and fifth nymphal instars and adult *R. marginatus* were the preferred food of second, fourth and fifth nymphal instars of *D. cingulatus*, respectively. The prey preference was different when *R. marginatus* was provided with life stages *S. litura* (second-, third-, fourth- and fifth-instar larvae). Each stage has its own preference (Sahayaraj and Sivakumar 1995). *Zelus longipes* preferred small-sized *Spodoptera frugiperda* followed by medium- and large-sized larvae (Cogni et al. 2002). Second, third and

fourth nymphal instars of *D. cingulatus* were preferred by third, fourth and fifth nymphal instar and adults of *R. fuscipes*, respectively. Both fifth nymphal instar and adults of *R. fuscipes* preferred fourth-instar larva of *S. litura,* but third and fourth nymphal instar predators preferred second- and third-instar larvae. Results revealed that life stages of reduviid predator prefer a particular stage of the prey. We arrived at the following conclusions: Larger-sized predator preferred larger-sized prey and the smaller-sized predator preferred smaller-sized prey. Reduviids preferred soft-bodied lepidopteron caterpillars. Specific prey could not elicit similar preference on different predators, and timely release of the predator into infested fields will lead to effective control of prey.

## 10.9 Bioefficacy

Reduviids are larger than other Hemipteran predators like *Nabis* (Nabidae), *Geocoris* (Lygaeidae), *Orius* (Anthocoridae), *Lygus* (Miridae) and *Podisus* (Pentatomidae), and they successfully attack and consume larger preys. Effectiveness of the biological control agent depends upon the number of prey it kills, quality of the prey and the reproductive capacity, searching time, prey handling time, digestion, hunger, prey preference of the predator and competition among predators (ref.). Among these various factors, prey capturing (acceptance and consuming) is of prime importance. Predators generally preferred to feed up on a particular life stage of the pest, and capturing success is considered to be the foremost act of a predator against any prey. Laboratory results revealed that *Rhynocoris marginatus* adults consumed more numbers of *Aproaerema modicella* (18.37 larvae/day) followed by *Helicoverpa armigera* (16.93 larvae/day), *Spodoptera litura* (16.12 larvae/day), *Amsacta albistriga* (4.06 preys/day) and *Aphis craccivora* (0.26 adults/day) (Sahayaraj 2000; Sahayaraj et al. 2003). However, nymphal instar of *Rhynocoris marginatus* consumed more *Aphis craccivora* (6.47 adults/day). *Rhynocoris fuscipes* consumed 10 *Pterophorus lienigianus (Z)* in a day under laboratory conditions (Anand and Chandral 2011). Hence, these predators should be mass reared and released, and evaluation of its biological control potential under field conditions is imperative. Predatory rate (Y) and attack ratio were generally increased from the early nymphal instars to the late nymphal instars. For instance, bioefficacy of *R. kumarii* was high in fourth nymphal instar (75.3 *A. gossypii*/predator) and low in first nymphal instar (24.0 *A. gossypii*/predator) (Sahayaraj and Asha 2010). Similar trend was observed when *R. fuscipes* was provided with *D. cingulatus* and *P. solenopsis*. However, a different trend was observed when *P. solenopsis* was offered to *R. kumarii*, with females always consuming more number of preys than males, and the predatory rate depends upon the type of the prey offered. Based on our studies we propose the following proclamation for bioefficacy of the reduviid predators: bioefficacy of a reduviid purely depends upon the type and stage of the prey offered, female reduviid predator consumed more number of preys than the males, and bioefficacy depends not only on the prey but also on the stage of the predator and its general habitat.

## 10.10 Mass Production

Mass production of the predators is an important prerequisite for the success of biological control programmes. Mass rearing is essential to obtain a large number of predators for release in the field. However, very little effort has been made to rear a few reduviids on small scale under laboratory conditions. Although the technology required for the large-scale production of reduviids is relatively straightforward, it is not adapted by small laboratories, and it appears that there is scanty information available on small-scale reduviid production. Currently, the reduviids are not commercially available for augmentative release programme in any part of the world. The basic requisite for a biological control agent is the availability of a sound, low-cost rearing and mass multiplication technique, which is lacking for the reduviids. Our studies revealed that the

following factors, namely, prey type, prey density, nature of preys (live or frozen or heat killed), quality of prey diets, and oligidic diets and sex ratio, influence mass rearing of the hunter reduviids. Adult sex ratio is an important factor, which determines the fecundity and hatchability of any polyandrous insect. Determining the suitable sex ratio is also a prerequisite for the mass production of the natural enemies like reduviids, and very limited studies have been undertaken on the subject.

Lakkundi and Parshad (1987) explained the mass rearing of the reduviid predators with frozen and immobilised larvae of *C. cephalonica*. Later Sahayaraj (1991) reported mass rearing of reduviids on head-crushed *C. cephalonica* by larval card method. This method prevents the entangling of the reduviids in the web of larvae undergoing metamorphosis. Furthermore, both alive and frozen larvae of *C. cephalonica* were used for mass rearing of *R. marginatus* (Sahayaraj and Jeyalekshimi 2002). In addition, substrata alteration and prey or predator density alteration (Kumaraswami 1991; Sahayaraj 2007; Sahayaraj et al. 2003) have been tested for the mass production of insects. Mass rearing of reduviid predator reduced the postembryonic developmental period and enhanced the adult longevity and female-biased sex ratio of *R. marginatus* and *R. fuscipes* (Kumaraswami 1991; Sahayaraj and Selvaraj 2003).

*Sinea diadema* (Fabricius) first instar nymphs were provided with free water, bean pod sections or glucose solutions have been studied (Petrakis and Moulet 1996) and results reveal that provision of water or glucose solutions significantly delayed the onset of conspecific predation. Sahayaraj (2004) proposed an innovative idea (artificial diet or oligidic diet) to mass rear the reduviids and evaluated the impact of both insect-based and meat-based artificial diets on the feeding behaviour of *Rhynocoris marginatus* (Sahayaraj et al. 2006). Results showed that *Rhynocoris marginatus* preferred pig liver-based artificial diet. Four types of objects, viz. cotton, foam, paraffin capsule and cavity slides, were used as carrier for the diet. Among the four materials tested, cotton was found to be the most suitable object for providing the liquid artificial diet. Feeding behaviour and biology of *Rhynocoris marginatus* was studied with meat-based artificial diet using cotton, and *Rhynocoris marginatus* fed with oligidic diet failed to lay eggs after mating (Sahayaraj et al. 2006, 2007). Employment of *Spodoptera litura*-based oligidic diet as an alternate method showed that all life stages of *R. marginatus* preferred *Spodoptera litura*-based oligidic diet. However, the diet prolongs the developmental period (148 days) and reduces the survival rate (46 %) of reduviids. Ingredients of oligidic diet were altered, and studies reveal that *Rhynocoris marginatus* laid eggs and offsprings emerged from the eggs (unpublished data). Later, Sahayaraj and Balasubramanian (2009) checked whether the biological control potential of the predator was affected by the oligidic diet and it was shown that the bioefficacy of *R. marginatus* rose on oligidic diet and was higher than that of predator reared on insects. It led to the conclusion that *Rhynocoris marginatus* can be mass reared with either insect hosts or insect- or meat-based artificial diet.

Bioefficacy of insect hosts as well as artificial diet reared *Rhynocoris marginatus* life stages on *Dysdercus cingulatus* (Fab.), *Spodoptera litura* (Fab.) and *Corcyra cephalonica* Stainton in terms of approaching time, handling time and predatory rate (weight gain and number of prey consumed) was evaluated under laboratory conditions. Results revealed that a specific stage of the predator preferred the desired stage of the pests studied. Moreover, young reduviids preferred younger prey and vice versa. The biological control potential of the reduviid showed that irrespective of the preys offered, females consumed greater numbers of preys than the males. Among the three preys tested, *S. litura* was the most preferred prey followed by *D. cingulatus* and *C. cephalonica*. Both the handling time and weight gain differed among the life stages of the reduviid reared with an artificial diet and insect prey (Sahayaraj and Balasubramanian 2009).

## 10.11 Augmentative Biological Control

The potential for using 'augmentative' or 'inundative' biological control to suppress insect pests has been recognised for many years. Augmentative biological control (or 'augmentation') is simply the release of large numbers of insectary-reared natural enemies with the goal of 'augmenting' natural enemy populations or 'inundating' pest populations with natural enemies. Field testing is an important step in evaluating the use of natural enemies as augmented biological control agents. Although many natural enemies were recommended in groundnut pest management, reduviids have not yet been utilised at the field level in any part of the world. Many species of beneficial insects and mites occur naturally on crop plants where they find food, moisture and shelter. Reduviids are well-known predators on insects belonging to various orders (see Figs. 10.1, 10.2 and 10.3). Production of the biological control agents at roughly one million times, the female progeny rate during the time required for the completion of one generation of biocontrol agents with an economical procedure involving minimum labour is a prerequisite for any successful biocontrol programme. Augmentation of the reduviid predator was attempted by Edward as early as 1962. Later, Rhyckman and Rhyckman (1996) tried it with *Rhynocoris carmelita* (Stål.), *Platymeris rhadamanthus* (Gerstaecker), *Reduvius sensiles* (Faust), *Reduvius vanduzeei* and *Reduvius sonoraensis* (Walker).

In 2002 (Grundy and Maelzer), third-instar nymphs of the Australian assassin bug, *Pristhesancus plagipennis* (Walker), were released into cotton plots at two release densities (0.51 and 1.38 nymphs per metre row) and two crop growth stages. Results revealed that over 70 % of nymphs died or emigrated within 2 weeks of release. Among the two release rates, 1.38 nymphs per metre row reduced the number of *Helicoverpa* spp. larvae in the plots for a 7-week period and significantly increased the cotton yield.

In India, an exotic reduviid predator *Platymeris laevicollis* (Distant) was colonised in laboratory and released in large numbers at the crowns of the coconut trees, and it led to the establishment of the predator population and control of the *Oryctes rhinoceros* beetle (Antony et al. 1979). Later, Sahayaraj (1999) released *Rhynocoris marginatus* (Fab.) at 5,000 predator's $ha^{-1}$ in groundnut field and evaluated its impact on two defoliators. The results showed that the incidence of *H. armigera* varied from 6.55 to 0.77 per plant and for S. *litura* variation was 5.66 to 0.88 per plant. Furthermore, the pod yield was highest in the reduviid released plot (1,867.77 kg $ha^{-1}$). Subsequently, Sahayaraj and Martin (2003) conducted a field experiment where all the life stages of the reduviid predator *Rhynocoris marginatus* (Fab.) were released into the groundnut field at 5,000/ha. *Rhynocoris marginatus* significantly reduced *Spodoptera litura* (85.89 %) followed by *Helicoverpa armigera* (67.65 %), *Aphis craccivora* (46.34 %), *Atractomorpha crenulata a*nd *Chrotogonous trachypterus* (42.86 %), but no impact on the *Mylabris* spp. populations observed during the study period. *Rhynocoris marginatus* did not affect the other predatory fauna such as coccinellids (*Menochilus sexmaculatus* Fab., *Coccinella septempunctata*), praying mantis, wasp, damselfly (*Agriocnemis femina femina* Brauer) and spiders (*Lycosa tista* Tikader and *Hippasa pisaurina* Pocock) present in control and predator-released field. Highest production of groundnut, net gain and the cost benefit ratio were recorded from *R. marginatus*-released groundnut field. Similarly, augmentative release of another assassin bug *Rhynocoris kumarii* Ambrose and Livingstone reduced the population level of the red cotton bug, *Dysdercus cingulatus* (Fabricius) (Claver and Ambrose 2001).

*Rhynocoris marginatus* Fab. (5,000 @ $ha^{-1}$), *Rhynocoris kumarii* Ambrose and Livingstone (5,300 @ $ha^{-1}$) and *Rhynocoris longifrons* Distant (5,600 @ $ha^{-1}$) (unpublished data) were released into the groundnut field, and it reduced the pest incidence and their infestation and increased groundnut production from 500 to 800 kg $ha^{-1}$. Similarly, biological control


**Fig. 10.1** *Rhynocoris marginatus* nymphs (**a–e**) and adults (**f–h**) feeding on *Nilaparvata lugens* (**a**), *Spodoptera litura* (**b**), *Achaea janata* (**c**), *Phenacoccus solenopsis* (**d** and **f**), *Pericallia ricini* (**e**), *Epilachna vigintioctopunctata* (**g**) and *Chrotogonus* sp. (**h**)

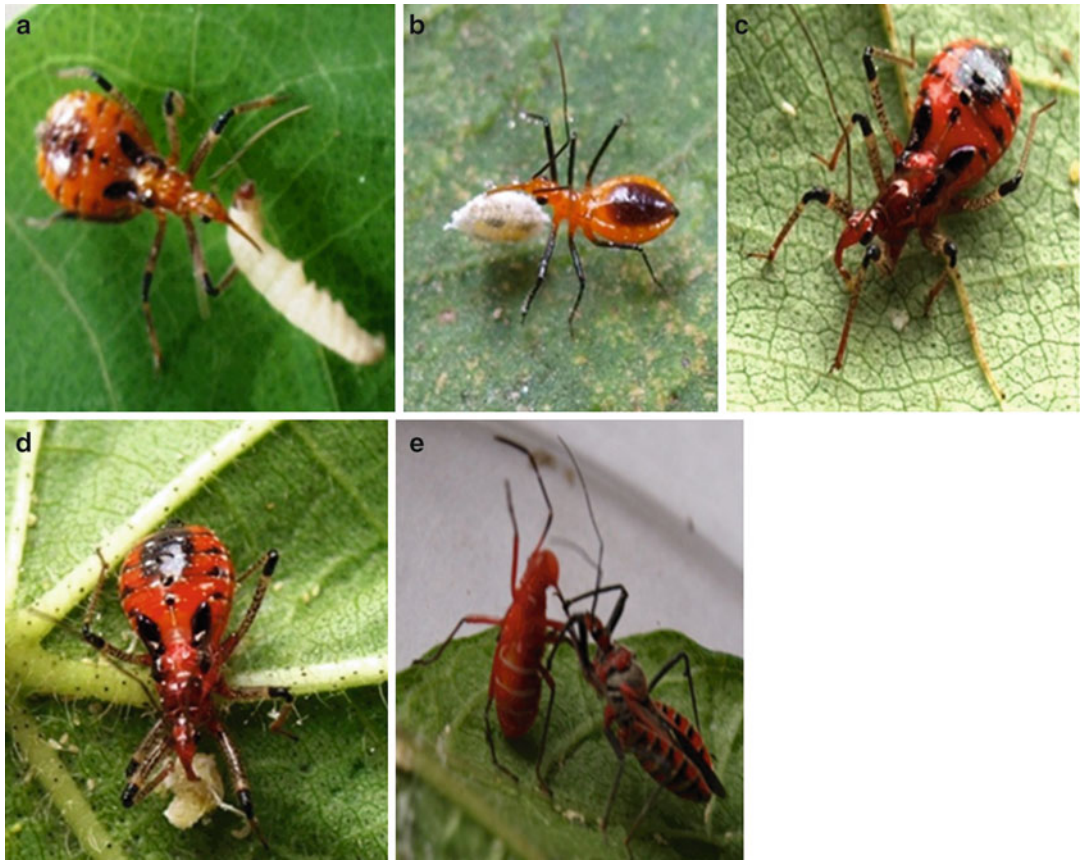


**Fig. 10.2** *Rhynocoris fuscipes* nymphs (**a–d**) and adults (**e**) feeding on *Corcyra cephalonia* (**a**), *Phenacoccus solenopsis* (**b**, **d**), *Aphis gossypii* (**c**) and *Dysdercus cingulatus* (**e**)

potential of *A. pedestris* on *H. armigera* was recorded in lady's finger orchards (Sahayaraj and Ravi 2007). Recent studies by Sahayaraj and Martin (2003) revealed that *R. marginatus* could be used as augmented biological control agent for groundnut.

Tactic predators use a wide range of tactics to catch prey, from sit-and-wait tactics, where the predator waits for prey to approach (e.g. snakes such as *Gloydius shedaoensis* that ambush their prey (Shine and Sun 2003)), to active tactics, where the predator approaches the prey (e.g. cougars, Puma concolor, that stalk and chase their prey). While some predators will use one tactic against all, or most, of their prey (e.g. crab spiders that ambush pollinating insects on flowers), others may flexibly alternate between tactics according to the type of prey, the environment or circumstances during the hunt. A predator that hunts dangerous prey may use specialised, prey-specific tactics. Stenolemus assassin bugs are also predators of web-building spiders. Whilst little is known of the predatory tactics used by *Stenolemus* species studied to date, they appear to have very narrow prey ranges. For example, *Stenolemus lanipes* has been reported hunting the tangle-web spider *Achaearanea tepidariorum* (Hodge 1984) and *Stenolemus edwardsii* has been reported to hunt spiderlings of the common house spider Ixeuticus robustus (Badumna insignis) although it will feed on other small spiders when these are unavailable (Hickman 1969). In sharp contrast to reports for these species, *Stenolemus bituberus* has a wide prey range and uses two distinct predatory tactics, 'stalking' and 'luring' (Wignall

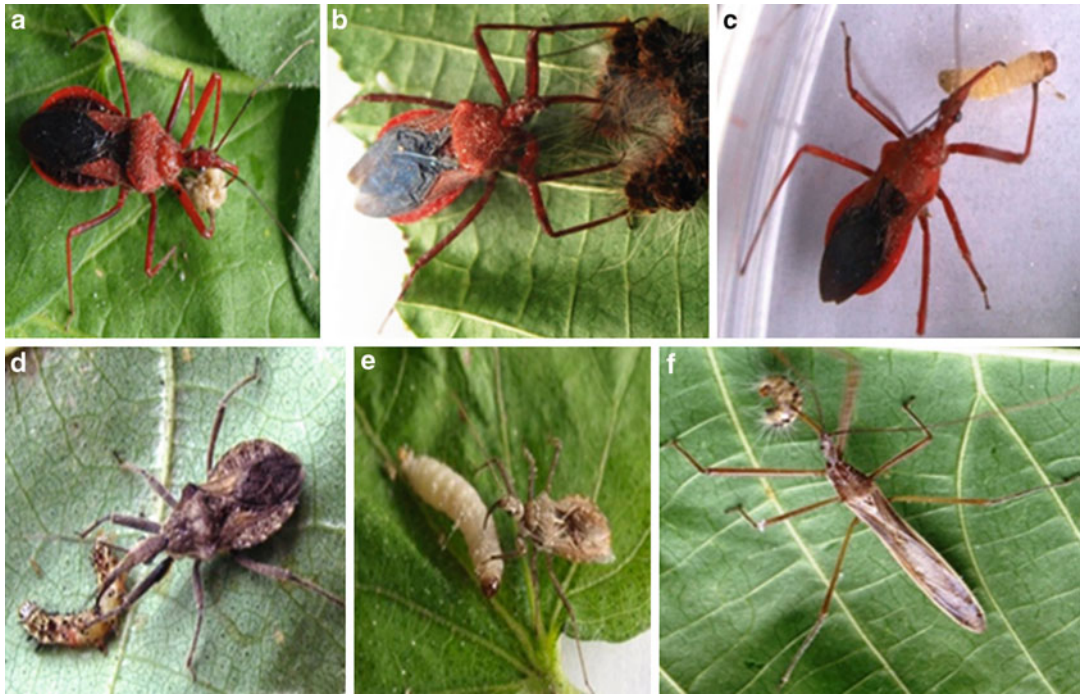


**Fig. 10.3** *Rhynocoris kumarii* (**a–c**), *Rhynocoris longifrons* (**d**), *Coronus caprilesi* (**e**) and *Euagoras plagiatus* (**f**) adults; *Helicoverpa armigera* (**a**), *Pericallia ricini* (**b**, **f**), *Corcyra cephalonia* (**c**, **e**) and *Spodoptera litura* (**d**)

and Taylor 2008). When stalking spiders, *S. bituberus* slowly approach the spider until within attacking range. When luring spiders, *S. bituberus* manipulate the silk of the webs, generating vibrations that attract the resident spider into range. We present in this chapter one of the first studies of how alternative predatory tactics are used by an araneophagic insect while hunting different prey spiders.

## 10.12 Conclusion

Reduviids (Hemiptera: Reduviidae) feed on preys larger than its body size. Larger-sized predator preferred larger-sized prey and small-sized predator preferred small-sized prey, and hence timely release of the predator into the pest-infested fields is necessary for effective control. *Rhynocoris marginatus* highly preferred lepidopteran larvae followed by nymphs and adults of both Hemiptera and Coleoptera. Its predatory rate varied from 1 to 18 prey/day and depends upon prey size. Reduviids killed more number of preys than they need to satiate, making it an effective biocontrol agent. Reduviids can be mass reared using natural hosts, factitious host and oligidic diets. Eggs and other life stages (nymphs and adults) can be stored at 20 °C and between 27.5 °C and 32.5 °C, respectively. Since reduviids reduced pest populations and increased groundnut production and biopesticides least affect the life stages of this predator, *Rhynocoris marginatus* can be utilised either alone or in combination with other bio-intensive integrated pest management (BIPM) components in the pest management of many agricultural crops.

## 10.13 Future Recommendations

1. It was proposed (Weirauch 2006) that some reduviids have a special structure, known as the ‘cave organ’, on the pedicel of their antennae, and there is morphological

evidence that these organs have a role in chemoreception. He further mentioned that, however, Harpactorinae is one of the reduviid subfamilies that apparently have no cave organs. On the whole, surprisingly, little is known about sensory systems of any reduviids; hence, this area can be undertaken in the future to explore about reduviid prey specificity.

2. Exploratory surveys should be intensified to identify new reduviid predators, and reduviids should be integrated with more BIPM components, and its role in BIPM should be evaluated.
3. Interaction of reduviid predators with resistant/tolerant/transgenic cultivars should be studied to improve the overall pest management efficiency.
4. Simple BIPM system evaluation techniques should be evolved to convince the end user (the farmer) of the system.
5. Since increasing the release rate of the predator did not significantly affect the target pest density, it is imperative to find out the release rate for each and every reduviid against target pest.

**Acknowledgement** I am very grateful to DST, CSIR, MEF and DBT for the financial assistance to carry out research projects related to biological control of reduviids. I also thank the authorities of St. Xavier's College (Palayamkottai) for laboratory facilities and encouragements.

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# Weaver Ants and Biocontrol of the Nuisance Pest *Luprops tristis* (Coleoptera: Tenebrionidae)

11

P. Aswathi and Sabu K. Thomas

**Abstract**

The utility of weaver ants (*Oecophylla smaragdina* Fabricius, 1775), and their potential in the control of agricultural pests, is highlighted. Comprehensive data on its habits, habitat requirements, foraging behaviour, social organisation involving polyethism and polydomous nesting behaviour, aggressiveness and recent establishment as an efficient biocontrol agent of the main insect pests in cashew, citrus and mango are listed. Identification of weaver ants as the sole arthropod predator that prey upon the nuisance pest *Luprops tristis* Fabricius, 1801, in rubber plantations and its possibility as an effective biocontrol agent to regulate the beetles are detailed. Lack of nesting trees in monoculture rubber plantations and the negative attitude of people towards weaver ants will hinder the establishment of weaver ants as an effective predator and biocontrol agent of *L. tristis* in rubber plantations.

**Keywords**

Weaver ants • Nuisance pest • Biocontrol potential

## 11.1 Introduction

Weaver ants of the genus *Oecophylla* are so called because of their habit of constructing nests by weaving leaves together, are one of the most familiar ant groups and are among the several most abundant and ecologically dominant elements of the arboreal ant fauna in the Afrotropical regions (Hölldobler 1983; Dejean 1990; Blüthgen and Fiedler 2002). They are important as one of the dominant species in the forest, a biological control agent of considerable economic importance, as food and medicine, and also as a potential pest itself. The combination of well-developed communication, aggressive territoriality and decentralised multiple nests allows *Oecophylla* to maintain absolute territories, which exclude most other ant species (Holldobler and Lumsden 1980).

Extant species of the weaver ants are *Oecophylla longinoda* Latreille, 1802 and *Oecophylla smaragdina* Fabricius, 1775. *Oecophylla longinoda* with eight subspecies occurs in a wideband across equatorial Africa and *O. smaragdina* with six

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_11, 

subspecies occurs in the Indian subcontinent, Indo-China, Sunda Shelf–Philippines and New Guinea–Melanesia bioregions of the Indo-Pacific terrestrial region and the northern margins of North Australia in the South Pacific (Bolton et al. 2007). The Australian subspecies of *O. smaragdina virescens* has a pale green abdomen, hence the common name 'green tree ant' (Dodd 1928), while Asian populations have a uniform reddish-brown colour (Wheeler 1922). The African species, *O. longinoda,* varies from reddish brown to dark brown (Lokkers 1990).

### 11.1.1 Origin

Likely origin of *Oecophylla* is in the early Palaeocene in the Palaearctic realm, radiating strongly during the climatic changes of the Eocene–Oligocene transition. Thirteen fossil species of weaver ants have been described, with *Oecophylla brischkei* and *O. crassinoda* (= *O. brevinodis*) from the Baltic amber and *O. bartoniana* (Cockerell 1920) from the Eocene Bagshot Beds, Bournemouth, UK (Dlussky et al. 2008), as the most ancient. Mitochondrial sequence divergence indicated that the two living species, *O. smaragdina* and *O. longinoda*, diverged 11.3–13.3 million years ago, in the Miocene (Azuma et al. 2006).

### 11.1.2 Food

Weaver ants are omnivorous in nature and feed on sugar and protein-rich food, but the major preference is proteins, most importantly for insects. They attack most arthropods, and the recorded prey items are termites, ants, honeybees, heteropterans, coleopterans, orthopterans, blattodeans, mantodeans, dipterans and araneans (Vanderplank 1960; Way 1954). They actively search for food, prey directly on a multitude of pests and will bring food back home to their nest to feed themselves as well as the larvae and others in the nest. Workers hunt diurnally in groups, detect prey visually from a relatively long distance, seize by an appendage and immobilise them. Long legs permit them to step from leaf to leaf, long antennae permit them to touch the ground, and comparatively large convex eyes provide stereoscopic vision for evaluation of distance between leaves. This behaviour permits the ants to capture small and large insects and even other animals (Hölldobler and Wilson 1990; Dlussky et al. 2008).

Weaver ants supplement their proteins with sugars like honeydew from sap-sucking insects or with plant nectar. When a new nest is made, weaver ants look for and prefer young leaves that have some honeydew-producing insects which will give them sugar and thus energy for the construction of their nest during the initial period. Honeydew is collected from trophobiotic interactions between weaver ants and honeydew-producing insects such as various homopterans, including Coccinellidae, Stictococcidae, Pseudococcidae, Aphididae, Margarodidae, Membracidae and Cicadellidae, and lycaenid butterflies (Vanderplank 1960; Way 1963; Hölldobler and Wilson 1990), some of which are specific to weaver ants (Seufert and Fiedler 1996; Eastwood and Fraser 1999). Many ant–homopteran interactions are mutually beneficial with the ants obtaining food rich in sugars and amino acids and the bugs obtaining some protection from predators and parasites (Way 1963). Many lycaenid butterfly larvae secrete ant-appeasement substances as well as sugary food solutions from epidermal glands which enable them to live within the protection of ant nests. Rather than actively searching for sugar, they prefer to keep their 'sugar pot', like aphids, scales or mealy bugs, close to their nests and sometimes even inside nests. But outbreaks of honeydew-producing insects never occur; on the contrary, the ants will even kill some of them if the amount of sugar produced by these insects is bigger than the amount required by the colony (Van mele and Cuc 2003).

### 11.1.3 Life Cycle

The life cycle of an *O. smaragdina* colony starts with a mated queen finding a sheltered site for a

first nest between leaves of a tree or shrub and laying a batch of about 35 eggs within 5–10 days after shedding her wings. Larvae emerge by eighth day, pupae follow after 17 days, and the adult worker appears after 28 days. First workers are intermediate in size, and the distinct minor and major castes appear later. As soon as last instar larvae arise (around day 15), they are used by the queen to seal the nest chamber with silk. Brood development strongly depends on temperature. Below 20 °C and above 35 °C brood development stalls mostly due to temperature sensitivity of the pupae, which inhibits colony growth in seasonal climates during the cooler months. When the queen dies, the workers activate their ovaries and produce a last set of male brood before the colony shrinks as worker numbers reduce over the following months.

New sexuals are produced during the wet season and released synchronously after heavy rainfall to take part in aerial swarm mating. The growth of a colony is highest during the wet season, when temperature, humidity and food availability (after flowering and leaf flushing of the trees and subsequent rise in the abundance of prey) are most suitable. Survival rates of new colonies are very low (Greenslade 1971a) because of high intra- and interspecific competition and disease. Those colonies surviving the founding stage will develop into colonies consisting of at least half a million individuals occupying several good-sized trees. The expansion of a colony is generally limited by the availability of trees and competition with other colonies of their own or a few other dominant ant species (Hölldobler and Wilson 1990; Hölldobler 1983). During wet season, colonies tend to spread out through their territory and then contract to fewer and bigger nests during the dry months. While larvae are present at all times of the year, pupae are absent in the cooler and drier season. The continuous presence of larvae may be favoured by selection to weave at all times (Lokkers 1990; Crozier et al. 2010).

### 11.1.4 Colony and Nest-Weaving Behaviour

*Oecophylla* are highly social insects that live in large colonies whose size may be in the range of 10,000 to 500,000 workers (Vanderplank 1960; Holldobler and Wilson 1978; Lokkers 1990). Each colony consists of queen, males, small workers and large workers in several nests. There is a pronounced size dimorphism between the two sexes. The queen is a lot larger and heavier than the males and is easily recognised by their bigger size and a big belly to produce many eggs. Males are much smaller than the queen and have a blackish body and a shorter life span. Their only task is to mate with the queen after which they die. The workers of *O. smaragdina* are highly polymorphic (Wilson 1953). The two worker castes show a clear division of labour with the minors staying inside the nest (caring for brood, etc.) and the majors performing outdoor tasks (e.g. foraging, defence) (Hölldobler and Wilson 1977a).

The colony of weaver ants is polydomous, where spatially discrete subgroups within a more or less genetically homogeneous colony occur. A colony is polydomous when individuals (workers and brood) of its constituent nests function as a social and cooperative unit and are regularly interchanged among nests. Unlike most polydomous ant species that have more than one reproductively active queen, mature *Oecophylla* colony is strictly monogynous with a single queen irrespective of the number of nests and strength of the colony. Age polyethism was also observed in weaver ant colonies where older workers are stationed in barrack nests along the colony periphery and act as guards and defenders (Hölldobler 1983). The loss of the single gravid queen signals the demise of the entire colony (Greenslade 1971a, Holldobler and Wilson 1977a; 1983). Average life span of colonies is eight years (Greenslade 1971b).

One of the most important characteristics of weaver ants is their nest-weaving behaviour, for which *Oecophylla* has been named the ‘weaver ant’ (Dejean 1990). The weaver ants have become experts in finding the most suitable leaves to construct a nest. They prefer plants with large, flexible leaves or with abundant smaller leaves and show less preference to trees which shed their leaves seasonally. To avoid disturbance, ants often prefer higher trees. However, when left undisturbed, smaller trees are also

**Fig. 11.1** Main nest of a weaver ant colony on a mango tree

preferred. The leaf nests are constructed by binding the leaves still attached to the nesting plant which are glued together by silken threads (Fig. 11.1) spun by the last instar larvae which the workers hold between their mandibles and move back and forth by spinning shuttles (Wilson and Holldobler 1980). Cooperation between larvae and adults is also seen in the weaving mechanism where larvae produce silk threads and adults use them in weaving.

Workers form living chains to cross gaps and bring leaves together; additional workers then hold final instar larvae in their mandible at the work sites and use the silk produced by the larvae to fasten together leaves to form the nest walls (Hölldobler and Wilson 1977b). Together, workers and larvae act as a living moveable sewing machine, a skill considered to play a significant role in the ability of *Oecophylla* to achieve such large colonies and ecological dominance (Hölldobler and Wilson 1990). One of the most distinctive behavioural attributes which can be shown as an example of high degree of cooperation is the manner in which nest-mates of multiple castes (including larvae) cooperate to construct arboreal silk nests. When a new nest is required, individual workers scout for suitable clusters of leaves, which they grab with their mandibles and attempt to draw together. Other workers are attracted to the site, presumably by the success of the first workers, and join the effort. Leaves in close proximity can be drawn together through the actions of multiple individuals aligning themselves along leaf perimeters and pulling the edges together. A large gap can be bridged by chains of ants, formed by each ant clasping the petiole of the ant in front with her mandibles. Chains of at least 10 ants, spanning over 5 cm in length, can be constructed which appears to be unique to the weaver ant genus (Crozier et al. 2010).

Weaver ants are highly territorial in nature. *Oecophylla* ants produce visible droplets from rectal sac fluids (anal spots) and deposit them on the substrate where they forage and they serve as territorial pheromones distributed

throughout the home range of the colonies. They mark their entire territories with visible and invisible trail pheromones produced in the rectal gland (Holldobler and Wilson 1978; Holldobler 1983; Beugnon and Dejean 1992). *Oecophylla* pheromones may serve as a long-lasting warning signal to herbivores, and the persistent nature of these pheromones lasting weeks in the field and even months in the laboratory (Beugnon and Dejean 1992) further strengthens the warning potential of these chemicals. Well-established trails between nests marked with rectal gland secretions remain effective for about three days (Jander and Jander 1979). Localised alarm and attack responses are invoked by volatile chemicals from the mandibular gland in the head, and poison and Dufour's glands in the abdomen (Bradshaw et al. 1975, 1979). Drops of faecal material are deposited randomly throughout the colony territory; workers can distinguish alien from friendly terrain using these territorial marks (Holldobler and Wilson 1977a, 1978).

## 11.2 Oecophylla as a Biocontrol Agent

*Oecophylla* ants have been acknowledged as one of the most efficient groups of ants in controlling plant pests (Way and Khoo 1992; Van Mele 2008). Competitive dominance of weaver ants over many other ant species affects the entire arboreal ant community. *Oecophylla* colonies defend mutually exclusive territories against conspecific colonies or competing ant species while permitting co-occurrence of certain other ant species (Hölldobler 1983). The earliest known example of biological control with *O. smaragdina* to control insect pests was in citrus orchards by Chinese farmers in 340 AD (Van mele and Cuc 2003). Use of weaver ants in agriculture has been practised in Asia especially in Vietnam and China in the early twentieth century (Van mele 2008). Ant husbandry practices involving collection and selling of ant nest and construction of bamboo bridges between citrus trees existed in Burma (Needham et al. 1986). Though the biocontrol potential of weaver ants was known to farmers of Asia, much of it was not known in Africa during the period. Scientists in Africa even considered weaver ants as injurious insects (Van mele 2008). *Oecophylla longinoda* was considered a nuisance to coffee pickers and pruners who avoided infested trees, and baits were used against *Oecophylla* in Congo (Steyaert 1946). Pioneering work of Way (1953, 1954) on *Oecophylla* provides great details on the biology, behaviour and ecology of the weaver ant and suggested how this knowledge can be used to improve the effectiveness of weaver ants in controlling pests.

Recent studies on the role of ants in pest management revealed that the tree-inhabiting weaver ant *Oecophylla smaragdina* known as the 'living pesticide' (Konishi and Itô 1973; Hölldobler and Wilson 1990) is an effective biocontrol agent against a wide range of potential pests and is utilised in biological control of numerous crop pests. Though they are predominantly arboreal, the highly organised aggressive predatory behaviour combined with extensive foraging throughout the area occupied by a colony explains its success in killing or driving away many pests or potential pests (Van mele 2000; Way and Khoo 1992). In addition to the predation, they also deter insect pests through direct or indirect encounter and thus effectively protect a wide variety of tree crops (Peng and Christian 2004; Van mele 2008; Offenberg et al. 2004) from insect pests.

In tropical tree crops and forest trees, weaver ants control over 50 pest species, including caterpillars, bugs, beetles, flies and thrips (Way and Khoo 1992; Peng et al. 2004). They have been identified as the efficient biocontrol agent of the following insect pests: seed weevil (*Sternochetus mangiferae*) (Peng and Christian 2007), red-banded thrips (*Selenothrips rubrocinctus*) (Peng and Christian 2004), mango leafhopper (*Idioscopus nitidulus*) (Peng and Christian 2005), the fruit-spotting bug (*Amblypelta lutescens*) (Peng et al. 2005), fruit fly (*Bactrocera jarvisi*) (Peng and Christian 2006) and mango seed weevil (*Sternochetus mangiferae*) (Peng and Christian 2007) in mango; citrus leafminer (*Phyllocnistis citrella*

**Fig. 11.2** Aggregated *L. tristis* beetles on the wall of a residential building

Stainton), aphids (*Toxoptera aurantii* and *T. citricidus*), citrus stink bug (*Rhynchocoris humeralis*) and citrus red mite (*Panonychus citri* and *Phyllocoptruta oleivora*) (Van Mele and Cuc 2000) in citrus; fruit-spotting bug (*Amblypelta lutescens*), tea mosquito bug (*Helopeltis pernicialis*), the shoot borer (*Penicillaria jocosatrix*) and leaf roller (*Anigraea ochrobasis*) (Peng et al. 1995) in cashew; capsids (*Distantiella theobroma* and *Sahlbergella singularis*) (Majer 1976) in cocoa; coreid bug (*Pseudotheraptus* (Way 1953), *Amblypelta cocophaga* (Greenslade 1971a) and *Pseudotheraptus wayi* (Vanderplank 1960)) in coconut; and shoot borer (*Hypsipyla robusta*) in mahogany (Peng et al. 2011).

### 11.2.1 Weaver Ants and Biocontrol of *Luprops tristis*

*Luprops tristis* (Coleoptera; Tenebrionidae), popularly known as Mupli beetle (Fig. 11.2), is an inconspicuous rubber litter-dwelling detritivorous beetle found in the rubber plantation belts in south India. Seasonal mass invasion of the beetle into residential buildings prior to the onset of southwest monsoon showers, nocturnal movements and subsequent aggregation in prolonged state of dormancy render them a serious nuisance pest (Sabu et al. 2008; Sabu and Vinod 2009).

Despite three decades of their widespread presence as a nuisance pest with astonishing abundance, no efficient strategies for controlling the population build-up of *L. tristis* have been developed and its presence in residential buildings during the rainy season and in the bottom layers of rubber litter during post rainy season makes insecticide-based control a tough task. The presence of many alternate host plants in addition to rubber indicates that it has the potential to spread into non-rubber belts (Sabu et al. 2012). Among the potential predators in the region, namely, house lizard (*Hemidactylus frenatus* Schlegel, 1836), huntsman spider (*Heteropoda venatoria* Latreille, 1802), domestic fowl (*Gallus gallus* Linnaeus, 1758) and weaver ants (*Oecophylla smaragdina* Fabricius, 1775), all except weaver ants are deterred by the defensive gland secretions of the beetle (Sabu et al. 2008; Aswathi and Sabu 2011).

Predatory experiments with weaver ants on *L. tristis* showed that weaver ants actively feed on the beetle despite the presence of the defensive secretion which deters most predator species (Aswathi and Sabu 2011) and has the potential to be used as an effective biocontrol agent to regulate the population build-up of *L. tristis*. Though the defensive gland secretion of *L. tristis* makes the initial attackers to move away from the beetle, the relentless confrontation by other ants from different fronts makes *L. tristis* defenceless, and the weaver ants take away the caught beetle (Fig. 11.3). It is possible that the glands become empty of secretions after the initial confrontation with initial attackers and make the beetle defenceless against the subsequent attackers. Active predation during day/night conditions indicates that

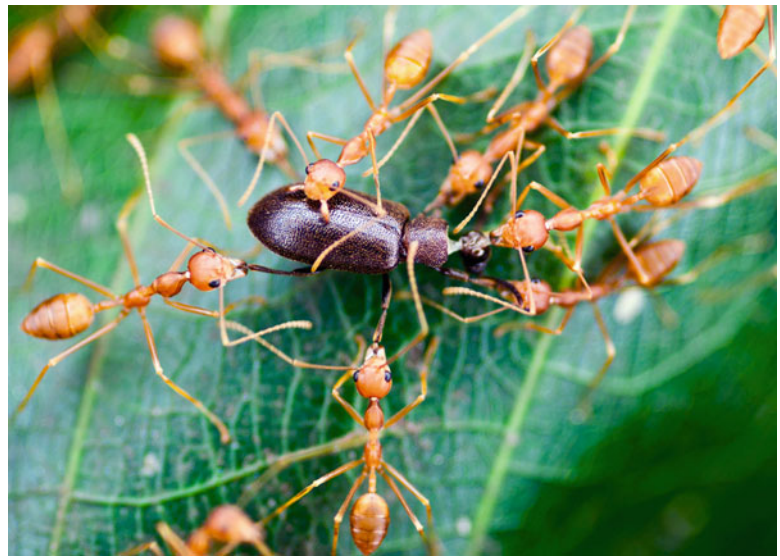

**Fig. 11.3** Predation of weaver ants *on L. tristis*

their predatory efficiency or prey-searching behaviour is not hindered by the diel periodicity in the behaviour of *L. tristis*. Also, repeated feeding on *L. tristis* by weaver ants did not lead to predator reluctance, which point out their potential as an effective biocontrol agent against the beetle (Nirdev et al. 2011).

Even though weaver ants are known as effective predator of many pests in a number of crops, it becomes the only native predator to be found effective against *L. tristis* (Aswathi and Sabu 2011). Their highly organised aggressive predatory behaviour, combined with extensive foraging throughout the area occupied by a colony, may lead to the killing or driving away of *L. tristis* as well as other potential pests inhabiting the rubber plantation litter and canopy. Nevertheless, the proposal to introduce weaver ants as a biocontrol agent of the beetle is less likely to be welcomed by the stakeholders as the weaver ant aggression has been an obstacle for its use in many parts of the world, mainly in plantations, and therefore *Oecophylla* has often been considered a pest (Way and Khoo 1992).

Negative attitude of people towards weaver ants is mainly arising from its aggression and bites which is a nuisance to the workers during harvest and other agricultural works. Unawareness of the beneficial effects of weaver ants as biocontrol agent in rubber belts will be the preliminary hurdle in its establishment as a biocontrol agent in the region. Deciduous rubber trees are without leaves for nearly a month during the leaf-shedding period of presummer season, and it would lead to lack of host plant leaves to support the weaver ant colony in monoculture rubber plantations and their disappearance from the rubber plantation. Lack of other host plants that could serve as nesting trees and would provide nectar and honey for weaver ants in the midst of monoculture rubber plantation linked to the removal of native trees that harbours the weaver ants, lesser incidence of prey resources in the canopy due to the near absence of herbivorous insects feeding on rubber leaves and litter arthropod prey resource in rubber plantation litter stands which are devoid of litter for a considerable period of time due to faster decomposition of rubber leaf litter (Vineesh 2007) are the other factors that would hinder the establishment of weaver ants in rubber plantations. Hence, introduction and maintenance of weaver ants in rubber plantations requires intercropping of rubber plants with host plant trees. Promoting intercropping of the native trees such as *Artocarpus heterophyllus* (jackfruit), *Psidium guajava* (guava), *Syzygium samarangense* (rose apple), *Citrus medica* (citron), *Annona reticulata* (custard apple) and *Mangifera indica* (mango) and mixed cropping with *Myristica fragrans*

(nutmeg), *Theobroma cacao* (cocoa), and *Coffea arabica* (coffee) that are used as nesting trees by weaver ants (Lim 2007) may solve the issue of lack of nesting trees in rubber plantations. Though weaver ant-mediated pest control is being successfully used for a variety of plantation crops and is found to increase the quality and production and reduce the application of chemical pesticides greatly, introduction of weaver ants as biological control agent for food crops has yet to be tested and proved.

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# Ground Beetles (Coleoptera: Carabidae): Their Potential as Bio-agents in Agroecosystems

12

R. Swaminathan

**Abstract**

The first step in any integrated pest management (IPM) system is a complete study of the agroecosystem. In biological agriculture, pest control depends largely on the activities of the natural enemies in the field habitat and on the use of various agricultural techniques. Technological advances have allowed productivity to increase, but not without consequences on the sustainability of the agroecosystem. In this chapter, we provided a brief account of the distribution and diversity by two case studies, prey record and biosafety of synthetic insecticides against carabids or ground beetles.

**Keywords**

Carabids or ground beetles • Distribution • Prey records • Mass production

## 12.1 Introduction

Simplification of the crop ecosystem often results in decreased predation pressure, which may cause pest outbreaks (Dempster and Coaker 1974; Potts 1977). Interaction between insects and plants is strongly influenced by the higher trophic levels, which include the predators and parasites. Augmentation of natural enemies, mass rearing and release at appropriate stage and condition are major components in IPM. Successful culturing of suitable prey in the laboratory or rearing predators and parasitoids depends on the biology, behaviour and reproductive fitness of natural enemies. In order to augment this approach in biological control, increased attention needs to be diverted towards factors involved in successful predation, prey suitability as well as survivorship. Duration of the postembryonic development, fecundity, longevity and number of prey consumed during the lifetime are of paramount importance in assessing the predatory efficiency of an individual (Ananthakrishnan 1996). The quality and quantity of nutrients of the prey influence the growth rate and survival of the predator (Ambrose and Subbarasu 1988; Ambrose et al. 1990; O'Neil and Wiedenmann 1990; Ambrose and Rani 1991). The fecundity and life table

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_12, 

characteristics, such as generation time as well as intrinsic rate of population increase (Awadallah et al. 1986), are also affected.

Generalist predators (vertebrate and invertebrate arthropod groups) are usually abundant in natural agricultural systems; however, they have more often been thought to be poor biocontrol agents. This prediction is based largely on theoretical considerations that generalist predators lack prey specificity, often have longer generation times than pests (Riechert and Lockley 1984) and interfere with other predators in addition to preying upon herbivorous pests (Polis and Holt 1992); besides, the high frequency of interference among predators, coupled with their often long development times, makes mass rearing of generalist predators economically unfeasible (DeBach and Rosen 1991). Therefore, much research has focused on identifying and manipulating characteristics of surrounding habitats to provide source populations of predators to migrate into agricultural fields (Best et al. 1981; Gravesen and Toft 1987; Nentwig 1988; Luff and Rushton 1989; Mangan and Byers 1989; Heidger and Nentwig 1989; Hance et al. 1990; Bedford and Usher 1994; Kajak and Lukasiewicz 1994; Barbosa 1998).

With a view to determine the extent to which interference among generalist predators limits their effectiveness as biocontrol agents, Snyder and Wise (1999) manipulated immigration of a guild of actively hunting generalist ground predators, carabid beetles and lycosid spiders, by intercepting them as they attempted to enter fenced 50-square-metre vegetable gardens. Immigration was blocked, allowed at the mean rate measured at the field site, or doubled. Altered immigration rates were maintained through a spring garden of cabbage, bean, eggplant and cucumber, followed by a summer garden of squash. Densities of carabids and lycosids were monitored to discover if altering their immigration rate changed their densities in the plots. Similarly, densities of other predators on the ground and in plant foliage, pest numbers and vegetable yields were monitored. Doubling the immigration rate of carabids and lycosids approximately doubled the densities of carabids inside the plots, but did not increase lycosid densities. Increasing the rate of immigration of carabids and lycosids depressed densities of non-lycosid ground spiders. In the spring gardens, manipulation of carabid and lycosid immigration did not influence numbers of predators or herbivores in the foliage and did not affect vegetable productivity. In contrast, in the summer gardens, foliage-dwelling predators were lower, pest densities were marginally lower, and squash productivity was higher in the carabid and lycosid immigration plots compared to the no-immigration treatment. Doubling carabid and lycosid immigration rate never increased the magnitude of their effects on other predators, pests or plant productivity. Predator interference limited lycosid establishment, reduced densities of other predator taxa and apparently prevented a doubling of carabid densities from having an increased impact on pest numbers. Nevertheless, despite widespread effects of predator interference, allowing immigration of lycosids and carabids increased squash productivity.

Despite theoretical misgivings, increasing evidence indicates that generalist predators can reduce pest populations in agroecosystems (Riechert and Lockley 1984; Chiverton 1986; Nyffeler and Benz 1988; Young and Edwards 1990; Wise 1993; Rosenheim et al. 1993; Nyffeler et al. 1994a, b; Lang 1997). The challenge is to reconcile the theoretical limitations of generalist predators as biocontrol agents with their reported effectiveness in some agroecosystems. The potential complexity of inter-predator interactions makes it difficult to answer these questions without conducting large-scale field experiments (Rosenheim et al. 1995).

## 12.2 Carabids: An Introduction

Carabids or ground beetles, as they are commonly known, are species rich and abundant in arable sites, but are affected by intensive agricultural cultivation. Carabids are negatively affected by deep ploughing, while enhanced by reduced tillage systems. No negative effects have

been found for mechanical weed control and even flaming. Proper organic fertilization and green manure application enhance carabid recruitment (Porhajasova et al. 2012). Intensive nitrogen amendment might indirectly affect carabids by altering crop density and microclimate. They are enhanced by crop diversification in terms of monocrop heterogeneity and weediness as well as by intercropping and the presence of field boundaries or farmscaping, although corresponding increases in their pest reduction efficacy have not yet been evidenced.

The role of arthropod predators, the carabids in particular, in natural pest control of cultivated crops has become increasingly clear. The ground beetles (Carabidae) are of great importance in the bio-regulation of insect pests, but their significance has not been assessed precisely. Most members of the family Carabidae are primarily carnivorous; larvae as well as adults are nocturnal and, hence, less well known. As early as 1883, Forbes reported aphids to be a component of the carabid diet, which was confirmed by Skurhavy (1958). The beetles exhibit polyphagy of diverse order (Davies 1953, 1959; Thiele 1977). Scherney (1959) indicated the possibility of carabid beetles being used in the biological control of crop pests and the idea that, they potentially reduce some pest populations, was corroborated by Coaker (1966). However, estimates of the effective diminution of the prey species vary considerably. Dunning et al. (1975) could not demonstrate any real effect of the different carabid numbers on aphid populations, but the results suggest that aphid numbers and virus incidence were being influenced by carabids. There has been increasing attention to carabids as predators of aphids (Hengeveld 1980; Hance 1987; Sunderland et al. 1987; Chiverton 1986, 1987; Helenius, 1990). The value of ground beetles to manage red cutworms (Frank 1971) and the black cutworm, *Agrotis ipsilon*, is also reported. The adult *Pterostichus melanarius* (Ill.) has been recorded to consume aphids, carrot weevil, bean weevil and codling moth in the field. In laboratory studies, *P. melanarius* can consume about 12 onion fly pupae a day. This species is very common, sometimes the most abundant, in some agricultural fields (Lys and Nentwig 1992). Many species breed in crop fields, and the fields can be a source of recruitment to the local populations. In Scandinavia, *Bembidion lampros* (Herbst) is abundant, univoltine adult overwintering species breeding in annual crops such as cereals (Wallin 1989).

## 12.3 Diversity and Distribution

Studies on the diversity and abundance of carabids in arable lands from the temperate and subtemperate zones have been well documented: 39 species of carabids were recorded from soybean with higher density and species diversity in June (Ferguson and Mc Pherson 1985), 45 species from pea (Novikov 1984), 29 species from sugar beet (Purvis and Curry 1984), 82 species from various field crops and crop rotations from the Poltava region of the former USSR (Brunner and Kolesnikov 1983), 54 species from cabbage fields (Hokkanen and Holopainen 1986), 26 species from a maize monocrop (Lövei 1982), 26 species from different field crops in Michigan (Dunn 1982) and 52 species belonging to 21 genera from the rotational intensive cropping systems of central non-chernozems in Russia. The dominant species being *Poecilus cupreus* Linnaeus forming 60.3 and 65.7 % of the total catch during 1991 and 1992, respectively (Swaminathan 1992; Isaichev and Swaminathan 1993).

In India, work on carabid faunal complex for forest ecosystems and of the Indian subcontinent is reported (Andrews 1929); however, work on carabids as bio-agents in pest management has been scanty. Rajagopal and Kumar (1988) have studied the predation potential of *Chlaenius panagaeoides* (Laferte) (Coleoptera: Carabidae) on cowpea aphid *Aphis craccivora* Koch (Homoptera: Aphididae). Rajagopal et al. (1992) have described the reproductive behaviour of certain carabid species. Vennila and Rajagopal (1999) have suggested the use of either 25 or 35 pitfall traps as optimum for assessing carabid diversity for the precise estimate of carabid species distribution. A diverse

**Table 12.1** Light trap catches of two dominant carabid genera during *kharif* 1998

| | Adult beetle genera (Numbers per week) | | Atm. temp. (°C) | | Rel. humidity (%) | | |
|---|---|---|---|---|---|---|---|
| Observation week (1998) | *Casnoidea* | *Chlaenius* | Max. | Min. | Morn. | Even | Total rainfall (mm) |
| 06–12/08 | 14 | 06 | 29.8 | 24.5 | 94 | 83 | 87.6 |
| 13–19/08 | 00 | 00 | 30.4 | 23.7 | 86 | 75 | 0 |
| 20–26/08 | 06 | 02 | 32.2 | 23.0 | 91 | 63 | 48.4 |
| 27/08–02/09 | 05 | 04 | 30.5 | 23.4 | 89 | 69 | 31.3 |
| 03–09/09 | 01 | 00 | 31.8 | 22.3 | 88 | 65 | 63.9 |
| 17–23/09 | 88 | 22 | 28.6 | 22.5 | 96 | 84 | 89.2 |
| 24–30/09 | 03 | 05 | 31.4 | 21.8 | 93 | 67 | 44.9 |
| Corr. coeff. **r** – values for *Casnoidea* | | | - 0.41 | −0.29 | +0.39 | +0.37 | +0.66 |
| Corr. coeff. **r** – values for *Chlaenius* | | | - 0.66 | −0.23 | +0.63 | +0.59 | +0.77 |

Note: *Crops associated in sampled area – green gram, black gram and soybean*

fauna of 14 carabid species belonging to 11 genera were collected during March through October 1998 (Table 12.1), of which two species were predominant: *Casnoidea indica* Thunberg and *Chlaenius viridis* Chaudoir. Adults of *C. indica* exhibited preference for the cotton aphid, consuming 250 aphids per day. Larvae of *C. viridis* were observed to prey upon the soybean leaf webber, *Lamprosema* sp. in the field, while under laboratory conditions, a single adult could consume 9-tobacco caterpillar (*Spodoptera litura* (F.)) larvae per day. An equal liking for the larvae of cotton leaf roller (*Sylepta derogata*) as prey was also observed (Swaminathan et al. 2001). Vennila and Rajagopal (2003) have classified the life cycles of 18 carabid species based on their phenology indicating 10 species as monsoon breeders having overlapping generations with adult gonad dormancy during winter and summer.

## 12.4 Conserving and Manipulating Carabids in Agroecosystems

It has become increasingly clear that ground beetles are important polyphagous natural enemies in agricultural landscapes that have the potential to suppress major insect pest species from reaching outbreak levels. Moreover, if augmented through their conservation, they will restrict minor insect pest species from becoming major ones. Many studies have analysed the importance of habitat characteristics, management practices and crop type on the conservation of carabid communities. As a general rule, while common agricultural practices such as pesticide applications and tillage frequently reduce carabid beetle abundance, organic and low-input production systems usually sustain more abundant beetle communities than conventional systems.

## 12.5 Ground Beetles' Association in Agroecosystem

The major factors conditioning the associations of ground beetles in agroecosystems are:

(a) The temporal stability of the habitat – with stable habitats providing suitable and sustainable environment.
(b) The type of tillage applied to the annual crop – lower input and reduced tillage enhance carabid diversity and abundance. Organic or biologically managed farms are more suited for Carabidae conservation.
(c) The type of crop – early crops and crops with greater cover favour carabid beetle abundance.
(d) Crop diversification through intercropping/multiple cropping, farmscaping and mulching positively affect the population build-up.

(e) Beetle activity is known to be correlated with hunger levels and availability of preferred prey.
(f) The soil hydrological regime, atmospheric humidity and temperature affect carabid diversity and numerical abundance.

## 12.6 Abundance of Predatory Carabids in Agroecosystems

Prior to taking up steps to conserve and augment these bio-agents in agroecosystems, extensive surveys to evaluate the diversity and abundance of predatory carabids in different crop ecosystems shall become necessary to identify the dominant species that can be conserved for the future. Besides, the surveys shall also enable one to know about the resident species. Studies on their biology must also be taken up for proper utilization of the species concerned as a bio-agent. Low-input farming with reduced tillage or otherwise biologically managing the farms shall become a prerequisite to enhance the predatory activity of these beneficial arthropods. Diversified cropping should be followed with good ground cover to harbour carabid beetles and their grubs for diurnal activity. Use of synthetic pesticides has to be avoided to safeguard these natural enemies.

The soil hydrological regime, soil treatment and crop cultivation determine the carabid population structure. The dominance structure and seasonal population dynamics of carabids vary according to the crop type and density or ground cover. Although the carabid beetles are well known to naturalists, there are only few papers on their food requirements. Hengeveld (1980) has reviewed the qualitative and quantitative aspects of the food of ground beetles. Efforts are therefore required to conserve and augment these non-specific epigeic predators, especially the carabid beetles to suitably fit in diversified agroecosystems based on IPM technology.

In its natural form, farmers who practice organic and other sustainable growing methods have used Bt formulations since the 1950s as a spray to kill pests without damaging beneficial and non-target insects or other wildlife. However, both the Cry1Ab and Cry1F Bt toxins produced by GM insect-resistant maize are significantly different: they are a shorter, or truncated, form of the protein. This truncated (or shortened) form is less selective than Bt sprays and therefore has potential to harm non-target insects in addition to the pests for which it is intended.

The possible effects of GM crops on entomophagous arthropods is a major concern, since these organisms play an important role in natural pest regulation and may affect the development of resistance towards the transgene product in the target pest. Thus, a good level of compatibility between GM-based strategies with biological control is necessary for a sustainable deployment of a GM crop (i.e. within an IPM framework).

Investigations on the effects of transgenic maize (*Zea mays*) expressing *Bacillus thuringiensis* toxin (Bt maize) on larval and adult *Poecilus cupreus* carabid beetles in laboratory studies showed that under no-choice trials, neonate *P. cupreus* larvae fed exclusively with *Spodoptera littoralis* caterpillars, which had been raised on Bt maize, and the mortality of carabids increased up to 100 % within 40 days. The experiment was repeated with 10-day-old beetle larvae, and Bt treatment resulted in fewer pupae than in both controls and in a higher mortality than in the *Calliphora* control. *Spodoptera littoralis* was suitable as exclusive prey in no-choice tests, at least for 40 days, although prey quality seemed to be low compared to *Calliphora* pupae. The observed effects are most likely indirect effects due to further reduced nutritional prey quality. However, direct effects cannot be excluded. In the second part of this chapter, exposure of *P. cupreus* to Bt-intoxicated prey was examined in paired-choice tests. Adult beetles were offered a choice between different prey conditions (frozen and thawed, freshly killed or living), prey types (*S. littoralis* caterpillars, *Calliphora* sp. pupae, cereal aphids) and prey treatments (raised on Bt or conventional maize). Living prey was preferred to frozen and dead prey. Caterpillars were only preferred to fly

**Table 12.2** Distribution of carabids in rotational intensive cropping systems, 1990

| Rotational cropping systems | Population abundance of carabid species (%) | | | | | | Shannon diversity index |
|---|---|---|---|---|---|---|---|
| | *Poecilus cupreus* | *Poecilus versicolor* | *Pterostichus melanarius* | *Harpalus affinis* | *Pseudophonus rufipes* | Other species | |
| Winter wheat | 59.17 | 12.68 | 15.01 | 1.89 | 2.52 | 8.73 | 1.2377 |
| Perennial clover II year | 75.14 | 17.20 | 0 | 2.40 | 1.00 | 4.26 | 0.7875 |
| Rye + *Vicia faba* | 74.12 | 12.00 | 1.25 | 7.45 | 0 | 5.18 | 0.8780 |
| Perennial clover I year | 47.75 | 10.09 | 12.61 | 5.63 | 4.95 | 18.97 | **1.4716** |
| Winter wheat | 61.44 | 13.02 | 7.60 | 1.50 | 3.00 | 13.44 | 1.1985 |
| Barley + clover | 58.7 | 12.46 | 9.56 | 3.72 | 4.24 | 11.32 | 1.2997 |
| Oats | 37.32 | 17.94 | 12.68 | 10.00 | 6.21 | 15.85 | **1.6327** |

pupae and aphids when living. Prey treatment seemed to be least important for prey selection. The tests showed that *P. cupreus* ingested caterpillars readily and there was no evidence of them avoiding Bt-containing prey, which means exposure in the field could occur (Meissele et al. 2005).

### 12.6.1 Case Study I

Investigations on the predatory carabids in rotational intensive cropping systems of the central non-chernozems of Russia during 1990–1991 revealed the occurrence of 52 species of carabid beetles belonging to 21 genera. The dominant carabid species included *Poecilus cupreus* L., *P. versicolor* (Pay), *Pterostichus melanarius* (Ill.) and *Pseudophonus rufipes* (DeG), which formed 80–85 % of the total catch, among which *P. cupreus* was predominant (60–66 %) and was recorded from all the seven rotational field crops. The diversity and abundance of predatory carabids significantly differed among the treatments; however, the maximum species diversity of carabids was recorded from perennial crops and winter wheat.

The seasonal population dynamics of ground beetles depended upon the few dominant species. In winter wheat, *P. cupreus*, *P. versicolor*, *Amara familiaris* (Duft.) and *P. rufipes* dominated with maximum numbers in the last week of May. In perennial clover as well as the other field crops, *P. cupreus*, *P. melanarius* and *P. rufipes* dominated at different periods of vegetative growth depending upon the availability of suitable prey. The Shannon diversity indices were the highest for the crop of oats followed by that for perennial clover (I year) during 1990 (Table 12.2) and similarly for perennial clover (I year) followed by barley with clover during 1991 (Table 12.3).

The dominant predatory carabids (*P. cupreus*, *P. versicolor*, *A. familiaris* and *P. rufipes*) specialized as zoophagous species that could be observed from their gut contents, which contained chitinous undigested parts of mostly arthropod prey (aphids, elaterids, true bugs and dipteran flies). Impact of commonly used pesticides (herbicides, Dialen and Lontrel; insecticides, Basudin, Fenvalerate, Cypermethrin and Decamethrin) at recommended doses on the carabid population showed both the herbicides to be safe to the carabids, while, among the insecticides, Basudin was the most toxic to all carabids under laboratory investigations as well as the mini-field trials with 80–100 % mortality after 7 days. Among the synthetic pyrethroids, Cypermethrin was relatively more toxic to the carabids, and Fenvalerate was the least toxic (Swaminathan 1992; Isaichev and Swaminathan 1993; Swaminathan and Isaichev 2000).

**Table 12.3** Distribution of carabids in rotational intensive cropping systems, 1991

| Rotational cropping systems | Population abundance of carabid species (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Poecilus cupreus* | *Poecilus versicolor* | *Pterostichus melanarius* | *Harpalus affinis* | *Pseudophonus rufipes* | Other species | Shannon diversity index |
| Barley | 58.81 | 2.35 | 5.85 | 7.76 | 3.42 | 21.81 | 1.2123 |
| Winter wheat | 69.01 | 3.65 | 3.17 | 7.63 | 1.96 | 14.58 | 1.0404 |
| Winter wheat | 74.14 | 1.34 | 8.52 | 3.74 | 2.78 | 9.48 | 0.9353 |
| Perennial clover II year | 58.58 | 4.12 | 6.71 | 4.47 | 8.21 | 17.91 | 1.2781 |
| Barley + clover | 52.03 | 1.54 | 17.44 | 2.92 | 7.12 | 18.95 | **1.3153** |
| Perennial clover I year | 50.44 | 1.42 | 9.17 | 6.11 | 7.13 | 25.73 | **1.3331** |
| Oats + *Vicia faba* | 78.55 | 4.12 | 2.34 | 2.28 | 5.75 | 6.96 | 0.8448 |

### 12.6.2 Case Study II

In the tropics and subtropical habitats, carabids are in plenty during the monsoon period from June through September and again in March and April. Usually, there is a significant increase in their populations after the monsoon rains. In field experiments on pulses (green gram, black gram) and oilseeds (soybean and groundnut) at Udaipur, Rajasthan, the light trap (at crop height level) collections yielded 14 carabid beetles belonging to 11 genera, which were identified as follows: *Abacetus* sp., *Bembidion* sp., *Brachinus limbicollis* Chaud., *Callistomimus chalcocephalus* Wied., *Casnoidea indica* Thunb., *Casnoidea* sp., *Chlaenius viridis* Chaud., *Chlaenius vulneratus* Dej., *Dioryche* sp., *Clivinia attenvirta* Herbst., *Pheropsophus lineifrons* Chaud., *Platymelopus* sp., *Stenolophus* sp. and *Stenolophus 5-pustulatus* Wied.

Adults of *Casnoidea indica* Thunberg exhibited great preference for *Aphis gossypii* on cotton leaves. The captive adults consumed more than 250 aphids per day. The occurrence of this beetle was more frequent. They are very agile and are good climbers reaching shoot tips in search of prey. The incidence coincides positively with the event of rains, and the species holds great promise for biocontrol of jassids and aphids on account of their preponderance and voracious feeding observed. Adults of this species have been observed feeding in the early hours on jassid nymphs (*Empoasca* sp.) on the underside of green gram leaves and, on the aphid, *Aphis craccivora*, infesting the developing pods during *kharif* 1998. Observations on the feeding potential of adult *Chlaenius viridis* Chaudoir showed that a single adult beetle could consume an average of 5.66 to 9.16 tobacco caterpillar (*Spodoptera litura* (Fab.)) larvae per day (Swaminathan et al. 2001).

## 12.7 Conclusion

Predatory carabids are considered as important biological control agents distributed in pulses (green gram, black gram) and oilseeds (soybean and groundnut). More than 14 carabid beetles belonging to 11 genera were recorded from India. *Spodoptera littoralis* and *P. cupreus* caterpillars, *Calliphora* sp. pupae and cereal aphids are the common preys for these predators. Synthetic pyrethroids, Cypermethrin, were relatively more toxic than Fenvalerate. Hence, this group of predators can be utilized in pest management.

## 12.8 Future Focus

Thorough knowledge about the distribution and diversity of predatory carabids, biology and life table studies are imperative and can be

undertaken in relation to biotic and abiotic factors in order to utilize them in pest management programme; mass production can be undertaken and laboratory, field cage and filed studies are imperative.

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# Eco-friendly Control of Three Common Mosquito Larvae Species by Odonata Nymphs

13

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**Abstract**

This chapter revealed the efficacy of three predominant dragonfly species found in a natural population where the survey of mosquito population was conducted. Nymphs of dragonflies belonging to family Libellulidae, *Neurothemis fluctuans*, *Orthetrum sabina*, and *Orthetrum chrysis*, were used as predators on the IV instar of mosquito larvae, *Aedes albopictus*, *Aedes aegypti*, and *Culex quinquefasciatus*. The daily feeding rates varied among predators and mosquito species. The mean numbers of mosquito larvae consumed by the predators were different between the mosquito species. *Aedes aegypti* was the most preferred prey for *Orthetrum sabina* and *Neurothemis fluctuans*. However, *Orthetrum chrysis* consumed more of *Culex quinquefasciatus* in contrast to other prey species. Feeding activities peaked during light-on in contrast to light-off. The results of variation factors that influenced the predation activities were significant and further discussed in this chapter. The factors that were assessed in the experiments included the water volume, predator species, predator density, and prey density and species. This chapter lends support to the potential use of Odonata species as an eco-friendly method of mosquito population eradication.

**Keywords**

Biocontrol • Dragonflies • Larvae • Libellulidae • Mosquitoes and predation activities

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## 13.1 Introduction

Mosquitoes play a significant role from the standpoint of human welfare because the females are bloodsucking in which many species bite people and at the same time serve as vectors in transmission of several fatal human diseases

K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_13, 

(Triplehorn and Johnson 2005). A number of *Aedes* (*Stegomyia*) spp. may act as a vector in these situations, depending on the geographic area, including *A. aegypti*, *A. albopictus*, *A. polynesiensis*, and other members of the *A. scutellaris* group. The serious transmission cycle from a public health standpoint is the urban endemic/epidemic cycle in large urban centers of the tropics. Humans are infected with dengue viruses when bitten by an infective mosquito. *A. aegypti*, the principal vector of dengue, is small in size with black-white body striations and a highly domesticated tropical mosquito that prefers to lay eggs in artificial containers commonly found in and around homes, for example, flower vases, old automobile tires, buckets, and trash that collect rainwater in general (Gubler 1998). In summary, demographic and societal changes, decreasing resources for vector-borne infectious disease prevention and control, and changes in public health policy have all contributed to increased epidemic dengue activity (Gubler 1998).

Normal chemical controls that were used in the eradication of adult mosquitoes were fogging with DDT and larvicide, for the larvae stages (Jatanasen 1997). Larviciding, the application of chemicals to kill mosquito larvae or pupae in the water, is generally more effective and target specific than applying chemicals to kill adult mosquitoes (adulticiding) but seemed less permanent. Adulticiding is usually the least efficient mosquito control technique. However, it is the only way to kill adult mosquitoes and is the last line of defense in reducing mosquito populations. Adulticides are typically applied as an ultralow-volume (ULV) spray where small amounts of insecticides dispersed by either truck-mounted equipment or aircraft. Adulticides labeled for mosquito control included the organophosphates malathion and naled, some natural pyrethrins, and synthetic pyrethroids (permethrin, resmethrin, and sumithrin). Insecticide selection and time of application should be based on the distribution and behavior of the target mosquito species (Dykstra 2008). Many synthetic insecticides are widely used for controlling adult and larval mosquito populations. However, the harmful effects of chemicals on nontarget populations and the development of resistance to these chemicals in mosquitoes along with the recent resurgence of different mosquito-borne diseases as reported by Milam et al. (2000) have prompted many researches to explore alternative, simple, sustainable methods for the potential control of mosquitoes.

Utilizing biological organisms to control mosquitoes had been proven, not only to be eco-friendly but as well constitute means by which more effective and sustainable control can be achieved. Control of mosquito larvae with biological agents that are their competitors and predators is more convenient and alleviates the need for frequent chemical applications (Kumar and Hwang 2006). There are various organisms that can act as biocontrol agents to control mosquito populations, thus avoiding the use of chemicals that are harmful to the environment. It is desirable to use biocontrol agents that can adapt to the mosquito breeding habitats, which are found naturally, and pose no danger to humans living in the area (Rishikesh et al. 1988, Spielman et al. 1993). Many biocontrol agents are able to disperse by themselves enabling them to spread and build up viable populations (Caltagirone 1981, Bellows 2001, Headrick and Goeden 2001).

Medlock and Snow (2008) identified five categories of possible mosquito habitats: permanent freshwater, temporary woodland pools or flooded habitats, brackish water salt marshes, artificial container habitats, and phytotelmatas. Each of these natural or artificial places would have their specific food webs including the mosquitoes populating these habitats that are preyed upon by varied predator types. The tree holes and bamboo nodes which are in the phytotelmata category are specialized habitats known to be exploited by *Aedes* mosquitoes (Yates 1979), not easily reached by normal predators, but there had been reports by Corbet (1999) and Juliano and Gravel (2002) that dragonflies were able to disperse to these specialized niche areas for oviposition behavior.

Dragonflies (Order: Odonata) are known not only for their dispersal capability but also for

their predaceous behavior as adults as well as immature stages. The females will seek water bodies to lay their eggs, and once hatched the Odonata larvae are voracious and known to be important predators of mosquito larvae living in sympatric. Water bodies that are utilized by females to lay eggs are not limited to large or flowing waters but instead could be confined in stagnant aquatic habitats, man-made or natural, that is simultaneously exploited by container-dwelling mosquitoes. The dragonflies and damselflies are true enemies of mosquitoes as the odonate larvae are able to prey on the mosquito larvae (Breene et al. 1990) and the adults are efficient predators of airborne adult mosquitoes. One example of a dragonfly that had been investigated by Singh et al. (2010) as biocontrol agent was the larvae of *Brachythemis contaminata* (Libellulidae) that efficiently preyed upon the larvae of *Anopheles stephensi, Culex quinquefasciatus*, and *Aedes aegypti*. The frequently cited success story of dragonflies as biocontrol agents was the work of Sebastian et al. (1990) that was conducted in a country, formerly known as Myanmar (now Burma). They released dragonfly larvae into water held containers in homes where there were known to accommodate *Ae. aegypti* mosquitoes. The resulting effect was the rapid disappearance of mosquito larvae with the introduction of the dragonfly predators in which led to a strategic management program of mosquito control by a systematic release of dragonfly larvae during the monsoon season (the time when dengue fever was being transmitted by the mosquito vectors). There was an impressive drop in mosquito infestations that would not be achieved even with the traditional use of chemical insecticidal treatments.

Although odonate larvae had not been investigated as much as the utilization of guppies or other predaceous aquatic insects as mosquito biocontrol, but their long life cycle, high predation capacity and sharing of habitats with mosquito immatures as well as adults made them highly appropriate for consideration as biological control agents.

## 13.2 Methodology Followed

The species of dragonfly nymphs that were used in these experiments came from the family group of Libellulidae, known to thrive in stagnant waters: *Orthetrum chrysis*, *Orthetrum sabina*, and *Neurothemis fluctuans* which were aptly the dominant species found in the natural study areas where mosquito monitoring studies were conducted, the work reported elsewhere (Saleeza et al. 2011). All the individuals used in the experiments were measured for their body lengths and widths using a digital caliper prior to experiments to ensure constant or standard selection for species group's body size. The mosquito larvae and their predator dragonfly nymphs were maintained and kept in laboratory aquariums separately.

Three species of dragonfly nymphs were exposed with all three species of mosquito larvae *Ae. albopictus*, *Ae. aegypti*, and *Cx. quinquefasciatus* within their individual isolated aquaria. Nine aquaria were used which contained pond water and were oxygenated using air pumps. During the experiment, three species of dragonfly nymphs *Orthetrum chrysis*, *Orthetrum sabina*, and *Neurothemis fluctuans* were allowed to feed on 100 IV instar mosquito larvae of *Ae. albopictus*, *Ae. aegypti*, and *Cx. quinquefasciatus*. During the duration of experiments, the water temperatures ranged from 24 to 29 °C, pH at levels of 6.1–6.3, and dissolved oxygen maintained at 5.2–6.3 mg/l. The number of mosquito larvae consumed by each of the dragonfly nymphs was counted at every 3 h interval for the total period of 24 h, in three replicates. The duration of time taken for first attack by each dragonfly nymph to approach or consume the prey was recorded. The numbers of mosquito larvae ingested by the dragonfly nymphs were obtained by pouring the aquaria water through a fine mesh sieve to collect all of the mosquito larvae that were not consumed and immediately transferred to a white pan for counting. After each 3 h interval, the aquaria were replenished

with the number of larvae that were eaten, along with the same volume of water, to maintain the same prey density. This experiment was conducted three times on three separate days (n = 3) with the same number of nymphs for accuracy. After 24 h, all remaining mosquito larvae and dragonfly predators were removed from the aquaria. These mosquito larvae and dragonfly nymphs were not used in subsequent experiments. The active period of dragonfly feeding on mosquitoes was determined by exposure to light and dark hours, that is, 12 h in the daylight and 12 h in the nighttime. The experiments conducted revealed the prey-predation relationships, feeding rate, predators active foraging times, as well as the exposures to all three species of the mosquitoes: *Ae. albopictus*, *Ae. aegypti*, and *Cx. quinquefasciatus* would enable preferred prey choice to be determined.

In a separate experiment to assess the predation efficiency when exposed to various conditions, a total of 36 aquaria were used, according to the protocols as listed below. This experiment was conducted in three replicates: Aquaria A, *Orthetrum chrysis* (1 × 1 × 100), single-dragonfly nymph with 1 L of water volume and 100 IV instar of mosquito larvae; Aquaria B, *Orthetrum chrysis* (1 × 2 × 100), single-dragonfly nymph with 2 L of water volume and 100 IV instar of mosquito larvae; Aquaria C, *Orthetrum chrysis* (2 × 1 × 100), two-dragonfly nymph with 2 L of water volume and 100 IV instar of mosquito larvae; Aquaria D, *Orthetrum chrysis* (1 × 1 × 200), single-dragonfly nymph with 2 L of water volume and 200 IV instar of mosquito larvae; Aquaria E, *Orthetrum sabina* (1 × 1 × 100), single-dragonfly nymph with 1 L of water volume and 100 IV instar of mosquito larvae; Aquaria F, *Orthetrum sabina* (1 × 2 × 100), single-dragonfly nymph with 2 L of water volume and 100 IV instar of mosquito larvae; Aquaria G, *Orthetrum sabina* (2 × 1 × 100), two-dragonfly nymph with 1 L of water volume and 100 IV instar of mosquito larvae; Aquaria H, *Orthetrum sabina* (1 × 1 × 200), single-dragonfly nymph with 1 L of water volume and 200 IV instar of mosquito larvae; Aquaria I, *Neurothemis fluctuans* (1 × 1 × 100), single-dragonfly nymph with 1 L of water volume and 100 IV instar of mosquito larvae; Aquaria J, *Neurothemis fluctuans* (1 × 2 × 100), single-dragonfly nymph with 2 L of water volume and 100 IV instar of mosquito larvae; Aquaria K, *Neurothemis fluctuans* (2 × 1 × 100), two-dragonfly nymph with 1 L of water volume and 100 IV instar of mosquito larvae; and Aquaria L, *Neurothemis fluctuans* (1 × 1 × 200), single-dragonfly nymph with 1 L of water volume and 200 IV instar of mosquito larvae.

## 13.3 Results

Figure 13.1 showed the number of mosquito larvae species consumed by three species of Odonata *Neurothemis fluctuans*, *Orthetrum sabina*, and *Orthetrum chrysis*. Overall, *Orthetrum sabina* consumed the highest number of mosquitoes. Both *Neurothemis fluctuans* and *Orthetrum sabina* preferred *Ae. aegypti* in contrast to *Orthetrum chrysis* which was skewed toward feeding on *Cx. quinquefasciatus* larvae. Overall, the dragonfly species showed daylight active feeding in contrast to dark hours (t test: $T(7,9) = 2.80$, $p = 0.01$) and this contrast was most apparent in *Aedes aegypti* (t test: $T(1,3) = 27.02$, $p = 0.01$) when compared to dragonflies consumption on other prey species.

Several interesting outcomes were apparent in the experimental setup where three species of Odonata were exposed and tested with multiple variables in terms of differences in mosquito species, water volume, number of predators, and mosquito densities. Overall, the predation activities that had significant result outcomes were for the differences in prey densities and when increased number of predators were present, resulting in obvious competition between them [multiple correlation coefficient (R): $Y = 72.44–25.92X1 + 14.22X2 = 0.72$, where Y = predation, X1 = mosquito densities, and X2 = number of predators]. Table 13.1 shows the regression equation for the experiment with different species of Odonata. Here, when the

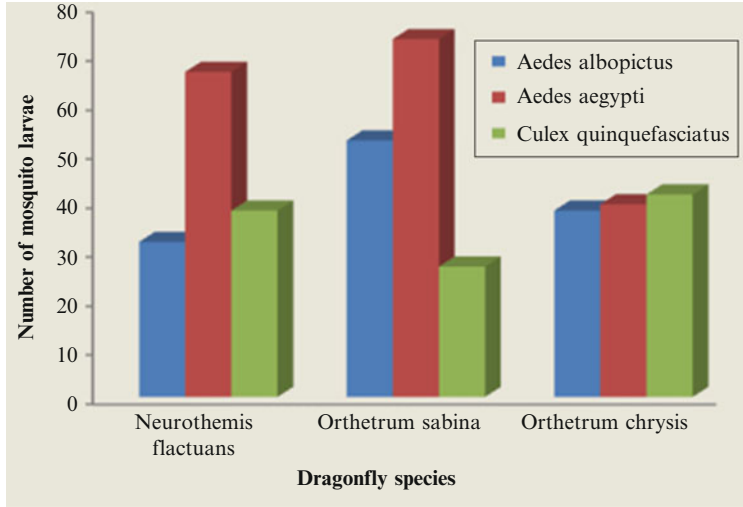


**Fig. 13.1** Number of three types of mosquito larvae species consumed by three species of dragonfly predators

**Table 13.1** Mean number ± SE (standard error) of predation by the different Odonata species on the prey, *Aedes aegypti*, for the different variables: water volume, number of predator counts, and mosquito densities

| Predator | Mosquito species | Water volume | No. of predators | Mosquito densities | Predation: mean number ± SE |
|---|---|---|---|---|---|
| *Neurothemis fluctuans* | *Aedes aegypti* | 1 | 1 | 100 | 64.67 ± 5.55 |
| | | 1 | 2 | 100 | 75.00 ± 8.08 |
| | | 1 | 1 | 200 | 79.33 ± 2.03 |
| | | 2 | 1 | 100 | 36.33 ± 1.33 |
| *Orthetrum sabina* | *Aedes aegypti* | 1 | 1 | 100 | 58.67 ± 1.20 |
| | | 1 | 2 | 100 | 88.00 ± 6.24 |
| | | 1 | 1 | 200 | 85.00 ± 7.09 |
| | | 2 | 1 | 100 | 38.67 ± 1.33 |
| *Orthetrum chrysis* | *Aedes aegypti* | 1 | 1 | 100 | 43.67 ± 3.53 |
| | | 1 | 2 | 100 | 70.00 ± 6.81 |
| | | 1 | 1 | 200 | 70.67 ± 4.91 |
| | | 2 | 1 | 100 | 38.67 ± 2.33 |

predators were *Orthetrum sabina* ($Y = 66.22–27.66\ X1 + 23.77\ X2$; $R = 0.82$) and *Orthetrum chrysis* ($Y = 61.00–19.88\ X1 + 16.33\ X2$; $R = 0.65$), the significant factors that influenced predation performance were water volume and number of predators. However, for *Neurothemis fluctuans* ($Y = 94.370–31.07\ X3$; $R = 0.77$), it was the prey densities that significantly affected predation activities, where there is higher predator feeding rate with increased prey densities ($R = 0.77$).

Tables 13.1, 13.2, and 13.3 reveal the mean number of predation activities ± SE by three different Odonata species on the three different types of prey (different mosquito species) with variations in water volume, number of predators, and mosquito densities. For the predators, *Orthetrum sabina* and *Orthetrum chrysis*, they showed increased predatory performances with high water volume and in contrast lowered their performances when in the presence of competitors (two individuals present). Interestingly, *Neurothemis fluctuans* predatory behaviors were affected by the increment of prey densities where they showed frenzy feeding acts with higher prey densities, from 100 to 200 mosquito larvae, and the larvae consumption increased until satiation levels were achieved.

**Table 13.2** Mean number ± SE (standard error) of predation by the different Odonata species on prey, *Aedes albopictus*, for the different variables: water volume, number of predator counts, and mosquito densities

| Predator | Mosquito species | Water volume | No. of predators | Mosquito densities | Predation: mean number ± SE |
|---|---|---|---|---|---|
| *Neurothemis fluctuans* | *Aedes albopictus* | 1 | 1 | 100 | 57.67 ± 3.48 |
| | | 1 | 2 | 100 | 63.67 ± 4.81 |
| | | 1 | 1 | 200 | 73.33 ± 2.96 |
| | | 2 | 1 | 100 | 33.33 ± 1.20 |
| *Orthetrum sabina* | *Aedes albopictus* | 1 | 1 | 100 | 49.00 ± 1.53 |
| | | 1 | 2 | 100 | 83.00 ± 3.60 |
| | | 1 | 1 | 200 | 78.67 ± 3.48 |
| | | 2 | 1 | 100 | 34.33 ± 1.76 |
| *Orthetrum chrysis* | *Aedes albopictus* | 1 | 1 | 100 | 34.00 ± 1.73 |
| | | 1 | 2 | 100 | 64.33 ± 6.89 |
| | | 1 | 1 | 200 | 48.33 ± 5.92 |
| | | 2 | 1 | 100 | 31.33 ± 2.03 |

**Table 13.3** Mean number ± SE (standard error) of predation by the different Odonata species on prey, *Cx. quinquefasciatus*, for the different variables: water volume, number of predator counts, and mosquito densities

| Predator | Mosquito species | Water volume | No. of predators | Mosquito densities | Predation: mean number ± SE |
|---|---|---|---|---|---|
| *Neurothemis fluctuans* | *Cx. quinquefasciatus* | 1 | 1 | 100 | 42.67 ± 2.333 |
| | | 1 | 2 | 100 | 56.33 ± 4.410 |
| | | 1 | 1 | 200 | 57.00 ± 2.309 |
| | | 2 | 1 | 100 | 27.00 ± 1.155 |
| *Orthetrum sabina* | *Cx. quinquefasciatus* | 1 | 1 | 100 | 41.67 ± 3.712 |
| | | 1 | 2 | 100 | 87.33 ± 8.192 |
| | | 1 | 1 | 200 | 61.00 ± 5.132 |
| | | 2 | 1 | 100 | 31.00 ± 2.309 |
| *Orthetrum chrysis* | *Cx. quinquefasciatus* | 1 | 1 | 100 | 61.33 ± 1.202 |
| | | 1 | 2 | 100 | 87.00 ± 6.658 |
| | | 1 | 1 | 200 | 86.67 ± 4.256 |
| | | 2 | 1 | 100 | 42.67 ± 1.202 |

## 13.4 Discussions

Investigations on aspects of biocontrol against mosquito larvae had been well documented, and the interests were not only confined to the tropical areas. A number of different predators were studied for their potentials as biological control agents, for example, *Rhantus sikkimensis* and larvae of *Toxorhynchites splendens* (Aditya et al. 2006; Aditya et al. 2007), *Diplonychus sp.* and *Anisops sp.* (Shaalan et al. 2007), odonate nymphs (Chandra et al. 2006; Mandal et al. 2008), *Acilius sulcatus* (Coleoptera: Dytiscidae) (Chandra et al. 2008), *Mesocyclops* (Copepoda: Cyclopoida) (Marten 1990), planaria (*Dugesia bengalensis*) (Kar and Aditya 2003), diving beetles (Ohba and Takagi 2010), and guppies (*Poecilia reticulata*) (Seng et al. 2008).

Some of the investigations were useful and practical as they were conducted in both the laboratory and the field. One such work was done by Marti et al. (2006) using three species of dragonfly nymphs, *Neurothemis fluctuans*,

*Orthetrum sabina*, and *Orthetrum chrysis*, as predators against the three species of mosquitoes *Ae. albopictus*, *Ae. aegypti*, and *Cx. quinquefasciatus*. The results revealed preferential prey choices by the predators where *Orthetrum sabina* and *Neurothemis fluctuans* consumed larger quantities of *Ae. aegypti* larvae in contrast to other mosquito prey species, while *Orthetrum chrysis* preferred *Cx. quinquefasciatus*. They concluded that the predation rates from highest to lowest performance were *Orthetrum sabina* > *Orthetrum chrysis* > *Neurothemis fluctuans*. These trends were in support for the current work as reported here. Mandal et al. (2008) investigated the prey-predation abilities of a number of Odonata species nymphs against the mosquito prey, *Cx. quinquefasciatus*, where they found that the daily feeding rates of these predator species had varied capacities. The outcomes in descending values of larvae quantities per day were *I. forcipata* (64), *A. flavifrons* (57), *R. ignipennis* (45), *S. durum* (25), and *C. kashmirum* (14).

The current work reported on specific prey preferences shown by odonate predators where dragonfly nymphs of *Orthetrum sabina* and *Neurothemis fluctuans* captured more of the *Ae. aegypti* larvae in contrast to the other two mosquito species, whereas *Orthetrum chrysis* consumed more of *Cx. quinquefasciatus* larvae. Two contributing factors that influenced the selectivity of preys by the predators would be the prey's escape strategies and the predatory abilities of predators. Observations conducted in this work, showed that *Orthetrum sabina* individuals were very active and aggressive when compared to the other two species, in which they consumed the most of the *Aedes* in contrast to the *Culex* mosquito larvae. The contributing factor to this high capture rates on *Aedes* compared to *Culex* could be deduced from work done by Yee et al. (2004) where they found different strategies in the mosquito larvae feeding behavior; the former prey species spent more of their activity time trashing below the water surfaces, and *Culex* spent more time at the surfaces. This evidential stratification in foraging areas made *Aedes* to be the targeted prey for dragonfly predators since dragonflies spent most of the time stalking for preys at bottom levels making *Culex* tendency to escape predation.

Additionally, the prey posture could be the contributing factor to the high success rate of capturing *Aedes*. Kar and Aditya (2003) worked on planaria as predators for mosquitoes where they found that planarians preferred *Anopheles* larvae rather than *Culex* species. This was explained to be due to the behavior of *Anopheles* larvae, in which they frequently were at rest and had resting postures in parallel to the water surfaces; this position made them easy targets for attack by the planarians. The Culex larvae were moving actively and thus impossible for captures by predators. Their work further reported exposure to different developmental stages of prey, where planarians selected larvae more than eggs or pupa regardless of mosquito species, whether *Anopheles* and *Culex*. Interestingly, planarians avoided the active 1st instars of mosquito species and foraged more on the less active 2nd and 3rd instars. Selection of prey based on profitability was apparent in studies done by Aditya et al. (2007), who found that the *Toxorhynchites splendens* consumed more of the prey *Armigeres subalbatus* compared to *Culex quinquefasciatus* larvae which were bigger in biomass. Thus, more predation efforts were directed toward *Armigeres subalbatus* as this would be the cost-effective foraging strategy. The rate of predation, however, dropped when prey turned to pupa. Similarly, work conducted by Ghosh et al. (2005) found that the predators used in their experiments ate greater quantities of mosquito prey larvae compared to pupa.

Predatory foraging decisions were also affected by dilution factors as displayed by *Orthetrum sabina* and *Orthetrum chrysis* where their attack behaviors decreased when water volume was increased. The tendencies for preys able to escape were enhanced with increased water volume and predators were less successful in their attacks in which led to waning of feeding rates. Such findings had been earlier reported by Mandal et al. (2008) for their experiments on dragonfly larvae predated on *Cx. quinquefasciatus*. Although increasing the water volume

seemed to be of positive impact for mosquito preys in being provided with increased escape areas or routes, such factor could also be translated as increased foraging area for the predators. Such perspective was adopted by Shaalan et al. (2007), who worked on Hemiptera (*Diplonychus sp*. and *Anisops sp*.) as predators on mosquito larvae and found that the adults of *Diplonychus sp*. regardless of increased water volume foraged efficiently with quick attack movements taking advantage of the increased foraging areas.

## 13.5 Conclusion

Biocontrol has gained serious attention evident by the extensive investigations that had been supported by various biocontrol agents expressed by several authors, for example, the use of fishes were recommended by Medlock and Snow (2008) being advantageous as able to be maintained in either natural or ornamental water bodies; Nyamah et al. (2011) promoted the use of *Toxorhynchites spp*. claimed to be efficient predators to the mosquito larvae as well as being environmental friendly; Chatterjee et al. (2007) vouched the use of dragonfly larvae, *Brachytron pratense*, that was proven in his studies to be more efficient than the well-known larvivorous fishes like the *Gambusia affinis* in suppressing the mosquito larvae populations. One ideal method proposed by Kumar and Hwang (2006), in using dragonflies as biocontrol agents, would be to select bodies of water known to be breeding grounds for mosquitoes and create miniature biotopes adjacent to these water bodies in which native dragonfly nymphs could be artificially introduced and once hatched would predate on the airborne adult mosquitoes, while the female dragonflies would lay eggs in the mosquito habitats to hatch out into voracious larvae exploiting the mosquito larvae.

Although it is difficult to be definitive in the possible prospect of natural predators as effective in mosquito control, but what is obviously essential is care must be taken not to reduce their numbers by environmental manipulation or intensive use of agricultural pesticides. Direct strategies to enhance their numbers by making habitats more suitable for their continued survival and existence in the ecosystems should be the essential actions taken (Medlock and Snow 2008). The current concerns are also pertaining to what had been proposed by Kumar and Hwang (2006); in order to achieve an acceptable range of control, a sound knowledge of various attributes of interactions between a pest population and the predator to be introduced is desirable.

## 13.6 Future Focus

Further work is necessary, to determine the proper methodology for the mass rearing and augmentative release of the biocontrol agents that would make this biocontrol procedure feasible for widespread application. It is also important for understanding under what set of environmental conditions a predator will be effective in reducing mosquito populations.

**Acknowledgments** We would like to thank the Institute of Medical Research for the mosquito larvae used in the experimentation, the Putrajaya Health Office and Kuala Selangor Health Office for sampling area permissions, and Mr. Mohaiyyidin who assisted in the fieldwork. This project was funded by UMRG grant RG209-13SUS and IPPP grants PS209/2009C and PV065/2011B.

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# Spiders as Potential Ecofriendly Predators Against Pests

14

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**Abstract**

Biological control has come a long way towards adapting to the changing needs of agricultural pest suppression. Current trends in agriculture towards reduced pesticide use and ecological sustainability have led to surge of interest in spiders as potential biological control agents. This is because spiders have the capacity to exist in various conditions, with wide-ranging food webs, and are able to exploit the various stages of their prey life cycles. These habit diversifications portray them as efficient predators; the web weavers skewed towards phytophagous pests mainly from Diptera and Hymenoptera, whereas the non-web weavers foraged for foliage-dwelling pests such as Coleoptera and Homoptera. Since different spider species play different predating roles for a specific pest in its life cycle, it may be reliable to sustain the diversity of spider species within the specific area. This will be further discussed in this chapter together with our current results obtained from the botanical garden, dragon fruit, and herbal garden plantations which are suggesting some potential bio-control agents for agricultural ecosystems belonging to the family groups of Araneidae, Lycosidae, Oxyopidae, Tetragnathidae, Thomisidae, and Salticidae. We further discussed the correlation of spider existence with the crop vegetation structures and architectural features.

**Keywords**

Spiders • Biocontrol • Behavior • Diversity • Agricultural ecosystems

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## 14.1 Why Biocontrol Alternatives?

The use of natural enemies to control pests originated as early as 324 BC in China where the citrus growers used the fire ants, *Oecophylla smaragdina*, to control the populations of large boring beetles. The farmers used bamboo runways between trees to encourage movements

K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_14, 

and migrations of the ant colonies within the orchard. The ant colonies were both harvested from the wild and moved into the orchards or even in those days could be purchased. Currently, biocontrol has moved far from the days of utilizing the fire ants in the orchards.

In more recent years, biological control is considered to be as a good alternative to chemical pest control for reasons summarized as below: the high costs of pesticide expenditures in pest management, fewer options of synthetic chemical pesticides due to the banning of some compounds and the pesticide treadmill that is associated with the development of pest resistance towards the synthetic chemical pesticides, the possible target pest resurgence and outbreaks of secondary pests, and human health hazards and serious environmental concerns.

Strategies for using biocontrol can be grouped into three major categories:

First is the *classical biocontrol* or the importation method also known as the "enemy release hypothesis" – this type is known for its environment friendliness, and here, the natural enemies are deliberately introduced into a new environment so that it will become established and will regulate the pest population in a natural cycle without further intervention (Van Driesche et al. 2010). The biocontrol agents were identified from the native areas of the pests' home ranges (Shanker et al. 2012). This method is inexpensive and can be long lasting (Cullen et al. 2008). It is well suited to permanent ecosystems, such as forests, natural areas, orchards, and perennial crops. Arthropod pests that are not hidden and are less mobile have been more successfully controlled because natural enemies have easier access to them (Hill and Abang 2005). Second, the augmentation biocontrol is further divided into two types – in general, augmentation means the supplemental release of biocontrol agents boosting the naturally occurring population to control the pest over an extended period. When relatively few natural enemies are released at a critical period in a season, this is known as the inoculative release. Thus, the natural enemies will be established in the habitat and subsequently increased their population within the area (Chanthy et al 2010). This provides a more long-term and self-sustained control than the other method which is inundative release. The inundative biocontrol is when very high quantities of natural enemies are released. This is a short-term strategy directed towards rapid control of pests over a short period of time. This protocol can again be repeated if the pest populations resurged over time. Third, the conservation biocontrol differs from the entire above where the natural enemies are not released but instead the resident populations of identified predator species are conserved or enhanced. Thus the biocontrol agents are already adapted to the area as well as to target pests, making conservation of the existing population simple and effective. The basic requirements for this strategy would be that the biology, behavior, and ecology of the pests and natural enemies must be understood to ensure success and viable resulting outcomes.

In agricultural pest management the main focus should be for the conservation and enhancement of the diversity of the beneficial organisms in which pest suppression could be achieved. Biocontrol is a pest management system provided by nature to sustain existing populations in a habitat to be in equilibrium; thus, applied biocontrol has been applying the fundamental principles of nature. Shanker et al. (2012) postulated that a sustainable agriculture is known to be successful when it has ultimately given rise to reduced inputs, high biodiversity index, reduced pest problems, and ultimately economically viable yields.

## 14.2 Spider Diversity in Agriculture Ecosystem

Spiders are one of the major groups in the class Arthropoda, to date, consisting of 40,024 species (Platnick 2012). This group has a widespread distribution and commonly exists in agricultural areas which have spurred many interests to research on their potential as pest control. The risks associated with using spiders to control pests are minimal but instead with far greater advantages as biocontrol agents for having the following characteristics: spiders exist in high

abundance but do not cause damages to vegetation or crops; they coexist naturally as diverse species in an agricultural system; they have a high diet variety preying on, for example, mites, aphids, thrips, and termites which are common pests of agroecosystems; they display different foraging modes and hunting tactics in prey capture due to differences in feeding behavior, either weaving webs for prey trapping, ambush, and chase or hunting for targeted preys; spiders have a long life cycle which varies from 9 months to 25 years and moreover are predaceous throughout their developmental stages, and not only adults but all also instars feed actively as predators; thus various stages of pests are preyed upon by a variety of spider species as, for example, minute prey items like thrips and mites are important food sources for the young spiderlings (Dippenaar-Schoeman 2006); they are able to occupy many microhabitats and niches within an ecosystem and most of them are polyphagous (can feed on a variety of prey items) predators, thus having a wide range of prey selection which are the available pests found in the agricultural areas (Wise 1993; Marc and Canard 1997). Moreover, spiders are purposed to be able to withstand treatments of broad-spectrum insecticide applications if utilized within the area, whereas all other beneficial enemies were impacted by the insecticidal treatments (Hoque et al. 2002). This makes them resistant beneficial species in which the populations are able to flourish successfully where needed. Biological control method is one of the strategies implemented in the Integrated Pest Management (IPM) to create sustainable agricultural areas. IPM prefers to use organisms or natural enemies to suppress pest populations rather than using pesticide which are known to be harmful to the environment and affect human health.

Spiders are one of the known successful groups of natural predators occupying the agriculture ecosystems, and as efficient predators they were able to suppress populations of major insect pests, at the same time significantly decreased crop damage while increased harvest of crop yields. Globally, they have been successfully adopted as biocontrol agents primarily in orchards and paddy fields. However, different strategies were adopted for the pest management in both agricultural scenarios. The orchard agroecosystems in Europe utilized the spider conservation approaches, while Asian countries reputed for their paddy fields implemented the augmentation method (Marc and Canard 1997).

The spider species composition varied with the different types of agriculture ecosystems, mainly because of the variable environmental conditions and different strata of the plant species communities or zonations in tree species, providing specific niches for different spider species to thrive (Norma-Rashid et al. 2009). This was supported by Noraina (1999) who revealed that spider assemblages divided themselves according to the plant stratifications to avoid competition and also foraged in different plant species in order to exploit the extensive available prey items. Sudhikumar et al. (2005) in their work reported the close relatedness of the plant species with the prey populations that depended on the plant hosts and the predators that exploit the prey species.

Various authors had investigated the beneficial roles of spiders in agricultural situations. The wolf spiders (Lycosidae) and jumping spiders (Salticidae) had been characterized as the two predominant biological control agents in paddy fields (Kiritani and Kakiya 1975; Tahir and Butt 2008). Motobayashi et al. (2007) proceeded to study the effects of spiders on the migrant skipper, *Parnara guttata guttata* (Lepidoptera), which is one of the major paddy field pests in Japan. They conducted the spider removal experiments in the paddy fields lending support to spider predation being the main cause of mortality in the migrant skippers; the late stage larvae were more susceptible to spider predation due to their foraging behavior on rice leaves above soil and nest construction behavior confined to the upper layer of rice crops where spider predators were mostly found. Tahir and Butt (2009, 2008) demonstrated the predatory potential of Lycosidae and Oxyopidae predating on larvae of stem borers, leaf folders, plant hoppers,

and grasshoppers in the rice ecosystems. Their work conducted in the rice fields in Punjab, Pakistan, revealed interesting behavioral partitioning between the two active lycosid predators, whereby *Lycosa terrestris* actively pursued preys found on the foliage, while *Pardosa birmanica* restricted their foraging behavior to the ground levels. The combined predatory roles of these two lycosids resulted in efficient pest control in rice fields (Tahir and Butt 2008). Although spiders are known to exist in a variety of species within a habitat structure (Norma-Rashid et al. 2009) with likely potential for competition and intraguild predation, it had been stressed by Nyffeler and Sunderland (2003) that a diverse sympatric living spider species would be more effective at reducing prey densities than a mono spider species. Riechert and Lawrence (1997) in their work on predation effects of spiders concluded that a diverse natural enemy fauna resulted in a more effective regulation of prey populations, where their results revealed for test plots that contained four spider species comprised of sheet-web weaver (*Florinda coccinea*), orb-web weaver (*Argiope trifasciata*), and two wolf spiders (*Rabidosa rabida* and *Pardosa milvina*) had lower prey densities in contrast to plots that contained only one of the listed species. Marc et al. (1999) concluded that diverse assemblages of spiders would be effective in pest control because species variability in habitat choices, activity rhythms, and foraging behavior would probably result in a specific or a number of species that will target a given pest.

Marc and Canard (1997) found that the high abundance of spider communities were beneficial and effective in removing herbivorous insects in apple orchards which included the beetle *Anthonomus pomorum* and Lepidoptera larvae in the family Tortricidae. More interestingly was the behavioral change of these lepidopteran larvae where they displayed avoidance behavior towards the spider by abandoning the apple tree branches when spiders were present (Marc et al. 1999). The spider avoidance behavior was also exhibited by tobacco cutworms, *Spodoptera litura*, towards spiders from the family Linyphiidae which prevented extensive damage to the tobacco plants (Riechert and Lockley 1984).

In order to augment spider populations in agricultural systems, the available structural complexity should be enhanced or manipulated in ways to benefit the spiders. Provision of refugia would be extremely important for the purpose of early colonization and conservation of potential targeted predators. Riechert and Lockley (1984) construed that high structural complexity of the agrosystem would be directly correlated to greater array of microhabitats with varied microclimatic features, alternative resources such as food, and nesting and retreat sites for elevated spider density and diversity. Costello and Daane (2003) found that ground cover affected the population density of spiders and leafhopper in the vineyard. Riechert and Bishop (1990) experimented on the habitat manipulation by adding mulch and flowers in the mixed vegetable plots to increase spider abundance which were successful in removing pests and decrease crop damage; furthermore, through direct observations they confirmed that 84 % of the predators that were foraging were spiders and 98 % of the prey captures were by spiders. Rice growers in China build straw or bamboo shelters to encourage web construction or spider retreat which could be transported to areas of pest outbreaks occurrence (Marc et al. 1999). Similarly, studies by Tanwar et al. (2011) had shown that placement of straw bundles in the sorghum fields to attract or trap spiders there and later transferred to the rice fields had the effect of great reduction in the pest population of common rice pests that were stem borers and leaf folders. Predator refugia could be in various forms, the common ground cover, straw bundles, or mulch, interspacing with intercrops, cover crops, field margins, bunds, and many other forms (Shanker et al. 2012; Luck et al. 2003).

Generally, the orb-web weavers predominate (could achieved to a maximum of 95 % of the spider population in an area) the agriculture ecosystem (Hogg and Daane 2011). Our previous work revealed that the common orb-web weavers were representatives from the family groups Tetragnathidae and Araneidae. Tetragnathidae

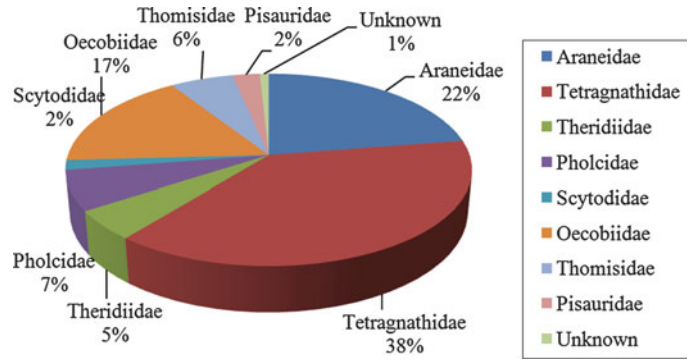


**Fig. 14.1** Relative abundance of the spider families in Rimba Ilmu Botanical Garden (Noraina 1999)

**Table 14.1** Spider species in Rimba Ilmu Botanical Garden (Noraina 1999)

| Families | Species |
|---|---|
| Araneidae | *Arachnura* sp. |
| | *Araneus mitificus* |
| | *Argiope aemula* |
| | *Argiope versicolor* |
| | *Cyclosa bifida* |
| | *Gasteracantha hasselti* |
| | *Nephila maculata* |
| | *Parawixia dehaani* |
| | *Poltys illepidus* |
| Tetragnathidae | *Leucauge argentina* |
| | *Leucauge fastigata* |
| | *Tetragnatha josephi* |
| | *Tylorida striata* |
| | *Tylorida ventralis* |
| Theridiidae | *Achaearanea mundulum* |
| | *Argyrodes argentatus* |
| Pholcidae | *Smeringopus pallidus* |
| Scytodidae | *Scytodes pallida* |
| Oecobiidae | *Oecobius* |
| Thomisidae | Unknown |
| Pisauridae | Unknown |
| Unknown | Unknown |

dominated the Rimba Ilmu Botanical Garden (Fig. 14.1 and Table 14.1) in which was similarly reported by Okuma (1968) who studied spiders in the rice filed. However our sampling in the dragon fruit plantation resulted in highest representative of Araneidae (Fig. 14.2 and Table 14.2). It seemed likely that the orb-web weavers had higher tendency to act as natural biological control agents in capturing the available prey items that included pests. This predominant group of Araneidae was also highly sampled during our spider trappings on an island in Peninsular Malaysia, which is called Carey Island, densely vegetated with palm oil trees, either young or matured palm trees, harvested for their fruits (Wan Azizi 2008). The results from the Carey Island revealed 40 species of Araneidae with 66 % of individual spiders captured within the areas sampled (Table 14.3). Interestingly when planted plots of young and matured palm trees were contrasted, the young plots revealed higher diversity indexes for Shannon-Wiener, H′ max, Pielou, and Margalef (Table 14.4). Could this be postulated to be an indication of higher available food resources or prey organisms for the spiders? This is a query needing more investigations in order to obtain possible answers.

Noraina (1999) reported that web weavers constructed webs of considerable sizes that were located within certain heights and found to be strategically placed to avoid strong winds and potential predators (mostly birds). This finding was further supported by Wan Azizi (2008) who found that the range of stratification between 120 and 140 m was where the webs are typically found in the palm oil estates. The structures of the webs that were further analyzed portrayed four types from complicated to simple patterns: three-dimensional, two-dimensional, rolled leaf, and simple threads evidential of web presence. Figure 14.3 illustrates contrasting differences between matured and young palm tree plots where the three-dimensional structures were higher in matured tree plot due to easy anchorage

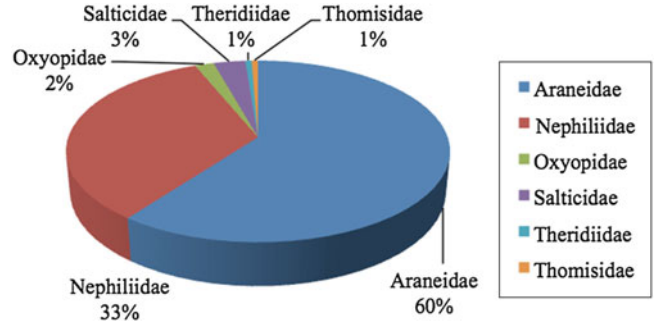


**Fig. 14.2** Relative abundance of the spider families in dragon fruit plantation (Dzulhelmi and Norma-Rashid 2014)

**Table 14.2** Spider species in dragon fruit plantation (Dzulhelmi and Norma-Rashid 2014)

| Family | Species |
|---|---|
| Araneidae | *Acusilas coccineus* |
| | *Araneus anapastus* |
| | *Araneus* sp.1 |
| | *Araneus* sp.2 |
| | *Araneus* sp.3 |
| | *Cyclosa nigra* |
| | *Cyrtophora* sp. |
| | *Neoscona theisi* |
| | *Neoscona* sp.1 |
| | *Neoscona* sp.2 |
| | *Neoscona* sp.3 |
| | *Parawixia dehaani* |
| | *Pronous tetraspinulus* |
| | *Zygiella laglaizei* |
| | *Zygiella medeleii* |
| | *Zygiella* sp.2 |
| | *Zygiella* sp.3 |
| | *Zygiella* sp.4 |
| | Araneidae sp. |
| Nephilidae | *Nephila* sp.1 |
| | *Nephila* sp.2 |
| Oxyopidae | *Oxyopes sikkimensis* |
| Salticidae | *Chrysilla lauta* |
| | *Chrysilla versicolor* |
| | *Chrysilla* sp. |
| | *Myrmarachne* sp. |
| | *Phintella ephippigera* |
| Theridiidae | *Theridiidae* sp. |
| Thomisidae | *Camaricus* sp. |

**Table 14.3** List of spider species belonging to the family Araneidae that were sampled in Carey Island, Peninsular Malaysia (Wan Azizi 2008)

| Species listings | Species listings |
|---|---|
| *Acusilas coccineus* | *Cyclosa bifida* |
| *Anepsion depressum* | *Cyclosa centrodes* |
| *Arachnura* sp. | *Cyclosa insulana* |
| Araneidae TH | *Cyclosa* sp. |
| Araneidae TH 2 | *Cyrtophora* A |
| Araneidae z | *Cyrtophora cicatrosa* |
| Araneidae? | *Cyrtophora hainanensis* |
| *Araneus* 1 | *Cyrtophora moluccensis* |
| *Araneus* B | *Gasteracantha mammosa* |
| *Araneus* IM | *Gasteracantha kuhli* |
| *Araneus* sp? | *Mangora hemicraera* |
| *Araneus anapastus* | *Nephilengys malabarensis* |
| *Araneus ancurus* | *Paraplectana* |
| *Araneus elongates* | *Prasonica* |
| *Araneus papulatus* | *Pronous* sp. |
| *Argiope aemula* | *Singa* sp. |
| *Argiope* sp. | *Thelacantha brevispina* |
| *Argiope versicolor* | *Zygiella calyptrata* |

of the webs with available spread branching of the leaf-frons and the rolled leaf web structures were frequently found in the young tree plots where these areas were exposed to high penetrating sun rays void of tree canopy shades. It seemed apparent here that even specific predator species that could be recommended for certain agriculture types but still need to be paired with the developmental features of the crops to maximize the efficiency of biocontrol agents.

The presence of wandering spiders seemed to be lower in abundance in contrast to the web weavers (Noraina 1999), but this is not common in all situations. Dippenaar-Schoeman (2006) in her extensive work in the agroecosystems in Africa reported high incidence of wandering spiders as beneficial predators, some of which

**Table 14.4** Diversity indexes calculated for Shannon-Wiener, H′ max, Pielou J index, and Margalef D index contrasting between matured and young palm tree plots (Wan Azizi 2008)

| Diversity index | Matured trees plot | Young trees plot |
|---|---|---|
| Shannon-Wiener, H′ | 2.432 | 2.797 |
| H′ max | 2.890 | 3.219 |
| Pielou's index, $J = H'/H'$ max | 0.841 | 0.869 |
| Margalef's index, D | 4.640 | 6.200 |

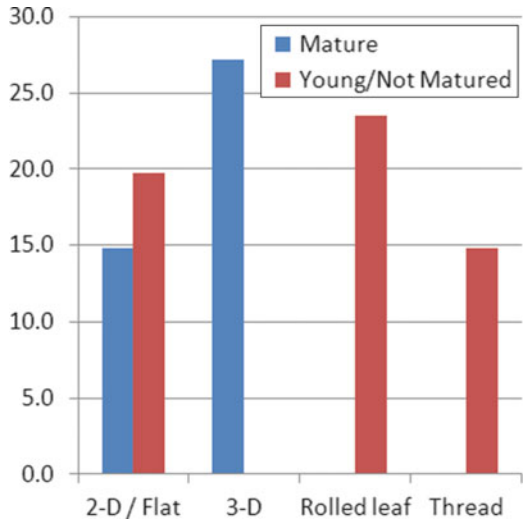


**Fig. 14.3** The number of web structure types divided into two-dimensional or flat, three-dimensional, rolled leaf, and simple web threads found in the matured and young palm tree plots (Wan Azizi 2008)

were the wolf spiders *Pardosa crassipalpis* that prey on red spider mites in strawberry hedges; jumping spiders (Salticidae) represented 73 % and preyed upon pests like thrips, mites, midges, and flies in macadamia orchards, whereas for the avocado orchards, the main predators for pest species such as aphids, red spider mites, and thrips were salticids that comprised of 31 % followed by crab spiders (Thomisidae) being the second highest group (24 %). It could probably be because the non-web weavers were sensitive to disturbances within the environment and had the capability to escape and hide in crevices or between leaves which resulted in failure to detect their presence and indirectly underestimate their counts in field samplings (Dzulhelmi and Norma-Rashid 2014). However, it is also possible that non-web weavers were low in abundance because of their cannibalistic habits (Nyffeler 1999). This being apparent during encounters when the bigger-sized individuals would overpower and eat the inferior victims (Jackson 1992). Their diet preference tendencies were selective rather than random choice contributed to their presence within specialized ecosystems (Maimusa et al. 2012).

In many instances spiders portrayed to be prey specialists, favoring prey of specific taxa, age (selection for certain stage prey instar), and size, and displayed behavioral specializations leading to effective elimination of specific pest populations. According to Marc and Canard (1997) the size class prey selection by spider predators was distinctly confined to consumption of prey items that were 50–80 % of their body sizes and ignoring others. Nyffeler (1999) found that web weavers were skewed at catching profitable larger prey and neglecting the smaller ones. Generally wandering spiders showed greater diet breadth than web weavers (Nyffeler 1999). Noraina (1999) found that wandering spiders foraged mainly on lower strata and prey items belonged to the groups of Coleoptera and Homoptera. She also collected and identified the web catches in her study area in the botanical garden, comprised of mixed vegetation with fruit orchard, herbal crops, citrus shrubs, ornamental plants, and rubber trees. The prey included a diverse assortment of arthropods but seemed biased towards flies (Diptera); the majority were fruit flies and Hymenoptera, while other family groups of prey items were in minor proportions (Fig. 14.4).

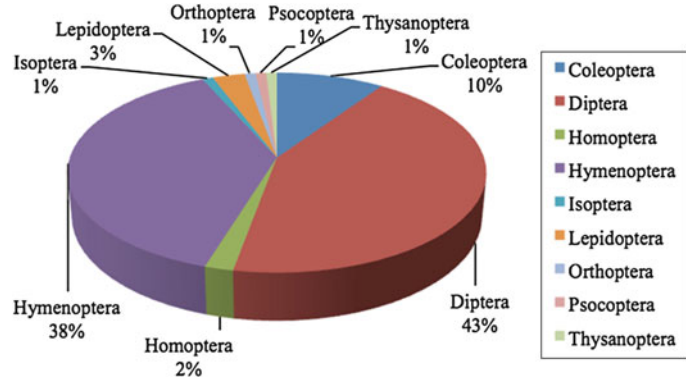


**Fig. 14.4** Prey captured by the web-weaver species at Rimba Ilmu Botanical Garden (Noraina 1999)

## 14.3 Spiders in Herbal Plots

Studies on spiders in herbal-based agricultural areas need a special mention, as it is important to recognize the need to use natural enemies to control pests in herbal farming (Wood 2002). This was perceived to be very important as herb-based products are utilized as health supplements, which should be void of chemical contamination due to synthetic pesticides. Research on herbal plots had been conducted to investigate the potential of spiders as biocontrol in which two types of herbal plots were selected: *Orthosiphon stamineus* commonly known as the cat's whiskers plant belonging to the family Lamiaceae and the mistletoe fig or *Ficus deltoidea* (family Moraceae). These study areas had different vegetation structures: one being bushy branching (*Orthosiphon stamineus*) and the other of simple stem structure and few branches with broad leaves (*Ficus deltoidea*). The spiders collected in both areas showed different domination (Table 14.3) due to the different architectural features of the crops, as well as related to the foraging behaviors of the spider predators on the prey. The *Orthosiphon stamineus* crop, which were covered by foliage, encouraged ambush-typed spiders (Oxyopidae) and small Araneidae to thrive well in such vegetation. Oxyopidae, known to be able to hunt with great agility, equipped with legs of large bristles to capture the insect pests, were found to exploit the bushy area which had the advantage for them to hide and ambush their victims at a close distance, sometimes using a unique foraging strategy to capture the prey in midair by hanging underneath the leaves. Oxyopidae comprised of 45.6 % of the spider population at the foliage level in this herbal crop.

*Ficus deltoidea* an herbal crop which has broad leaves with few branches attracted Salticidae (26.1 %), Araneidae (23.6 %), and Tetragnathidae (22.9 %). The two conditions that influenced the presence of these family groups were as follows: (1) the broad leaves provided a large surface area which were suitable for the Salticidae that utilized the jumping strategy to forage for the prey and (2) the few number of branches available made it possible for the orb weavers to anchor their silk threads to build large and wider webs which were more efficient in trapping flying prey items. The orb-web spiders made up 46.5 % of spider population collected at the foliage level in *Orthosiphon stamineus*. Two dominant species collected in *Ficus deltoidea* plots were *Tylorida ventralis* and *Cyclosa bifida*. Others include Araneidae (21.5 %) and Salticidae (19.6 %).

Wise (1993) reported that the population of spiders was influenced by the architecture features of habitat and changes of the surrounding environment. Vegetation structures would affect the distance to anchor the silk threads for orb-web spiders. The variety of vegetation structures would also affect the microclimate in the

agricultural area impacting on the spider populations in a particular habitat (Turnbull 1973). Whittaker (1975) stated the heterogenous habitat would increase the abundance of natural enemies including spiders.

## 14.4 Summary

In summary, spiders, by virtue of their top-down effects, were able to decrease and stabilize pest populations, and conclusively it was revealed that plant damage due to insect herbivores was lowered with the presence of spiders than when they were absent. Gone are the days when spiders were thought to be an insignificant component of agroecosystems (Riechert and Bishop 1990). Literature search would reveal many other biological control agent success stories that were utilized for pesticide alternatives and were able to suppress pest populations. Such results on the use of biological control in decreasing pest damage should more importantly be able to increase crop yield as well as quality and, in the long run, improve the economic status for agriculturists that would reflect the ultimate success. Ideal overall biocontrol strategies for IPM are still scarce, and there are urgent needs for more groundwork research (Jonsson et al. 2008).

Farmers are getting disappointed with the high cost for health and environmental concerns in keeping up with the pesticides treadmill (Altieri et al. 1997). Initiatives had been taken in a number of approaches including support from governments and NGOs, community organizations, and farmer-to-farmer networks to encourage farmers to utilize biological applications (Altieri et al. 1997) for the betterment of all concerned. However, biological control application requires in-depth knowledge on the natural enemies and their communities of which they came from (Jonsson et al. 2008). Most of the time, biological control only seeks to "balance" in controlling specific pests in a specific agriculture (Altieri et al. 1997). Some biological control practices had resulted in unpredictable and irreversible impact that may cause negative perceptions by farmers. But one must also consider the cost, benefit, and risk value that are involved to test for biological control efficiency in comparison to economic loss of plants to pests (Simberloff and Stiling 1996). The performance inconsistency in different environmental practices from biotic to abiotic factors does not blend well in this situation. Meanwhile, mass rearing and import-export from countries had been practiced in previous years to ensure stock supply for specific biological control agents.

## 14.5 Future Challenges

The greatest challenge of all is to enhance local interests to conduct initial groundwork research and providing information for the baseline to biological control practice for farmers. It is obvious from the current scenario that most literature and research in biological control and pest management were mainly obtained from industrialized countries. Thus it is timely for local researchers to embark on this challenge that will be of beneficial contribution to the homeland. It is crucial to stress that scientific research alone cannot guarantee the adoption of biocontrol since what would enable transition to implementation of biocontrol would be economic incentives to reward farmers for undertaking the challenge of adoption.

**Acknowledgements** Norma-Rashid would like to acknowledge the financial aids from the Malaysian Higher Education Ministry (FP045-2013A) and the University of Malaya (PG096-2012B and RP001G-13SUS).

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# Genetic Improvement of Biocontrol Agents for Sustainable Pest Management

15

Ramesh Arora and P.S. Shera

**Abstract**

Genetic improvement involves directed purposeful genetic alterations to enhance the efficacy of natural enemies for biological control. This may be achieved by conventional approaches as well as through recombinant DNA techniques. The conventional methods include strain selection, serial passage through hosts, mutation, conjugation, transduction, selective breeding, hybridisation, etc., whereas the genetic engineering approaches involve gene transfer utilising various methods. Entomophagous insects may be improved for climatic tolerance, sex ratio, host-finding ability, host preference, increased host range, increased pesticide resistance, etc. The main objectives in genetically altering microbes are to increase host range, virulence and persistence. The *cry* genes from *Bacillus thuringiensis* have been cloned and expressed in a wide variety of organisms (baculoviruses to cyanobacteria) as well as in plants in attempts to improve their delivery and efficacy against insect pests. Apart from *B. thuringiensis*, binary toxin from different *B. sphaericus* strains has been expressed in different hosts like *Escherichia coli*, non- or low-toxic *B. sphaericus* and crystal minus *Bt israelensis* as well as in *Caulobacter crescentus* or cyanobacteria *Anabaena* sp. Insect viruses, especially baculoviruses, are mostly specific viruses which can replicate only in hosts. The recombinant DNA technology has its current applications in inserting foreign genes into insect baculoviruses and achieving their rapid and efficient expression in the recipient host systems. Candidate genes for hyperexpression in the baculoviruses include those encoding insect-specific enzyme genes (juvenile hormone esterase gene), hormone genes

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_15, 

(eclosion hormone gene, diuretic hormone gene) and insect-specific foreign toxic genes (scorpion venom toxin genes, predatory mite toxin gene, predatory spider toxin gene, parasitic wasp venom gene and *Bt* δ-endotoxin genes). It is worthwhile to mention that genetic engineering of BCAs is potentially very promising and has led to the development of more effective entomopathogens with desired pathogenicity, virulence, broad host range and persistence, providing a valuable tool for sustainable pest management.

**Keywords**
Biological control • Entomopathogens • Genetic improvement

## 15.1 Introduction

The global population in 2011 has crossed the seven billion mark and is projected to increase to 9.6 billion in 2050. The population of developing countries would increase from 5.7 billion in 2011 to 8.3 billion in 2050 (PRB 2011). Obviously, escalating human population especially in developing countries necessitates augmentation in food, feed and fibres and that too from a limited agricultural land base. The average crop loss resulting from animals (mostly insects), diseases, viruses and weeds has been reported as 32.1 % of the potential production of all crops with a higher percentage of 40 % for potato and the lowest being 26 % for soybean (Oerke 2006). But losses are comparatively higher in tropical and subtropical areas. India loses about 30 % of its crops due to various insect pests and diseases each year resulting in estimated annual revenue losses of Rs. 100,000 crores (Anonymous 2009). Synthetic pesticides, as major agro-inputs and integral part of modern crop-management practices, have significantly contributed to the improved agricultural production in the country by minimising yield losses. However, the indiscriminate and broadly unscientific application of a number of different recalcitrant synthetic chemicals to control insect pests during the last four decades has led to many ecological backlashes. These include emergence of high levels of pesticide resistance in many pest species, environmental toxicity, fishery losses, ground water and surface water contamination, depletion of rhizosphere microflora, food safety hazards and human health concerns (Shetty and Sabitha 2009; Singh 2012). Besides growing public concern over potential health hazards of synthetic pesticides in the management of insect pests, a steep increase in cost of cultivation due to high cost of these chemicals is also major concern. Due to this, there is renewed interest in eco-friendly approaches to pest management, and rationale for biological control of insect pests is irrefutable (Koul 2009; Arora et al. 2012).

Biological control refers to the destruction or suppression of undesirable insect pests by the introduction, encouragement or artificial increase of biological control agents (BCAs) that include both macro- (predators, parasites, parasitoids) and microorganisms. This control based upon naturally derived pest control processes that are more easily biodegradable, more target specific and eco-friendly is argued to be preferable to conventional chemical pesticides. Natural (biological control) is constantly active in all world terrestrial ecosystems and keeps most of the potential arthropod pests under control. Most of the potential arthropod pests (95 %, 100,000 arthropod species) are under natural control; all other control methods used today are targeted at the remaining 5,000 arthropod pest species (Van-Lenteran 2008). The three basic strategies for the exploitation of biocontrol agents in insect pest management include importation or classical biological control, augmentation and conservation. Classical biological control involves intentional introduction of exotic usually co-evolved biocontrol agents to control a pest in an area where it has

been accidentally or speciously introduced. Conservation biological control refers to modification of the environment or existing practices to protect and enhance natural enemies or other organisms to reduce the effects of pests. Augmentation involves two approaches, viz. inoculative and inundative releases, designed to increase the population of biocontrol agents by mass multiplication and periodic releases. These approaches have different applications depending on the adaptability of natural enemy and environment in which they must operate (Eilenberg et al. 2001). Classical biological control is applied on 350 million hectares, which is about 8 % of land under culture, and has very high benefit-cost ratios of 20–500: 1. However, augmentative, commercial biological control is utilised only on 16 million hectares, which is 0.4 % of land under culture, and has a benefit-cost ratio of 2–5: 1, which is similar to or better than chemical pest control (Van-Lenteran 2008). The main reason for the limited success achieved with augmentative biological control is that biocontrol agents suffer from a number of important limitations, viz. narrow spectrum of activity, susceptibility to adverse environmental stress and slow speed of kill. However, recent advances in molecular biology techniques have offered opportunities to enter into a novel realm in overcoming these limitations through genetic improvement of biocontrol agents. It has broadened available techniques for genetic manipulation for diverse traits in species of interest (Atkinson et al. 2001; Kramer 2004).

## 15.2 Genetic Improvement of Arthropod Natural Enemies

Genetic improvement involves directed purposeful genetic alterations to enhance the efficacy of biocontrol agents for biological control. It may be achieved by artificial selection, hybridisation to achieve heterosis effects or use of recombinant DNA techniques. Biotechnological interventions can offer opportunity to improve beneficial arthropods for climatic tolerance, sex ratio, host-finding ability, host preference, increased host range, increased pesticide resistance, etc. There is tremendous scope for developing natural enemies with genes for resistance to pesticides and ability to withstand adverse weather conditions (Hoy 1992). However, genetic improvement of arthropod natural enemies has received little attention because of the concern that breeding and prolonged rearing under artificial condition would necessarily result in laboratory-adapted strains that would perform poorly in the field. Biotechnological approaches can be helpful in understanding the genetics and physiology of reproduction and control of sex ratio in natural enemies, which can be used to improve their rearing for biological control (Sharma 2009).

### 15.2.1 Climatic Tolerance

Genetic transformation can be exploited to augment tolerance to extreme hot or cold conditions in arthropod natural enemies. The superior strains of *Trichogramma chilonis* Ishii, an important egg parasitoid of lepidopterans have been developed by selection technique for adaptation to high as well as low temperature regime at National Bureau of Agricultural Important Insects (NBAII), Bangalore. The strain adapted to high temperature regime can be utilised in temperatures above 35 °C. This strain has been found to give increased longevity and parasitism at 36 °C and 60 % relative humidity. High temperature-tolerant strain of *T. chilonis* is useful against various insect pests of sugar cane, cotton and vegetable crops during hot months (Singh 2003). This strain has also proved superior in reducing the incidence of sugar cane stalk borer, *Chilo auricilius* (Dudgeon), and early shoot borer, *Chilo infuscatellus* (Snellen), as against local strain under Punjab conditions (Singh et al. 2007a, b). Similarly, low temperature-adapted strain, developed through selection for 30 generations under laboratory conditions at 18–24 °C, has shown better host searching ability and can be successfully exploited under low temperature agrosystem (Jalali et al. 2006a).

### 15.2.2 Tolerance to Insecticides

Insecticide tolerance is a key desirable trait for parasitoids and predators which can be genetically improved for use in integrated management programmes. Phytoseiid mite, *Metaseiulus occidentalis* (Nesbitt), the most important predator of spider mites in orchards and vineyards in the USA, has been improved through artificial selection to pesticide-resistant strain called COS strain (carbaryl-OP-sulphur-OP-resistant strain) and has also been documented in its field effectiveness. It acquired resistance to organophosphorus insecticides such as azinphos-methyl, diazinon and phosmet through selection in orchards and vineyards. Resistance to sulphur was also discovered in native California vineyard populations. To augment this naturally acquired resistance, *M. occidentalis* was artificially selected in the laboratory with permethrin and carbaryl and then selected to obtain multi-resistant strains. This laboratory-selected strain of *M. occidentalis* successfully met the criteria for a field release. The carbaryl-OP and permethrin-OP strains have also established in apple, pear and almond orchards in California, Oregon and Washington in the USA. The resistant strains were released in the field and multiplied, overwintered and survived pesticide application in the field. Mass rearing and commercial releases of this strain in California almond orchards have resulted in annual savings of about $ 21,56,000 to the almond growers alone (Hoy 1990). It has been reported that mortality of first instar of common green lacewing, *Chrysoperla carnea* (Stephens), selected in the laboratory for resistance to carbaryl decreased from 98 to 10–20 %. Further, tests using the oxidase inhibitor piperonyl butoxide and the esterase inhibitor phenyl saligenin cyclic phosphonate suggested that both oxidase and esterase enzymes contribute to the resistance (Grafton-Cardwelu and Hoy 1986). A parasitoid, *Aphytis melinus* DeBach, of the California red scale, *Aonidiella aurantii* (Maskell), has been selected successfully for resistance to carbaryl (Rosenheim and Hoy 1988). Similarly, *Trioxys pallidus* Haliday, an effective parasitoid of the walnut aphid, *Chromaphis juglandicola* Kaltenbach, has been selected for resistance to azinphos-methyl (Hoy and Cave 1991).

An endosulfan-resistant strain of *T. chilonis* termed as 'endogram' has been developed by sequentially exposing adult parasitoids to various concentrations of endosulfan (0.004–0.09 %) for 341 generations. The tolerant strain was found to parasitise 56 % of *Helicoverpa armigera* (Hübner) eggs immediately after insecticide spray as compared to 3 % by susceptible strain (Jalali et al. 2006b) in net house potted cotton plants. Endogram strain has been further improved for multiple resistance to monocrotophos and fenvalerate to obtain multiple insecticide resistance strain through selection process for over 72 generations. Field efficacy of this resistant strain has shown higher parasitism in *H. armigera* eggs on tomato by 260.4 % as compared to susceptible strain under sprayed conditions (Jalali and Venkatesan 2011). In Punjab, 11–13 releases of this multiple insecticide-resistant strain of *T. chilonis* at 1,50,000 per ha at weekly interval during July to October along with 6–9 insecticide applications have been reported to be effective for the control of cotton bollworms and resulted in increasing egg parasitism (Brar et al. 2007). Similarly, a strain of *Chrysoperla zastrowi arabica* Henry et al. (PTS-8) having tolerance to endosulfan, acephate and fenvalerate has been developed. PTS-8 acquires higher activity of detoxifying enzymes (esterase and glutathione S-transferase activity) as against susceptible population. It has been found to be effective against sucking pests of cotton under pesticide sprayed conditions (Venkatesan et al. 2011). These strains could be useful biocontrol agents for the suppression of insect pests in different crop ecosystems.

It will be desirable to select pesticide-resistant strains of natural enemies to overcome the problems caused by the use of pesticides (Dhaliwal and Arora 2006). However, concerns have been raised that insecticide resistance also may confer fitness disadvantages (Georghiou and Taylor 1977) that would reduce the stability of the trait within field populations in the absence of selection pressure. Carbaryl resistant strain of

*C. carnea*, when reared in the absence of carbaryl exhibited lower larval and pupal survival and produced fewer females than the colony from which it was derived. However, its fecundity was significantly higher and adult longevity slightly higher, which compensated in part for reduced survival of immature stages (Grafton-Cardwelu and Hoy 1986). Conversely, the COS strain of *M. occidentalis* exhibited life table parameters comparable to those of other strains, suggesting that the reproductive attributes of this predator were not altered as result of artificial laboratory selection (Bruce and Hoy 1990). Spollen and Hoy (1992) measured relative fitness components for a genetically improved strain of *Aphytis melinus* DeBach with increased resistance to carbaryl. Except for a difference in progeny sex ratio, developmental parameters were not significantly different between the resistant and susceptible strains. Baker et al. (1998) reported no fitness costs associated with malathion-resistant strain of the solitary parasitoid, *Anisopteromalus calandrae* (Howard), parasitising on immature rice weevils, *Sitophilus oryzae* (L.), in stored wheat.

In addition to selection, the resistance genes with potential importance have also been cloned through biotechnological interventions. These include cyclodiene resistance gene ($GABA_A$) from *Drosophila*, a cytochrome P450-B1 gene (*CYP6A2*) associated with DDT resistance in *Drosophila*, parathion hydrolase gene (*opd*) from *Pseudomonas diminuta* Leifson and Hugh and *Flavobacterium*, *β-tubulin* genes from *Neurospora crassa* (Draft) and *Septoria nodorum* (Berk.) conferring resistance to benomyl, acetylcholinesterase gene (Ace) from *Drosophila melanogaster* Meigen, glutathione S-transferase gene (*GST*) from *Musca domestica*, esterase B1 gene from *Culex* responsible for resistance to organophosphates (Atkinson et al. 2001) and a cytochrome P450-B1 gene (*CYP6F1*) from deltamethrin-resistant *Culex pipiens pallens* (Gong et al. 2005).

### 15.2.3 Tolerance to Multiple Traits

A strain of *T. chilonis* has been developed for tolerance to multiple traits known as MITT (multiple insecticide and temperature tolerant) strain for effective control of the pest even in harsh climatic conditions and under high insecticide pressure in different economically important crops. This strain has showed tolerance to three major groups of insecticides which include endosulfan (organochlorine), monocrotophos (organophosphate) and fenvalerate (synthetic pyrethroid) and to high temperature (32–38 °C) through selection for 81 generations. Increase in parasitisation from 35 to 90–95 % and decrease in mortality from 100 to 57–70% after 6 h of constant exposure to three insecticides and high temperature have been reported (Kumar et al. 2008).

### 15.2.4 Altered Biological Traits

Altering different biological traits such as enhancing fecundity, shortening developmental period, sex ratio, host or habitat preferences could enhance the effectiveness of biological control agents (Hoy 1976). Altering longevity of certain arthropods might be beneficial, and research on mechanisms of ageing may provide useful genes in the future (Sharma 2009). NBAII, Bangalore, has developed superior strains of *T. chilonis* such as Bio SC1 for graminaceous tissue borers, Bio H3 for *H. armigera* and Bio C1 for cotton bollworms. These strains are 60–100 % more prolific than the previously used strains. Hybridisation has also been attempted as a method for increasing vigour or fitness through heterosis. It was observed that the developmental rate, diapause attributes, sex ratio and parasitisation rate of gypsy moth parasite *Apanteles melanoscelus* (Ratzeburg) in the insectary were improved through hybridisation (Hoy 1975). However, limited evaluation conducted with hybrid strain did not indicate that it was more effective than parent strains under field conditions.

## 15.3 Genetic Improvement of Entomopathogenic Microbes

The entomopathogens have attained a special status in biopesticide umbrella and have the potential to provide economically viable and

environmentally safe alternative to chemical pesticides in many ecosystems. However, concerns related to their susceptibility to adverse environmental stress, less persistence under field conditions, limited host range, slow activity, deprived quality and limited storage stability have restricted their widespread use commercially. The main objectives in genetically altering microbes are to make them more effective by increasing host range, virulence and persistence, adapting to extreme temperature conditions and overcoming resistance. For the first time, cloning and expression of *Bacillus thuringiensis* (Berliner) *delta*-endotoxin gene in *Escherichia coli* Migula demonstrated the potential of genetic engineered technology for microbial control in the early 1980s. Based on selection, hybridisation, mutagenesis and DNA recombinant technology, microbial agents have now been genetically engineered for a number of traits.

### 15.3.1 Entomopathogenic Bacteria

Most of the insect pathogenic bacteria occur in the orders Bacillales (Bacillaceae, Paenibacillaceae), Lactobacillales (Enterococcaceae, Streptococcaceae), Enterobacteriales (Enterobacteriaceae), Pseudomonadales (Pseudomonadaceae), Neisserales (Neisseriaceae), Rickettsiales (Rickettsiaceae, Anaplasmataceae) and Entomoplasmatales (Spiroplasmataceae) (Jurat-Fuentes and Jackson 2012). Of these, the pathogen that has received special attention and attained considerable success is the Gram-positive spore-former and crystalliferous *B. thuringiensis* which accounts for nearly 90 % of the sale of bioinsecticides. Other bacterial pathogens do exist, but only few have been successful pest control agents such as *Lysinibacillus sphaericus*, *B. cereus*, *Paenibacillus popilliae*, *P. lentimorbus*, *Xenorhabdus* spp., *Photorhabdus* spp. and *Serratia entomophila* enumerating their growing commercial importance.

#### 15.3.1.1 *Bacillus thuringiensis*

*Bacillus thuringiensis* (*Bt*) is a motile, Gram-positive spore-forming phylloplane and soil-inhabiting bacterium and, in addition to endospores, produces a proteinaceous parasporal crystal in the sporangium at the time of sporulation. Due to its remarkable activity against a wide range of nefarious insect pests, *Bt* has been extensively studied and excellent reviews are available on its insecticidal properties (Baum 1998; Glare and O'Callaghan 2000; Nester et al. 2002; Sanchis 2011; Jurat-Fuentes and Jackson 2012). The ability to identify and clone *Bt* cry genes and the characterisation of the specific activities of individual cry proteins, as well as the availability of recombinant DNA technology, led to the development of new strategies for improving the exploitation of *Bt* for increasing its entomopathogenic potential. The first step towards improving *Bt* strains naturally involved the isolation of new strains with new or higher insecticidal activity against targeted insect pests. Until the 1970s, it was generally accepted that lepidopteran insects (moths and butterflies) were the only targets of *Bt*. In 1976, Goldberg and Margalit reported that a new *Bt* subspecies found in the Negev Desert, called *israelensis* (or *Bti*), killed mosquito and black fly larvae; both are from the order Diptera. This was the first documented case of a *Bt* strain killing an insect other than a caterpillar (Goldberg and Margalit 1977). The dipteran-active *Bt* subsp. *israelensis* was used extensively for vector control, particularly of black flies and mosquitoes, providing both medical and environmental benefits. In 1983, another new subspecies of *Bt*, subsp. *morrissoni* var. *tenebrionis*, was isolated (Krieg et al. 1983). This isolate, discovered in Germany, had excellent activity against the larvae of certain coleopteran species, and enhanced commercial development of this organism as a bioinsecticide. More recently, *Bt* crystal proteins were screened for activity against the free-living larval stages of nematode pests that infect animals and plants, and some of them were identified with significant activity in inhibiting larval development, thus demonstrating that the phylum Nematoda was also a target of *Bt* crystal proteins (Wei et al. 2003). To date, several thousand natural strains have been isolated from various geographical areas and from different sources, including grain dust, soil, insects and plants (Martin and Travers 1989; Smith and Couche 1991). These

**Table 15.1** Commercial genetically engineered *Bt* products for pest control

| *Bt* strain | Trade name | Company | Target insects |
|---|---|---|---|
| *Kurstaki* recipient, *aizawai* donor | Condor, Cutglass (transconjugant) | Ecogen | Lepidoptera |
| *Aizawai* recipient, *kurstaki* donor | Agree, Design, Turex (transconjugant) | Thermo Trilogy | Lepidoptera (*Bt*-resistant *Plutella xylostella*) |
| *Bt kurstaki* ED 7841 | CRYMAX | Ecogen | Lepidoptera |
| ED7826 | Leptinox (recombinant) | Ecogen | Lepidoptera |
| EQ7673 | Raven (recombinant) | Ecogen | Lepidoptera, Coleoptera |
| delta-endotoxin encapsulated in *P. fluorescens* | MVO, MATCH, M-Trak (Cell cap®) | Mycogen | Lepidoptera, Coleoptera |
| *Bt israelensis* | Acrobe | Cyanamid | Diptera |
| *Bt israelensis* | Skeetal | Novo-Nordisk | Mosquito |
| *Bt israelensis* | Teknar, Teknar HPD | Zoecon | Black flies, mosquito |
| *Bt israelensis* | Vectobac G, Gnatrol, Bactimos | Abbott | Mosquitoes, black flies |
| *Bt israelensis* 187, CS-8 | MieJueLing Preparation | Huazhong Agri. University | Diptera |

Source: modified after Sharma (2009)

isolates have been classified into about 85 serotypes based on biochemical properties and flagellar antigens or H-antigens (de Barjac and Frachon 1990; Lecadet et al. 1999; Jurat-Fuentes and Jackson 2012), producing several hundred crystal proteins that are active against most orders of insects (>575 species) and some other invertebrates and recently, leukemic cells (Ohba et al. 2009). A full listing and nomenclature of *B. thuringiensis* toxins is maintained by *Bt* toxin Nomenclature Committee headed by Neil Crickmore at www.lifesci.sussex.ac.uk/home/Neil_Crickmore/*Bt*. It is also important to note that most *Bt* strains produce more than one type of crystal protein that can act in combination (Sanchis 2011). Detailed information on bioassays and range of activity for specific cry toxins is available in the *Bt* specificity database (www.glfc.cfs.nrcan.gc.ca/bacillus/bt search.cfm).

Amongst the critical milestones was the discovery that the genes coding for the toxin crystals were located on transmissible plasmids enabling exchange of genetic information between *Bt* strains (Gonzáles et al. 1982). This opened up the way to manipulation of genes, including transfer between *Bt* strains. Conjugation was used to develop strains with optimised activity against a given insect pest or strains with a broadened toxicity spectrum (Sanchis 2000). *Bt* toxin genes have been cloned and expressed in a wide variety of organisms (baculoviruses to cyanobacteria) as well as in plants in attempts to improve their delivery and efficacy against insect pests. Several commercial products and novel strains of *Bt* have been developed through biotechnological intervention (Table 15.1). Breeding strategies involving both conventional (conjugation, transduction, electroporation, classical mutation) and genetic engineering methods have been employed for *B. thuringiensis* improvement. Recombinant DNA technology offers the possibility of transferring specific genes from one cell to another giving rise to a new array of new products with desired characteristics. An important delivery system was the encapsulation of the *cry* genes in a non-pathogenic *Pseudomonas fluorescens* (Flugge). This approach has been used to produce two commercial products: MVP for controlling lepidopterans and M-Trak for controlling coleopterans in which bacterial cells are killed by means of a physical chemical process after

fermentation and the toxins remained enclosed in the cell wall of the dead microorganisms as crystalline inclusions. These two products were registered by US Environmental Protection Agency in 1991. The process results in an active, stable biotoxin encapsulated within nonviable cells. The microencapsulation of *Bt* crystal, i.e. biopacking by *P. fluorescens* cell wall, thus protects the endotoxin from environmental factors. This process significantly increased the efficacy of the Cry proteins, increasing their persistence in the environment by protecting them against degradation and inactivation by UV irradiation (Gaertner et al. 1993).

The development of transformation protocols (Bone and Ellar 1989) and the availability of *Bt* shuttle vectors with multiple cloning sites (Baum et al. 1990; Arantis and Lereclus 1991) have greatly advanced the potential to produce improved strains. The use of vectors containing *Bt* replication origins and promoter sequences to derive expression of the introduced toxin gene (Gamel and Piot 1992; Sanchis et al. 1996), and site-specific recombination systems (Baum et al. 1996), has allowed elimination of foreign DNA sequences in the generated strains to result in a non-transgenic product (Jurat-Fuentes and Jackson 2012).

**Improvement in Host Range:** The efforts to improve insecticidal activity have been based on the transfer of *Bt* genes into nonhomologous isolates of *Bt*, a means of combining *delta*-endotoxins to produce either additive or synergistic effects and thus expand the host range. A self transmissible *cry1A* plasmid was transferred via conjugation from *Bt aizawai* strain to *Bt kurstaki* to design the commercial product Condor® (EG2348) to target lepidopteran pests as it combined the insecticidal properties of both the parents. An *rDNA* modified variant (ECX9399) of EG2348, the active ingredient in the bioinsecticide Condor®, has also been developed, which showed superiority to its parent strain against fall armyworm *Spodoptera frugiperda* (Smith) on corn (All et al. 1994). Similarly, two commercial products Agree® and Design® were developed by the transfer of cry1 plasmid from *Btk* to *Bta* strains. A unique combination of coleopteran- and lepidopteran-active insecticidal crystal proteins Cry 1Ac and Cry 3A was achieved by conjugation of *Btk* with *Bt tenebrionis*. This commercial product Foil® had an expanded host range to control Colorado potato beetle *Leptinotarsa decemlineata* (Say) in potatoes and European corn borer *Ostrinia nubilalis* (Hübner) in the corn (Carlton and Burke 1993; Baum 1998). The cloned genes from *Bt tenebrionis* were introduced into *Bt israelensis*, thus increasing host spectrum. The resulting organism demonstrated activity against mosquitoes and beetles as expected, but also showed unexpected additional activity against *Pieris brassicae* Linnaeus, a property which neither parent strain possessed (Crickmore et al. 1990).

A new type of biocide GCSC-*Bt*A based on 'Germany-China Scientific Cooperation' research has been developed by conjugation of *delta*-endotoxin from *B. thuringiensis* with abamectin, a toxin of *Streptomyces avermitilis* (ex Burg et al.). The conjugated GCSC-*Bt*A biocide had a broader host spectrum and a faster killing speed than either the *Bt* crystal or abamectin alone for the control of agricultural pests (Liu and Sengonca 2003). GCSC-*Bt*A has been recommended for use in the integrated pest control programmes in the vegetable fields as it not only proved to be highly effective against *Plutella xylostella* (Linnaeus), *Myzus persicae* (Sulzer), *Phyllotreta vittata* Fabricius, *Tetranychus cinnabarinus* (Boisd.), *Frankliniella occidentalis* (Pergande), *Aphis fabae* Scopoli and *Cameraria ohridella* (Deschka & Dimić) but also displayed safety to some predators (Zhu and Sengonca 2006).

**Improvement in Photostability and Activity:** The use of *B. thuringiensis* as insecticide is limited in field applications because of the rapid inactivation of toxins and spores after exposure to sunlight (Pusztai et al. 1991). Ultraviolet light is mainly responsible for this inactivation because of its impact on cells by direct DNA damage (e.g. pyrimidine dimers, cross-linking with proteins) or by producing reactive oxygen-derived free radicals (Zhang et al. 2008). *B. thuringiensis* mutants producing melanin from *Btk* and other strains obtained by inducing mutagenesis utilising

ethyl methanesulphonate (EMS) were found to be more resistant to UV irradiation (Saxena et al. 2002) and also showed highly insecticidal activities against potato tuber moth, *Phthorimaea operculella* (Zeller) (Mohamed et al. 2010). Melanin is a natural product, easily biodegradable in nature, and has been found to be excellent UV-protective agent (Liu et al. 1993). *Bt tenebrionis* strain NB-125, a mutant strain NB-176, was developed by gamma irradiation which contains two to three copies of Cry 3A. The commercial product Novodor® (Abbott) based on this mutant strain was able to produce large thromboid crystals composed primarily of Cry 3A toxin thus enhancing the field activity (Gurtler and Peterson 1991).

**Improvement in Activity Through Alternate Delivery System:** The insecticidal activity of *B. thuringiensis* has also been improved by delivering the toxins to target insects through alternate means of delivery. *Bt* genes have been cloned and expressed in endophytic, epiphytic and/or aquatic bacteria as well as eukaryotic plants to improve their delivery and residual activity. The rationale for using live endophytic or epiphytic bacteria as hosts is to prolong the persistence of Cry proteins in the field by using a host that can propagate itself at the site of feeding and continue to produce crystal protein (Sharma 2009).

**Endophytic Delivery of *Bt* Gene:** Crop Genetics International (CGI) transferred *cry 1Ac* gene from *Btk* ND-73 into *Clavibacter xyli* var. *cynodontis* (Cxc), an endophytic bacterium, that colonises the vascular system of various plants including maize. The transgenic Cxc Incide® infects the internal tissues of growing plants, and gene introduced into this bacterium encoded a protein toxic to the larvae of the European corn borer (Lampel et al. 1994). Endophytic isolates of *B. cereus* have been used as hosts for *cry 2A* gene, and *B. megaterium* isolates that persist in phyllosphere have been used as host for *cry 1A* genes (Mahaffee et al. 1994; Bora et al. 1994). Plant colonising bacteria such as *P. fluorescens*, *P. cepacia* Burkholder, *Rhizobium leguminosarum* Jordan and *Azospirillum* spp. have also been used to produce and deliver *Bt* proteins (Udayasuriyan et al. 1995). Two endophytes, *Rhizobium meliloti* Dangeard and *R. leguminosarum* (Frank) (nodule-forming bacteria), were introduced with δ-endotoxin *cry 3A* gene from *Bt tenebrionis* for the control of two coleopteran insects, *Sitona hispidulus* (Fabricius) and *S. lineatus* (Linnaeus), respectively (Bezdicek et al. 1991). A strain RSI identified as *Bacillus* sp. has been isolated, which is capable of colonising in cotton leaves. It has been found to be an excellent coloniser of cotton phyllosphere. *Cry 1Aa* gene of *Bt* was introduced into it by conjugal transfer. It has been shown that the resultant transconjugant colonises cotton plants for prolonged period and also protects the plant from *H. armigera* attack for more than 30 days (Narayanan 2002).

**Epiphytic Delivery of *Bt* Genes:** The major problem with *Bt* toxins is that they are not stable in soil, i.e. their residual activity is very low. Thus microbes, which normally occur in close association with roots of the crop plants, can be used for engineering these bacteria to produce the appropriate *Bt* toxin(s). A cloned *Bt* endotoxin gene (*cry 1Ab*) has been expressed in the corn root colonising Gram-negative bacterium, *P. fluorescens*, to deliver lepidopteran-active toxin beneath the soil surface for root-feeding insects. The recombinant bacteria were subsequently killed by heat and iodine, and thus the pseudomonad cell protected the *Bt* protein from environmental degradation, thus providing longer residual activity. The symbiotic relationship between pigeon pea, *Cajanus indica* Adans and nitrogen-fixing bacterium *Bradyrhizobium* has also been utilised for improving delivery system for soil insects. Nambiar et al. (1990) expressed dipteran-active *cry 4D* gene from *Bt israelensis* into *Bradyrhizobium* spp. that nodulate pigeon pea. The transfer of plasmid by conjugative mobilisation into this species provided protection against root nodule damage by *Rivellia angulata* (Hendel) on pigeon peas. Similarly, cloned genes from coleopteran active *Bt tenebrionis* have been transferred to *R. leguminosarum* (nodule-forming bacteria) by conjugation. Pea and white clover plants showed reduced root and nodule damage by larvae of

*Sitona lepidus* Gyllen when inoculated with toxin gene containing *Rhizobium* strains (Skot et al. 1990).

**Aquatic Delivery of *Bt* Genes:** The major limitation of *Bt israelensis* used for mosquito control is the rapid sedimentation of the spores and insecticidal crystals as most of the larvae feed near the water surface. So alternate delivery system has been sought for increasing their persistence in aquatic environment by expressing the *Bt* toxin genes in the hosts such as *Caulobacter crescentus* Poindexter, cyanobacteria, *Agmenellum quadruplicatum* (Menegh.) and *Synechococcus* spp. that are found predominantly in regions at or close to the water surface where larvae of many mosquito species feed. *Cry 4B* toxin gene from *Bt israelensis* was cloned into the broad host range plasmid Prk 248 and was expressed in *C. crescentus* CB15 (a motile ubiquitous bacterium living in upper layer of aquatic habitat). The recombinant *Caulobacter* can provide the potential for prolonged mosquito control (Thanabalu et al. 1992).

**Insertion of *Bt* δ-Endotoxin into Eukaryote Plants:** Remarkable progress based on recombinant DNA technology has been made over the past last three decades for developing crops with novel genes for resistance to insects, plant pathogens and herbicides. Development of insect-resistant transgenic crops has mainly focused on the integration of bacterial genes encoding for the production of toxic proteins, especially from *B. thuringiensis*. To date, *Bt* genes have subjugated the commercial scene very influentially for the last 15 years, at least in cotton and corn crops. More than 150 cry toxins have been cloned and tested for their toxicity to various insect pests belonging to different orders (Crickmore et al. 2011). Several *Bt* genes encoding cry toxins have been introduced either alone or stacked with other *Bt* genes or with herbicide resistance genes in different crops for imparting resistance to different insect pests. The crops in which *Bt* genes have been inserted for producing insect-resistant transgenic crops include cotton, maize, brinjal, rice, sorghum, tomato, cabbage, cauliflower, sugar cane, chickpea, alfalfa, broccoli and poplar (Table 15.2).

#### 15.3.1.2 *Lysinibacillus sphaericus*

*Lysinibacillus sphaericus* (syn. *Bacillus sphaericus* Meyer and Neide) is a highly heterogeneous species that contains both saprophytic and pathogenic species. It has attracted attention due to its pathogenicity to several mosquito species. A defining feature of this bacterium is the production of a spherical spore that is located in a terminal position within the swollen sporangium (Jurat-Fuentes and Jackson 2012). *L. sphaericus* spores are less sensitive to inactivation by UV radiation than other *Bacillus* spp. spores owing to high concentrations of small acid-soluble protein and DNA repair systems (Myasnik et al. 2001). The cloning and characterisation of binary toxin genes have been done from many *B. sphaericus* strains with different level of toxicity. Besides these, the *mtx* genes have also been detected in some toxic *B. sphaericus* strains. With the aim of expanding host range, increasing virulence and overcoming resistant colonies, the binary toxin from different strains has been expressed in different hosts like *E. coli*, non- or low-toxic *B. sphaericus* and crystal minus *Bt israelensis* as well as *C. crescentus* or cyanobacteria *Anabaena* sp. which naturally occurs in every aquatic habitat at or close to water surface.

**Expanding Host Range:** Cry 11A from *Bt israelensis* and Cry 11Ba from *Bt jegathesan* introduced, separately and in combination, into the chromosome of *B. sphaericus* 2297 by in vivo recombination resulted in recombinant strains toxic to *Aedes aegypti* (Linnaeus) larvae to which the parental strain was not toxic (Servant et al. 1999). It also overcame the resistance of *Culex pipiens* Linnaeus and *C. quinquefasciatus* Say to *B. sphaericus* strain 2297 partially. It has been reported that recombinant *E. coli* strain expressing a plasmid encoding a combination of mosquito-larvicidal genes from *Bt israelensis* (Cry 4A, Cry 4B and Cry 11A) and binary toxin genes from *B. sphaericus* exhibited broad range larvicidal activity against all *Aedes*, *Culex* and *Anopheles* larvae (Tanapongpipat et al. 2003). Recombinant *Bt israelensis* IPS-82/*B. sphaericus* 2362 showed high potency against 4th instars of *C. quinquefasciatus* and also improved efficacy

**Table 15.2** Insect transgenic crops having *Bt genes* from *Bacillus thuringiensis*

| Crop | *Bt* gene | Target pests |
|---|---|---|
| Cotton | *cry 1Ac, cry 1Ab/cry 1Ac, cry 1Ac + cry2Ab, cry1C* | *Helicoverpa armigera*, *Pectinophora gossypiella*, *Earias* spp., *Heliothis virescens*, *H. zea*, *Trichoplusia ni*, *Spodoptera* spp. |
| Rice | *cry 1 Ab, cry 1 Ac, cry 1Ab/cry1Ac, cry2a* | *Chilo suppressalis*, *Cnaphalocrocis medinalis*, *Scirpophaga incertulas* |
| Corn | *cry 1Ab, cry 9C, cry 3Bb, cry 1 F, cry 34Ab1/ cry 35 Ab1, cry 1 Ab + cry 3Bb, cry 1 F + cry 34Ab1/cry 35 Ab1* | *Ostrinia nubilalis*, *Chilo partellus*, *Busseola fusca*, *H. zea*, *Diatraea grandiosella*, *D. saccharalis*, *S. frugiperda*, *Diabrotica undecimpunctata howardi*, *D. virgifera virgifera* |
| Poplar | *cry 1Aa* | *Lymantria dispar* |
| Soybean | *cry 1Ac* | *H. virescens*, *H zea* |
| Sorghum | *cry 1Ac* | *C. partellus* |
| Sugar cane | *cry 1Ab* | *D. Saccharalis* |
| Groundnut | *cry 1Ac* | *Elasmopalpus lignosellus* |
| Chickpea | *cry 1Ac* | *H. armigera* |
| Tobacco | *cry 3, cry 2a5, cry 1Aa, cry 1Ab, cry 1Ac* | *H. virescens*, *Manduca sexta*, *H. armigera*, *H. zea*, *Leptinotarsa decemlineata* |
| Potato | *cry 3, cry 3a, cry 3b, cry 2a5, cry 1Ab, cry 1Ac9, cry 5* | *Leptinotarsa decemlineata*, *Phthorimaea operculella* |
| Tomato | *cry 1Ab, cry 1Ac* | *M. Sexta*, *H. armigera* |
| Brinjal | *cry 1Ac, cry 3b* | *Leucinodes orbonalis*, *Leptinotarsa decemlineata* |
| Chinese cabbage | *cry 1Ab, cry 1Ac* | *Plutella xylostella* |
| Broccoli | *cry 1C* | *P. xylostella*, *T. ni*, *Pieris rapae* |
| Alfalfa | *cry 1C* | *S. littoralis* |
| Canola | *cry 1C* | *H. zea*, *S. exigua* |

Source: Modified after Sharma (2009), Dhaliwal and Koul (2010) and Gujar and Dhillon (2011)

against larvae of *C. tarsalis* than *Bt israelensis* IPS-82 and *B. sphaericus* 2362 alone (Park et al. 2005). Moreover, it also suppressed resistance to *B. sphaericus* 2362 in *C. quinquefasciatus*.

**Enhanced Virulence:** Isolation of two *B. cereus* strains, Ae10 and Cx5, from mosquito larval guts and transformation with a recombinant plasmid, pBS373, harbouring binary toxin genes from *B. sphaericus* 2297 showed very high toxicity against *C. quinquefasciatus* larvae (Luxananil et al. 2003). Recombinant *Bt israelensis* Bti IPS-82/*B. sphaericus* BsB showed high potency against fourth instars of *C. quinquefasciatus*, being 21-fold as potent as *Bt israelensis* and 32-fold as potent as *B. sphaericus* alone (Park et al. 2005). The expression of chitinase gene *chi Ac* from *B. thuringiensis* in *B. sphaericus* 2297 using binary toxin promoter yielded a recombinant strain that was 4,297-fold more toxic than strain 2297 against *C. quinquefasciatus* thereby synergising the toxicity of binary toxin and may be useful in managing resistance to *B. sphaericus* (Cai et al. 2007).

*Caulobacter crescentus* Poindexter naturally occurs in every aquatic habitat and is found predominantly in regions at or close to the water surface. The *bin* gene from strain 2297 and *mtx 1* gene from SSII-1 were separately linked with the broad host range plasmid pRK248 and then electroporated in *C. crescentus* CB 15. The resulting recombinants expressing binary toxin were very active to *Culex*. sp. with $LC_{50}$ value of $2 \times 10^5$ cells/ml, which was similar to the toxicity of natural strain SSII-1 (Zhimming 2002). Like *Caulobacter*, various species of cyanobacteria are widely found near the water surface in both freshwater and saltwater environments. The effective expression of binary toxin in a single-cell cyanobacterium

*Anabaena* sp. 7120 resulted in recombinants with very high toxicity to targets and might be a potential agent for mosquito control with the persistence of one month (Xu et al. 2000).

**Overcoming Resistant Colonies:** Digestion of total genomic DNA from *B. sphaericus* LP1-G; ligation of purified products into vector pUc18 and then transformation in competent *E. coli* resulted in recombinant *E. coli* (E-UL68) which showed toxicity against both susceptible and two resistant colonies having the same level of toxicity as that of wild strain Lp1-G (Shi et al. 2003). The introduction of the mosquitocidal toxin gene *mtx 1* from *B. sphaericus* strain SSII-1 into an acrystalliferous strain of *B. thuringiensis* individually had moderate toxicity to binary toxin-susceptible and toxin-resistant *C. quinquefasciatus*; however, in combination with cytolytic protein gene *cyt1Aa* from *Bt israelensis*, the activity of *mtx 1* to target mosquito larvae enhanced, suggesting a synergism between Cyt 1Aa and mtx1 toxins (Zhang et al. 2006). The conjugal transfer of toxin-encoding megaplasmid from *Bt israelensis* to *B. sphaericus* produced the transconjugant bacteria that were significantly more toxic to *Aedes* sp. and were able to overcome resistance to *B. sphaericus* in resistant colony of *C. quinquefasciatus* (Gammon et al. 2006).

#### 15.3.1.3 *Bacillus cereus*

Most strains of *Bacillus cereus* Frankland & Frankland consistently associated with insects are saprophytic or symbiotic bacteria occupying the digestive tracts. While genetically highly similar to *Bt*, *B. cereus* does not produce parasporal crystalline toxins, which limit its virulence against insect hosts (Jurat-Fuentes and Jackson 2012). However, *B. cereus* strains have been found to be adequate hosts for expression of binary toxin genes from *B. sphaericus* as well as *cry4B* gene from *Bt israelensis* resulting in higher larvicide efficiency along with preventing mosquito population for obtaining resistance against these strains at early stages. Transformation of recombinant plasmid pBS373 harbouring binary toxin genes from *B. sphaericus* 2297 into two *B. cereus* strains, Ae10 and cx5, isolated from mosquito larval gut revealed the production and presence of 51 kDa toxin protein in both strains, and these two recombinant strains showed very high toxicity against *C. quinquefasciatus* larvae (Luxananil et al. 2003). A recombinant sphingomyelinase C, purified toxin, isolated from larvae of *Myrmeleon bore* (Tjeder) expressed in *E. coli* was as potent as the native protein in killing the German cockroaches, *Blattella germanica* (Linnaeus) (Nishiwaki et al. 2004).

#### 15.3.1.4 *Xenorhabdus*

*Xenorhabdus nematophila* (Poinar and Thomas), a facultative anaerobic bacterium, is engaged in mutualistic relationship with specific soil nematode *Steinernema* sp. Lee et al. (2004) identified and cloned a novel toxin gene (*tccC1*/*xptB1*) from *X. nematophila* strain isolated from Korea-specific entomophagous nematode *Steinernema glaseri* (Steiner) and expressed in *E. coli*, and the recombinant toxin protein caused a rapid mortality (80 % death within 2 days) of wax moth, *Galleria mellonella* (L.) larvae.

#### 15.3.1.5 *Serratia entomophila*

*Serratia entomophila* (Grimont et al.), a non-spore-forming bacterium, offers promise for the control of New Zealand grass grub, *Costelytra zealandica* (White), an important pest of pastures causing amber disease in target insect (Jackson 2007). Hurst et al. (2004) observed that 155-kb amber disease-associated plasmid (pADAP) carries the genes *sepA*, *sepB* and *sepC*, which are essential for the production of amber disease symptoms by *S. entomophila* in grass grub. Based on deletion analysis of pADAP and subsequent sequence data, a 47-kb clone was constructed, which when expressed in either an *E. coli* or a *Serratia* background exerted strong antifeeding activity and often led to rapid death of the infected grass grub larvae.

### 15.3.2 Baculoviruses

Baculoviruses are among the best known and most thoroughly studied insect pathogens and belong to family Baculoviridae. The baculovirus genome consists of a single circular double-stranded DNA molecule which is

covalently closed and supercoiled. Baculovirus replication is distinguished by the production of two different virion phenotypes: the occlusion-derived virions, which occur within proteinaceous viral occlusions and initiate infection of host, and the budded virions, which spread infection to other cells and tissues within the host. Baculoviruses have been isolated exclusively from insects and possess many of the attributes required in ideal biopesticides for use in insect pest management (Harrison and Hoover 2012). The major successes with baculoviruses have taken place in forestry, particularly for sawflies control in Europe and North America, and in agriculture for velvet bean caterpillar control on soybean in Brazil. However, narrow host range and slow action are the major obstacles in the widespread use of entomopathogenic viruses (Battu et al. 2002). Genetic engineering provides an effective tool for improving the activity of baculoviruses as biocontrol agents (Table 15.3). To improve the efficiency of viral insecticides, focused on increasing virulence, expanding host range and enhancing photostability, several strategies comprising both conventional (strain selection, serial passage and mutation) and DNA recombinant technology have been employed. These include expressing physiological effectors (hormones, enzymes or antisense), interrupting the normal metabolism of the insect, expressing insect-specific toxins such as neurotoxin and *Bt* crystal toxins or deleting baculoviral genes that may interact with the reaction of the viruses to the insect (Bonning et al. 2003; Hu et al. 2003; Narayanan 2003).

#### 15.3.2.1 Expanding Host Range

Baculovirus infections have been described in over 700 species of insects belonging to orders Lepidoptera, Hymenoptera, Diptera, Coleoptera, Trichoptera, Thysanura and Neuroptera. But most baculoviruses have limited host rages, usually restricted to one or a few closely related species within the same genus (Battu et al. 2002). An old-fashioned methodology of cross infectivity testing of baculoviruses that includes screening of long recognised strains for possible encroachments on generic, tribal, subfamilial, familial or even ordeal taxonomic frontiers in their host relationships may prove to be highly rewarding. Variants of baculoviruses may arise when virus infects alternate hosts. An isolate of spruce budworm, *Choristoneura fumiferana* NPV, was fed to the neonate cabbage looper larvae and the wax moth larvae. After passage, virus was able to infect new hosts. Baculovirus, hitherto restricted to one genus in Noctuidae, could also provoke lethal virus disease in various locusts and grasshoppers. An ELISA test showed that the baculovirus (from a moth) that spread systematically in the locust and killed it, sometimes within 4 days, was homologous to the virus extracted from the infected caterpillar. A reciprocal transmission of the virus from the locust back to the caterpillar, produced the same syndrome and the same causal agent (Harpaz 1987). Thus, it is very important that cross infectivity testing of baculoviruses should be undertaken to identify baculoviruses that improved from their original parent stock. After passage through the alternate host, the viruses can be further evaluated for enhanced virulence to their original (in back-feeding trials) as well as alternate insect hosts (Arora et al. 2000).

The recombinant DNA technology has its current applications in inserting foreign genes into insect baculoviruses and achieving their rapid and efficient expression in the recipient host systems. There are many avenues to explore the further development of baculovirus-based viral insecticides with extended host range, and detailed methods for genetic manipulations are available. The effective host range of baculoviruses might be expanded by inserting insect-specific neurotoxin and/or behaviour-modifying gene into the baculovirus genome so that the gene is under the control of promoter that expresses early on virus entry into cells. This would not actually alter the host range for virus replication but could alter the ability of virus to influence host behaviour and thereby increase the effective host range of virus as pesticide. The necessity for application of a variety of viruses to protect those crops affected by a multitude of pests could be avoided by specifically broadening the host range of one virus. It is envisaged that this may be achieved by engineering specific genes from

**Table 15.3** Genetically engineered baculoviruses for insect pest management

| Insect species | Baculovirus | Gene/toxin | Impact on biological activity | References |
|---|---|---|---|---|
| *Autographa californica* | AcNPV | Scorpion, *Androctonus australis* toxin gene (*AaIT*) | Falling off infected *H. virescens* larvae 5–11 h before death and unable to climb plant to continue feeding, less foliage consumption | Hoover et al. (1995) |
| | AcMNPV | *AaIT* | Reduces time to kill (25–40 %) of *S. eridania*, *T. ni* and *H. virescens* | Stewart et al. (1991), McCutchen et al. (1991), Carbonell et al. (1988) |
| | AcNPV | Enhancin gene from *Trichoplusia ni* granulovirus | Increase in infection by 21-fold in *S. exigua* | Hayakawa et al. (2000) |
| | AcMNPV | *Agelenopsis aperta* (*Mu-Aga-IV*) | Increase in effectiveness to kill of *S. frugiperda* and *T. ni* | Prikhodko et al. 1996 |
| | | *Anemonia sulcata* (*AsII*) | | |
| | | *Stydactyla* (*sh1*) | | |
| | AcMNPV | Juvenile esterase from *Heliothis virescens* | Increase in speed of kill by 20 % | Hammock et al. (1990), Bonning et al. (1999) |
| | AcMNPV | Viral gene (*egt*) – deletion | Increase in speed of kill, reduced feeding | O'Reilly and Miller (1991) |
| | AcNPV | Mite toxin | Increase in speed of kill | Tomalski and Miller (1991) |
| | AcNPV | Scorpion *Leiurus quinquestriatus hebraeus* toxin gene (*LqhIT1*, *LqhiT2*) | Improvement in speed of kill (32 %), decrease in median survival time (34 %) in *H. virescens* | Gershburg et al. (1998), Harrison and Bonning (2000) |
| | AcNPV | Spider *Diguetia canities* toxin gene (*DTX9.2*) | Induced paralysis and improved speed of kill | Hughes et al. (1997) |
| *Rachiplusia ou* | RoMNPV | Scorpion *L. quinquestriatus hebraeus* toxin gene (*LqhiT2*) | Improvement in speed of kill (40 %)in *O. nubilalis* and *H. zea* | Harrison and Bonning (2000) |
| *Bombyx mori* | BmNPV | *AaIT* | Increase in biological activity | Maeda et al. (1991) |
| | BmNPV | Diuretic hormone | Haemolymph of the infected larvae was decreased by 30 % with an increased mortality | Maeda (1989) |
| *Heliothis zea* | HzNPV | *AaIT* | Mortality at faster rate | Treacy et al. (2000) |

one NPV into the genome of other. A future approach would be to synthesise biologically active and authenticated insect pheromones and other behaviour-modifying proteins that are used in IPM by way of expressing the genes responsible for pheromones in insect cells through baculovirus vector instead of depending on in vitro production technique, which is cost prohibitive and time consuming. Engineering viruses by insertion of insecticidal proteins in many cases should also result in expanded host range. This is because, in many lepidopteran host species that do not develop disease upon inoculation with conventional baculoviruses, there can be limited viral replication, and these less susceptible hosts infected by an engineered virus that expresses a potent insecticidal protein will likely succumb as the virus need not replicate

extensively to paralyse or kill the larva (Narayanan 2003). Some of the baculoviruses genes identified to date that influence the host range are *p143* (putative DNA helicase), apoptotic suppressor genes (*p35* and *iap*) and some line transcriptional factors like *lef-7*, *hrf-1*, *hcf-1*, etc. These represent future DNA segments for genetic engineering of baculoviruses.

**Helicase Gene *(p143)*:** In case of AcMNPV, the putative helicase gene p143 is essential for DNA replication as reported by Lu and Carstens (1991). BmNPV and AcMNPV infect *B. mori* and *S. frugiperda* cells, respectively, but cannot replicate in heterologous cell line, but the recombinant AcMNPV carrying helicase gene from BmNPV was capable of replicating both in *B. mori* and in *S. frugiperda*.

**Apoptotic and Anti-apoptotic Gene *(p35)*:** Apoptosis may be defined as 'a process where the cell dies in a controlled manner in response to specific stimuli, apparently following an intrinsic programme'. A specific gene *p35* which is required for AcMNPV late gene expression and virus DNA replication in Sf-21 cells was identified as being responsible for blocking the apoptotic response (Clem and Miller 1994).

**Host Range Factor Gene *(hrf 1)*:** The broad spectrum AcMNPV does not infect either gypsy moth larvae or its cell line IPLB-Id 6527, but the recombinant AcMNPV carrying *hrf-1* gene from LdMNPV was able to replicate both in gypsy moth larvae and in its cell line.

#### 15.3.2.2 Improvement in Speed of Kill/Virulence

It has been known for sometime that different isolates of same viral species from different geographical locations can vary significantly in efficacy against the same target pests. Moreover, a virulent NPV sprayed at an appropriate rate will kill the first and second and in some cases the third instar within 2–4 days, but the later instars may live for a week or more, causing further damage to the crop. Current approaches improving the efficacy of viral insecticides, therefore, are aimed at developing broad spectrum viruses that will cause cessation of feeding within 24–48 h. NPVs isolated from different populations of fall armyworm, *S. frugiperda*, in Louisiana have been shown to vary by 16-fold in their $LC_{50}$ against these pests (Fuxa et al. 1988). Rabindra (1992) reported that among the isolates of NPV of *H. armigera* collected from three agroclimatic regions of India, there existed tremendous variation in virulence. Arora et al. (1997) reported that PAU isolate of HaNPV was more virulent than isolates from other parts of India. Similarly, Padmanaban et al. (2002) observed significant variation in virulence of different isolates of HaNPV collected from different areas of Punjab. These findings suggest that natural variations in pathogenic populations and virulence of viruses isolated from different geographical areas should receive more attention towards the selection of isolates to be used as viral insecticides.

In 2003, decreased susceptibility in several *Cydia pomonella* populations to *C. pomonella* granulovirus (CpGV) products in apple and pear orchards has been reported from Germany and from France (Fritsch et al. 2005; Sauphanor et al. 2006; Asser-Kaiser et al. 2007). The resistance was observed to be 60,000-fold in resistant colonies as against susceptible ones (Berling et al. 2009). The resistance to CpGV was found to be based on a single, dominant gene that was located on the Z-chromosome (Asser-Kaiser et al. 2010). Resistance to Mexican isolate (CpGV-M) was also observed when budded virus of CpGV was directly injected into the haemocoel of resistant individuals, thereby circum-passing the midgut which clearly indicated that not a change in the midgut but a cellular factor must be responsible for the CpGV not being able to replicate in resistant *C. pomonella* individuals (Jehle et al. 2010). Intensive search for alternatives to the conventionally used CpGV isolate resulted in the finding of new isolates for overcoming CpGV resistance. Several of these isolates have been tested in the field and are being registered for codling moth control in different European countries. Among them, two isolates (112 and NPP-R1) presented an increased virulence to resistant codling moth larvae. CpGV-I12 identified from Iran was found to be as effective against resistant codling moth larvae in Germany and was observed to

partially overcome the resistance (Jehle et al. 2006; Eberle et al. 2008). However, isolate NPP-R1 showed an even higher pathogenicity on resistant CpGV than other isolates. In addition, CpGV virus (Madex Plus) was also selected by subsequent passage of CpGV-M through resistant codling moth larvae. The 2016-r4 isolate obtained from four successive passages of NPP-R1 in RGV larvae had a sharply reduced proportion of the CpGV-M-like genotype and an increased virulence to against insects from the resistant colony (Berling et al. 2009).

Serial passage of viruses leads to rapidly accumulating mutants also known as 'the passage effect' which results in changing of virus efficacy. Increase in virulence of *Helicoverpa zea* SNPV against *H. zea* after repeated passage through the host has been reported (Shapiro et al. 1997; Ignoffo et al. 1995). Similarly, 15-fold increase in virulence of *Autographa californica* (Speyer) MNPV has been observed after repeated passages through *P. xylostella* (Klondy-Hirsch and Beck 1997). The direct application of conventional genetics through chemical mutagens so as to create variants of viruses has also been documented. Reichelderfer and Benton (1973) reported ninefold increase in virulence of *S. frugiperda* NPV after 4 treatments of virus with a chemical mutagen 3-methylcholanthrene. Similarly, Wood et al. (1981) obtained variants of *A. California* NPV with enhanced virulence after treating virus with 2-aminopurine.

The strategies used for genetically modifying the baculoviruses include (a) use of very late gene promoters like the polyhedron and p10 promoters to drive gene expression, (b) use of alternative promoters to produce relatively an early expression of the foreign gene, (c) use of multiple expression vectors to produce polyhedron-positive recombinant viruses and (d) deletion of gene ecdysteroid UDP-glucosyltransferase (*egt*) which the virus has acquired to protect the host from premature death. Candidate genes for hyperexpression in the baculovirus insecticide system include those encoding insect-specific enzyme gene (juvenile hormone esterase gene), hormone genes (eclosion hormone gene, diuretic hormone gene) and insect-specific foreign toxic genes (scorpion venom toxin genes, predatory mite toxic gene, predatory spider toxin gene, parasitic wasp venom gene and *Bt* δ-endotoxin genes). Recombinant baculoviruses expressing such genes have been constructed and their insecticidal activity assessed in vivo (Sharma 2009).

**Expression of Juvenile Hormone Esterase Gene:** The metamorphic changes of insect caterpillars into pupae and ultimately into adults are regulated by the juvenile hormone (JH). A reduction in the titre of JH is associated with drastic increase in the levels of juvenile hormone esterase (JHE) (Bonning and Hammock 1994). The speed of kill of AcMNPV was improved by 20 % by the expression of JHE (Hammock et al. 1990). First instar larvae of *H. virescens* or *T. ni* were killed 20 % faster when these were infected with AcJHE-KK (AcMNPV expressing mutant JHE-KK) as compared to control larvae infected with recombinant AcMNPV expressing authentic JHE (AcHE) (Bonning et al. 1999); however, survival time was only marginally reduced in older AcJHE-KK-infected instars.

**Expression of Hormone Genes:** Neurohormones are the master regulatory hormones of insects and affect critical physiological processes that include moulting, metamorphosis, reproduction and general homeostasis and kill or debilitate the treated insects. Further, neurohormones are proteins which are unstable and unsuited for application. So the use of insect viruses as highly efficient cloning-expression vectors for neurohormone gene comes to the rescue for their efficient utilisation in the management of insect pests.

**Eclosion Hormone Gene:** The eclosion hormone triggers the shedding of the old cuticle. Eldrigde et al. (1991) expressed eclosion hormone in *Manduca sexta* through AcMNPV baculovirus expression system with high level of biological activity.

**Diuretic Hormone Gene:** Diuretic and antidiuretic hormones are considered to play important roles in maintaining the water balance in insects. This balance might be disrupted if a recombinant virus produced elevated levels of either hormone in the infected larva. Maeda

(1989) expressed a synthetic diuretic hormone gene of tobacco hornworm in a recombinant baculovirus, viz. *Bombyx mori* NPV. The silkworm larvae infected with recombinant baculovirus showed alteration in the larval fluid metabolism; the haemolymph of the infected larvae was decreased by 30 % with an increased mortality in comparison with wild-type-infected larvae.

#### 15.3.2.3 Expression of Insect-Specific Foreign Toxic Genes

Arthropod venom offers a rich source of insect-selective toxins. Carbonell et al. (1988) were the first to attempt to improve the insecticidal activity of baculovirus by expressing biologically active scorpion toxin, insectotoxin-1 of *Buthus eupeus* Koch. Unfortunately, biological activity was not detected in insect bioassays using larvae of *Trichoplusia ni* (Hübner), *G. mellonella* and *Sarcophaga* sp. with any of the constructs. However, the first genes that were inserted into baculoviruses which successfully altered their biology were insect-selective toxin genes derived from other arthropods, viz. scorpion *Androctonus australis* (Linnaeus) and the predatory mite, *Pyemotes tritici* (LaGreze-Fossat & Montagne). The scorpion neurotoxins are an ideal choice to improve the efficacy of baculoviruses owing to their high selectivity as toxin proteins bind to sodium channel proteins and affect neuronal membranes (Lester et al. 1982; Zlotkin 1988). Genes that code for scorpion and mite venom have been engineered into the AcNPV from *A. californica* (Speyer) to increase the effectiveness of this virus (Tomalski and Miller 1991). It has been reported that recombinant AcMNPV expressing gene-encoding insect-specific toxin (*AaIT*) from scorpion significantly reduces time of kill by 25–40 % in southern armyworm, *Spodoptera eridania* (Cramer); cabbage looper, *T. ni*, and tobacco budworm, *Heliothis virescens* (Fab.) (Carbonell et al. 1988) and also acted more quickly and significantly reduced the crop damage by cabbage looper *T. ni* on cabbage under field trials. The *AaIT* gene expressed under the control of various promoters has also been inserted into NPV of mint looper, *Rachiplusia ou* (Guenee) (RoMNPV) (Harrison and Bonning 2000), cotton bollworms, *H. zea* (HzNPV) (Treacy et al. 2000) and *H. armigera* (HaSnPV) (Chen et al. 2000; Sun et al. 2004). Expression of *AaIT* under late p6.9 promoter of AcMNPV by recombinant RoMNPV resulted in 34, 37 and 19 % improvements in speed of kill in comparison to control larvae infected with RoMNPV when tested on neonates of *O. nubilalis*, *H. zea* and *H. Virescens*, respectively (Harrison and Bonning 2000). Treacy et al. (2000) evaluated insecticidal properties of corn earworm *H. zea* (Boddie) NPV (Hz*AaIT*) genetically altered with toxin from *A. australis* against Heliothine species and found that Hz*AaIT* killed the larvae of *H. virescens* and *H. zea* at faster rate than non-transformed HzNPV. In addition to *A. australis*, venom of yellow Israeli scorpion, *Leiurus quinquestriatus hebraeus* (Birula), also contains insect-selective toxins (*LqqIT1*, *LqhIT1*, *LqhIT5*, *LqhIT2* and *LqqIT2*). Gershburg et al. (1998) showed that recombinant AcMNPV expressing the excitatory LqhIT1 toxin from scorpion resulted in improvement in speed of kill by 32 % as against wild AcMNPV. Harrison and Bonning (2000) observed 34 % decrease in median survival time though expression of LqhIT2 as compared to control larvae in *H. virescens*. Similarly, recombinant RoMNPV expressing LqhIT2 gene construct showed 40 % improvement in speed of kill as against wild-type virus on larvae of European corn borer, *O. nubilalis*, and *H. zea*. Maeda et al. (1991) achieved a significant increase in activity by expression cDNA of *AaIT* in NPV from silkworm, *Bombyx mori* L.

*Helicoverpa zea* (Boddie) NPV has been improved by way of inserting mite toxin gene and expressed toxin during infection resulting in 50 % mortality within 40 h after virus treatment. The toxins NPS-901 (Krapecho et al. 1995) and *DTX9.2* (Hughes et al. 1997) discovered from spider *Diguetia canities* (McCook), when injected, induced paralysis that was specific to insects, and cloning it into a baculovirus enhanced the virus activity. Likewise, spider toxins *μ-Aga-IV* from *Agelenopsis aperta* (Gertsch) and *TalTX-1* from *Tegenaria agrestis* (Walckenaer) also showed an improved speed of kill (Prikhodko

et al. 1996; Hughes et al. 1997). The toxins from sea anemones, *Anemonia sulcata* (Pennant) (*AsII*) and *Stichodactyla helianthus* (Ellis) (*ShI*), have also shown 38 and 36 % improvements in speed of kill in neonates of *T. ni* and *S. frugiperda*, respectively. Quistad et al. (1994) purified and characterised insecticidal toxin from the venom of the parasitic wasp, *Bracon hebetor* Say, and had shown that the toxin paralyses insects. It has shown 400-fold higher biocidal activity against *Spodoptera* when compared to scorpion toxin and 100-fold higher activity against *Galleria* when compared to mite toxin.

#### 15.3.2.3.1 Expression of Proteases

Expression of basement membrane degrading proteases is one of the most remarkable improvements in speed of kill of recombinant baculoviruses. The baculovirus faces several obstacles within insect midgut, and the final midgut barrier is basement membrane. Expression of basement membrane degrading protease cathepsin L from flesh fly, *Sarcophaga peregrina*, in AcMNPV resulted in recombinant which showed 51 % faster killing speed in comparison to AcMNPV in neonates of *H. virescens* (Harrison and Bonning 2001).

#### 15.3.2.3.2 Expression of *Bt* Toxin Genes

*Bt* toxin as the fused product, i.e. polyhedron-Cry 1Ac-green fluorescent protein (GFP), has been expressed in baculovirus AcMNPV. The recombinant AcMNPV (Color*Bt*rus) expressing this fused product produced polyhedra that occlude *Bt* toxin and GFP and released toxin and GFP proteins in the insect midgut. Bioassay using second and third instar larvae of *P. xylostella* showed 100-fold reduction in $LD_{50}$, and $ST_{50}$ decreased by 60 % as compared to wild-type AcMNPV (Chang et al. 2003).

#### 15.3.2.3.3 Deletion of Ecdysteroid UDP-Glucosyltransferase (*egt*) Gene

Baculoviruses have acquired a gene known as *egt*, which encodes for enzyme ecdysteroid UDP-glucosyltransferase. The enzyme can transfer either glucose or galactose from UDP-sugar to ecdysteroids such as 20-OH-ecdysone, the hormone of insects which triggers moulting by governing gene expression. The deletion of the *egt* gene from viral genome accelerates the virus-induced mortality by allowing the infected larvae to begin moulting, resulting in feeding cessation during infection and premature degeneration of Malpighian tubules. Larvae infected with *egt*-deleted AcNPV displayed approximately 40 % reduction in larval feeding and early mortality with 20 % reduction in $LT_{50}$ as compared to wild-type AcMNPV-infected larvae. An AcNPV lacking *egt* was the first genetically improved recombinant baculovirus to be approved by USEPA for field testing in the USA and was first field tested by American Cyanamid in 1993 (Black et al. 1997).

Some of the baculoviruses genes identified to date that influence the virulence include chitinase gene (*chi A*) and enhancin genes which can be potential DNA segments for genetic engineering of baculoviruses.

**Chitinase Gene (*Chi A*):** The chitinous cuticle of the insect covers virtually all external surface, even extending through the foregut, hindgut and tracheal tubes, constituting the first line of passive defence in insects. The median time for mortality of fourth instar larvae of *S. frugiperda* infected with a recombinant virus containing chitinase gene was approximately 20 h shorter than that for insects infected with a wild-type virus (Gopalakrishnan et al. 1995). Such engineered baculoviruses possessing the chitinolysis activity, in addition to their standard infectivity, should be more effective in the field against pests, rather than directly applying chitinase-based insecticidal spray formulations (Mazzone 1987).

**Enhancin Genes:** Wang and Granados (1997) have identified an invertebrate intestinal mucin (IIM) from a lepidopteran insect *T. ni* similar to the mucus layer found in mammals, protecting the digestive tract from microbial infections. It has been shown that *T. ni* granulosis virus (TnGV) can overcome this IIM barrier and disrupt the integrity of peritrophic membrane due to the presence of 'enhancin gene' or viral enhancing factors (VEF). Since baculovirus 'enhancing' protein genes are virus coded, they

can be better utilised to improve the efficacy of viral pesticides in the future. A virus enhancing factor from armyworm, *Pseudaletia unipuncta* (Walker) (=*Mythimna separata*), has been successfully expressed in *E. coli*, and the resulting lysates of transformed *E. coli* cells enhanced PsunMNPV infection when treated with either trypsin or thrombin. The VEF enhanced PsunMNPV infection in larvae of armyworm (Hukuhara et al. 2001).

#### 15.3.2.3.4 Improvement in Photostability

One of the major obstacles to expansion in use of commercial baculovirus preparations for the control of crop pests is their sensitivity to photodegradation. The exact mechanism by which UV radiation inactivates the virus is not fully understood, although it has been opined that hydrogen peroxide produced by the near UV irradiation (300–380 nm region of solar spectrum) of one or more amino acids reduced both the vitality and pathogenicity of baculoviruses. A number of formulations containing sunlight protectants and application techniques have been used to improve photostability. However, genetic methods offer the greatest potential in this regard also.

Insect viruses have been selected for resistance to UV irradiation and for an increased rate of vertical transmission. One such strain of GV of codling moth (CpGV) was quite resistant to the effect of UV irradiation both under natural sunlight and in laboratory conditions (Brassel and Benz 1979). The modified strain was 5.6 times as resistant to UV light as the original isolate and remained infective for twice as long in the field. A UV-resistant strain of NPV of *Lymantria dispar* (Linnaeus) has also been obtained (Shapiro and Bell 1984). The genes that might be responsible for the effective DNA repair system, if isolated, inserted and expressed into the genomes of baculoviruses, can produce UV-resistant baculoviruses. Considering the available biodiversity in the tropics, such investigations in tropical agroecosystems will yield fruitful dividends. The biotechnological tools provide exciting opportunities for improving photostability, but thorough understanding of the mechanisms of photodegradation is a prerequisite for using these techniques (Arora et al. 2000). In an attempt to reduce UV inactivation of baculoviruses, Petrik et al. (2003) have developed a recombinant AcMNPV, vHSA50L, that expresses an algal virus pyrimidine dimer-specific glycosylase, cv-PDG, which is involved in the first steps of the repair of UV-damaged DNA. Although the polyhedra of vHSA50L showed no differences in UV inactivation in comparison to AcMNPV, the BV of vHSA50L was threefold more resistant.

### 15.3.3 Fungal Entomopathogens

Of the estimated 1.5–5.1 million species of fungi in the world, approximately 100,000 have been described (Blackwell 2011). Of these, approximately 750–1,000 species are entomopathogens placed in over 100 genera (St. Leger and Wang 2010). However, based on the number of cryptic species revealed by recent molecular phylogenic studies, it is evident that these estimates are low (Vega et al. 2012). The fungi infect individuals in most of the orders of insects including Hemiptera, Diptera, Coleoptera, Lepidoptera, Orthoptera and Hymenoptera. The majority of entomopathogenic fungi identified to date belong to fungal orders Entomophthorales (class, Zygomycetes; division, Zygomycota) and Hypocreales (class, Sordariomycetes; division, Ascomycota). Entomophthorales are chiefly parasitic on lower animals, and host death occurs by tissue colonisation with little or no use of toxins. Important entomopathogenic genera include *Entomophaga*, *Entomophthora*, *Erynia*, *Massospora*, *Pandora*, *Zoophthora* and *Neozygites* (Webster and Weber 2007). Although some of these genera cause frequent epizootics in insect pest populations, they are hard to mass produce. On the other hand, the members of Hypocreales are generally considered as opportunistic pathogens infecting many species of several insect orders, and host death is commonly associated with toxin production overwhelming host defence mechanisms. Important entomopathogenic genera include *Beauveria*, *Cordyceps*, *Isaria*, *Metarhizium*, *Nomuraea*, *Lecanicillium*, *Paecilomyces* and *Sorosporella*.

Besides these groups, microsporidia have also been included among fungi based on recent phylogenetic studies (Hibbett et al. 2007). Most of the more than 1,300 described species in approximately 180 genera are pathogens of invertebrate animals, with insects being type hosts of nearly half of the described genera (Becnel and Andreadis 1999). The major obstacles in the successful utilisation of entomopathogenic fungi as biocontrol agents include relative instability, requirement of moist conditions for spore germination, invasion and growth and also slow rates of kill. The optimisation of entomopathogenic fungi by genetic engineering is in its infancy because of a limited knowledge of the molecular and biochemical basis of fungi. Moreover, due to large size of the fungal genome and most fungal toxins being complex molecules determined by several genes, their genetic manipulation still remains a knotty task. The major objectives in genetic improvement of entomopathogenic fungi are to increase virulence and improve photostability. In addition, they have also been improved for resistance to fungicides. Breeding techniques like mutagenesis, transformation using plasmid vectors and protoplasts fusion are used for field stability and effectiveness.

#### 15.3.3.1 Enhancing Virulence

Protoplast fusion technique has been utilised to develop hybrid strains that possess more pathogenicity than that of the parents. Protoplast fusion of diauxotrophic mutants of *B. bassiana* (entomopathogenic strain) and *B. sulfurescens* (toxicogenic strain) resulted in hybrids that were more pathogenic than parents, and some possessed very high virulence and killed the insects more rapidly (Viaud et al. 1998). Entomopathogenic fungi such as *B. bassiana* invade insects by direct penetration of host cuticles via the action of diverse hydrolases including proteases and chitinases coupled to mechanical pressure. In order to better target cuticle protein-chitin structures and accelerate penetration speed, a hybrid protease (CDEP-BmChBD) was constructed by fusion of a chitin-binding domain BmChBD from *Bombyx mori* chitinase to the C-terminal of CDEP-1, a subtilisin-like protease from *B. bassiana*. Compared to the wild type, the hybrid protease was able to bind chitin and released greater amounts of peptides/proteins from insect cuticles. The insecticidal activity of *B. bassiana* was enhanced by including proteases, CDEP-1 or CDEP-BmChBD produced in *Pichia pastoris*, as an additive; however, the augment effect of CDEP-BmChBD was significantly higher than that of CDEP-1. Expression of the hybrid protease in *B. bassiana* also significantly increased fungal virulence compared to wild type and strains overexpressing the native protease (Fan et al. 2010).

Apart from cuticle-degrading proteinases, a strain of *M. anisopliae* was identified that produced an acute protein toxin active at 0.7 μg/100 mg, and other toxins from *M. anisopliae* and *B. bassiana* are being isolated (Quesada-Moraga et al. 2006). A toxic protein from *B. bassiana* (bassiacridin) had an $LT_{50}$ of 3 μg per insect when injected into fourth instar locust nymphs *Locusta migratoria* (Linnaeus) (Quesada-Moraga and Vey 2004). The various toxins from *M. anisopliae* and *B. bassiana* affect different aspects of insect biology and, therefore, could be used synergistically to increase the magnitude of hypervirulence and to reduce the probability of resistance evolving to a single transgene product (St. Leger and Wang 2010). The molecular and biochemical bases of pathogenicity of *M. anisopliae*, which causes green muscardine disease, have been particularly well studied. Various genes relating to formation of the appressorium, virulence and nutritional stress have been cloned from *M. anisopliae*. *Pr1*gene, which encodes a subtilisin-like protease, is involved in insect host cuticle penetration and has been found to activate trypsin, which in turn activates the phenoloxidase system involved in the initiation of the melanisation process and tyrosine metabolism. Additional copies of this gene were introduced into the genome of *M. anisopliae* with the objective to enhance the efficacy as *M. anisopliae*, which in general takes 5–10 days to kill a host. Infection with recombinant strain resulted in partial hydrolysis of haemolymph proteins and extensive melanisation of insect (St. Leger et al. 1996). As a result, the larvae infected with the recombinant strains were killed

at 25 % faster rate in comparison to larvae infected with wild-type *M. anisopliae*, and feeding damage was reduced by 40 %. Moreover, the melanised cadavers were poor substrates for fungal sporulation, which would restrict dissemination of the recombinant fungus, thereby limiting the environmental impact and necessitating repeat sales of the control agents. Although it would be possible to incorporate other insecticidal genes into *M. anisopliae*, the use of a homologous gene such as *Prl* may be more acceptable from a regulatory viewpoint (St. Leger 2007). *M. anisopliae* has also been genetically improved for increased pathogenicity and virulence through expression of insect-specific neurotoxin from scorpion *A. australis* under the control of promoter that is active only in the presence of insect haemolymph. The modified fungus exhibited same mortality rate in tobacco hornworm at 22-fold lower doses and, at certain concentrations, reduces survival of infected mosquitoes by > 40 % as compared to wild-type fungus (Wang and St. Leger 2007).

Epiphytic fungus, *Erwinia herbicola* (Lohnis) Dye, has been successfully transformed with a *Bt* toxin gene *cry1Aa1* present in plasmid pUN4. The pUN4 was expressed into three isolates of *E. herbicola*. The transformed *E. herbicola* strains Eh4, Eh5 and Eh6 expressed the toxin protein and conferred insecticidal activity. One of the transformed *E. herbicola* strains, Eh4, when sprayed on cabbage leaves for colonisation to test its insecticidal efficacy and persistency against the diamondback moth, *P. xylostella*, showed 36.7–60 and 40–83.3 % mortality after 48 and 72 h, respectively (Lin et al. 2002).

#### 15.3.3.2 Resistance to Fungicides

Benomyl- or carbendazim-resistant isolates of entomopathogenic fungi have been obtained by mutagenesis as well as by DNA-mediated transformations so that they can be used along with these pesticides in insect control programme. The susceptible strains of *M. anisopliae* have been treated with ultraviolet light and mutagenic agents to obtain benomyl- or carbendazim-resistant isolates (Tsai et al. 1993). A *β-tubulin* gene from the fungus *Neurospora crassa* (Draft) encoding resistance to benomyl was transferred to *M. anisopliae* via cosmid $p^{50}$. The transformed fungi showed normal cell division when cultured on nonselective agar and retained the ability to infect and kill the larvae of tobacco hornworm, *Manduca sexta* (Bernier et al. 1989). Another gene *benlate* A3 from *Aspergillus nidulans* has also been used to transform *M. anisopliae* via cosmid $p^{50}$ as vector (Goettel et al. 1990). The transformants were pathogenic to *M. sexta* in the presence of 50 μg/ml of benomyl, producing infection structures (appressoria) and enzyme chymoelastase which dissolve insect cuticle. The gene encoding the cuticle-degrading protease (Pr1) has also been inserted into genome of the same fungus. *B. bassiana* has also been transformed using electroporation for methyl 1, 2-benzimidazole carbamate (MBC) resistance with *N. crassa* '*β-tubulin*' gene. The transformants were stable and able to grow in the presence of 5 μg MBC/ml. Similarly, *B. bassiana* transformed with conventional protoplasting and electroporation and polyethylene glycol treatment via pSV50 harbouring 'β-*tubulin*' gene from *N. crassa* grew well on benomyl concentrations at 10 μg/ml. The transformants were found to be mitotically stable on wither-selective or non-selective medium (Sandhu et al. 2001). These studies demonstrated that the genetically engineered strains of entomopathogenic fungi having tolerance to fungicides can be used along with other components of pest control, thereby promoting their utility in integrated management programmes.

#### 15.3.3.3 Improving Photostability

The efficacy of mycoinsecticides is significantly influenced by environmental abiotic factors including solar UV radiation, temperature and humidity (Rangel et al. 2008). Various UV protectants have been employed to protect fungal conidia against UV radiation, especially UV-B (280–320 nm) (Jackson et al. 2010). To improve resistance to UV damage, Shang et al. (2012) used agrobacterium-mediated transformation to engineer *B. bassiana* with an exogenous tyrosinase gene. Tyrosinases are type-3 copper-containing

monooxygenases involved in the conversion of L-tyrosine or L-Dopa to form melanin (Ito et al. 2000). Melanin absorbs light at all wavelengths, especially the UV range. The mitotically stable transformants produced larger amounts of yellowish pigments than the wild-type strain, and these imparted significantly increased UV resistance. Moreover, the virulence of the transgenic isolate was also significantly increased against the silkworm *B. mori* and the mealworm *Tenebrio molitor* (Linnaeus).

### 15.3.4 Entomopathogenic Nematode

Nematodes comprise a tremendously diverse phylum whose members exploit habitats more varied than any other groups of animals except arthropods. The nematodes associated with insects are diverse phylogenetically, belonging to 13 different suborders of the Nematoda (Lewis and Clarke 2012). Nematodes in the families Steinernematidae and Heterorhabditidae have the ability to quickly infect their insect hosts. Their unique association with symbiotic bacteria (*Xenorhabdus* for Steinernematidae and *Photorhabdus* for Heterorhabditidae) makes them effective biocontrol agents. Entomopathogenic nematodes (EPNs) are persistent and recycle inside host causing long-term and sustainable effects on pest populations. They can be cultured easily in vitro and have a high reproductive potential. However, susceptibility to environment stress and diverse host-finding behaviour limits their efficiency. Genetic improvement in entomopathogenic nematodes may increase their searching capacity, virulence and resistance to environmental factors. Techniques such as selective breeding, hybridisation, mutagenesis and gene transformation have been employed for genetic manipulation in nematodes.

#### 15.3.4.1 Improvement in Host-Finding Ability and Infectivity

A successful breeding programme must have for a desired trait either a moderately high heritability value or a very large genetically diverse base population from which to select. Artificial selection for enhancement of entomopathogenic nematode host finding and infectivity has been demonstrated in several selection programmes. A 72-fold increase in host-finding ability of a strain of *Steinernema carpocapsae* Weiser against grubs of Japanese beetle, *Popillia japonica* (Newman), was obtained, but when assayed in field and laboratory, no difference in infectivity was detected when compared to wild-type strains (Gaugler and Campbell 1991). Similarly, an increase of 20–27-fold for host-finding ability has been demonstrated in *S. carpocapsae* towards wax moth, *Galleria mellonella* (Linnaeus), after 13 rounds of selection (Gaugler et al. 1989; Gaugler and Campbell 1991).

#### 15.3.4.2 Adaptability to Environment Stress

Selection has also been successful in enhancing infectivity at low and high temperatures. Screening natural entomopathogenic nematode populations for desirable biological traits, while very laborious, is feasible and has shown some success in isolating heat- and cold-tolerant strains (Glazer et al. 1996; Mracek et al. 1998). Grewal et al. (1996) showed that selecting nematodes for cold tolerance enhanced their virulence by 5.3 and 6.6 times after six and twelve passages at 8 °C. A high variability in tolerance among strains and comparatively high heritability for the adapted heat tolerance were observed in 36 natural populations and 18 hybrid or inbred strains of *Heterorhabditis bacteriophora* Poinar tested for response to high temperature (Mukuka et al. 2010a). Similarly, variability in desiccation tolerance has also been documented in 43 strains of *Heterorhabditis* and 18 hybrid/inbred line strains of *H. bacteriophora* (Mukuka et al. 2010b). Desiccation-tolerant mutants of *Heterorhabditis megidis* Poinar have been isolated after exposing to ethyl methanesulphonate (EMS). Mutagenesis induced by exposing young *H. bacteriophora* hermaphrodites to EMS has been also documented (Koltai et al. 1994). Hybridisation can also offer powerful tool for genetic

improvement of entomopathogenic nematodes to produce superior strains. A trait for heat tolerance was transferred from *H. bacteriophora* strain designated IS5 discovered in the Negev desert in Israel to the wild-type *H. bacteriophora* HP88 strain with no loss of fitness in hybrid progeny as compared to wild strain (Glazer and Segal 2003). Hybridisation of heterogeneous population of *S. feltiae* for desiccation tolerance and host-seeking ability resulted in a survival rate of 80–90 % for tolerance to rapid desiccation, more than 85 % for tolerance to slow desiccation and more than 75 % host-seeking ability after 10, 20 and 25 selection cycles, respectively (Salame et al. 2010).

Genetic engineering provides significant advantage over conventional methods in improving the efficacy of EPNs. This approach involves microinjection of foreign DNA into the gonads of a young adult female or a hermaphrodite or through biolistic DNA bombardment. For the first time, successful transformation of *H. bacteriophora* was made by microinjection of plasmid vectors carrying hsp-16 genes coding for 16-kDa heat-shock protein as well as *rol-6* gene coding for roller phenotype (Hashmi et al. 1995). *H. bacteriophora* has also been genetically improved for thermotolerance by transfer of gene from *Caenorhabditis elegans*, *hsp70A*, encoding heat-shock protein which enables cells to eliminate or renature proteins damaged by high temperature. The transgenic nematodes exhibited 18-fold tolerance to heat shock as against wild ones (Hashmi et al. 1998). Transformation of *tps-1* (trehalose-6-phosphate synthetase) gene and heat-inducible promoter derived from *C. elegans* into *Steinernema feltiae* resulted in transformed nematodes showing tolerance to desiccation and osmotic loss (Vellai et al. 1999). Currently, genome of TTO1 strain of *H. bacteriophora* is being sequenced at the Washington University Genome Sequencing Center in St. Louis. The completion of the genome sequence will establish a solid foundation for the much needed functional genomic studies on the genes that are involved in critical biological processes of nematodes, viz. dauer formation, stress resistance, sex determination, etc., and functions of these genes will be elucidated using RNA interference technology (Jindal et al. 2010).

#### 15.3.4.3 Tolerance to Nematicides

Entomopathogenic nematodes have also been selected for enhanced resistance to nematicides and other insecticides. Genetic selection of *H. bacteriophora* strain HP88 to nematicides resulted in 70-fold increase in resistance to oxamyl and eight- to ninefold increase in resistance to phenamiphos and avermection (Brey and Hashmi 2003).

## 15.4 Conclusions

The exploitation of biocontrol agents (parasitoids, predators and pathogens) could provide an excellent alternative to chemicals for pest management as they are economically viable and ecologically safe. However, limited success has been realised with augmentative biological control due to limited research efforts. The major obstacles in their widespread use include sensitivity to adverse environment conditions, narrow spectrum of activity and slow speed of kill. Therefore, increasing efforts need to be devoted to overcome these limitations so that these can be utilised for the efficient biological control programmes. This may be achieved by conventional as well as genetic engineering or recombinant DNA techniques. The conventional methods include strain selection, serial passage, mutation, conjugation, transduction, natural selection, selective breeding, hybridisation, etc., whereas the genetic engineering approaches comprise indirect gene transfer methods (vector mediated) by using *Agrobacterium tumefaciens* as vector and direct gene transfer (vectorless methods) such as microprojectile bombardment with DNA or biolistics, direct DNA transfer into isolated protoplasts, electroporation, microinjection, etc. It is worthwhile to mention that the tools of molecular biology have led to the development of more effective, faster acting baculoviruses, bacteria, fungi and nematodes that could

provide a valuable tool for sustainable pest management. This has led to the improvement in their genetic makeup providing broad range insect control with desired pathogenicity, virulence, host range and persistence. Among these entomopathogens, genetically modified bacteria have reached the commercial sector, and recombinant baculoviruses are expected to mark their appearance in the near future. The engineering of entomopathogenic nematodes and fungi is in its infancy, slowed in part by the greater genetic complexity of these organisms. However, genetic improvement of arthropod natural enemies has received little attention and is only restricted to selection process for improved strains with tolerance to pesticides and high temperature. The thoughtful study of genetics and physiology of reproduction through biotechnological interventions could play an important role in improving mass production of arthropod natural enemies. The prospects for genetic improvement of biocontrol agents are exciting; however, as with all other initiatives that involve release of genetically modified bioagents, there is significant concern. The potential environment risks associated with the release of these genetically manipulated bioagents need to be addressed at all four trophic levels if we are to really exploit these biotechnological interventions in improving the biocontrol agents for sustainable pest management.

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# Part IV

# Semio-Chemicals and Biotechnology

# Kairomones for Increasing the Biological Control Efficiency of Insect Natural Enemies

16

Pathipati Usha Rani

**Abstract**

Insect natural enemies exploit a variety of chemical signals from different trophic levels as kairomones and synomones to locate their herbivorous host and their habitat. Herbivore-induced plant odor synomones; prey-/host-related kairomones from materials such as eggs, larval cuticular extracts, and insect frass; or even host pheromones lead natural enemies to their hosts which further results in successful parasitization. This chapter provides an overview of the biocontrol agents and their strategies in host location utilizing the chemical signals from various sources associated with hosts and host plants. The interactions between plant kairomones and insect sensory organs and their role in biocontrol were discussed. The author describes her own work with plant-produced chemicals and other kairomones for the control of different pests and provides an overview of other research in this area.

**Keywords**

Kairomones • Plant defense • Egg parasitoid • Semiochemicals • Sensilla • Host location

**Objective** The main objective of this chapter is to review the plant responses in emission of volatile organic compounds on pest/predator attack and their role in attracting the herbivore enemies in locating the infested plants (host habitat location) and to kill the pest or predator. This chapter also focused on in-depth explanation of biocontrol agents like egg and larval parasitoid attraction towards the infested plants using these volatiles as cues. This chapter leads to various possibilities in selective plant breeding to induce the plant for the production of parasitoid oviposition stimulants from plant to improve biocontrol efficiency of the insect natural enemies.

## 16.1 Introduction

Kairomones as defined by Brown et al. (1970) are the chemical substances released by one insect species which evoke behavioral and physiological response in the receiver such that the subsequent

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_16, 

actions are favorable to the receiver and not the emitter. Discoveries of kairomones for several parasitoids increased their exploration for the possibility of using them in biological control programs. The fact that the rate of parasitism could be increased by spraying crops with the kairomones and their use in behavior manipulation of biocontrol agents was explained as early as in the 1970s itself (Lewis et al. 1972, 1975a, 1977). Later the studies of Gross et al. (1975), Nordlund et al. (1976, 1984), Lewis et al. (1982), Elzen et al. (1984), and Nordlund (1987) showed that kairomones originating from both the hosts and their food sources influence the searching, attacking, and retention of entomophagous insects which have tremendous advantage in biological control programs. Parasitoids are considered as the most effective biocontrol agents as they manage the herbivore at the egg stage itself, before they attain larval stage which is usually the most destructive period. Host eggs can be used in conjunction with kairomones to improve the performance of these important beneficials.

It is already established that the process of host location by parasitoids consists of at least 4 phases – host habitat location, host location, host recognition, and finally the host acceptance (Nordlund et al. 1988; Vinson 1998). Parasitoids employ visual cues at a distance along with long-range volatile cues emitted by the infested plants in an attempt to locate host habitat (Turlings and Wäckers 2004). Once landed on a plant, multiple non- or low volatile searching stimulants that are linked to the host are used for retrieval (Rostás et al. 2008). A number of plant secondary compounds have been identified as nonvolatile kairomones of relatively high molecular weight. These are active in modifying insect behavior as arrestants, feeding stimulants, and oviposition stimulants. *Trichogramma*, the common egg parasitoid, use the plant volatiles as host location cues from the damaged plant parts (Vet and Dicke 1992).

## 16.2 Kairomones from Host Plant

Plants, particularly the flowering plants (angiosperms), have colonized vast majority of the terrestrial surfaces, mainly due to their exponentially bigger contribution to the terrestrial biomass by volume and weight, high levels of specialization, and elaborate relationships with other organisms. Plants are principal food source for many organisms; particularly herbivores are the most frequent dependants on plant parts in a crop ecosystem. Most herbivore insects cause extensive damage to plant tissues while feeding. Therefore, plants require effective defense mechanisms. In spite of so many attackers, plants not only survive but thrive in this environment only because of their remarkable ability and diverse defense mechanisms. These sophisticated and complex mechanisms are explored vigorously in recent years, and the inventions of modern analytical tools made the identification easier.

## 16.3 Induced Plant Defenses

One category of plant defenses which are relatively newly discovered is those features that indirectly protect the plant by enhancing the probability of attracting the pest's natural enemies. Several semiochemicals are involved in this kind of defense. Plants can respond to feeding or egg deposition by herbivorous arthropods by changing the volatile blend that they emit – a phenomenon that is called indirect defense. Herbivore damage induces production of volatile chemicals through the leaf surface in many plants (Pare and Tumlinson 1999). These are volatile organic compounds (VOCs) involved in interaction between organisms (Fig. 16.1). The volatiles emitted from the plants due to induction of herbivore feeding act as synomones, which are involved in indirect defense because they attract their natural enemies (Dicke 1999b; Dicke and van Loon 2000). Different organisms respond rather specifically to these cues generated by herbivory.

Plants emit volatile hydrocarbons through their leaf surfaces. Volatile compounds emitted by undamaged plants are much lower in rate than the herbivore-damaged plants. The emission of plant volatiles can increase substantially following insect damage. These induced volatile chemicals are highly specific to the herbivore

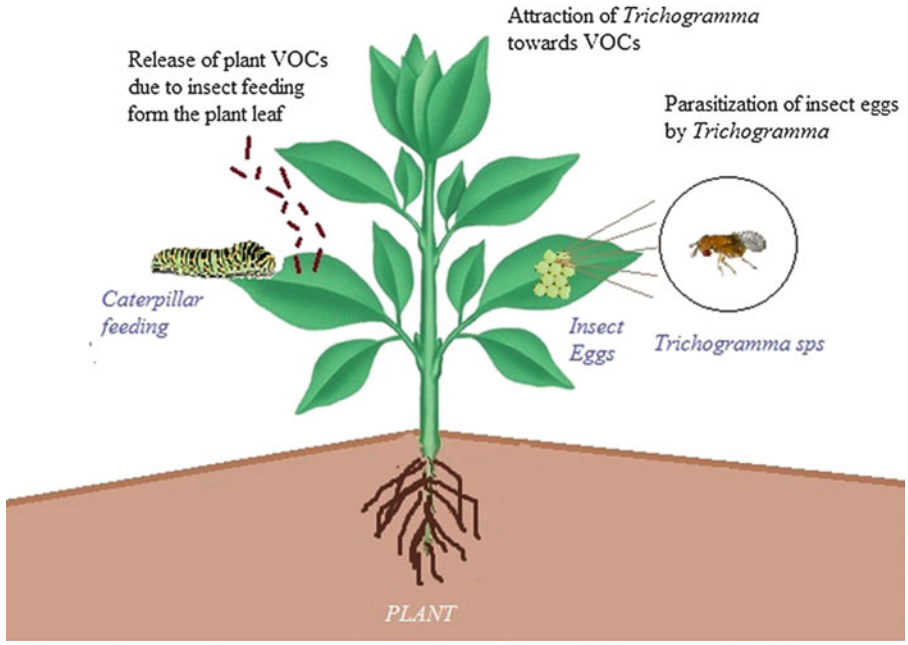


**Fig. 16.1** Schematic diagram of plant defense volatile chemical (VOC) emissions and the egg parasitoid attraction

and its plant system (De Moraes et al. 1998; Karban and Baldwin 1997; Takabayashi and Dicke 1996; Sabelis et al. 2007). Plant-derived stimuli are assumed to be more detectable from a distance (Geervliet et al. 1994). The herbivore-induced plant volatile (HIPV) blend is very complex, sometimes consisting of hundreds of compounds (Mumm and Dicke 2010). In response, the herbivore-damaged plants are known to emit more than 1,000 different VOCs, including alkanes, alkenes, alcohols, ketones, aldehydes, ethers, esters, and carboxylic acids (Dudareva et al. 2004; Niinemets et al. 2004). Some VOCs are taxon specific, such as the glucosinolate breakdown products in *Brassica* species (Mattiacci et al. 1995), whereas others appear to be common to many different plant families (Van Den Boom et al. 2004).

The most common VOCs are the "green leaf volatiles" (C6 aldehydes, alcohols, and derivatives), cyclic and acyclic terpenes, phenolic compounds, and nitrogenous compounds (Dicke 1999b; Pare and Tumlinson 1999). Most of the HIPVs consist of terpenoids, e.g., ((E)-β-ocimene (3,7-dimethyl-1,3,6-octatriene), (E, E)-a-farnesene, (E)-4,8-dimethyl-1,3,7-nonatriene) (Fig. 16.2), green leaf volatiles (e.g., hexanal, (Z)-3-hexen-1-ol, (Z)-3-hexenyl acetate), phenylpropanoids (e.g., methyl salicylate, indole), and sulfur- or nitrogen-containing compounds (e.g., isothiocyanates or nitriles, respectively) (Mumm and Dicke 2010). Terpenoids are widely used as host location cues by wandering parasites or predators (Turlings et al. 1990, 1995).

Damaged corn seedlings release high levels of volatile terpenoids within several hours of infestation. These induced chemicals serve two functions. Firstly, they affect the feeding of the herbivore by making the leaf less palatable (Turlings et al. 1991a, b), and secondly they attract the parasitoids that feed on caterpillars (Turlings et al. 1990). Artificially damaged plants did not produce these volatiles, but addition of caterpillar regurgitates or oral secretion to the wounded site induced the release.

The female parasitoid while foraging displays characteristic sequences of walking orthokinetic (changes in speed) and klinokinetic (changes in frequency of turning) responses, a behavior that

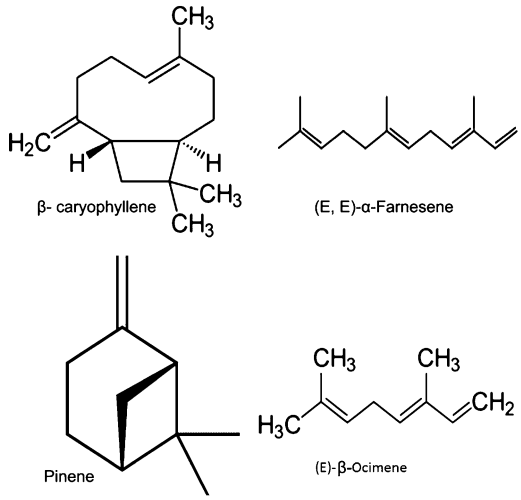


**Fig. 16.2** Some of the plant terpenoids that are used as host location cues

results in restricted area/patch search. Plant infochemicals play a major role in the tritrophic interactions of the plant–host–parasitoid interactions, especially during host selection process by parasitic wasps in general, including *Trichogramma* (Vinson 1976; Nordlund 1994).

D'Alessandro and Turlings (2006) tested the attraction of naive and experienced females of the two parasitoids *Cotesia marginiventris* and *Microplitis rufiventris* to partially altered volatile blends of maize seedlings (*Zea mays* var. Delprim) infested with *Spodoptera littoralis* larvae and specified that parasitoids with a comparable biology may employ different strategies in their use of plant-provided cues to locate hosts. Results from similar experiments with modified odor blends of caterpillar-infested cowpea (*Vigna unguiculata*) indicate that key VOCs in different plant species vary greatly in quality and/or quantity. Finally, experienced wasps were more strongly attracted to a specific blend after they perceived the blend while ovipositing in a host.

It was demonstrated that the egg parasitoid *Trichogramma chilonis* (Ishii) responds positively to the leaf surface chemicals from the castor (*Ricinus communis*) plants infested with the semilooper, *Achaea janata* (L.). The crude dichloromethane extracts of the castor leaves from the pest infested and the normal healthy plants, applied on to the surface of the host eggs, resulted with the enhanced parasitization of *T. chilonis* (Usha Rani and Lakshiminaryana 2008). Parasitoid's oviposition rate is higher in the eggs treated with the *A. janata*-infested castor leaf extracts, and they showed a lower or nil attraction to the normal healthy (pest-undamaged) castor plants. Isolation and purification of the crude extracts using chromatographic techniques and identification by GC and GC-MS revealed several terpenoids, hydrocarbons, acids, and other chemicals (Fig. 16.3, Table 16.1). It is interesting to find that tricosane and pentacosane, the straight chain hydrocarbon chemicals that commonly occur in kairomones from the adult female moths, are present only in semilooper-damaged plants and not in intact leaf extracts. Similarly the acids 9-hexadecanoic acid, 9-octadecanoic acid, and dimethyl-ester-ethanedioic acid have been found in the pest-infested leaves (Usha Rani and Lakshiminaryana 2008).

However, when the leaf surface chemicals of *R. communis*, damaged due to the feeding of the host insect, *Achaea janata* (L.) (castor semilooper) (Lepidoptera: Noctuidae), and a nonhost insect, serpentine leaf miner *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae), were evaluated for their influence on host location, host acceptance, and ovipositional behavior against the egg parasitoid *T. chilonis* (Usha Rani et al. 2008), the parasitoid behavior was quite different to each of the plant chemicals. *A. janata*-damaged leaf emissions had synomonal effects on the parasitoid and induced orientation and increased oviposition, whereas the surface chemicals from the plant infested with nonhost *L. trifolii* ceased to produce any such effects. Maximum egg parasitization was observed in *A. janata*-infested castor leaf extracts compared to the leaf miner-infested or normal healthy castor leaf extracts. The results are interesting in the context of tritrophic interactions between the pest, the parasite, and the host plant and are useful in biological control of insect pests (Usha Rani et al. 2008) (Fig. 16.4).

Studies with plants like lima beans (Dicke et al. 1990, 1993), corn (Turlings and Tumlinson 1992),

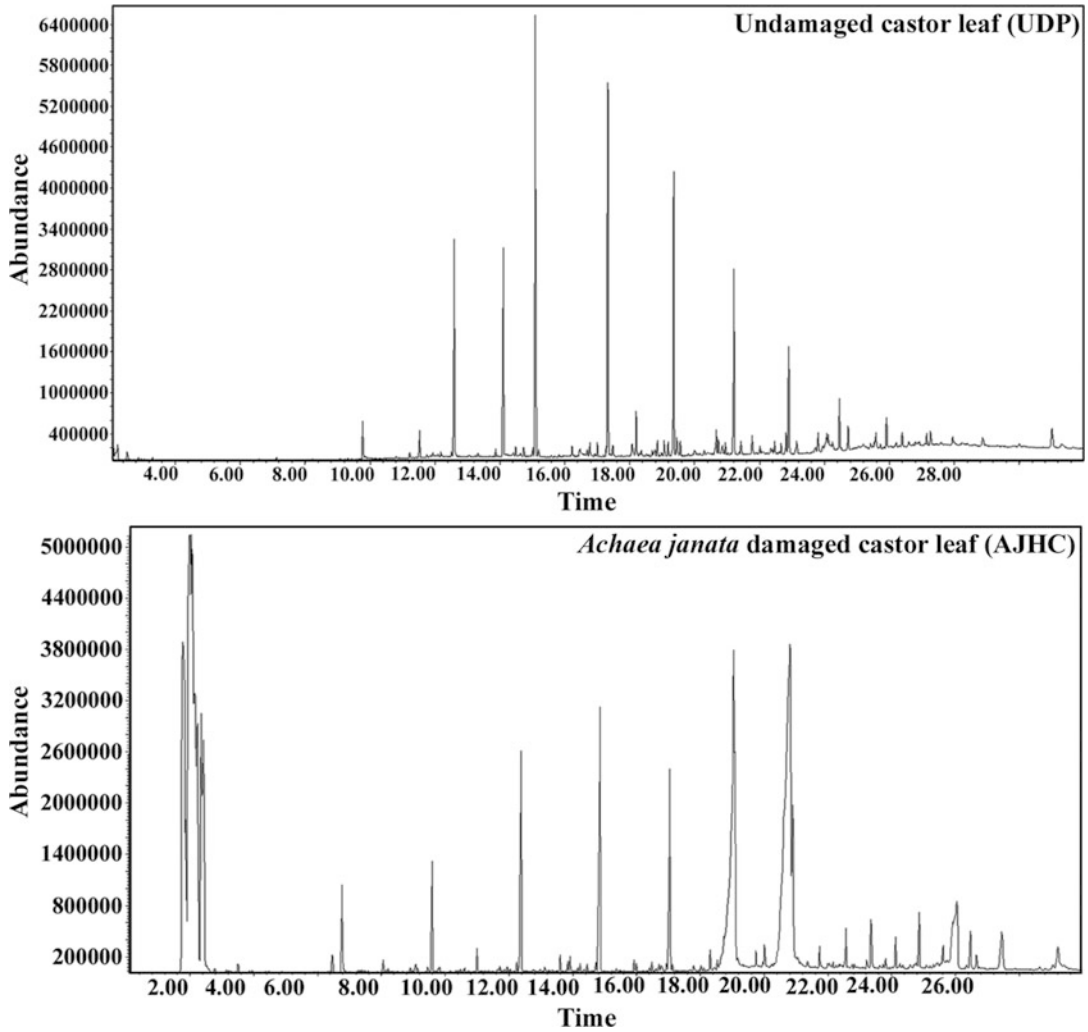


**Fig. 16.3** Gas chromatogram of the castor plant surface chemicals from undamaged and *Achaea janata*-damaged plant

**Table 16.1** List of chemicals present in *A. janata*-damaged castor plants

| S. no | Chemicals |
|---|---|
| 1 | Dodecane |
| 2 | Tetradecane |
| 3 | Hexadecane |
| 4 | Octadecane |
| 5 | Pentacosane |
| 6 | Hexacosane |
| 7 | *N*,*N*-dimethylformamide |
| 8 | Lupenol |
| 9 | 9-Octadecenoic acid |
| 10 | Unidentified chemical |

and recently cotton (Turlings et al. 1995; Rose et al. 1996) have shown that induced volatiles were released not only locally by the damaged leaf but also systemically in undamaged parts of the plant. The yellow stem borer *Scirpophaga incertulas* Walker (YSB)-infested rice plants emit chemicals through the surface of their infested stems which trigger attractant and arrestment responses and ovipositional stimulation in its egg parasitoid, *Trichogramma japonicum* Ashmead, apart from acting as long-range kairomones (Usha Rani and Sandhyarani 2012). Stem borer-infested plant extracts had enhanced

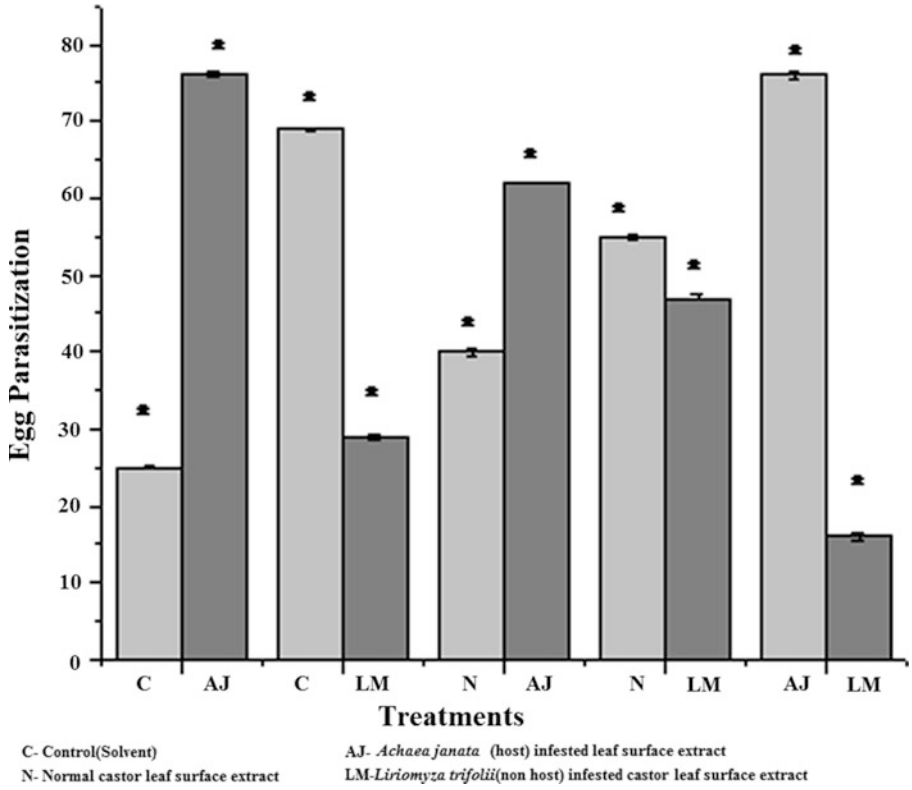


**Fig. 16.4** Effect of host (AJ)- and nonhost (LM)-infested castor plant surface extracts on parasitization rates of *Trichogramma chilonis*

the parasitization rate of *T. japonicum*, whereas host eggs treated with the extract from undamaged stems or solvent-treated control failed to evoke changes in the parasitoid's behavior. A preliminary GC-MASS analysis indicated the presence of several organic compounds. The analysis also revealed qualitative and quantitative differences between the chemical profiles of the infested and uninfested plants (Table 16.2). The hydrocarbons – decane, dodecane, tetradecane, hexadecane, octadecane, docosane, and eicosane – were detected in both the pest-undamaged and pest-damaged plants, and also there are quantitative differences between these samples. The quantitative variation of the $C_{10}$ to $C_{14}$ hydrocarbons between the pest-damaged and pest-undamaged stem extracts was negligible; however, the variation was more significant between $C_{16}$ and $C_{30}$ hydrocarbons. Nearly twice as many hydrocarbons (hexadecane, octadecane, docosane, and triacontane) were present in the surface extracts of the pest-damaged stems compared with normal healthy or undamaged plants (Usha Rani and Sandhyarani 2012).

The maize stem borer endoparasitoid *Cotesia flavipes* was attracted to the volatile chemicals from maize seedlings infested with *Chilo partellus* larvae, and the uninfested maize failed to induce any response in these parasitoids (Ngi-Song et al. 2000). Coupled gas chromatography-electroantennographic detector (GC-EAD) analysis of the volatiles from larvae-infested maize revealed six electrophysiologically active compounds on the antennae of the female parasitoid. These compounds were identified by GC-MS as (Z)-3-hexenyl acetate, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, heptanal, (*E*)-β-ocimene, and a C-5 aliphatic compound. Green and Ryan (1972) and later Arimura et al. (2009) showed that many of these induced responses to herbivore damage are

**Table 16.2** List of chemical compounds along with Rt values, detected by GC-MS from the stems of rice yellow stem borer-infested rice plants

| S. no | Chemicals | RT | S. no | Chemicals | RT |
|---|---|---|---|---|---|
| 1 | Tetradecane | 2.52 | 20 | a-Terpinene | 12.20 |
| 2 | Hexanal | 3.79 | 21 | Tetracosane | 12.74 |
| 3 | 2-Pentanone | 3.83 | 22 | Alloaromadendrene | 12.98 |
| 4 | p-Xylene | 4.51 | 23 | Octadecane | 13.14 |
| 5 | a-Pinene | 5.03 | 24 | Docosane | 13.69 |
| 6 | Styrene | 5.09 | 25 | Heneicosane | 14.09 |
| 7 | Decane | 5.96 | 26 | Hexadecane | 14.47 |
| 8 | Beta-myrcene | 6.21 | 27 | Heptadecane | 15.01 |
| 9 | d-3-Carene | 6.42 | 28 | Neryl acetate | 17.89 |
| 10 | Limonene | 6.83 | 29 | Pentacosane | 20.05 |
| 11 | Sabinene | 6.94 | 30 | Octadecanoic acid | 21.05 |
| 12 | 1,8-Cineole | 7.05 | 31 | Neophytadiene | 21.37 |
| 13 | Trans-b- ocimene | 7.60 | 32 | 2-Propanoic acid | 21.60 |
| 14 | Nonanal | 8.90 | 33 | Erucic acid | 22.38 |
| 15 | Dodecane | 9.20 | 34 | Hexanedioic acid | 23.20 |
| 16 | Tricosane | 10.30 | 35 | 9-Octadecenamide | 23.92 |
| 17 | Eicosane | 10.98 | 36 | Squalene | 26.28 |
| 18 | 2-Decanal | 11.48 | 37 | Hexacosane | 27.83 |
| 19 | Piperitone | 11.66 | 38 | Triacontane | 27.90 |

systemic. The damaged plant tissue may produce signal that is transmitted systemically throughout undamaged parts of the plant (Heil and Bueno 2007; Usha Rani and Sandhyarani 2012). In addition, it is also evident that the volatile blends emitted by different plant species infested by the same herbivore species show large qualitative differences, whereas blends emitted by plants of the same species, but infested by different herbivore species, are mostly qualitatively similar with quantitative variation and the pest's natural enemies can discriminate between blends that differ qualitatively and/or quantitatively (Dicke 1999a). Neveu et al. (2002) showed that water stressed or artificially damaged turnip plants do not emit volatiles that can influence the specialist endoparasitoid *Trybliographa rapae* Westwood (Hymenoptera: Figitidae) of the root feeder *Delia radicum* L. (Diptera: Anthomyiidae). The parasitoids were highly attracted to volatiles emitted by roots infested with *D. radicum* larvae, by undamaged parts of infested roots, and by undamaged leaves of infested plants (Fig. 16.5).

All these plant-derived chemicals are perceived by parasitoid's well-developed sensory system. Insect responses to these kairomones present in plant tissues and on surfaces are produced by direct contact with chemoreceptors present on tarsi or maxillary palpi or ovipositor (Schoonhoven 1985; Stadler 1984).

## 16.4 Kairomones from Host Insects

Almost all natural enemies (parasitoids and predators) use different by-products of the hosts to distinguish the host from nonhost. After reaching the host-infested plant by using various plant-derived and other cues, the parasitoid intensifies the search for the host-related chemicals or products. Host-derived cues are the most reliable cues for parasitoids at the last stages of host location, i.e., host recognition and acceptance, as they provide the most authentic information about the host's presence (Takabayashi and Takahashi 1989; Vet and Dicke 1992). Egg parasitoid females can exploit semiochemicals arising from the host eggs, from the interaction of the plants and the eggs, or from stages other than the one attacked (Powell 1999). Host larval body chemicals, larval feces,

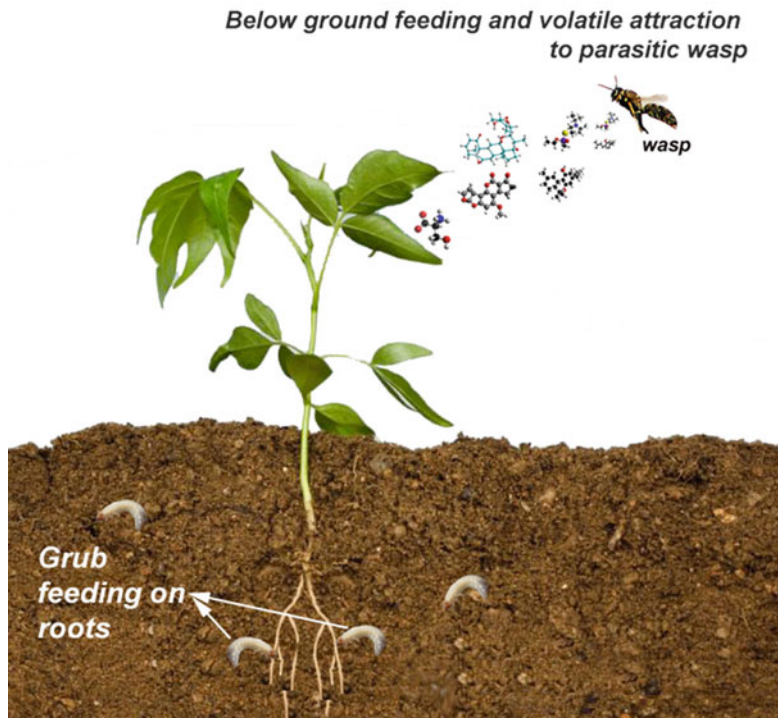


**Fig. 16.5** Root herbivory-induced volatile chemicals and parasitoid attraction

pheromones, silk, and oviposition participate in producing attractants (Hilker et al. 2000; Boo and Yang 2000; Paul et al. 1997). Hilker and Meiners (2006) demonstrated the attraction of parasitoids to the chemicals released by egg deposition on the surface of leaf. Mandibular glands of several Lepidopteran larvae attracted many parasitoids towards their hosts (Vinson et al. 1975).

Among the different by-products, frass is the most commonly reported source for parasitoids. Many insect natural enemies use frass kairomone to find their hosts, as frass consists of volatile and contact kairomones acting as a strong kairomone source for the respective parasitoid (Afseen et al. 2008). The female *Cotesia marginiventris* (Cresson) responded positively to the materials derived from the fall armyworm (FAW) larvae, *Spodoptera frugiperda* (J. E. Smith), more intensely to the frass (Loke and Ashley 1984). While studying the semiochemicals that influence the host selection behavior of this parasitoid, the highest percentage of ovipositor probing was caused by frass (100 %) and moth scales (90 %). The corn leaf damage combined with larval frass and oral secretions elicited strong responses in parasites than the larval damaged plants alone, confirming the importance of insect kairomones in parasitoid's behavior. Another interesting phenomenon recorded while working with tritrophic interactions of this parasitoid, FAW and corn plant complex is that parasitoid response was somewhat better to frass derived from FAW larvae feeding on corn and peanut leaves than from larvae feeding on the foliage of soybeans, Bermuda grass, cowpeas, or laboratory diet (Loke and Ashley 1984).

Parasitoids also use chemical footprints left by hosts while walking over the substrate (Conti et al. 2003; Colazza et al. 1999). Colazza et al. (1999) showed the chemical footprints left behind by the true bug *Nezara viridula* (L.) are perceived as contact kairomones by the scelionid egg parasitoid *Trissolcus basalis* (Wollaston). Furthermore, they also proved the wasp's ability to discriminate host male and female footprints is mainly based on the presence/absence of nonadecane $C_{19}$.

Such nonvolatile kairomones are definitive in regulating insect behavior and can be important in the applied ecology of insect pest control. Plants exposed to multiple bouts of herbivory showed stronger induced responses than plants that were exposed only once (Walner and Walton 1979). Predators or parasitoids hunting for their caterpillar prey also use the footprints or the walking track left on the epicuticular wax layer of a plant to locate them. The braconid wasp *Cotesia marginiventris* can locate potential hosts from a distance by orienting towards the scent of herbivore-damaged plants. Upon landing on the caterpillars' food plant, the female parasitoid searches for further cues (kairomones) that confirm the presence of a suitable host. Studies on host location behavior of *C. marginiventris* reported that the parasitoids show a characteristic antennation behavior towards chemical track left by the larvae of *Spodoptera frugiperda* for up to 2 days and confirmed that both hexane extracts of caterpillar footprints and of the larvae's ventral cuticle induced antennation and contained almost identical long-chain hydrocarbons. A series of linear C (21) to C (32) alkanes accounted for ca 90 % of all identified compounds. They also presumed that minor compounds, such as monomethyl branched alkanes, which were also found, might contribute additionally to host recognition.

The scelionid egg parasitoid *Telenomus isis* (Polaszek) is one of the most important biocontrol agents of the noctuid maize stem borer *Sesamia calamistis*. The role of various sources of contact kairomones (male or virgin or mated female moths) and of the moth's oviposition substrate in host location and oviposition behavior of *T. isis* was investigated (Olaye et al. 2001). Traces left by both the male and female moths acted as contact cues, which elicited an arrestment response in the parasitoid.

It is very difficult to control wood borers, important forest insect pests, because of their concealed nature of living. However, parasitic wasps can effectively ascertain and parasitize wood borers as well as other concealed pests by using special searching, finding, and attacking mechanisms. Xiaoyi and Zhongqi (2008) studied their host-searching mechanisms in locating their concealed hosts and found that the parasitic wasps can accurately find the location of their hidden hosts and then parasitize them, by using olfactory semiochemicals from hosts (larvae and adults) as well as host larval frass. The ovipositing adult moths often leave scales behind on the oviposition substrates, which in most cases are the leaf surfaces and are freely exposed to the foraging parasitoids. Several parasitoids use these moth scales as cues for finding the host eggs. The egg parasitoid *Trichogramma evanescens* West. responded to the chemicals from the host *Heliothis zea* Boddie adult scale extracts, and the gas chromatographic and mass spectrometric examination showed docosane, tricosane, tetracosane, and pentacosane as the active compounds. Among these, tricosane induced parasitization in laboratory and small-field tests (Jones et al. 1973). Kairomones in the scales of *H. zea* increased the percent parasitization, the number of progeny produced, and the longevity of female *Trichogramma pretiosum* Riley (Nordlund et al. 1976).

Male and female host cuticular extracts and scale extract of *Corcyra cephalonica* St. produced kairomonal activity to *Trichogramma brasiliensis* (Ashmead) and *T. japonicum* Ashmead (Paul et al. 1997). However, among these samples, highest attraction and enhanced oviposition were noted in scale extract treatments which consisted of the hydrocarbon chemicals pentadecane, heneicosane, eicosane, tetracosane, pentacosane, and hexacosane. In several cases, host sex pheromones are also shown to act as kairomones to find them successfully. The egg parasitoids *Trichogramma chilonis* is attracted to the sex pheromone components of *Helicoverpa assulta*. Among four components of its sex pheromone, (Z)-11-hexadecenyl acetate was the most attractive. *T. chilonis* was also highly attracted to (E)-12-tetradecenyl acetate, a component of the sex pheromone of *Ostrinia furnacalis*, another host. It is indicated that airborne chemicals from egg masses of Asian corn borer (ACB), *Ostrinia furnacalis* (Guenee), (E)-12-tetradecenyl acetate (E 12–14: Ac, a component of ACB sex pheromone), mated females before their first

oviposition and their accessory glands stimulated an intensive search behavior by *Trichogramma ostriniae* Pang et Chen females; however, volatiles from virgin females or females after their first oviposition and their accessory glands did not elicit the parasitoid's movement (Shu-Xiong et al. 2004). It is interesting to note that foraging egg parasitoids rely on a variety of chemical cues originating from the adult host, host products, or the host plant rather than from the attacked host stage – the insect egg itself. The host eggs do not emit attractive stimuli or long-range volatile chemicals to attract parasitoids. Hence, egg parasitoids have evolved several strategies such as exploiting long-range kairomones of the adult hosts (host aggregation and sex pheromones, plant synomones induced by egg deposition, or host feeding) or short-range contact cues derived from the adult host or the host plant.

The eggs of the diamondback moth (DBM) *Plutella xylostella* (L.) are parasitized by the egg parasitoids *Trichogrammatoidea bactrae* Nagaraja and *Trichogramma confusum* Viggiani. Saturated hydrocarbons from host eggshells and adult scales of *P. xylostella* were highly attractive to the parasitoids in laboratory behavioral bioassays (LU Yan-qing 2010).

Predatory hemipterans are another significant group of insects that are used widely in biological control programs. The carnivorous pentatomids attained important status due to their ability to hunt and consume their prey body in a very efficient way and also due to their ability to use different prey-originated materials as kairomones. Some preys make use of kairomone chemicals originating from predators and use them as cues as an indicator of the level of predation risk. Reduviids (Hemiptera: Reduviidae) are also popular predacious hemipterans that have considerable agricultural importance as they are biological control agents for several economically important pests. They are abundant in agroecosystems, semiarid zones, scrub jungles, and tropical rainforest ecosystems (Ambrose 1999). Their morphological and physiological adaptations in predation and extra oral digestion can be attributed to their successful survival in different ecosystems.

The potential of the reduviid predator *Rhynocoris marginatus* (Fab.) as biological control agent was shown in laboratory (Irudayaraj et al. 2003) as well as in field (Sahayaraj and Ravi 2007), and this entomophagous bug is known to feed on more than twenty economically important insect pests in India (Sahayaraj 2007). Some recent studies on nature of the venom from certain Reduviidae indicated that the saliva of *Rhynocoris marginatus* consists of insecticidal activity to a few lepidopteran pests. The effects included reduction of food consumption, assimilation, and utilization (Sahayraj and Muthukumar 2011). Semiochemical importance in prey location and the role of chemicals in this process were shown in reduviid predators also. Anti-predator adaptations have an important direct effect of info-chemical-based phenomenon of prey and predator as well as prey density. The predatory efficiency of the reduviid predator *Rhynocoris fuscipes* (Fab.) has been shown to be influenced by the info-chemicals released by their prey, the red cotton bug *Dysdercus cingulatus* (Fabricius), tobacco caterpillar *Spodoptera litura* (Fab.), and castor semilooper pest *Achaea janata* Linn. (Sujatha et al. 2012). The water fraction of the mixture of chloroform and methanol extract of *A. janata* elicited maximum feeding responses over the other two insects tested. Also the predators preferred the older stages of larvae and nymphs than the younger stages. These kinds of research findings are beneficial while designing the biocontrol programs involving application of *R. fuscipes* as a biocontrol agent in the management of cotton, castor, and groundnut pests. *R. marginatus*, a predatory hemipteran, utilizes prey-related chemicals as cue for prey location and these chemicals emanating from the prey *Helicoverpa armigera* (Hub.) (Lepidoptera: Noctuidae), *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae), and *Mylabris pustulata* (Thunberg) (Coleoptera: Meloidae) act as kairomones (Sahayaraj 2008). The predators exhibit typical prey location behaviors such as approaching and protrusion of rostrum to the hexane extracts of all these three groundnut pests. Predator's response was more intense towards lepidopteran pests

than the coleopteran pest. A GC-MS study indicated the occurrence of standard cuticular hydrocarbons (Sahayaraj 2008) and corroborates with the studies of *E. furcellata* (Usha Rani and Wakamura 1993).

However, the effects of kairomonal chemicals in increasing the predatory potential of the coleopterans are lesser known than the other orders. The checkered beetle, *Thanasimus formicarius* (L.) (Coleoptera: Cleridae), is a common predator of European conifer bark beetles, such as the pine shoot beetle *Tomicus piniperda* (L.) and *I. typographus* (Weslien 1994). Its prey range is mostly restricted to conifer bark beetles. Nonhost volatiles, two strongly antennally active molecules, 3-octanol and 1-octen-3-ol from bark beetle pheromones, had a strong inhibitory effect, both in the laboratory (walking bioassay) and in the field (flight trapping), thereby creating a kairomonal impact. Physiological evidence from electroantennography (EAG) and from single-cell recordings (SCRs) shows that *T. formicarius* has olfactory receptor cells specialized to bark beetle pheromone components and to prey host plant volatiles, with sensitivity and specificity similar to that of its prey (Hansen 1983). Poland et al. (2004) evaluated blends of semiochemical disruptants, which included nonhost volatiles and verbenone, for their ability to disrupt attraction of *T. piniperda* to traps baited with the attractant α-pinene and to Scots pine, *Pinus sylvestris* L., trap logs; a single blend of nonhost volatiles alone (comprised of 1-hexanol, (Z)-3-hexen-1-ol, (E)-2-hexen-1-ol, 3-octanol, and 1-octen-3-ol) or the nonhost volatile blend combined with verbenone significantly reduced attraction of *T. piniperda* to attractant-baited traps by 68–77 %.

The well-known predator of the stored product pests, including *Stegobium paniceum* (L.), *Lasioderma serricorne* (F.), *Anagasta kuhniella* (Zell.), *Pyralis farinalis* L., and *Tribolium* spp., is the *Peregrinator blannulipes* Montrouzier et Signoret (Hemiptera: Ruduviidae) (Awadallah et al. 1990). The solvent extract of *Tribolium confusum* (Jacquelin du Val) larvae as kairomones was individually and collectively tested for probing behavior of *Peregrinator blannulipes* Montrouzier et Signoret. Four fatty acid methyl esters, namely, methyl palmitate, methyl linoleate, methyl oleate, and methyl stearate, were identified from the larval extracts, and all the four chemicals exhibited characteristic kairomonal probing behavior of *P. blannulipes* towards the tested compounds indicating the feeding stimulants and kairomones in their prey body components (Tebayashi et al. 2003). The predatory beetle *Trogossita japonica* (Coleoptera: Trogossitidae) feeds on wood-boring insects in the forests. The Japanese pine sawyer *Monochamus alternatus* (Coleoptera: Cerambycidae) that transmits the pine wood nematode, *Bursaphelenchus xylophilus*, the pathogen of the pine wilt disease of Japanese red pines (*Pinus densiflora*) and black pines (*Pinus thunbergii*), is one of its prey species. *M. alternatus* utilizes monoterpenoids, especially α-pinene, which are emanated from nematode-infected pine trees, to orientate towards the host trees for oviposition (Nakamuta et al. 2002).

Zhang and Schlyter (2010) made an important discovery. They hypothesized that specific chemical cues from prey nonhosts and non-habitats, which are not part of the trophic chain, are also recognized by predators and would inhibit attraction to the host/prey kairomone signals. They studied the olfactory physiology and behavior of a predaceous beetle, *Thanasimus formicarius* (L.) (Coleoptera: Cleridae), in relation to specific angiosperm plant volatiles that are nonhost volatiles (NHVs) for its conifer-feeding bark beetle prey and suggested that some NHV chemicals for herbivores are part of specific behavioral signals for the higher trophic level and not part of a background noise. Such bypass-trophic signals could be of general importance for third trophic-level players in avoiding unsuitable habitats with nonhost plants of their prey (Zhang and Schlyter 2010).

## 16.5 Kairomone Perception by Insect Natural Enemies

The reproductive success of a biocontrol agent is determined by their ability to effectively locate their hosts/prey. Insects use their sense of taste or smell to detect the presence of semiochemicals.

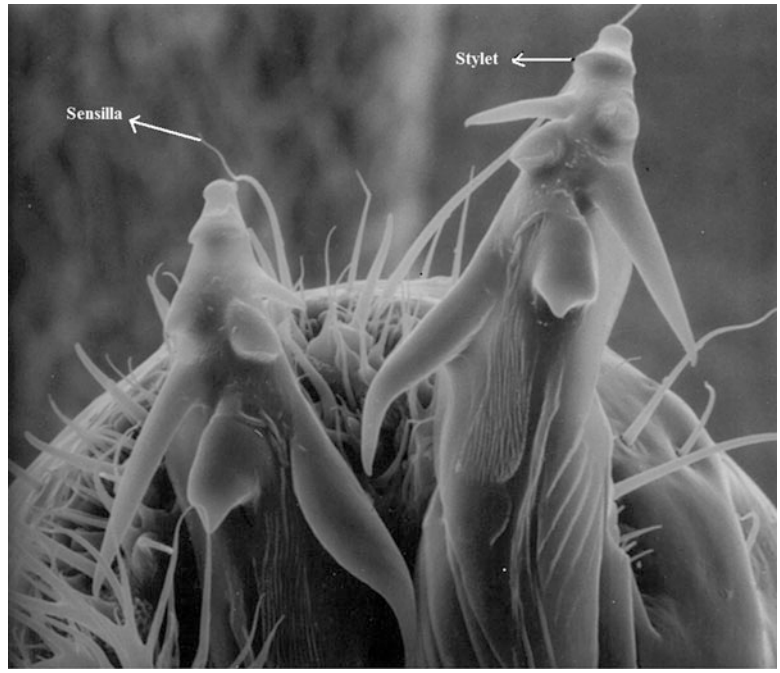


**Fig. 16.6** Rostral tip of the carnivorous stinkbug *Eocanthecona furcellata* with sensilla around the proboscis

Specialized receptors may be located anywhere on the body but are especially common on the feet, antennae, palps, and ovipositor. Perception of host-related semiochemicals occurs on specialized sensilla on the antennae, rostral tip in the case of predatory bugs, and ovipositor in several hymenopterans. Antennae of parasitic Hymenoptera (Norton and Vinson 1974) and predatory Hemiptera (Usha Rani and Madhavendra 1995) play a major role in host/prey location. Olfactory sensilla (located mainly on the antennae) play a major role in detecting semiochemicals with low molecular weight that are volatile enough to become airborne from a distance, while the sense of taste (gustation) is used for contact chemoreception – detecting molecules that adhere to a substrate or to the outside of an insect. *Eocanthecona furcellata* (Wolff) is a predatory hemipteran attacking and feeding on several species of Lepidoptera. The scanning electron microscope (SEM) studies on morphology of labial tip sensilla (Usha Rani 2008) and the mouthpart structures of this carnivorous stinkbug (Usha Rani et al. 1994) showed interesting anatomy (Fig. 16.6). Structures of both maxillary and mandibular stylets are well adapted for predation, the mandibular tip consisting three recurved hooks to hold the prey while sucking on it. Sen et al. (2005) studied external morphology and peripheral olfactory responses of antennal chemoreceptors of *Trichogramma chilonis* Ishii (Hymenoptera: Trichogrammatidae) to host-related stimuli and demonstrated that this egg parasitoid uses its antennal sensilla to perceive host plant-derived chemicals and suggested that using plant information enhances its parasitization efficiency. The carnivorous beetle, *Trogossita japonica* Reitter (Coleoptera: Trogossitidae), a general predator of wood-boring insects, is also shown to perceive kairomones and other stimuli associated with the host tree *Pinus densiflora* Siebold and Zuccarini (Japanese red and black pines), through the olfactory receptors present on the terminal club sensilla on the antennae (Usha Rani and Nakamuta 2001). Sense organs are found on the ovipositor of *Trichogramma maidis* that involved in host examination and recognition (Le Ralec and Wajnberg

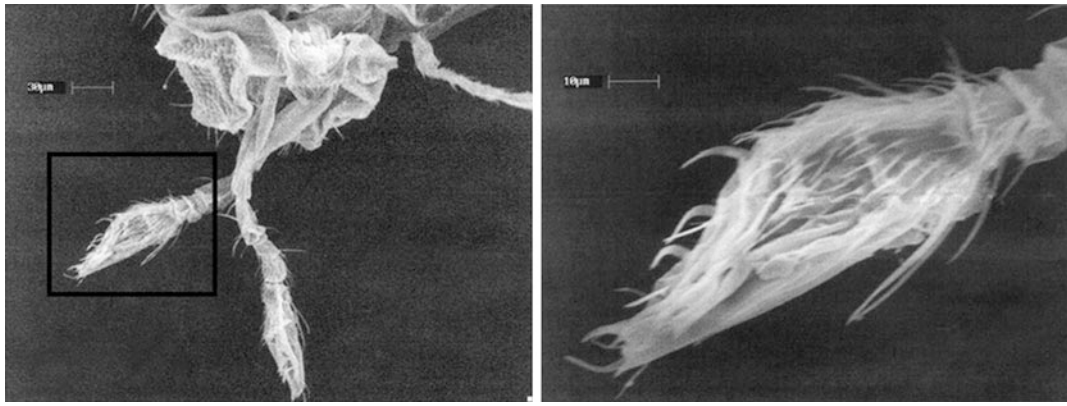

**Fig. 16.7** Antennal sensilla of *Trichogramma japonicum*

1990). Often parasitoids have to recognize parasitized hosts from unparasitized hosts among a number of hosts. Already-parasitized hosts are often of poorer quality than healthy hosts. It is therefore usually advantageous for parasitoid females to recognize and reject them, and for this task they use various physical or chemical marks present on the surface or inside the hosts or their surroundings in the case of concealed host (Goubault et al. 2011). The egg parasitoid *Trichogramma japonicum* has several olfactory and gustatory receptors at their antennal surface, which aid in recognizing their concealed host, *Scirpophaga incertulas* or rice stem borer (Fig. 16.7). The endoparasitoid wasp *Diadromus pulchellus* Wesmael (Hymenoptera: Ichneumonidae) utilizes contact receptors present on the host cocoon of *Acrolepiopsis assectella* (Lepidoptera: Yponomeutoidea) that act as kairomone for host acceptance (Benedet et al. 2002).

## 16.6 Field Use of Kairomones

James (2003a) was the first to demonstrate attraction of predators to synthetic HIPVs. In an agricultural system, traps baited with (2)-3-hexenyl acetate caught more predatory mirids, *Deraeocoris brevis* (Uhler), and anthocorids, *Orius tristicolor* (white), than unbaited traps, whereas traps baited with MeSA (methyl salicylate) attracted more *Geocoris pallens* Stall and hover flies (James 2003b). James and Price (2004) obtained and showed similar results in juice grape vineyards with sticky traps in MeSA-baited blocks attracting greater number of predatory insects than traps in unbaited blocks. The mass-trapping technique to control codling moth populations was investigated Light et al. (2006) using a dual lure, a combination of the pear-ester kairomone and codlemone pheromone. Lewis et al. (1975b) showed that the efficiency of *Trichogramma* sp. released into the field was significantly improved by the application of the kairomone, tricosane. Field studies were conducted at Kawanda Agricultural Research Institute, Uganda, to develop a kairomone trapping system based on processed banana tissues that would be used for delivering *Beauveria bassiana* to control the banana weevil (Tumuhaise et al. 2003). Several advantages associated with kairomones, mainly their effectiveness over distances and around corners, inexpensive as only small quantities are required, and their usefulness all the time without a barrier of day and night, make them promising in the field. Most of the kairomones are often simple, commercially available chemicals. Because these attractants are used in ways that do not injure other animals or humans or result in residues on foods or feeds, they can be used in an environmentally sound manner in pest management programs. A combination of kairomone usage on the crop along with good understanding of the pest's behavior is very important when testing them in the field.

## 16.7 Conclusion

The increasing knowledge on the plant's ability to self-defense is attracting more agriculturalists and pest control scientists to develop alternate and safer pest control tactics with less or no usage of pesticides. This phenomenon is also made possible over years to breed pest- and pathogen-resistant crop plants. Biocontrol programs like releasing egg parasitoids, mainly those belonging to Trichogrammatidae, Scelionidae, and Mymaridae families, are used widely all over the world. Biological control is the most desired method of pest control, it is essential to increase its success rate. Kairomones can be used in a similar manner to sex pheromones, bait traps for monitoring, or to disrupt host-finding behavior. Several insect sex pheromones also act as kairomonal cues to the searching parasites. The mated female parasitoids particularly *Trichogramma* apart from moth scales use moth pheromones as cues to locate the eggs laid by the female moths, thus succeeding in finding a host to parasitize. These overlapping roles of chemical cues as pheromones for the male moths and host-searching kairomones for the female adult of *Trichogramma* open exciting possibilities for integration of augmentation–manipulation of entomophages into a potentially powerful pest management system for several lepidopteran pests.

Crops that produce more quantities and/or different kairomones can be developed through genetic engineering and selective breeding, so that more insect natural enemies can be directed towards the crop and parasitize the pest larvae/eggs, thus killing the pest in the younger stage itself. This is one of the potential avenues for the environmentally friendly sustainable pest control of the future. Consequences of extensive use of these kinds of technologies could be very important in reducing the level of contamination caused by the pesticide use in the environment. Kairomones are already proven effective in pest management. Interestingly even for the control of stored pests, the kairomones are being used. Dried food odors are reported to be good kairomones for moths that infest stored products.

Research on semiochemicals demonstrates the importance of fundamental and interdisciplinary research dealing with the chemistry of biologically active compounds in combination with biological studies on their action. Organic chemistry, both analytical and synthetic, plays an essential role in such studies.

Future focus or recommendations: There have been promising developments in pest control strategies utilizing semiochemicals for improving the performance of biological control agents. Many of these chemical lures attract beneficial insects and can be used to monitor or directly reduce insect populations. These attractant chemicals or kairomones do not leave any residues on the crop; hence, they can be used in an environmentally sound manner in pest management programs. The identification and synthesis of these kairomonal compounds and their field use may lead to the newer and environmentally safer pest control operations and increase the biocontrol potential of predators and parasites, thus making the biological control a more effective method of pest control. The future focus needs to be on identification of kairomones from more pest parasite/predator systems and the chemical synthesis of the identified compounds. There is a tremendous potential for these semiochemicals as they can play a vital role in IPM.

**Acknowledgments** The process of writing a book is a collaborative experience involving the efforts and responses of many people. I want to express my sincere gratitude to my students, Jyothsna Yasur, Sandhyarani Kurra, Joish Madhusudhanamurthy, and Pratyusha Sambangi at Biology and Biotechnology Division of Indian Institute of Chemical Technology, Hyderabad, India, whose support and goodwill kept me going through the writing of this edition. I am grateful to Dr. Sahayraj for his faith in me and giving me this opportunity.

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# Classical Practices and Pheromones in Biopesticides

17

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**Abstract**

Application of biopesticides is a globally rising phenomena on yearly basis, and the use of traditional insecticides is on the decline. North America uses the largest percentage of the biopesticide market share at 44 %, followed by the Europe with 20 %, each South and Latin American countries with 10 %, and about 6 % in Asia and India. However biopesticide growth is projected at 10 % annually; it is highly variable among the regions constrained by factors such as regulatory hurdles, public and political attitudes, and limitations for market expansion. Microbial biopesticides have been registered globally for 35 years, but the number of registrations for commercial restricted industry and domestic uses has significantly increased over the past 10 years.

The early Canadian biopesticides registered by pest control category were *Bacillus thuringiensis* in 1972 as the first bioinsecticide, *Agrobacterium radiobacter* in 1989 as the first biobacteriocide, *Colletotrichum gloeosporioides* sp. *malvae* in 1992 as the first bioherbicide, and *Streptomyces griseoviridis* in 1999 as the first biofungicide. Between 1972 and 2008, the Pest Management Regulatory Agency approved registration of 24 microbially active substances with 83 formulations. The majority of the registrations (55/83) occurred since 2000, and at the beginning of 2008, there were 10 new products (a combination of new active substances, strains, formulations, and uses) under regulatory evaluation. This chapter examines the evolution of microbial biopesticides illustrating how the actions of the government, the people, and the industry have led to changes in legislation, policy, and programming that spurred momentum for new microbial pest control products in recent years and created a model safe system for future microbial biopesticide discovery, development, and implementation that could be adopted throughout the world. Pheromones present new environmentally safe strategies used for insect control. Pheromones follow the process of mating disruption through chemical communication

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_17, 

inhibitors, pheromones, and plant-based volatiles, and attractant-and-kill and push–pull strategies. Important successes have been obtained particularly in mating disruption with significant reduction in pesticide use in low to moderate pest infestations. One important factor of concern is the high cost of semiochemicals and formulations containing them in comparison to the conventional insecticide treatments, and a combined effort of researchers, producers, and farmers should be made to reduce the cost of application of these semiochemicals.

**Keywords**
Biopesticides • Classical practice • Pheromones • Insect control • Semiochemicals

## 17.1 Introduction

In principle and through strategy, the microbial control viruses, bacteria, and pheromone constitute the part of biopesticide management system. These are the key components of integrated pest management. Increased problems of resistance and contamination with conventional insecticides have assured the bright future for their substitute in the form of biopesticidal use. However the advances in the biotechnology should help bring the microbial cost decrease and its efficacy to increase, and it may be achieved through (a) fastidious production of microorganisms on artificial media, (b) single host cell can be used to accelerate the production of pathogens, (c) raising the killing, and (d) enhancing environmental stability. Ecogen Incorporation and Mycogen Corporation have used later two points and engineered two products combining more than one toxin of bacterium *Bacillus thuringiensis* when one of these products is effective in controlling gypsy moth and spruce budworm in forest and others against Colorado potato beetle and European corn borer on potatoes (Benhamou et al. 1994; Maddox 1994; Pedigo 1996; Lacey and Kaya 2000; Bailey et al. 2010). Similarly Mycogen Corporation has developed a technique to improve *B. thuringiensis* stability by engineering a rhizobacterium *Pseudomonas fluorescens* and toxin and then killing the cell. *P. fluorescens* encapsulate the toxin and make it much more resilient and useful to environmental factor. Apart from the genetic engineering of *B. thuringiensis*, similar strategy is used toward use of virus efficacy. Virus is engineered to produce juvenile hormone esterase JHE, and thus engineered virus is used to infect lepidopteran that profoundly reduces the feeding and growth of these larvae. The future of microbial control in the form of bacterial and viral insecticides or biopesticides is promising safe and would be reasonably low cost through the advancement of genetic engineering. In the past, myriad methods have been used in applying the microbial control, and all of them have culminated or are aimed at increasing mortality of the pest by increasing natural enemy to prey ratio, and another measure to raise the efficacy of biopesticide management through modifying crop production to enable the conservation of natural enemies and saving them from destruction (Sheck 1991; Kalra and Khurana 2007).

Once chemists knew that the communications among a variety of organisms are based on chemical substances termed pheromones, they isolated, identified, and synthesized hundreds of pheromones to use them practically for pest control. The same idea is at the heart of a number of initiatives to control a range of stock pests and to control a range of insects that present a risk to human health either directly or as a result of the agents of disease that they transport. The key to all of these behavioral chemicals is that they leave the body of the first organism, pass through the air (or water), and reach the second organism where they are detected by the receiver. Pheromones are a class of semiochemicals that

insects and other animals release to communicate with other individuals of the same species. Classical practices used in the application of biopesticides are grouped in three categories: introduction, augmentation, and conservation. However natural products or biochemical products are generally used as in the use of conventional insecticides. This chapter is a step forward to disseminate the information in favor of the use of biopesticide for a safe and healthy human environment. Earlier classical practice used the process of importation that include the identification of the natural enemy in their original location and then importing them to new places hence natural enemies are associated with their host with an anticipation that microbial organism would establish themselves permanently to bring the average population below the economic injury level.

*Classical practices:* Since distant past classical practice used the process of importation that included the identification of the natural enemy in their original location and then importing them to new places, natural enemies are associated with their host with an anticipation that microbial organisms would establish themselves permanently to bring the average population below the economic injury level.

*Augmentation:* It is accomplished by increasing the number of microorganisms or their effectiveness. Generally it is used temporarily for the suppression of the pest population peaks rather than effectively changing the general equilibrium position. Due to seasonal use, this practice is repeated periodically and these may be inundative or inoculative.

*Inundative:* Most successful releases with pathogenic microorganisms like *B. thuringiensis* mostly against lepidopteran and coleopteran larvae, mosquitoes, and blackflies showed extensive results, but there was little subsequent suppression after initial release. In case of additional outbreaks during the season, more applications of the microbes should be required.

*Inoculative:* These techniques have been successful against Japanese beetle in turf grass. Two bacteria cause milky disease in this insect. Beetle population is suppressed by commercial formulations of spore powder deposited on the surface and washed manually or by rainfall. In successes thus achieved, epidemic is caused in their larval population density, for example, *Bacillus thuringiensis var. san diego* for Colorado potato beetle; *B. thuringiensis var. israelensis* for mosquitoes and blackflies; *B. thuringiensis var. kurstaki* for army worm and other moths; and *B. thuringiensis var. aizawi* for wax moth.

*Environmental manipulation:* It is very important to reduce pest population that is obviously achieved by planting cover crops broad bean *Vicia faba* in apple orchards. Maintaining cover crop, periodically moving it, and leaving the mulch reduces the number of aphids, leafhoppers, and codling moths. In fact, this practice confuses the pest to find the host (Gould 1991; Copping 2009; Kalra and Khurana 2007).

## 17.2 Pheromones

Apart from the use of microbes, pheromones are also an integral part of biopesticidal strategy; it is practiced in different styles and ways. Application of the potential for using odorants in this way has targeted the control of leaf-cutting ants and the red imported fire ant (Blums and Ross 1965; Vander Meer 1996). Use of pest control or suppressing their population dates back to the early 1900s in the form of attracting baits treated with insecticides or on food and killing them outright, but the current paradigm remains largely confined to improving the performance of toxic baits (Billen and Morgan 1998; Rust et al. 2004). This kind of control is very much in practice in the control of household pests and other places where the conventional use of insecticide causes unnecessary risk through direct exposure to the dwellers. In fact, characterization of ant trail pheromones over the last several years and little use of these compounds has been implemented in pest management (El-Sayed 2012). New application technologies that deliver pheromones against invasive pest ants could help reduce our reliance on the use of insecticides for ant pest control in sensitive

ecosystem or where insecticides are undesirable. Synthetic trail pheromone was applied in combination with insecticidal bait (hereafter "bait") in an attempt to develop a novel strategy for controlling medically important ants in a small treatment area. Trail pheromone disruption that affects recruitment is an example of a novel tactic for ant pest management. Trail pheromones are sex- and species-specific chemical compounds that affect insect behavior and bioactivity. They are active or effective in extremely low doses, one millionth of an ounce, and are used to bait traps or confuse a mating population of insects. Pheromones can play an important role in integrated pest management for household pests.

*Agricultural or forest pest:* Pheromones are a class of semi- or semiochemicals that insects and other animals release to communicate with other individuals of the same species. The key to all of these behavioral chemicals is that they leave the body of the first organism pass through the air (or water) and reach the second organism where they are detected by the receiver. In insects, these pheromones are detected by the antennae on the head. The signals can be effective in attracting faraway mates and in some cases can be very persistent, remaining in place and active for days. Long-lasting pheromones allow marking of territorial boundaries or food sources. Other signals are very short lived and are intended to provide an immediate message such as a short-term warning of danger or a brief period of reproductive readiness. Pheromones can be of many different chemical types to serve different functions. As such, pheromones can range from small hydrophobic molecules to water-soluble peptides (hydrophilic) suiting its diversified applications. Majority of research have been carried out on sex pheromones followed by aggregation pheromones due to their effective utilization and usefulness in pest management. To date, approximately 50 companies are involved on commercially producing synthetic pheromones (parapheromones) for more than 50 species of the dangerous pests. Among thus produced parapheromones, 80 % are for controlling Lepidoptera, 10 % for Coleoptera, and the remaining 10 % for Orthoptera, Diptera, and Hymenoptera (Ridgway et al. 1990).

Parapheromones are used in three ways: (1) in sampling and detection, (2) to attract and kill, and (3) to disrupt mating. Some of these techniques have been applied to control other animal pests including vertebrate herbivores such as deer. A major strength of pheromones is their effectiveness as part of integrated pest management (IPM) schemes because of their compatibility with biological control agents and other beneficial invertebrates such as bees and spiders. Pheromones fit neatly into the *virtuous* spiral, for example, in greenhouse IPM where the use of one biological control agent such as a predatory spider mite encourages (or requires) moving away from conventional pesticides for other pests (van Lenteren and Woets 1988).

Affluent attractants apart from the food lures are available in the form of pheromones absolutely unknown in the past. Advanced methods of their application and new formulations (products) have raised the interest in these compounds. In this chapter, we describe some important information on trail pheromone including source, optimum dose, longevity, specificity, and synthetic trail pheromone and the possibility to apply in pest control. Knowledge of insect attracting the insect of the same species was known centuries ago. This pheromone (trans, *cis*-10, 12-hexadecadien-1-ol) was isolated from silk worm, and it was a sex attractant resulting in the successful mating in the same species. To date, hundreds of pheromones have been isolated so far ranging from algae to primates due to the research advancement in analytical chemistry.

*Sex pheromones:* Generally sex pheromones are secreted by females to attract their counterpart males, but these may also be secreted by some males. Although removal of adult males unless at a very high proportion of the population is unlikely to have a large impact on the size of subsequent generations compared to removal of females (Lanier 1990). Pheromones for many insect pests have been identified. For example, a website "Pherolist" cites more than 670 genera from nearly 50 families of Lepidoptera in which female sex pheromones have been identified (Tumilsom et al. 1977; Arn et al. 1995; Fónagy et al. 2011). These are secreted by eversible

gland at the tip of the abdomen of the lepidopteran insects through a very complex process depending on the maturity of the insects, photoperiod, and light. This is perceived by the males through the sensillae on the antennae of the males. This is used in diverting the males away from the females and thus suppressing their numbers as a control method. These pheromones are commonly used as attractants to facilitate contact with and dispersal of pathogens in pest populations (Pell et al. 1993).

*Trail-marking pheromones:* Pheromones are the specialty of foraging ants and mites and used to communicate the food requisites to the other members of the community, and it can be used to deceive and deprive them from the food and suppress their population. Chemical trail communication allows group foragers to exploit conspicuous food sources efficiently and represents the most prevalent form of recruitment behavior (Mashaly 2010, 2011; Mashaly et al. 2008; Sillam et al. 2007). Trail communication is more commonly based on a multicomponent system where the secretions of different glands (or a blend of pheromones produced by the same gland) may contribute to the structure of the trail and regulate different behaviors in the process of recruitment (Hölldobler and Wilson 1990; Jackson et al. 2006; Mashaly et al. 2008, 2010, 2011; Mashaly and Al-Khalifa 2012).

These pheromones are utilized by animals as navigational aids in passing directional hints to other members of the colony to a distant location, varying in length from hundreds of meters in bees to few meters in terrestrial insects. The reasons for orienting members of the colony to a distant point may vary. In most cases, trails are laid by foraging workers as they return from a food source. These trails are then used by other foragers (Wilson and Pavan 1959). In other cases, however, trails may be laid to recruit workers for slave raids, colony emigration, or the repair of a breach in the nest wall (Wilson and Pavan 1959). Different types of trail marking are found in terrestrial insects and flying insects. The terrestrial insects appear to lay a continuous or nearly continuous trail between points. Wilson (1962) had shown that the fire ant *(Solenopsis saevissima)* drags its sting and lays a trail in a manner similar to a pen inking a line. If the food source is of good quality, other workers choose to reinforce this trail and a highway several centimeters wide may be formed.

*Aggregation pheromones:* Some of the coleopterans are known to attract their community members on food sites, reproductive habitats, and also hibernation sites, an example of which is bark beetle. Aggregation pheromones lead to the formation of animal groups near the pheromone source either by attracting animals from a distance or stopping ("arresting") passing conspecifics (Wyatt 2003). In contrast to sex pheromones (which attract only the opposite sex), aggregation pheromones by definition attract both sexes (and/or possibly larvae). Nymphs of the German cockroach *Blattella germanica* (Sakuma and Fukami 1990). Their ability to attract females makes these pheromones well suited for the attract-annihilate method (Lanier 1990). Aggregation pheromones have been used successfully for controlling various Coleoptera including the cotton boll weevil *Anthonomus grandis* in the USA (Hardee 1982) and bark beetles in North America and Europe (Lanier 1990). Innocenzi et al. (2001) characterized a male-produced aggregation pheromone of *Anthonomus rubi* as a 1:4:1 blend of grandlure I, grandlure II, and lavandulol (note that "grandlure" is the name given to four components in the aggregation pheromone lure of the cotton boll weevil *Anthonomus grandis* Boh).

*Alarm pheromones:* This pheromone is peculiar in the ants and bees, and the remaining sting apparatus of a honeybee in the victim's body releases this pheromone to attract other members of the same community for attack in defense. These are used in killing the pest through entangling them on sticky substance or kill them by use of microbes or chemicals. It is achieved by trapping the pests by slow release of pheromones and insecticides in the form of small particles. The pest control is also attempted by mating disruption through air permeation with synthetic pheromone; thus, mating is disrupted because they are unable to reach their counterpart or opposite sex. Mode of action of this type of pheromone is to induce alert in conspecifics to

raise a defense response and/or to initiate avoidance (Rechcigl and Rechcigl 1998). Weston et al. (1997) showed a dose response of attraction and repellency for several pure volatiles from the venom of the common and German wasps *Vespula vulgaris* and *V. germanica*. The compounds are usually highly volatile (low molecular weight) compounds such as hexanal, 1-hexanol, sesquiterpenes (e.g., (E)-β-farnesene for aphids), spiroacetals, or ketones (Francke et al. 1979). Alarm pheromones of aphids have been used commercially to increase the effectiveness of conventional pesticides or biological control agents such as the fungal pathogen *Verticillium lecanii* (Howse et al. 1998). The synthetic alarm pheromones and the increased activity of the aphids in response to their alarm pheromone increase mortality because they contact more insecticide or fungal spores (Pickett et al. 1992).

The trail pheromone is secreted by the poison gland, and Cross et al. (1982; Sillam-Dussès et al. 2007) have reported it in the genus *Atta* such as *A. sexdens arbropilosa* Forel; in *Monomorium* such as *M. niloticum*, *M. najrane*, *M. lepineyi*, and *M. bicolor*; and in *Tetramorium* such as T. *simillimum*. Pygidial gland is the synonym of anal gland and it can be found in all other subfamilies except the Formicinae. In Dolichoderinae, the pygidial gland is usually very large and serves the purpose in defense and alarm (Morgan 2008). Termite predation by the ponerine ant *Pachycondyla laevigata* is responded by a recruitment trail pheromone originating from the pygidial gland that was previously reported from the hindgut. Pygidial gland opens between the 6th and 7th abdominal terga and is covered with a special cuticular structure serving as a glandular applicator (Hölldobler and Traniello 1980).

## 17.3 Chemical Composition of Pheromones

Pheromones are chemically classified into different categories to serve different functions, and this range includes small hydrophobic molecules to water-soluble peptides (Sunamura et al. 2011). The main ways of utilizing an understanding of pheromones to control pests are monitoring mating disruption, "lure and kill" or mass trapping, and other manipulations of pest behavior (Billen and Morgan 1998; Blum and Ross 1965; Fónagy et al. 2011). Some of these techniques have been applied to control other animal pests including vertebrate herbivores such as deer. A major strength of pheromones is their effectiveness as part of integrated pest management (IPM) schemes because of their compatibility with biological control agents and other beneficial invertebrates such as bees and spiders. Pheromones fit neatly into the *virtuous* spiral, for example, in greenhouse IPM where the use of one biological control agent such as a predatory spider mite encourages (or requires) moving away from conventional pesticides for other pests (van Lenteren and Woets 1988; Tangchitphinitkan et al. 2007).

Pheromones are a class of semiochemicals that insects and other animals release to communicate with other individuals of the same species. The key to all of these behavioral chemicals is that they leave the body of the first organism, pass through the air (or water), and reach the second organism where they are detected by the receiver (Free 1987). In insects, these pheromones are detected by the antennae on the head. The signals can be effective in attracting faraway mates and in some cases can be very persistent, remaining in place and active for days. Long-lasting pheromones allow marking of territorial boundaries or food sources. Other signals are very short lived and are intended to provide an immediate message such as a short-term warning of danger or a brief period of reproductive readiness. Some of the chemicals secreted by respective insect of economically important pest are listed with their finder's references in the Table 17.1.

*Epideictic pheromones:* These are also known as spacing pheromones, and their main endeavor is to repel from the crowded food source and reduce their numbers. These are used to reduce competition between individuals and are known from a number of insect orders (Hummel and Miller 1984). These are mainly produced by the bark beetles and other insects belonging to Lepidoptera, Homoptera, Hymenoptera, and Orthoptera. Its best example is the apple maggot *Rhagoletis pomonella* (Tephritidae). Females ovipositing in

**Table 17.1** Chemical nature of the pheromones

| S. no. | Pheromone | Name of the insect species | Chemical formula | Reference |
|---|---|---|---|---|
| **Alcohols** | | | | |
| 1 | Seudenol | *Dendroctonus pseudotsugae* | $C_7H_{12}O$ | McKnight (1978) |
| 2 | Sulcatol | *Gnathotrichus sulcatus* | $C_8H_{16}O$ | Borden et al. (1976) |
| 3 | Grandisol | *Anthonomus grandis* | $C_{10}H_{18}O$ | Mori et al. (1978) |
| **Acetates and propionate** | | | | |
| 1 | Acetate of (Z)-6-isopropenyl-3-methyl-3 9-decadien-1-1 | *Aonidiella aurantii* | $C_{16}H_{26}O_2$ | Roelofs et al. (1978) |
| 2 | Acetate of (E)-6-isopropyl-3 9-dimethyl-58-decadien-1-1 | *Aonidiella citrina* | $C_{17}H_{30}O_2$ | Roelofs et al. (1982) |
| 3 | Propionate of (Z)-6-isopropenyl-3 9-dimethyl-3 9-decadien-1-$_0$1 | *Pseudaulacaspis pentagona* | $C_{18}H_{30}O_2$ | Heath et al. (1980) |
| **Aldehydes and ketones** | | | | |
| 1 | Franal | *Monomorium pharaonis* | $C_{17}H_{30}O$ | Kobayashi et al. (1980) |
| 2 | Trogodermal | *Trogoderma granarium* | $C_{17}H_{32}O$ | Levinson and Mori (1980) |
| 3 | 311-Dimethyl-2-nonacosanone | *Blattella germanica* | $C_{31}H_{62}O$ | Mori (1981) |
| **Acids esters and lactones** | | | | |
| 1 | 4-Hexanolide | *Trogoderma glabrum* | $C_6H_{10}O_2$ | Ravid et al. (1978) |
| 2 | Callosobruchusic acid | *Callosobruchus chinensis* | $C_{10}H_{16}O_4$ | Mori et al. (1983) |
| 3 | Dominicalure 1 | *Rhyzopertha dominica* | $C_{11}H_{20}O_2$ | Williams et al. (1981) |
| **Acetals and epoxides** | | | | |
| 1 | Dominicalure 2 | *Rhyzopertha dominica* | $C_{12}H_{22}O_2$ | Williams et al. (1981) |
| 2 | (Z)-5-Tetradecen-4-olide | *Popillia japonica* | $C_{14}H_{24}O_2$ | Tumlinson et al. (1977) |
| 3 | Frontalin | *Dendroctonus frontalis* | $C_8H_{14}O_2$ | Wood et al. (1976) |
| 4 | exo-Brevicomin | *Dendroctonus brevicomis* | $C_9H_{16}O_2$ | Wood et al. (1976) |
| 5 | Lineatin | *Trypodendron lineatum* | $C_{10}H_{16}O_2$ | Slessor et al. (1980) |
| 6 | α-Multistriatin | *Scolytus multistriatus* | $C_{10}H_{18}O_2$ | Elliott et al. (1979) |
| 7 | Disparlure | *Porthetria dispar* | $C_{19}H_{38}O$ | Iwaki et al. (1974), Vitae et al. (1976) |

fruit mark the surface to deter other females. This behavior has also been studied in the related cherry fruit fly (*Rhagoletis cerasi*). Egg laying is a key stage determining subsequent population density so it is perhaps not surprising that there is considerable evidence of such pheromones affecting gravid females of herbivores. There is also exploitation of prey host marking and sex pheromones by parasitoids which use the signal persistence of these intraspecific cues to find their hosts. Mating-deterrent pheromones are also known from a number of insects, including tsetse flies, houseflies, and other Diptera. These pheromones are released by unreceptive females to deter males from continuing mating attempts (Rechcigl and Rechcigl 1998; Taymour 2012).

*Source of trail pheromones:* Generally the source of trail pheromones are hind tibia,

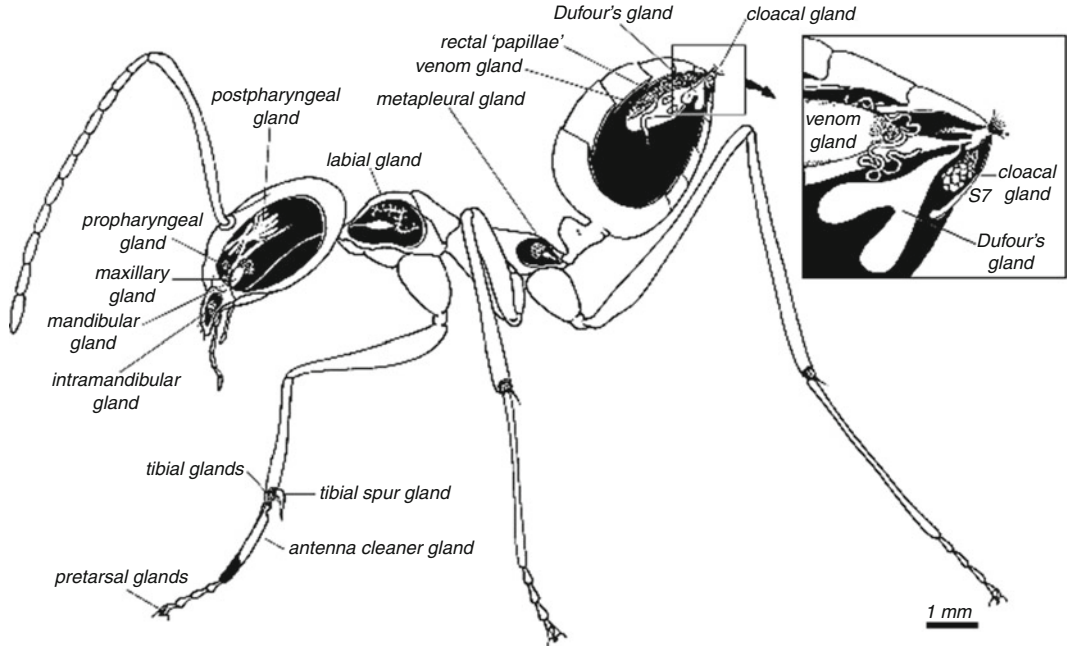


**Fig. 17.1** Diagrammatic sketch of the worker ant showing the distribution of different exocrine glands. *Inset* is the enlarged abdominal tip showing cloacal gland, Dufour's gland and venom gland (Wenseleers et al. 1998)

Dufour's gland, and venom glands in Myrmicinae; Pavan's gland in Dolichoderinae; pygidial gland in Ponerinae; post pygidial gland in Aenictinae; and the hind gut in Fig. 17.1. Dufour's gland of at least a portion in Myrmicinae, Dolichoderinae, Ponerinae, and Formicinae secretes and contains mixture of straight chain of hydrocarbons ranging between C9 and C27 (Morgan 2008).

*Commercial pheromones:* Some of commercially available 209 brands of sex pheromones are produced by Pest Mall, USA, and Yingkou Tanyun Chemical Research Institute Corporation, Liaoning, China, and many other companies. Basically sex pheromone are microchemical matter secreted outside their bodies by female insects to be used for luring male insect mating; it is also called the sex information hormone or sex luring agent; it is mainly used in pest for casting, preventing, and curing (the luring and extinguishing method or disturbing mating method, namely, the loosing bearing method); insect sex pheromone is a new drug for eliminating pest which is efficient nonpoisonous harmless to beneficial insect not polluted to environment, and its activity is highly beneficial and remarkable; this technical achievement had been awarded the first-class prize of research achievements on science and technology in Yingkou City, China in 1980. Some of these brands are listed in Table 17.2.

## 17.4 Role of Biopesticides in Integrated Pest Control

In health-conscious society, the use of biopesticide in the coming 5 years would reach 10 %. Though in the year 2000 the use of biopesticides was just 0.2 %, in 2005 it reached 2.5 % and in 2010 it climbed to 4.5 % (Burges 1998; Thakore 2006; Taymour 2012). The 40 % of the biopesticides are used in the USA and 20 % in European countries. The prospects of biopesticides are excellent due to its low-cost research, easy availability, and health-friendly environment. Today communities are more health conscious than ever (Gould 1991; Sheck 1991; Rechcigl and Rechcigl 2000;

**Table 17.2** Commercially available brands of pheromones

| S. No. | Pheromone | Brand name | Category | Manufacturer |
|---|---|---|---|---|
| 1 | Indian meal moth pheromone | 4 allure pack | Biopesticides/biological pesticides | Pest Mall, USA |
| 1 | *Carposina sasakii* sex pheromone | Tanyun | Biopesticides/biological pesticides | Liaoning, China (Mainland) |
| 2 | Hot sale insect sex pheromone | Tanyun | Biopesticides/biological pesticides | Liaoning, China (Mainland) |
| 3 | Tomato leaf miner | Tanyun | Biopesticides/biological pesticides | Liaoning, China (Mainland) |
| 4 | Pink bollworm | Tanyun | Biopesticides/biological pesticides | Liaoning, China (Mainland) |
| 5 | *Feromona spodoptera* | Tanyun | Biopesticides/biological pesticides | Liaoning, China (Mainland) |
| 6 | *Pectinophora* insect sex pheromone | Tanyun | Biopesticides/biological pesticides | Liaoning, China (Mainland) |
| 7 | *Leptinotarsa decemlineata* | Tanyun | Biopesticides/biological pesticides | Liaoning, China (Mainland) |
| 8 | Manufacturer of sex pheromone traps | Tanyun | Biopesticides/biological pesticides | Liaoning, China (Mainland) |
| 9 | High-quality insect sex pheromone | Tanyun | Agrochemicals and pesticides | Liaoning, China (Mainland) |
| 10 | Supply sex pheromone of *Grapholita molesta* | Tanyun | Biopesticides/biological pesticides | Liaoning, China (Mainland) |

Tangchitphinitkan et al. 2007; Sinha and Biswas 2008; EPA 2013).

*Merits:* Merits of using biopesticides are nontarget species, are substantially safe, and show least impact; harmful residues are nonoccurring; locally produced biopesticides are low cost and cost saver, and comparatively more use of these have more impact in the long-term strategy (suggested by the LUBILOSA Programme). One of the demerits of the pheromones is that the cost is high.

## 17.5 Conclusion

The concept of biopesticides to modify insect development and behavior exerts unique approach for the management of insect population. These products and methods of their application are based on the principle to provide safety to the environment and human. Extensive research is on the way and more is required to achieve improvement in the future. The future of the biopesticides would entirely depend on the adoption of their application. Versatile use of these must meet the standard of the established management procedures and means that they must be able to manage and suppress the harmful insect pest and prevent them from their colonization, relatively in a compatible manner to that of conventional methods. Researchers, farmers, and producers should extensively explore their utility and findings for the safe environment and more production of their crops.

## 17.6 Future Recommendations

1. Multiple or mixed cropping should be encouraged or followed in the practice of biopesticides in the field.
2. Provide means to develop better understanding about managing pest by the end users through seminars conferences and publications.
3. Researchers, producers, and farmers need to evolve strategy to ensure regular availability of biopesticides in the market or to stabilize their market, ensuring their prevalence to us.

**Acknowledgments** The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project no. 340.

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# The Use of Transgenic Plants in Insect Control

18

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**Abstract**

Over the last decade, the research data about the importance of the transgenic crops – named *Bt* crops – has increased commercially, leading them to take the second place among genetically modified (GM) crops most used and distributed. In 2006, total *Bt* crops reached 19 million hectares worldwide, and the *Bt*-corn already presented 16 commercial approvals, given that the first commercial *Bt*-rice plantation was made in Iran, in 2005. The clean t.echnology development, such as GM cultivars, presents several advantages in comparison to formulated insecticides, which depends on appropriated application methods according to the crop culture or the insect behavior and the impacts over the nontarget organisms and the environment. In this sense, the *Bt*-rice has the potential to increase productivity, decrease the pesticide application, and thus improve the environmental quality on the agricultural systems, which are highly related to environment conservation areas such as flooded regions of Brazil. The potential benefits that *Bt*-rice can offer, based on already obtained results with *Bt*-cotton and *Bt*-corn, should motivate new researches and the development of different varieties of *Bt*-rice. As a result, it could accelerate the approval and release of this technology to the rice farmers.

**Keywords**

Transgenic • Bt • *Bacillus thuringiensis* • Biological control • Insect pests

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## 18.1 Introduction

One of the difficulties that agriculture faces and that contributes to the reduction in its productivity is the attack by phytophagous insects. So it has been a constant need, for sustainable development of agriculture, the search for alternative methods of controlling these insects, with less

K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_18, 

environmental impact (Ferry et al. 2006). The control of insect pests has been accomplished primarily through the application of chemical products such as carbamates and pyrethroids (SOSBAI 2005). Considering the problems related to the action of these insecticides on nontarget organisms, contamination of water, and food waste (Schulz 2004; Kamel et al. 2007), it has became necessary studies that provide alternative methods of control, which can be applied on Integrated Pest Management (IPM).

An alternative in the biological control of insects is the application of entomopathogens, which can be bacteria, viruses, and fungi (Alves 1998). The practice of biological control of insects represents a viable option that allows the reduction in the use of chemicals (SOSBAI 2005; Crickmore 2006). Among the entomopathogens used for biological control, studies with the bacterium *Bacillus thuringiensis* became promising in the search for insecticidal proteins and, consequently, in obtaining their genes (Bobrowski et al. 2003; High et al. 2004; Crickmore 2006). Several research groups assess the insecticidal potential of this bacterium to insect pests that attack different crops, such as soybean, maize, cotton, and rice, among others (Schnepf et al. 1998; de Maagd et al. 2001; Clark et al. 2005).

Among the cultivated plants, rice culture is targeted for several breeding programs because it is the basis of the diet for about two billion people worldwide and, moreover, is the most important food for half the Asian population (Khush 1997; Datta 2004). About 114 countries grow rice, and more than 50 have an annual production of 100 thousand tons of cereal. Brazil stands out because it is one of the ten countries with the highest production of rice in the world. It produced, in 2005, about 13 million tons of this grain (IBGE 2012). The State of Rio Grande do Sul is the national leader in the production of rice, totaling 67.2 % of the national production (IBGE 2012). Despite the high yield, the rice production in Rio Grande do Sul often suffers from attack by pests, which have been noticed in elevated levels of density, a fact that leads to losses in productivity of 10–35 % (SOSBAI 2005).

The widespread application of chemical insecticides not only increases the cost of rice production but also contributes to concerns such as the health of rice farmers and the deterioration of agroecosystems (Datta 2004; High et al. 2004; Huang et al. 2005). The use of products based on *B. thuringiensis* for the control of insect pests of rice is already recommended in Integrated Pest Management (IPM) of this culture (SOSBAI 2005). Furthermore, researches involving genetic engineering of various crops, including rice, have been attractive due to the success of some genetically modified (GM) plants with *cry* genes from *B. thuringiensis* to control insects (Wang et al. 2002). This is the second most common feature after herbicide resistance, sought on genetically modified cultures (O'Callaghan et al. 2005).

In this context, this chapter presents data on the bacterium *B. thuringiensis* toxins, describes how its toxins act, studies the use of *cry* genes in genetic engineering of plants, and clarifies aspects of biosafety regarding *Bt*-plants.

### 18.1.1 The Bacterium *Bacillus thuringiensis*

*Bacillus thuringiensis* is a Gram-positive bacterium characterized by the production of parasporal crystals inside the parent cell during its sporulation, which contains delta-endotoxins active against several orders of insects (Hofteand Whiteley 1989; Schnepf et al. 1998). These toxins are highly specific against the target insects but are harmless to humans, plants, and vertebrates, besides being completely biodegradable. Considering these facts, *B. thuringiensis* becomes a safe alternative for controlling insect pests of agricultural importance as well as important vectors of human diseases (Bravo et al. 2005).

Thousands of strains of *B. thuringiensis* have already been isolated, which mostly produce one or more δ-endotoxins with specific activity to evaluated insect orders (Schnepf et al. 1998; Pinto and Fiuza 2002; Monnerat et al. 2007; Gao et al. 2008). The mode of action of these proteins, called Cry, to susceptible larvae has been studied mainly in lepidopterans, as illustrated in Fig. 18.1.

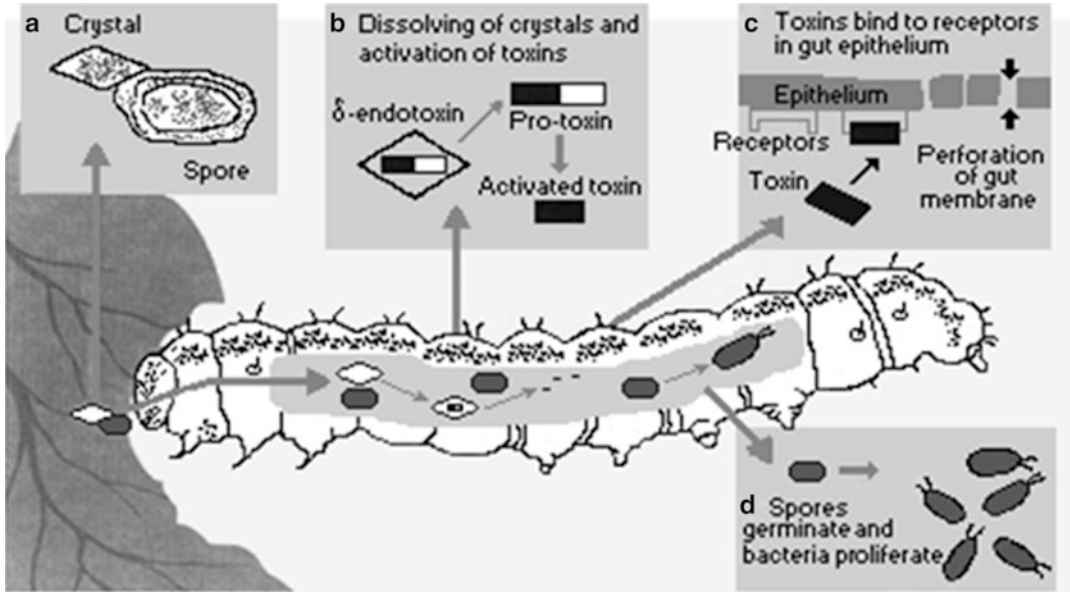


**Fig. 18.1** Mechanism of action of *Bacillus thuringiensis* (WHO 1999)

The primary action of these proteins occurs after ingestion and solubilization of the parasporal inclusions in the alkaline environment of the intestine. Then these solubilized protoxins are cleaved by proteases in the insect midgut, yielding peptides resistant to proteases that are able to bind to the receptors of the microvilli of the midgut columnar cells. The proteases produced by the various insect groups represent one of the factors in the spectrum of toxicity achieved by different strains of *B. thuringiensis* (Lightwood et al. 2000; Bravo et al. 2004). Such insertion causes the formation of pores in the apical membranes and the subsequent lysis of microvilli and rupture of the midgut epithelium, which then releases its cellular contents, providing a way for the germinating of the spores of *B. thuringiensis*. These events lead to septicemia and death of the insect (de Maagd et al. 2001; Fiuza 2004; Bravo et al. 2007).

### 18.1.2 The *cry* Genes of *Bacillus Thuringiensis*

The *cry* genes have been first classified by Hofte and Whiteley (1989) according to their molecular structure as well as their host spectrum. At the time, the authors mentioned the classification of 13 *cry* genes, which were divided into four classes. In 1998, a study was undertaken to revise the nomenclature of *cry* genes, indicating that there are approximately 100 of these genes that were grouped into 22 classes (Crickmore et al. 1998). In this nomenclature, the Roman numerals that appeared first after the acronym *cry* were replaced by Arabic numerals in order to accommodate the growing number of new proteins. Currently, the authors updated the data for the *cry* genes of *Bacillus thuringiensis* in the site http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt. To date, over 400 *cry* genes are contained in the database, which are distributed in 55 classes.

The genome of *B. thuringiensis* varies from 5.2 (GenBank AE017355) to 6.7 (GenBank ACNK00000000) million base pairs, and most of the isolates show linear or circular extrachromosomal elements (Carlson et al. 1994). The *cry* genes are located on plasmids, and many isolates have several *cry* genes responsible for the synthesis of different insecticidal proteins (Lereclus et al. 1993), which were classified from Cry1 to Cry55, depending on the host specificity and the degree of homology of their amino acids (Höfte and Whiteley 1989; Crickmore 2006).

During its development, *B. thuringiensis* undergoes two phases: vegetative growth and stationary phases, similar to the development of *Bacillus subtilis*. The first phase is characterized by exponential growth of bacterial cells, when there is large availability of nutrients in the medium. The stationary phase occurs when the environment becomes hostile and the bacterium adapts to the decrease of nutrients through genetic mechanisms, ceasing cell reproduction. During this period, the endospore becomes mature. The expression of the *cry* genes of *B. thuringiensis* usually occurs in the stationary phase cell, accumulating the product in its mother cell in the form of a crystalliferous inclusion, which is released to the medium at the end of sporulation (Lereclus et al. 2000). This addition can represent about 25 % of the dry weight of already sporulated cells (Agaisse and Lereclus 1995). Although the expression of the *cry* genes may be closely related to the event of sporulation, there are *cry* genes that are expressed independently of sporulation (Agaisse and Lereclus 1995).

## 18.2 Genetically Modified Plants with *cry* Genes

The biggest breakthrough in the use of *B. thuringiensis* has been the conversion of various agricultural cultivars aiming at the expression of Cry toxins. Since 1996, the importance of these processed cultivars has grown commercially, leading them to stand in the second place among the genetically modified plants with higher employment and distribution, followed by herbicide-resistant plants (Crickmore 2006). These cultivars transformed with *cry* genes, also known as "*Bt*-plants," present several advantages over *B. thuringiensis* formulated products because it does not require foliar spray for the control of certain insects, since the toxin is expressed by the plant itself. Moreover, it decreases the amount of chemical insecticides released into the environment as well as the gases emitted by agricultural machinery used in its application (James 2011).

In 2011, crops of GM plants totaled 160 million hectares planted in the world, of which *Bt*-plant crops reached23.9 million hectares (15 %) (James 2011). The most significant growth was the *Bt*-corn and *Bt*-cotton. These plants produce a truncated form of Cry proteins, which resembles those toxic peptides activated after its cleavage in the intestine of susceptible insects (Crickmore 2006). Thus, the toxin is synthesized in its toxic form or soluble, preferably the crystalline inclusions, which have yet to be solubilized at alkaline pH of the intestine. Table 18.1 shows the transformed plants with genes from *B. thuringiensis*, adapted from Sources et al. (2002). It should be noted that the insect-resistant maize (*Bt*-maize) and cotton (*Bt*-cotton) have several approvals in worldwide business. These transformed crops with *cry* genes from *B. thuringiensis*, as shown in Table 18.2 (adapted from Que et al. 2010).

## 18.3 The *cry* Genes in Rice Plants

China is a recognized world leader in production and consumption of rice (Chen et al. 2006). In 1998, it began the first field trials with *Bt*-rice resistant to lepidopterans (Shu et al. 2000), which is followed by India in 2001 (Ye et al. 2003). The varieties of rice transformed with *cry* genes of *B. thuringiensis* were resistant to one or more species of insect pests of rice grown in these countries, indicating an increase in grain yield between 6 and 9 % (Huang et al. 2005). Tests conducted in China since 1998 show that *Bt*-rice effectively controls three species of stem borers (*Chilo suppressalis*, *Scirpophaga incertulas*, and *Sesamia inferens*) and phytophagous insects (*Cnaphalocrocis medinalis*). In the field evaluations, the *Bt*-rice caused 90–100 % mortality and 84–100 % to defoliators (Chen et al. 2005). This fact evidences an action significantly more effective in controlling the borers, when compared to most insecticides used by farmers in China.

In 2005, *Bt*-rice was grown commercially for the first time in Iran, in approximately four acres,

**Table 18.1** Plants modified with *cry* genes from *Bacillus thuringiensis*, resistant to insect pests of agricultural importance

| Plant | *cry* gene | Insect target | References |
|---|---|---|---|
| Poplar | *cry1Aa* | *Lymantria dispar* (L.) (Lep.) | McCown et al. (1991) |
| | *cry3Aa* | *Chrysomela tremulae* F. (Col.) | Cornu et al. (1996) |
| Alfalfa | *cry1Ca* | *Spodoptera littoralis* (Boisduval) (Lep.) | Strizhov et al. (1996) |
| Cotton | *cry1Ab* | *Heliothis virescens*, *Helicoverpa zea* | Perlak et al. (1990) |
| | *cry1Ac* and *cry2Ab* | *Spodoptera exigua*, *Pseudoplusia includens* (Walker) (Lep.) | Adamczyk and Hardee (2001) |
| Potato | *cry1Ab* | *Phthorimaea operculella* (Zeller) (Lep.) | Peferoen (1992) and Rico et al. (1998) |
| | *cry1Ab* | *Heliothis armigera* (Hübner) | Chakrabarti et al. (2000) |
| | *cry3Aa* | *Leptinotarsa decemlineata* | Adang et al. (1993), Perlak et al. (1993), and Coombs et al. (2002) |
| Eggplant | *cry1Ab* | *Leucinodes orbonalis* Guenée (Lep.) | Kumar et al. (1998) |
| | *cry3A* | *Leptinotarsa decemlineata* (Say) (Col.) | Jelenkovic et al. (1998) |
| Broccoli | *cry1C* | *Plutella xylostella* (L.) (Lep.) | Zhao et al. (2001) |
| Canola | *cry1Ac* | *Trichoplusia ni* (Hübner) (Lep.), *Spodoptera exigua* (Hübner) | Stewart et al. (1996b) |
| | *cry1Ac* | *Heliothis virescens* (Fabr.) | |
| | | *Helicoverpa zea* (Boddie) (Lep.) | |
| | | *Plutella xylostella* | |
| Tobacco | *cry1Aa* | *Manduca sexta* (L.) (Lep.) | Barton et al. (1987) |
| | *cry1Ab* | *Manduca sexta* | Vaeck et al. (1987) |
| | *cry1Ab* | *Manduca sexta* | Perlak et al. (1991) |
| | *cpTI* | *Manduca sexta* | Williams et al. (1993) |
| | *cry1Ab* | *Heliothis virescens*, *Helicoverpa zea*, *Spodoptera littoralis* | McBride et al. (1995) |
| | *cry1Ac* | *Spodoptera littoralis* | Strizhov et al. (1996) |
| | *cry1C* | *Helicoverpa armigera* | Selvapandiyan et al. (1998) |
| | *cry2A* | *Heliothis virescens*, *Helicoverpa zea* | Kota et al. (1999) |
| | *cry2A* | *Spodoptera exigua* | |
| Maize | *cry1Ab* | *Ostrinia nubilalis* (Hübner) (Lep.) | Koziel et al. (1993) |
| | *cry9C* | *Ostrinia nubilalis* | Jansen et al. (1997) |
| Cabbage | *cry1Ab* | *Plutella xylostella* | Bhattacharya et al. (2002) |
| Soybean | *cry1Ac* | *Heliothis virescens*, *Helicoverpa zea* | Stewart et al. (1996a) |
| | | *Pseudoplusia includens* | |
| Tomato | *cry1Ab* | *Heliothis virescens* | Fischoff et al. (1987) |
| | *cry1Ac* | *Helicoverpa armigera* | Mandaokar et al. (2000) |

for many farmers. Currently, Iran and China are the most advanced countries in the development of *Bt*-rice. According to a research by Huang et al. (2005), held in China, GM rice farmers apply the same type of pesticide that conventional rice farmers use, but with a significantly lower frequency compared to the use of these products by producers of conventional rice. The quantity and cost of pesticides per hectare of conventional rice producers were 8–10 times higher, respectively, compared to the use of these chemicals by producers of GM rice. This reduction also contributes to a reduction of its adverse health effects of the producers themselves. Huang et al. (2005) also showed that there was an increase, although small, of 3.5 % in the yield of GM rice cultivars, compared to the conventional rice.

**Table 18.2** Transgenic plants, with insect resistance, currently on the market (Que et al. 2010)

| Trait developer(s) | Crop | Product name | Transgenic event(s) | Trait genes | Trait targets |
|---|---|---|---|---|---|
| Bayer CropScience | Cotton | FiberMax LibertyLink Bollgard II® | LLCotton25, MON15985 | *bar*, Cry1Ac, Cry2Ab | Lepidopteran pests, weeds |
| Dow AgroSciences | Cotton | WideStrike® | DAS-21023-5, DAS-24236-5 | *pat,* Cry1Ac, Cry1Fa | Lepidopteran pests, weeds |
| Dow AgroSciences | Cotton | WideStrike®/ Roundup Ready® | DAS-21023-5, DAS-24236-5, MON01445-2 | *pat,* Cry1Ac, Cry1Fa, CP4 EPSPS | Lepidopteran pests, weeds |
| Dow AgroSciences | Cotton | WideStrike®/ Roundup Ready® Flex | DAS-21023-5, DAS-24236-5, MON88913-8 | *pat,* Cry1Ac, Cry1Fa, CP4 EPSPS | Lepidopteran pests, weeds |
| Monsanto | Cotton | Roundup Ready®, Bollgard® | MON531, MON1445-2 | Cry1Ac, CP4 EPSPS | Lepidopteran pests, weeds |
| Monsanto | Cotton | Bollgard II/ Roundup Ready® Flex | MON88913-8, MON15985 | CP4 EPSPS, Cry1Ac, Cry2Ab | Lepidopteran pests, weeds |
| Dow AgroSciences and Pioneer Hi-Bred | Maize | Herculex® CB | TC1507 | Cry 1Fa, *pat* | Lepidopteran pests (European corn borer), weeds |
| Dow AgroSciences and Pioneer Hi-Bred | Maize | Herculex® RW | DAS-59122-7 | Cry34Ab1/Cry35Ab1, *pat* | Coleopteran pests (Corn rootworm), weeds |
| Dow AgroSciences and Pioneer Hi-Bred | Maize | Herculex® XTRA | TC1507, DAS-59122-7 | Cry 1Fa, Cry34Ab1, Cry35Ab1, *pat* | Lepidopteran and coleopteran pests, weeds |
| Dow AgroSciences and Pioneer Hi-Bred | Maize | Herculex® XTRA/Roundup Ready® 2 | DAS-59122-7, TC1507, NK603 | *pat,* CP4 EPSPS, Cry34Ab1, Cry35Ab1, Cry1Fa2 | Lepidopteran and coleopteran pests, weeds |
| Monsanto | Maize | Yieldgard® VT Pro® | MON89034 | Cry1A.105, Cry2Ab2 | Lepidopteran pests |
| Monsanto | Maize | Yieldgard® VT | MON88017 | CP4 EPSPS, Cry3Bb1 | Coleopteran pests (corn rootworm), weeds |
| Monsanto | Maize | Yieldgard® VT Triple | MON810, MON88017 | Cry1Ab, Cry3Bb1, CP4 EPSPS | Lepidopteran and coleopteran pests, weeds |
| Monsanto | Maize | Genuity® VT Triple Pro® | MON89034, MON88017 | Cry1A.105, Cry2Ab2, Cry3Bb | Lepidopteran and coleopteran pests, weeds |
| Monsanto and Dow AgroSciences | Maize | Genuity® SmartStaxTM | MON89034, TC1507, MON88017, DAS-59122-7 | PAT, CP4 EPSPS, Cry1Fa2, Cry1A.105, Cry2Ab, Cry3Bb1, Cry34Ab1, Cry35Ab1 | Lepidopteran and coleopteran pests, weeds |
| Syngenta | Maize | Agrisure® GT/CB/LL | Bt11, GA21 | Cry1Ab, *pat,* mutant maize EPSPS | Lepidopteran pests (European corn borer), weeds |
| Syngenta | Maize | Agrisure® CB/LL/RW | Bt11, MIR604 | Cry1Ab, mCry3Aa, *pat* | Lepidopteran and coleopteran pests, weeds |
| Syngenta | Maize | Agrisure® 3000GT (GT/ CB/LL/RW) | GA21, Bt11, MIR604 | *pat,* Cry1Ab, mCry3Aa, mutant maize EPSPS | Lepidopteran and coleopteran pests, weeds |

## 18.4 Biosafety of *Bt*-Plants

Controversies about the benefits and the ecological risks of transgenic cultivars exist since its introduction, but have increased after the release of the first commercial *Bt*-plants in 1996 (Shelton and Sears 2001). In Brazil, the National Technical Commission on Biosafety (CTNBio) established conclusive safety standards and technical advice regarding the protection of human health, living organisms, and the environment for all activities with GM organisms and products.

Normative Instruction #3 CTNBio Biosafety Note (site: http://www.ctnbio.gov.br/upd_blob/0000/8.pdf) specifies "Standards for the Environment in Planned Release of Genetically Modified Organisms." This Normative sets out the procedures that apply to the release, in Brazil, of genetically modified organisms (GMOs) into the environment (including imported GMOs) – for either field experiments or other means. The advancement of the production of GM crops has driven research addressing questions about the impact of this technology on biodiversity, especially its effects on nontarget organisms such as natural enemies (predators and parasitoids) and in humans.

According to Romeis et al. (2006), to study the effect of environmental toxins of *B. thuringiensis* synthesized by GM plants, a detailed analysis of the potential effects of GM plants prior to commercial release must be performed. The ecological risk assessment for regulatory purposes should be based on a number of approaches, in which the evaluation increases in complexity, according to the knowledge acquired during previous tests. For the risk assessment on nontarget organisms, a series of tests is performed in laboratory to determine whether the organism in question is susceptible to the toxin in the harshest conditions. In this case, the organism is directly exposed to high doses of the toxin (Dutton et al. 2003).

These tests are simple to design and standardize and produce results easy to interpret. If the results identify risks to organisms tested, new experiments should be conducted under conditions closer to reality (Romeis et al. 2006). Chen et al. (2006) published a survey of the field evaluation of the effects of *Bt*-rice with *cry*1Ab and *cry*1AcI genes, which have demonstrated specific lepidopteran insecticidal action in nontarget populations of Homoptera and Hemiptera. After two years of monitoring, the obtained data indicated that the *Bt*-rice had no significant impact on the composition and density of insects evaluated throughout the sample period and did not affect the population dynamics of the species predominant site.

Several studies also show that *Bt*-rice has no adverse impact on natural enemies compared with areas planted with non-GM rice (Liu et al. 2002, 2004; Bai et al. 2005; Chen et al. 2006). Another aspect that must be evaluated is important to decrease the release of insecticide in agroecosystems in areas with GM plants (Velkov et al. 2005; James 2011; Brookes and Barfoot 2006). In a study by Huang et al. (2005), it was found that farmers have significantly decreased the amount of insecticide applied in the field using 16.77 kg less of pesticide per hectare, compared to conventional rice producers, representing a comparative reduction of 80 %. This decrease reflects the use of pesticides directly on environmental quality and human health, as farmers reduce exposure to chemicals, which generally have high toxicity (Breitler et al. 2004; Huang et al. 2005).

## 18.5 Final Considerations

The reduction in agricultural productivity due to the attack of insects is a significant factor limiting food production in the world. The resistance to insect pests, mediated by plants transformed with genes from *B. thuringiensis*, has been one of the great successes of technology corresponding to the genetic engineering of crop plants (Ferry et al. 2006). To date, no data reporting the resistance of insects to *Bt*-plants under field conditions has been published, which reinforces the importance of biotechnological tool. More than 50 field studies with different lengths, sizes, and sampling methods were conducted in commercial and experimental evaluation of the

impact of *Bt*-plants on natural enemies, and no negative effect was revealed, compared to conventional plants (Chen et al. 2006; Romeis et al. 2006). Moreover, several studies with *Bt*-rice cultivars, conducted in a greenhouse, revealed that most of these cultivars can effectively control the lepidopteran targets. Thus, the research also reveals that *Bt*-rice reduces the use of chemical insecticides, with significant benefits to the environment and the health of rice farmers (Chen et al. 2006). Currently, it is evident the need for additional strategies durable to the use of *Bt*-plants, to ensure the benefits achieved with obtaining them. For this, a new generation of *Bt*-plants is emerging, with cultivars that express simultaneously two or more Cry proteins of *B. thuringiensis* (Cohen et al. 2000; Bates et al. 2005; Ferry et al. 2006). These new *Bt*-plants aim to achieve this goal because, by its greater number of expressed proteins, they extend the reach to other insects, as well as delay the onset of resistance to susceptible insects.

This chapter reveals that *Bt*-plants can be considered another tool in the strategies of Integrated Pest Management as they have the potential to increase productivity and reduce the application of pesticides and thereby improve environmental quality in cultivation areas. These benefits of plant genetic engineering, combined with the *cry* genes of *B. thuringiensis*, are motivating the expansion of this technology cultivated plants of economic interest nationally and regionally, such as rice, in search of the sustainability of agroecosystems.

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# Part V

# Equipments, Bioinformatics Tools and IPM

# Equipment for Application of Biopesticides

19

Samuel Gan-Mor, Eric Palevsky, and Graham A. Matthews

**Abstract**

Many types of application systems are needed to meet the individual application requirements of commercially available biopesticides, because a wide variety of products are involved. Commercial use of low-toxicity biopesticides can be accomplished by means of very uniform spraying; it possibly could be increased by the use of improved systems such as air-assisted spraying in conjunction with electrostatic technologies. Effective technologies for deposition of granular formulations utilize air assistance and accurate feeding systems. Special equipment is used for spreading small living organisms, such as predators. Many biopesticide products that exhibit good control capabilities in laboratories or small-scale tests have not successfully been scaled up for full-scale field use. Effective application of the new biological control agents will necessitate development of new equipment, technologies, and formulations.

**Keywords**

Biopesticides • Equipment • Sprayer • Deposition

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## 19.1 Introduction

Biopesticide application presents different problems from those of chemical pesticides. In particular, some are living organisms and great care is needed to maintain their viability. As the biopesticides include a large variety of biological products, the application systems will vary depending on the biopesticide. To meet these requirements, for example, standard sprayers are used to apply liquid biopesticides on crop canopies and soil, tillage equipment may be used

K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_19, 

to incorporate entomopathogenic nematodes in the ground, refrigerated applicators with special cells are used to deliver and deposit predatory mites and pupae, and paraffin-wax capsules have been used to disperse pheromones.

Good application of a biopesticide in a greenhouse or a field requires an adequate system to spread and deposit the agents to fit the specific feature of the agent and to guarantee optimal efficacy of the technology. Biopesticides as defined by Steinke and Giles (1995) are biological products or organisms, which are produced from a biological source and may include viruses, bacteria, fungi, predators, parasites, and pheromones. The design of a system for applying a biopesticide depends strongly on the type of material and its mode of action, as well as physical aspects. Thus, close collaboration is needed between the biologist, formulation specialist, and equipment engineer, to develop the most adequate delivery system, which is economically acceptable (Gan-Mor and Matthews 2003).

When an insect pest is feeding on a sprayed leaf, the efficacy of an insecticide is primarily determined by the uniformity of spray coverage, amount deposited and its toxicity, as well as the movement of the pest and its feeding pattern (Hall et al 1995; Navon et al 1991; Alchanatis et al 2000). Thus, the majority of research groups involved in developing biopesticides endeavor to form a liquid formulation of the active material, but in general biopesticides are usually particulate suspensions. Biopesticides have limited mobility and their radius of influence is very small, unlike certain pesticides with systemic activity through plants. Therefore, optimal coverage and deposition uniformity of the application equipment is needed for their effective performance (Gan-Mor et al. 1996; Gamliel 2010). Application of biopesticides with existing spray technologies potentially offers low investment for the farmers, but, often its efficacy is inadequate. The older types of spray applicators have been commonly used with high volumes of spray being applied. Once a surface has been wetted, the surplus is lost by dripping off the foliage, which is wasteful when the biopesticide is expensive. However, some biopesticides can be applied with adequate existing equipment, but there is insufficient development of a suitable formulation or lack of information on efficacy under field conditions.

Improved distribution and suitably uniform deposition throughout the crop canopy can often be fulfilled using air-assisted spraying. Improving deposition uniformity can also be achieved by utilizing electrostatic charging, which is currently under development by research laboratories and commercial companies. In contrast to the uniformity difficulties involved in foliar application, an example of less demanding uniformity is the application techniques for entomopathogenic nematodes to soil (Grinstein et al 1995; Gamliel et al 1998), because of nematode mobility. Techniques to overcome this uniformity problem as well as several application systems and solutions for deposition problems are discussed below.

In this chapter, systems for delivering biopesticides, including natural enemies that have been developed to control pests in commercial crops, are discussed. These materials present significantly different problems for growers compared with conventional chemical pesticides, so require more work to adapt or more research to develop appropriate equipment and application techniques.

## 19.2 Advantages and Difficulties in Biopesticide Application

### 19.2.1 Advantages in Biopesticide Application

Biopesticides are distinguished from conventional chemical pesticides as many are very selective and are nontoxic toward nontarget organisms (Mendelsohn et al 1995). With chemical pesticides, much attention has been given to prevent traces of toxic pesticides from reaching water surfaces due to drift or runoff from fields and preventing waste materials from reaching the ground (Balsari et al. 2008; Mickle 1995; Teske et al. 1995). Thus, larger droplets are recommended as drift increases with smaller

droplets, although smaller droplets often offer better coverage. Certain biopesticides require droplet size sufficiently large to contain the organism or carry sufficient ingredient to where the biopesticide is needed. Thus, the optimal droplet spectrum needs to be carefully considered. As the biopesticides are more selective, narrow buffer zones may be used as required for application of less toxic pesticides. Their use presents a lower hazard to operators, consumers, and ecosystems.

### 19.2.2 Difficulties in Biopesticide Application

Certain difficulties which involve application of biopesticides are listed above; a large variety of application systems that are needed to meet the individual application requirements cause extra expenses on growers. Great care is needed to maintain the viability of the living organisms. While handling certain biopesticides, sterile conditions are required (Jin et al. 2012). High uniformity and dense deposit are often required while applying these pesticides.

## 19.3 Droplet Size and Application Equipment Design Requirements

The technique of applying biopesticides generally has to be more accurate than that required for application of synthetic pesticides. Thus, for example, a minimum droplet size is required when applying entomopathogenic nematodes. In addition, the uniformity of the spray coverage where the target pest is located is more important, as translocation with systemic pesticides does not occur.

Deposition uniformity is usually perceived as high coverage and dense deposition. The coverage can be measured by the percentage of leaf covered and cover density by the number of droplets per unit area. High-quality deposition often depends also on providing sufficient amount of active material per unit area (Bateman 1999). Small droplets can provide higher coverage and cover density; however, a large droplet carries more material. Actually, a droplet of twice the diameter carries eight times [$2^3$] more material. The application thus may have to compromise between avoiding too small a droplet liable to drift and, at the other extreme, avoiding too large a droplet that may not impact on a leaf surface, may provide lower uniformity, or be lost from a leaf surface due to runoff. Table 19.1 provides important data for droplet size for several application techniques.

**Table 19.1** Typical droplet sizes of sprayers referred to in the text

| Sprayer | Droplet size, μm |
|---|---|
| ESS – air/liquid atomizer with electrostatics | 20–40 |
| Cold fogger | 10–20 |
| Grooved spinning disc | 70–200 |
| Hydraulic nozzles | 110–200 |
| Air blast orchard sprayer | 130–200 |
| Knapsack mistblower | 100–200 |

Grooved spinning disc is commonly associated with narrow band of droplet sizes. This narrow band can be maintained at the lower ranges mentioned in Table 19.1 or the higher one, to meet the requirement of the particular operation. Other nozzles are associated with wider band but also can provide droplet sizes at lower or higher part of the abovementioned sizes. Although hydraulic nozzles have many shortcomings, it is the most commonly used technology for field crops (Bateman 1999). They are adaptable with different spray patterns and volume application rates and by careful design of equipment can be positioned to optimize spray distribution. When selecting or designing a delivery system, the above droplet size should be considered to achieve high-quality deposition and reduced hazard. Additionally toxic pesticides are being phased out and softer pesticides, mainly from botanical and other natural sources, are being developed. These softer materials are essentially of minimal toxicity. The commercial use of the soft pesticides depends on the development of high-quality effective application systems (Gamliel 2010).

## 19.4 Deposition Utilizing Commercial and Specially Designed Applicators

### 19.4.1 Experiments on Application with Commercially Available Sprayers

Steinke and Giles (1995) refer to several reports of the more successful field applications of biopesticide utilizing commercially available sprayers. Chapple et al. (2000) have recently discussed many of the problems associated with application of microbial insecticides, while Bateman and Alves (2000) and Gan-Mor and Matthews (2003) have described equipment that has been used to apply biopesticides.

#### 19.4.1.1 Bt Application

Development of *Bacillus thuringiensis* (Bt) as a biopesticide has been particularly important as it was possible to develop formulations that could be easily diluted in water for application through hydraulic sprayers. Being less toxic to natural enemies, their role was important in integrated pest management (IPM) systems. Poor efficacy that occurred was often due to a poor quality formulation or inadequate deposition where the pest was located. The latter was shown by Perez et al. (1995) who conducted laboratory and field tests to determine the effect of application technology, plant age, and Bt subspecies on the mortality of one susceptible and one resistant population of diamondback moth. There was less variation among different sections of plants when sprays were applied with an electrostatic sprayer, providing some underleaf coverage, than with a knapsack sprayer or a hydraulic sprayer fitted with hollow-cone nozzles. The hydraulic sprayer was fitted with droplegs so that each row was sprayed via three nozzles, one above the row and two on the droplegs on either side of the row, the lateral nozzles also achieving some underleaf coverage. Figure 19.1 shows the ability of electrostatic forces to bend the trajectory of charged spray droplets so that it is deposited on surfaces that are not facing the oncoming spray cloud (Gan-Mor et al. 2010c).

In contrast to liquid spraying, when dry granular particles, approximately 70 μm in diameter, containing Bt were applied on cotton and

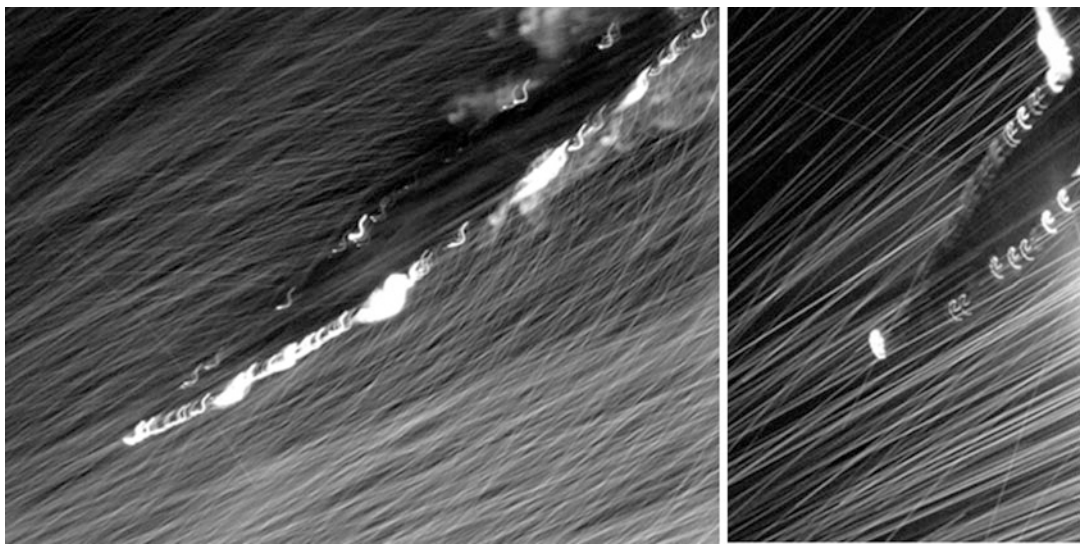

**Fig. 19.1** Sufficiently long exposure photography shows the curved trajectories of charged spray droplets around grounded leaf, due to electrostatic forces (*left*) and straight trajectory with no charging (*right*)

dates by Navon et al. (1997, 1999), as dry powder hardly sticks to leaves, a relatively high percentage of the material was deposited on the plant with the aid of electrostatic charging. Laboratory tests had determined that at least 1,500 particles per $cm^2$ were needed to provide good control, but it was difficult to achieve such a high density in the field, even when electrostatic charging was used. However, results were sufficiently encouraging and thus worthy of further investigation.

#### 19.4.1.2 Entomopathogenic Nematodes (EPNs)

As EPNs are large organisms, relative to the spray droplet diameters, Chapple et al. (2000) have argued that there is no "optimum" droplet size. Certainly there is a minimum droplet volume that can hold a nematode and very small droplets may be incapable of conveying EPNs. While for soil-applied treatments a high volume may be advantageous to get nematodes beyond the soil surface and provide moisture for survival, high volumes applied to foliage are wasteful. In an effort to develop a technique to apply EPNs to foliage, certain studies have included investigations on the use of spinning discs (Mason et al. 1998a, b, 1999). The addition of a number of adjuvants, containing nematode juveniles, to sprays was examined. The mean number of infecting nematodes was significantly enhanced by the use of some of the adjuvants. Two types of spinning discs were also considered. Both discs produced similar spray spectra that were found to be unaffected when various adjuvants were added to the spray solution. The mortality among the target pests generally increased as the flow rate was increased. Piggott (2000) found that spinning discs do not provide good delivery technology for EPNs. Piggott et al. (2000) also showed an advantage in adding a polymer (polyacrylamide) to the spray to improve the EPN survival on foliage. Brusselman et al. (2011b) examined the volumetric distribution pattern of EPNs beneath hydraulic flat-fan nozzle, air-induction nozzle, and TwinJet spray nozzle. Nozzle type significantly influenced the number of nematodes deposited on a horizontal target and their distribution within the droplets. Brusselman et al. (2011a) also tested these nozzles for deposition of EPNs on cabbage and cauliflower. They found that the nozzle type has a minor effect on the number of nematodes delivered on difficult-to-reach targets but concluded that modification of the spray application techniques is necessary to increase the deposition efficiency.

In other experiments, the viability of an EPN was significantly decreased as the pumping period of a high-pressure hydraulic sprayer increased (Nilsson and Gripwall 1999). Fife et al. (2007) found that temperature increases due to pump recirculation are more detrimental to entomopathogenic nematodes (EPNs) than mechanical stress induced by the pump. Particularly, extensive recirculation of the tank mix increases the liquid temperature, while diaphragm and roller pumps contribute less heat and are better suited for use with biopesticides compared to the centrifugal pump. Moreira et al. (2013) found that under moderate spraying conditions, the EPN (*Steinernema feltiae*) viability and pathogenicity were not significantly affected. In assessing the effectiveness of foliar sprays of the EPN *Steinernema carpocapsae* against the apple sawfly *Hoplocampa testudinea* and the plum curculio *Conotrachelus nenuphar*, two early-season pests in apple orchards, Belair et al. (1998) found significantly less damage at harvest where the nematode had been sprayed via a commercial handgun sprayer. However, where a commercial air blast orchard sprayer was used, no significant difference in the damage was observed. The differences here may be that the handgun applied a much higher volume and with the air blast sprayer many of the smaller droplets failed to carry sufficient EPNs.

#### 19.4.1.3 Baculoviruses

One of the major difficulties with applying *baculoviruses* is that they are liable to be inactivated by sunlight, so formulation with sunscreens has been investigated by Killick (1986) and Arguer and Shapiro (1997). Nevertheless with a suitable formulation, it is important that the deposition occurs primarily where the target pest is feeding. In attempting to control cotton bollworms,

Parnell et al (1999) compared the distribution of a *baculovirus* applied at a medium volume (MV) using a motorized knapsack mistblower, with a very low volume (VLV) application from a spinning disc. Field tests showed that the spinning disc VLV application gave better control than the commonly used mistblower MV application, presumably because the more uniform droplet spectrum deposited more at critical areas where young larvae would feed. In similar trials in Egypt against *Spodoptera littoralis*, better results were obtained when nozzles were placed between the rows and directed up to the lower leaf surfaces where eggs were laid.

#### 19.4.1.4 Mycoinsecticide

One of the largest research programs has sought a biological control of locusts. For logistical reasons, preference has always been given to ultralow volume sprays, so the first study was to determine whether the conidia of *Metarhizium anisopliae* var. *acridum* could be applied in an oil formulation. Fortunately, the spores are lipophilic and successful formulation led to effective applications using rotary atomizers to optimize droplets at 70–100 μm, even under arid conditions in Africa (Bateman 1997). This *Mycoinsecticide* is intensively used in Australia and other countries (Hunter 2005). Other strains of the fungus have since been effective in sprays formulated for dilution in water, but where an oil adjuvant has been also included in the spray (Bateman and Alves 2000). Backpack sprayer application of a fungus against Colorado potato beetle was significantly more effective than a conventional insecticide treatment, and especially in comparison with the no-treatment control (Poprawski et al. 1997). Although the specific mechanism remained unclear to the researchers, the fungal application evidently yielded significant reduction in larval densities and provided substantial foliage protection.

#### 19.4.1.5 Fungicides

Not many field tests applying fungi have been reported. The difficulties in developing a biofungicide are discussed by Hofstein and Chapple (1999), giving the development of a hyperparasitic antagonist *Ampelomyces quisqualis* as an example. In relation to application, they point out that the equipment used in small trials must match that of the end user as the size distribution of particles in the spray tank was markedly different after passage through a pump. Any clumping of particles will decrease the efficiency of an application. They also note the difficulty in selecting a surfactant system in which to suspend the spores that are not fungitoxic. Specially adapted secateurs were used to apply a suspension of *Trichoderma viride* to the cut surface (Jones et al. 1994). Hidalgo et al. (2003) conducted a field investigation into delivery systems for fungal agents in oil adjuvants. Application techniques compared were motorized mistblowers fitted with rotary atomizers and hydraulic sprayers fitted with cone nozzles giving a narrow angle of spray. Overall, directional hydraulic nozzles were better than the motorized mistblower with rotary atomizers. The fungal conidia distribution of BotaniGard® 22WP on chrysanthemum plants, using different spray nozzles, was assessed by Goulia et al. (2011). A flat-fan nozzle (TeeJet 8001XR) provided the optimal overall fungal application to plants in a greenhouse.

### 19.4.2 Development of Application Techniques for Parasitoid Fungi and Predators

Giles and Wunderlich (1998) developed an electrically controlled delivery system for spraying beneficial insect eggs in liquid suspensions. Large droplets (*ca.* 2 mm diameter) were released via a pulse-width-modulated valve which monitored the application rate and the spacing between the discharged eggs. A uniform suspension of eggs in liquid was delivered with no significant reduction in viability (Wunderlich and Giles 1998). Giles et al. (1995) developed a delivery system especially for the gentle release of predatory mites, for biological pest control in strawberries. The mites were easily injured by the agitation of the mixture, so the handling system consisted of an insulated storage reservoir that kept the chilled mixture stationary and a rotating metering plate. As this plate rotated, the formulation filled cylindrical cells in the

**Fig. 19.2** Corn pollen on citrus leaf and eggs of *Euseius scutalis* during augmentation of the predatory mite (From Gan-Mor et al. 2010a)

plate and each cell's contents were released as the cell passed above an opening.

Bouse et al. (1981) and Bouse and Morrison (1985) applied *Trichogramma pretiosum* pupae aerially by incorporating a refrigeration system to prevent wasp emergence before delivery, but with this technique many pupae are lost on the soil. Weintraub et al. (2003) tested a slow release delivery system (Thripex, Koppert, Holland) for control of the broad mite (*Polyphagotarsonemus latus* (Banks)) on organic greenhouse sweet peppers (*Capsicum annuum* L.) with the predatory mite *Neoseiulus cucumeris* (Oudemans). The predatory mites were released twice (on days 1 and 5, or 15 days later) on each plant, every second plant, or every fourth plant. The predatory mite successfully controlled the broad mite.

Santosa et al. (2012) developed a high-volume formulation that in conjunction with high-volume sprayers was found to be appropriate for dispersion of entomopathogenic fungi *B. bassiana* conidia in commercial fields. Their work included determining surfactants' properties and finding those that cause minimal damage to the biopesticide. Jin et al. (2012) tested a new laboratory size specially designed sterile spray tower made of enclosed chamber where filters remove more than 99.9 % of particles that are larger than 0.2 mm. Claiming that biopesticide application is sensitive to unsterile conditions, the effectiveness of the spray tower was assessed by spraying entomopathogenic fungi against imported fire ants.

#### 19.4.2.1 Predator Population Augmentation

Technology for in-field population augmentation of predatory mites (Phytoseiidae) by food supplementation was developed by Gan-Mor et al. (2010a). Corn pollen was selected as a suitable food for the generalist predator *Amblyseius swirskii* and *Euseius scutalis*. The cost of the pollen was significantly reduced by the development of a dedicated corn pollen harvester. To increase the percentage of deposited pollen on the foliage, a dedicated electrostatic applicator was developed. Figure 19.2 shows corn pollen deposited on citrus branch. The target pests were *Frankliniella occidentalis* and *Tetranychus urticae* and the model crop was sweet pepper. The technology can become economically viable since more predators and fewer pests were found where corn pollen was applied.

### 19.4.3 Bait Sprays Using Pheromones

Typically, the research in which agricultural engineers are involved does not extend into the formulation aspects related to the problem. However, sometimes the formulation and the suspension in which the agent is carried have an important role, and they affect the application technology. Such a case was reported by Atterholt et al. (1994), who found that the carrier and the application technology significantly affected the

**Fig. 19.3** A sprayer for cordoned crops capable of applying the spray upward for improved underleaf coverage (*left*) and equipped with electrostatic charging system (*right*)

release and degradation rates of the insect sex pheromones, which formed the control agent.

Chandler and Sutter (1997) reported on a development of a high-clearance field sprayer and spraying methods for the application of baits. Semiochemical-based baits were applied in cornfields using water volumes of 19 and 37 l/ha. A sprayer fitted with over-the-canopy nozzles was compared with two drop-line sprayers, one having nozzles mounted on drop lines on every row and the other having drop lines on alternate rows. No significant differences in pest population reduction were found among the nozzle configurations; therefore, the over-the-canopy spraying technique is preferable because of its mechanical robustness. Stelinski et al. (2006) developed a tractor-mounted mechanized applicator to deposit the pheromone for mating disruption of oriental fruit moth, *Grapholita molesta*, incorporated in paraffin-wax dispensers. They found that the 0.04 ml wax per drop required too many applications. Larger drops are expected to prolong pheromone release for extended efficacy and desirable overall economics.

### 19.4.4 Botanical Oils: Technology to Facilitate Field Application

Neem oil has been applied commercially worldwide but requires a relatively high amount of oil for active material to be deposited. Fenigstein et al. (2001) showed that common cooking oils, such as soy and peanut oils, can provide good efficacy but require even higher amounts than neem oil. Fortunately these cooking oils are inexpensive; thus, to reduce handling and preservation costs and to provide an economically viable process, a new technology for on-site emulsifying of edible oils was developed (Gan-Mor et al. 2010b). This technology can be effective provided sprayers apply a uniform deposition and in sufficient amounts. A substantial number of growers are already using this technique commercially. Recently developed applicators, such as the Chico sprayer (Degania Sprayers Ltd.), can provide improved spray deposition and are favored for application of low-toxicity agents as those mentioned above (Fig. 19.3). This sprayer is available with improved deposition capability utilizing electrostatic charging.

## 19.5 Concluding Remarks and Future Scope

Effective use of commercially available biopesticides requires a large variety of application equipment to meet the specific needs of each product. Effective deposition of granular formulations can be achieved by using accurate feeding systems and air assistance, whereas low-toxicity pesticides require uniform-application sprayers.

Under optimal laboratory conditions, many successful experiments with biopesticides have been carried out, but the results are seldom repeated under the harsh conditions experienced in the field. This has undoubtedly been due to lack of investment in the development of effective formulations and delivery systems, which are needed to enable the commercialization of the more promising biopesticides. A major challenge is to develop effective formulations that will enable biological control agents to be easily applied by farmers, but this also requires investment in development of appropriate equipment. Although formulation of control agents as dry granular particulates and baits frequently improves their stability, and thereby ensures a longer period of activity of an active ingredient, the use of granular formulations has declined because farmers prefer to spray liquid formulas when possible. Improvement in deposition is possible by means of electrostatic technologies, and these are more effective when used in conjunction with air assistance. Further development of this technology needs to be explored, as well as the application of granular formulations, in order to increase the effectiveness of low-toxicity pesticides, so that their commercial use can be increased. The abovementioned problem of biopesticides that have shown good efficacy in the laboratory and limited effect in the field calls for R&D related to delivery systems for biopesticides and for focus on filling this gap between laboratory tests and field use, in order to meet the individual application requirements of specific biopesticides. Especially with regard to biological control, a major effort needs to be dedicated in developing equipment for spreading the relevant small living organisms, such as predators.

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# Relevance of Bioinformatics in Biopesticide Management: A Comparative Comprehensive Review

20

P.K. Ragunath, P.A. Abhinand, and K. Archanna

**Abstract**

Bioinformatics is conceptualizing biology in terms of macromolecules (in the sense of physical chemistry) and then applying "informatics" techniques (derived from disciplines such as applied mathematics, computer science, and statistics) to understand and organize the information associated with these molecules, on a large scale (Luscombe NM, Greenbaum D, Gerstein M, What is bioinformatics? A proposed definition and overview of the field. Methods Inf Med 40:346–358, 2001).

**Keywords**

Biopesticide • Bioinformatics • Gene expression profiling • Micro arrays

## 20.1 Overview of Bioinformatics

### 20.1.1 Introduction to Bioinformatics

Bioinformatics is the science of conceptualizing biology in terms of macromolecules (in the sense of physical chemistry) and then applying "informatics" techniques (derived from disciplines such as applied mathematics, computer science, and statistics) to understand and organize the information associated with these molecules, on a large scale (Luscombe et al. 2001).

Bioinformatics deals with algorithms, databases and information systems, web technologies, artificial intelligence and soft computing, information and computation theory, software engineering, data mining, image processing, modeling and simulation, signal processing, discrete mathematics, control and system theory, circuit theory, and statistics. Bioinformatics generates new knowledge of biology and medicine, improving and discovering new models of computation. Bioinformatics encompasses three important subdivisions: the development of new algorithms and statistics with which to assess relationships among members of large data sets; the analysis and interpretation of various types of data including nucleotide and amino acid sequences, protein domains, and protein structures; and the development and implementation of tools that enable efficient access and management of different types of information.

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_20, 

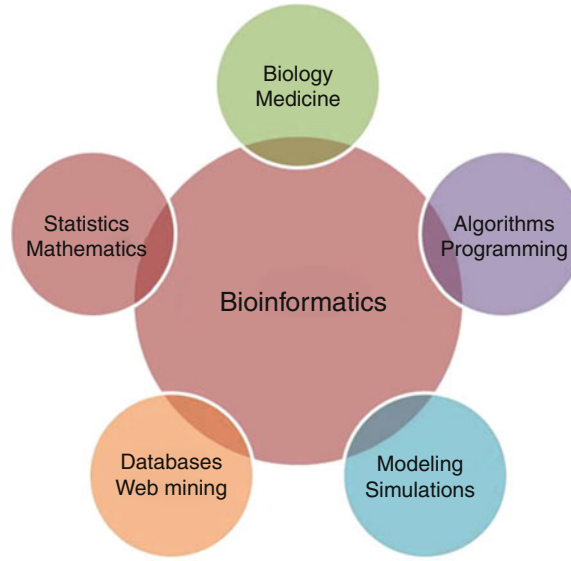


**Fig. 20.1** Overview of bioinformatics

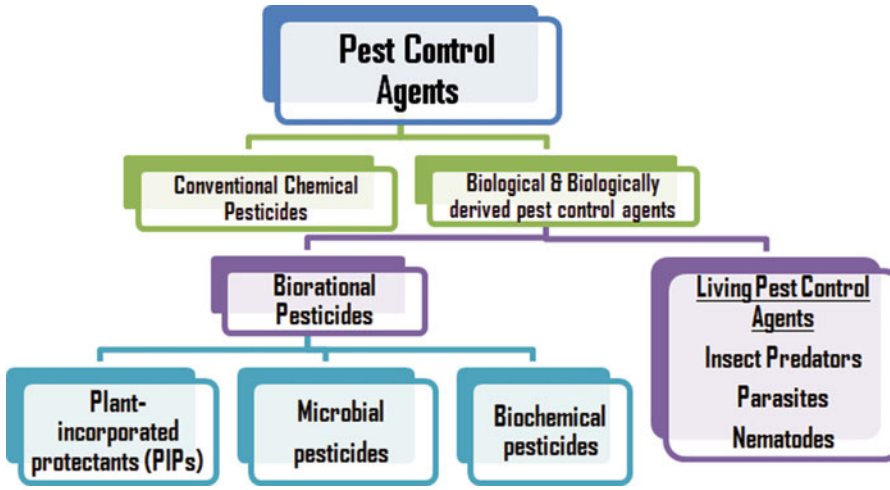


**Fig. 20.2** Detailed classification of biopesticides

### 20.1.2 Bioinformatics: Major Components

The multidimensional nature of bioinformatics makes it imperative that it encompasses various ancillary sciences. A plethora of "omics" sciences are an integral part of bioinformatics: genomics, proteomics (in strict sense, should be used with the prefix computational), computer-aided drug design, bio databases and data mining, molecular phylogenetics, microarray informatics, and systems biology. We will briefly touch upon their scope in the ensuing paragraphs (Figs. 20.1, 20.2, 20.3, 20.4, 20.5, and 20.6).

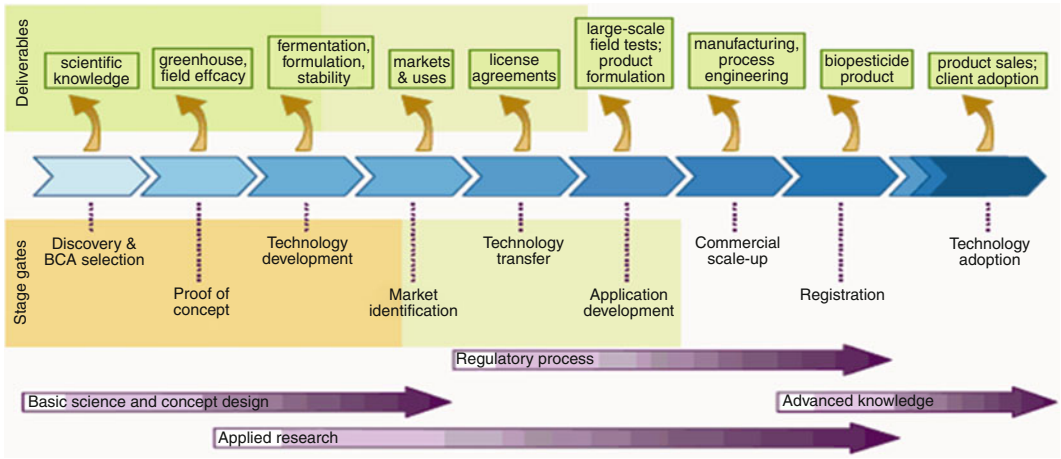


**Fig. 20.3** Sequential process of biopesticide discovery (Agriculture & Agri-Food Canada 2005)

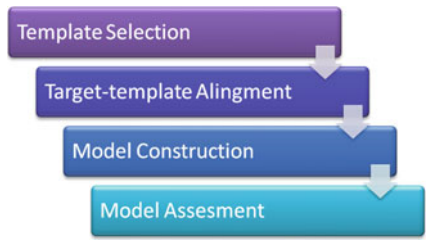


**Fig. 20.4** The steps involve in homology modeling

There are two fundamental ways of modeling a biological system (e.g., living cell) both coming under bioinformatics approaches: (1) static (sequences (proteins, nucleic acids, and peptides), structures (proteins, nucleic acids, ligands (including metabolites and drugs), and peptides), and interaction data among the above entities including microarray data and networks of proteins and metabolites) and (2) dynamic (systems biology comes under this category including reaction fluxes and variable concentrations of metabolites and multiagent-based modeling approaches capturing cellular events such as signaling, transcription, and reaction dynamics).

Genomics analyzes the context of genes or complete genomes (the total DNA content of an organism) within the same and/or across different genomes. Genomics is a discipline in genetics concerned with the study of the genomes of organisms. The field includes efforts to determine the entire DNA sequence of organisms and fine-scale genetic mapping. The field also includes studies of intragenomic phenomena such as heterosis, epistasis, and pleiotropy and other interactions between loci and alleles within the genome. In contrast, the investigation of the roles and functions of single genes is a primary focus of molecular biology or genetics and is a common topic of modern medical and biological research. Research of single genes does not fall into the definition of genomics unless the aim of this genetic, pathway, and functional information analysis is to elucidate its effect on, place in, and response to the entire genome's networks (http://www.genome.gov/19016904).

Proteomics is the subdivision of genomics concerned with analyzing the complete protein complement, i.e., the proteome, of organisms, both within and between different organisms. Proteomics is the large-scale study of proteins, particularly their structures and functions (Anderson and Anderson 1998; Blackstock and Weir 1999). Proteins are vital parts of living organisms, as they are the main components of the physiological metabolic pathways of cells. The term "proteomics" was first coined in 1997 (Blackstock and Weir 1999) to make an analogy with genomics, the study of the genes. The word "proteome" is a blend of "protein" and "genome" and was coined by Marc Wilkins in 1994 while working on the concept as a PhD student

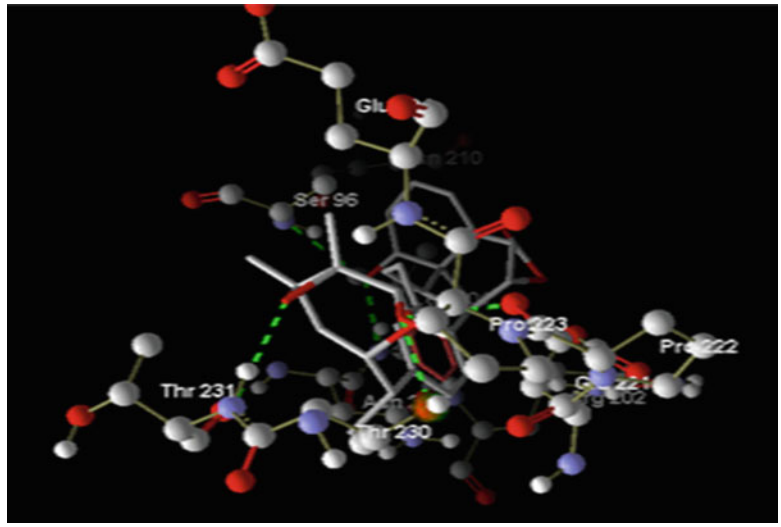


**Fig. 20.5** Docking of ligand to receptor

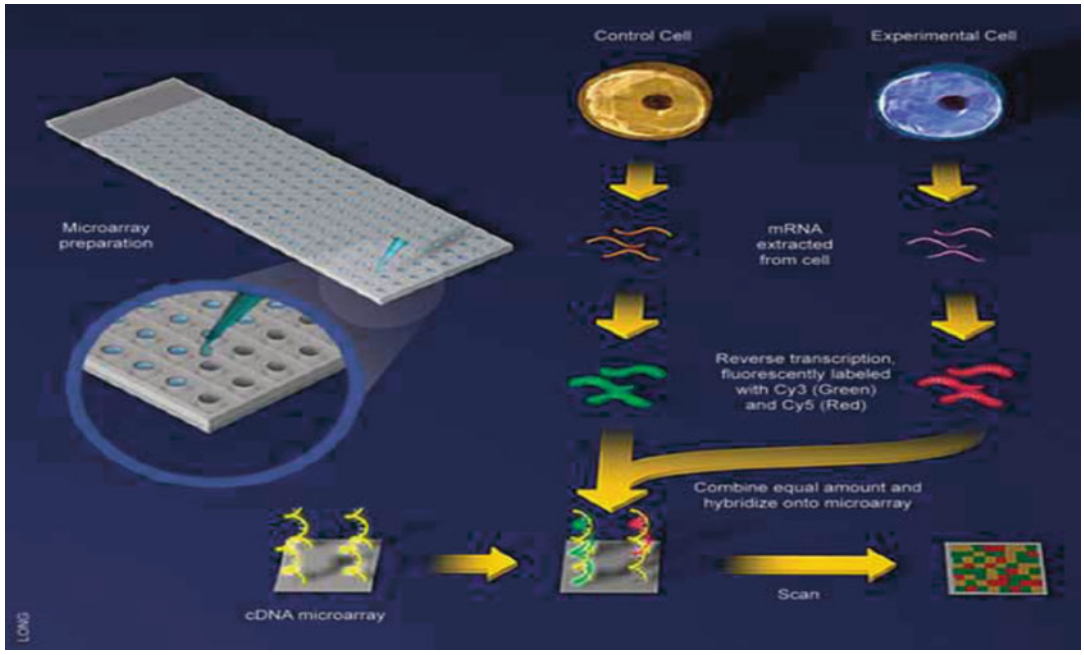


**Fig. 20.6** This schematic portrays an experiment using a spotted or cDNA microarray (The Science Creative Quarterly, *artist: Jiang Long*)

(Blackstock and Weir 1999; James 1997). The proteome is the entire complement of proteins (Wilkins et al. 1996), including the modifications made to a particular set of proteins, and produced by an organism or system. This will vary with time and distinct requirements, or stresses, that a cell or organism undergoes.

Systems biology is a term used to describe a number of trends in bioscience research and a movement which draws on those trends.

Proponents describe systems biology as a biology-based interdisciplinary study field that focuses on complex interactions in biological systems, claiming that it uses a new perspective. Particularly from year 2000 onward, the term is used widely in the biosciences and in a variety of contexts. An often stated ambition of systems biology is the modeling and discovery of emergent properties, properties of a system whose theoretical description is only possible using techniques which fall under the remit of systems biology. These typically involve cell signaling networks, via long-range allostery.

## 20.2 Bioinformatics Application

### 20.2.1 Sequence Analysis

A sequence alignment is a way of arranging the sequences of DNA, RNA, or protein to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships between the sequences (Mount 2004). Aligned sequences of nucleotide or amino acid residues are typically represented as rows within a matrix. Gaps are inserted between the residues so that identical or similar characters are aligned in successive columns. A comparison of genes within a species or between different species can show similarities between protein functions or relations between species (the use of molecular systematics to construct phylogenetic trees). With the growing amount of data, it long ago became impractical to analyze DNA sequences manually.

### 20.2.2 Genome Annotation

In the context of genomics, annotation is the process of marking the genes and other biological features in a DNA sequence. This includes protein-coding genes as well as RNA genes, but may also include prediction of other functional elements such as regulatory. Gene finding is one of the first and most important steps in understanding the genome of a species once it has been sequenced.

### 20.2.3 Computational Evolutionary Biology

Evolutionary biology is the study of the origin and descent of species, as well as their change over time. Informatics has assisted evolutionary biologists in several key ways; it has enabled researchers to trace the evolution of a large number of organisms by measuring changes in their DNA, rather than through physical taxonomy or physiological observations alone; more recently, compare entire genomes, which permits the study of more complex evolutionary events, such as duplication, horizontal gene transfer, and the prediction of factors important in bacterial speciation; build complex computational models of populations to predict the outcome of the system over time; and track and share information on an increasingly large number of species and organisms.

### 20.2.4 Analysis of Gene Expression

The expression of many genes can be determined by measuring mRNA levels with multiple techniques including microarrays; expressed cDNA sequence tag (EST) sequencing; serial analysis of gene expression (SAGE) tag sequencing; massively parallel signature sequencing (MPSS); RNA-seq, also known as "whole transcriptome shotgun sequencing" (WTSS); or various applications of multiplexed in situ hybridization. All of these techniques are extremely noise-prone and/or subject to bias in the biological measurement, and a major research area in computational biology involves developing statistical tools to separate signal from noise in high-throughput gene expression studies. Such studies are often used to determine the genes implicated in a disorder: one might compare microarray data from cancerous

epithelial cells to data from noncancerous cells to determine the transcripts that are upregulated and downregulated in a particular population of cancer cells.

### 20.2.5 Analysis of Regulation

Regulation is the complex orchestration of events starting with an extracellular signal such as a hormone and leading to an increase or decrease in the activity of one or more proteins. Bioinformatics techniques have been applied to explore various steps in this process. For example, promoter analysis involves the identification and study of sequence motifs in the DNA surrounding the coding region of a gene. These motifs influence the extent to which that region is transcribed into mRNA. Expression data can be used to infer gene regulation: one might compare microarray data from a wide variety of states of an organism to form hypotheses about the genes involved in each state. In a single-cell organism, one might compare stages of the cell cycle, along with various stress conditions (heat shock, starvation, etc.). One can then apply clustering algorithms to that expression data to determine which genes are co-expressed. For example, the upstream regions (promoters) of co-expressed genes can be searched for overrepresented regulatory elements.

### 20.2.6 Analysis of Protein Expression

Protein microarrays and high-throughput (HT) mass spectrometry (MS) can provide a snapshot of the proteins present in a biological sample. Bioinformatics is very much involved in making sense of protein microarray and HT MS data; the former approach faces similar problems as with microarrays targeted at mRNA; the latter involves the problem of matching large amounts of mass data against predicted masses from protein sequence databases and the complicated statistical analysis of samples where multiple, but incomplete peptides from each protein are detected.

### 20.2.7 Analysis of Mutations in Cancer

In cancer, the genomes of affected cells are rearranged in complex or even unpredictable ways. Massive sequencing efforts are used to identify previously unknown point mutations in a variety of genes in cancer. Bioinformaticians continue to produce specialized automated systems to manage the sheer volume of sequence data produced, and they create new algorithms and software to compare the sequencing results to the growing collection of human genome sequences and germline polymorphisms. New physical detection technologies are employed, such as oligonucleotide microarrays to identify chromosomal gains and losses (called comparative genomic hybridization) and single-nucleotide polymorphism arrays to detect known *point mutations*. These detection methods simultaneously measure several hundred thousand sites throughout the genome and, when used in high throughput to measure thousands of samples, generate terabytes of data per experiment. Again the massive amounts and new types of data generate new opportunities for bioinformaticians. The data is often found to contain considerable variability or noise, and thus hidden Markov model and change-point analysis methods are being developed to infer real copy number changes.

### 20.2.8 Comparative Genomics

The core of comparative genome analysis is the establishment of the correspondence between genes (orthology analysis) or other genomic features in different organisms. It is these intergenomic maps that make it possible to trace the evolutionary processes responsible for the divergence of two genomes. A multitude of evolutionary events acting at various organizational levels shape genome evolution. At the lowest

level, point mutations affect individual nucleotides. At a higher level, large chromosomal segments undergo duplication, lateral transfer, inversion, transposition, deletion, and insertion. Ultimately, whole genomes are involved in processes of hybridization, polyploidization, and endosymbiosis, often leading to rapid speciation. The complexity of genome evolution poses many exciting challenges to developers of mathematical models and algorithms, who have recourse to a spectra of algorithmic, statistical, and mathematical techniques, ranging from exact, heuristics, fixed parameter and approximation algorithms for problems based on parsimony models to Markov chain Monte Carlo algorithms for Bayesian analysis of problems based on probabilistic models.

### 20.2.9 Modeling Biological Systems

Systems biology involves the use of computer simulations of cellular subsystems (such as the networks of metabolites and enzymes which comprise metabolism, signal transduction pathways, and gene regulatory networks) to both analyze and visualize the complex connections of these cellular processes. Artificial life or virtual evolution attempts to understand evolutionary processes via the computer simulation of simple (artificial) life forms.

### 20.2.10 High-Throughput Image Analysis

Computational technologies are used to accelerate or fully automate the processing, quantification, and analysis of large amounts of high-information-content biomedical imagery. Modern image analysis systems augment an observer's ability to make measurements from a large or complex set of images, by improving accuracy, objectivity, or speed. A fully developed analysis system may completely replace the observer. Although these systems are not unique to biomedical imagery, biomedical imaging is becoming more important for both diagnostics and research. Some examples are high-throughput and high-fidelity quantification and subcellular localization (high-content screening, cytohistopathology, bioimage informatics), morphometrics, clinical image analysis and visualization, determining the real-time air-flow patterns in breathing lungs of living animals, quantifying occlusion size in real-time imagery from the development of and recovery during arterial injury, making behavioral observations from extended video recordings of laboratory animals, and infrared measurements for metabolic activity determination.

## 20.3 Structural Bioinformatics Approaches

### 20.3.1 Prediction of Protein Structure

Protein structure prediction is another important application of bioinformatics. The amino acid sequence of a protein, the so-called primary structure, can be easily determined from the sequence on the gene that codes for it. In the vast majority of cases, this primary structure uniquely determines a structure in its native environment. (Of course, there are exceptions, such as the bovine spongiform encephalopathy – a.k.a. mad cow disease – prion.) Knowledge of this structure is vital in understanding the function of the protein. For lack of better terms, structural information is usually classified as one of the *secondary*, *tertiary*, and *quaternary* structures. A viable general solution to such predictions remains an open problem. As of now, most efforts have been directed toward heuristics that work most of the time. One of the key ideas in bioinformatics is the notion of homology. In the genomic branch of bioinformatics, homology is used to predict the function of a gene: if the sequence of gene *A*, whose function is known, is homologous to the sequence of gene *B*, whose function is unknown, one could infer that B may share A's function. In the structural branch of bioinformatics, homology is used to determine which parts of a protein are important in structure formation and interaction with other proteins. In a technique called homology modeling, this information is used to predict the structure of a

protein once the structure of a homologous protein is known. This currently remains the only way to predict protein structures reliably.

### 20.3.2 Molecular Interaction

Efficient software is available today for studying interactions among proteins, ligands, and peptides. Types of interactions most often encountered in the field include protein–ligand (including drug), protein–protein, and protein–peptide. Molecular dynamic simulation of movement of atoms about rotatable bonds is the fundamental principle behind computational algorithms, termed docking algorithms for studying molecular interactions.

### 20.3.3 Docking Algorithms

In the last two decades, tens of thousands of three-dimensional protein structures have been determined by X-ray crystallography and protein nuclear magnetic resonance spectroscopy (protein NMR). One central question for the biological scientist is whether it is practical to predict possible protein–protein interactions only based on these 3D shapes, without doing protein–protein interaction experiments. A variety of methods have been developed to tackle the protein–protein docking problem, though it seems that there is still much work to be done in this field.

## 20.4 Biopesticides

### 20.4.1 Biopesticide Overview

Biopesticides are agents for biological pest control, which are derivatives of natural materials such as animals, plants, microbes, and minerals that can excise pests by mechanisms – nontoxic to humans. In comparison, conventional pesticides are usually synthetic substances that are capable of killing the pests or rendering them inactive. Biopesticides include naturally occurring substances that control pests (biochemical pesticides), microorganisms that control pests (microbial pesticides), and pesticidal substances produced by plants containing added genetic material (plant-incorporated protectants) or PIPs (http://www.epa.gov/opp00001/biopesticides/). According to FAO biopesticide is "A compound that kills organisms by virtue of specific biological effects rather than as a broader chemical poison. Differ from biocontrol agents in being passive agents, whereas biocontrol agents actively seek the pest. The rationale behind replacing conventional pesticides with biopesticides is that the latter are more likely to be selective and biodegradable" (http://www.fao.org/biotech/spec-term-n.asp?id_glo=4875&id_lang=TERMS_E).

As depicted in the image, biopesticides fall into three major classes:

1. Microbial pesticides which consist of bacteria, entomopathogenic fungi, or viruses (and sometimes includes the metabolites that bacteria or fungi produce) and entomopathogenic nematodes are also often classed as microbial pesticides, even though they are multicellular.
2. Plant-incorporated protectants (PIPs) have genetic material from other species incorporated into their genetic material (i.e., GM crops).
3. Biochemical pesticides are naturally occurring substances that control pests by nontoxic mechanisms.

Biopesticides, key components of integrated pest management (IPM) programs, are receiving much practical attention as a means to reduce the load of synthetic chemical products being used to control plant diseases. In most cropping systems, biological pesticides should not necessarily be viewed as wholesale replacements for chemical control of plant pests and diseases, but rather as a growing category of efficacious supplements that can be used as rotation agents to retard the onset of resistance to chemical pesticides and improve sustainability. In organic cropping systems, biopesticides can represent valuable tools that further supplement the rich collection of cultural practices that ensure against crop loss to diseases.

#### 20.4.1.1 Microbial Pesticides

Microbial pesticides consist of a microorganism (e.g., a bacterium, fungus, virus, or protozoan) as the active ingredient. Microbial pesticides can control many different kinds of pests, although each separate active ingredient is relatively specific for its target pest[s]. For example, there are fungi that control certain weeds and other fungi that kill specific insects. The most widely used microbial pesticides are subspecies and strains of *Bacillus thuringiensis*, or Bt. Each strain of this bacterium produces a different mix of proteins and, specifically, kills one or a few related species of insect larvae. While some Bt's control moth larvae found on plants, other Bt's are specific for larvae of flies and mosquitoes. The target insect species are determined by whether the particular Bt produces a protein that can bind to a larval gut receptor, thereby causing the insect larvae to starve.

#### 20.4.1.2 Plant-Incorporated Protectants

Plant-incorporated protectants (PIPs) are pesticidal substances that plants produce from genetic material that has been added to the plant. For example, scientists can take the gene for the Bt pesticidal protein and introduce the gene into the plant's own genetic material. Then the plant, instead of the Bt bacterium, manufactures the substance that destroys the pest. The protein and its genetic material, but not the plant itself, are regulated by EPA.

#### 20.4.1.3 Biochemical Pesticides

Biochemical pesticides are naturally occurring substances that control pests by nontoxic mechanisms. Conventional pesticides, by contrast, are generally synthetic materials that directly kill or inactivate the pest. Biochemical pesticides include substances, such as insect sex pheromones that interfere with mating as well as various scented plant extracts that attract insect pests to traps. Because it is sometimes difficult to determine whether a substance meets the criteria for classification as a biochemical pesticide, EPA has established a special committee to make such decisions.

Biochemical pest control agents include four (4) general biologically functional classes:

*Semiochemicals:* These are chemicals emitted by plants or animals that modify the behavior of receptor organisms of like or different kinds. They include pheromones, allomones, and kairomones. Pheromones are substances emitted by a member of one species that modify the behavior of others within the same species. Allomones are chemicals emitted by one species that modify the behavior of a different species to the benefit of the emitting species. Kairomones are chemicals emitted by one species that modify the behavior of a different species to the benefit of the receptor species.

*Hormones:* These are biochemical agents synthesized in one part of an organism and translocated to another where they have controlling, behavioral, or regulating effect.

*Natural Plant Regulators*: These are chemicals produced by plants that have toxic, inhibitory, stimulatory, or other modifying effects on the same or other species of plants. Some of these are termed "plant hormones" or "phytohormones."

*Enzymes:* In this regard, enzymes are protein molecules, which are the instrument for expression for gene action and catalyze biochemical reactions.

### 20.4.2 Discovery and Development of Biopesticide

Discovery and improvement of biopesticide is a multistep process and follows a sequence of steps that are distinctive for every individual target pest–biopesticide system and is termed the "innovation chain." The innovation chain progresses from initial discovery and technology development to the later stages of application

development, scale-up at commercial levels, and transfer of technology to the industry and ultimately reaches the end consumer. Successful biopesticide development is a blend of science, art, and entrepreneurship that can take over 10–15 years.

## 20.5 Relevance of Bioinformatics in Biopesticide Research

The ever increasing use of biopesticides makes it imperative that newer horizons are explored and breakthroughs are made rapidly in the area of biological pest control. Exploring different biological cycles and identifying viable hot spots (ideal protein targets) are essential in this kind of research. A series of detailed observation and literature investigation makes it very clear that the role of bioinformatics in research and development of novel biopesticides is very vital. It is very obvious that the application of various bioinformatics methods can comfortably reduce the time frame involved in the initial stages of the research, especially in screening suitable protein targets. We were able to recognize few of the important bioinformatics techniques which can be employed with precision and considerable ease.

### 20.5.1 Bioinformatics Tools That Can Be Applied in Biopesticide Research

#### 20.5.1.1 Homology Modeling

Homology modeling, also known as comparative modeling of protein, refers to constructing an atomic-resolution model of the *target* protein from its amino acid sequence and an experimental three-dimensional structure of a related homologous protein (the *template*). Homology modeling relies on the identification of one or more known protein structures likely to resemble the structure of the query sequence and on the production of an alignment that maps residues in the query sequence to residues in the template sequence. It has been shown that protein structures are more conserved than protein sequences among homologues, but sequences falling below a 20 % sequence identity can have very different structure and reach what is called, in homology modeling, the twilight zone (Chothia and Janin 1975). Model building by homology is a multistep process. At almost all steps choices have to be made. The modeler can virtually never be sure that she makes the best choices, and thus a large part of the modeling process consists of serious thought about how to gamble between multiple seemingly similar choices. As this process resembles very strongly what goes on in the mind of a professional gambler who visits the Loutraki Casino, some introduction in game theory seems in place. Modeling of protein targets for which 3D structure is not available can be highly useful in biopesticide discovery. In silico protocols such as de novo biopesticide development can be initiated with a modeled protein structure as the starting point.

#### 20.5.1.2 Molecular Docking

Molecular docking is a computational method to find out binding modes of ligands to their receptors rapidly. Molecular docking can be thought of as a problem of "lock-and-key," where one is interested in finding the correct relative orientation of the "key" which will open up the "lock" (where on the surface of the lock is the keyhole, which direction to turn the key after it is inserted, etc.). Here, the protein can be thought of as the "lock," and the ligand can be thought of as a "key." Molecular docking may be defined as an optimization problem, which would describe the "best-fit" orientation of a ligand that binds to a particular protein of interest. However, since both the ligand and the protein are flexible, a "hand-in-glove" analogy is more appropriate than "lock-and-key" (Jorgensen 1991). During the course of the process, the ligand and the protein adjust their conformation to achieve an overall "best fit," and this kind of conformational adjustments resulting in the overall binding is referred to as "induced fit" (Wei et al. 2004). The focus of molecular docking is to computationally simulate the molecular recognition process. The aim of molecular docking is to achieve an optimized

conformation for both the protein and ligand and relative orientation between protein and ligand such that the free energy of the overall system is minimized. Molecular docking can be employed to analyze in silico the affinity between hypothesized compound to be used as a biopesticides and a target protein.

#### 20.5.1.3 Gene Expression Profiling: Microarray

A DNA microarray (also commonly known as gene chip, DNA chip, or biochip) is a collection of microscopic DNA spots attached to a solid surface. Scientists use DNA microarrays to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome. Each DNA spot contains picomoles ($10^{-12}$ moles) of a specific DNA sequence, known as *probes* (or *reporters*). These can be a short section of a gene or other DNA element that are used to hybridize acDNA or cRNA sample (called *target*) under high-stringency conditions. Probe–target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target.

### 20.5.2 Case Study: Practical Application of Gene Expression Profiling in Investigating Efficacy of *Helicoverpa armigera* Single-Capsid Nucleopolyhedrovirus as a Biopesticide

Quan Haung Nguyen et al. at the University of Queensland took up a study "Transcriptome sequencing of and microarray development for a *Helicoverpa zea* cell line to investigate in vitro insect cell-baculovirus interactions." The *Helicoverpa armigera* single-capsid nucleopolyhedrovirus (HaSNPV) can be propagated using *H. zea* insect cell cultures, for use as a biopesticide against Heliothine agricultural pests. This study sequenced, assembled, and functionally annotated 29,586 transcript sequences from cultured *H. zea* cells using Illumina 100 bps and paired-end transcriptome sequencing (RNA-seq). From these sequences, a genome-scale microarray platform was constructed and validated for effective expression analysis of *H. zea* genes. This array also included probes for all HaSNPV genes, thereby allowing virus and host gene changes to be monitored simultaneously (Nguyen et al. 2012).

## 20.6 Conclusion

The comprehensive literature survey undertaken makes it quite clear that bioinformatics can play a pivotal and paramount role in speeding up the biological pest control research process. Gene expression profiling can be a highly powerful tool for understanding the pathological mechanism behind every pest control agent or methodology with a biological rationale. Bioinformatics and agricultural research can go hand in hand for a faster and efficient development of biopesticide leads. Employing in silico screening protocols can be highly successful in reducing attrition of biopesticide lead molecules at final stages of research.

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# Pest Management of Soybean: Sustainable Production Techniques

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**Abstract**

Diverse pests cause losses in agriculture. Among them, insects are noted for inhabiting all the environments and for quickly adapting to the environmental adversities. From one million insects, around 10 % of them cause some kind of damage in agriculture, representing severe or even total losses, depending on the culture. The agricultural production losses reach up to 37 %, which 13 % are caused by insects. In agroecosystems of high economic expansion, such as soy in Brazil, any factor that interferes in the reduction of production becomes of great importance. However, it is necessary to control the populations in economical levels, considering that their total elimination may cause the appearance of secondary pests. In this context, the specific knowledge of the interaction of each species in the culture is needed, as well as the development of strategies in management to maintain the maximum of natural balance in the cultivated area.

Despite the existence of Integrate Pest Management (IPM) in Brazil for three decades, only 35 % of the cultivated areas adopt the pest management. On the other areas, the control is performed through regular and non-planed applications of pesticides, which has been causing technical, economical, environmental, and toxicological problems. This chapter aims to bring a bibliographic survey of the economical importance of lepidopterans and hemipterans, considered pests in the soy culture in Brazil; to characterize the advancements of techniques of control and the utilization of microorganisms (bacteria, fungi, and viruses); and also to make a perspective of transgenic soy in biotechnology programs utilized to perform culture management in Brazil.

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_21, 

**Keywords**
Soybean • Biological control • Insect pests • Integrated pest management • Brazil agroecosystems

## 21.1 Introduction

In monoculture areas, the substitution of native plants, adapted to the local conditions and favored by intraspecific relations, for another that disrupts the natural succession, may cause large environmental impacts. This may cause the appearance of pests and diseases (Panizzi and Parra 2008). According to Gallo et al. (2002), among one million species of insects, about 10 % cause some kind of damage to agriculture, representing severe or even total losses, depending on the culture. Losses in worldwide agriculture production, due to insect attacks and diseases, reach up to 37 %, which 13 % are caused by insects.

In agroecosystems of high economic expansion, such as soy in Brazil, any factor that interferes in the reduction of production becomes of great importance. Losses caused by pests are among the main factors that reduce the production (Hoffmann-Campo et al. 2000; Embrapa Soja 2011). Due to the geographic extension of the country and the high diversity of ecosystems where soy is cultivated, some species are considered main pests, such as plant defoliators and suckers. Besides these, some can be considered as "regionally important" and also "secondary," due to their frequency, extent, and damage caused in the culture (Embrapa Soja 2011). However, it is necessary to control the populations in economical levels, considering that their total elimination may cause the appearance of secondary pests. In this context, the specific knowledge of the interaction of each species in the culture is needed, as well as the development of strategies in management to maintain the maximum of natural balance in the cultivated area. In this way, this chapter aims to bring information about the economical importance of the insect pests in soy culture of Brazil and to characterize the advancements of the control techniques for the pest management in the culture in this country.

## 21.2 Soybean in Brazil

The soybean [*Glycine max* (L.) Merril 1917] is a millenary culture that gained prominence from the Second World War. Currently, it is considered as indispensable feedstock to promote several agro-industrial complexes, being stated as the most important oleaginous plant cultivated in the world, with a relevant socioeconomic role due to the crescent demand and the need for its oil and protein. From the dicotiledonean class, the soy possesses an expressive genetic variability, both vegetative and reproductive, having strong influence in the environment, showing high diversity in its cycle, depending on the region (CI Soja 2011).

In Brazil, the soy culture reached 130 years old of presence in 2012. Introduced in the south of the country, it encountered favorable conditions for its development due to the similar edaphoclimatics, mostly regarding the photoperiod of the original ecosystem (south of the United States) (Schnepf et al. 2001). Currently, the cultivated area, around 24.35 million of hectares, presents a production of 71.5 million tons (USDA- ERS 2011). This oleaginous is one of the three main cultures which, added together, represent 90.9 % of the production of grains, leguminous and oleaginous in Brazil. Among the cultures of rice, maize, and soy, they correspond to 83.6 % of the harvested area (IBGE 2012), with soy being the responsible for half of the planted area of grains in the country (Embrapa Soja 2012).

In worldwide scale, Brazil is the second largest producer of soy, followed by the United States and in front of Argentina. The country is also the world's biggest exporter of soy and soybean meal, with a participation of 33 % in the world market (USDA 2012). The agro-industrial context of soy drives around 30 billion dollars per year and has a big role in the commercial balance,


**Fig. 21.1** Soybean pests: *Anticarsia gemmatalis*: natural (**a**), infected by *Nomuraea rileyi* (**b**), and by Nuclear polyhedrosis virus (*Ag*NPV) (**c**); *Euschistus he*ros infected by *Nomuraea rileyi* (**d**); *Bacillus thuringiensis* (**e**); *Ag*NPV (**f**)

contributing with 26 % of the exportations in the agribusiness and 11 % of the total exportations in the country (SECEX 2009).

In its production history, the two first decades were characterized by low sustainability of the culture in the environmental aspect, yet contradictory to the social aspect, because its economic value grew 56 times in 20 years and its expansion in area was 22 % per year. The environmental problems were marked by the high soil erosion because of, by the excessive use of pesticides, the inappropriate use of fertilizers, which cause soil contamination and siltation of rivers lakes and ponds, as well as recurrent cases of intoxication by the pesticide applicators. In the decade 80, soy leaded the implantation of a new civilization in central Brazil, taking the progress and the development to these regions, once unpopulated and devalued. One decade later, soy became the main culture in the Brazilian agribusiness, due to the growth in cultivated area and the productivity increase by the new technologies applied in its cultivation.

Soybean is also the raw material readily available for the immediate production of biodiesel. The biofuels produced from grains are a rising interest in the production and consumption of renewable and clean energy in the world (Fontes 2010). According to data from the *Agência Nacional do Petróleo, Gás Natural e Biocombustiveis* (National Agency of Oil, Natural Gas, and Biofuels) (ANP 2012), the raw material mostly utilized for the production of biodiesel in Brazil is the soy, around 75 % from total.

## 21.3 Insects

The insects inhabit all the environments and quickly adapt to environmental adversities. These organisms may be benefic (natural predators and pollinators) but also can damage agricultural cultures, causing production losses. Considering the ecological importance of the environment, about two-thirds of the plants depend on insects for pollination. Among the advantages some can be emphasized, such as the seed dispersal, the nutrient recycling, the biological control of pests, and other disease vectors (Gullan and Cranston 2007). The agroecosystems hold ideal conditions for the appearance of insect pests, because the plants possess limited duration and all have the same cultivar and age. It does not occur in the natural environment, for having a larger diversity of species, which interact all the time. The phytophagous stinkbugs of the Pentatomidae family, *Nezara viridula* (Linnaeus 1758) (Hemiptera: Pentatomidae), *Piezodorus guildinii* (Westwood 1837) (Heteroptera: Pentatomidae), and *Euschistus heros* (Fabricius 1974) (Heteroptera: Pentatomidae), together with the velvetbean caterpillar *Anticarsia gemmatalis* (Hübner, 1818) (Lepidoptera: Noctuidae), constitute the main pests of the soy culture in Brazil, while the others can be considered secondary pests (Hoffmann-Campo et al. 2003) (Table 21.1).

From the most occurring pests and that mostly cause damages, *A. gemmatalis*, popularly known as velvetbean caterpillar, is the key pest of soy culture in Brazil. This insect occurs in all soy-producing regions of the American continent (Pedigo 2002). The caterpillars attack the leaves, scraping the leaflets at young age (Sosa-Gómez 2000), and, as they grow up, they become voracious and destroy the leaves completely (Praça et al. 2006). The same authors mention that the velvetbean caterpillar consumes about 120 $cm^2$ of the leaves to complete their development. The control of this pest may occur naturally, in favorable conditions, through the presence of natural enemies. When this does not happen, the use of pesticides, in high applications per crop, is accomplished to avoid losses in the grain production (Moscardi 1998). The phytophagous stinkbugs, during their nymph stage (3rd to 5th instars) or adults, may significantly reduce the culture yield and are responsible for losses in the quality and quantity of grains and seeds. The population pressure registered in the last years reflects the high numbers of bugs that have been occurring in the soy farming of many producing regions from Brazil and, consequently, the intense damages found on the grains and seeds during the harvest (Bueno et al. 2007; Corrêa-Ferreira et al. 2009).

The stinkbugs feed from the seeds, inserting their proboscis and sucking the liquid or liquefied parts through the saliva. The insect histolytic agents, which liquefy the solid and semisolid portions of the cells (Panizzi 1991). The attacked plants become reduced in size, darkened, and wrinkled (Hoffmann-Campo et al. 2000). In the seeds they may cause biochemical alterations in the protein and lipid fractions, pod and/or seed abort, reduction on the weight and size of the grains, and, consequently, reduction on the productivity, as well as smaller force to germinate power of the seeds and leaf retention of soy. Furthermore, the bugs are also responsible for the transmission of pathogens, such as the *Nematospora coryli* yeast (Boethel et al. 2000). As the diversity of stinkbug species that attack the soybean culture in Brazil is high, studies show that their damage may vary according to the specie, population level, soy cultivar, its developing stage, and other factors (Corrêa-Ferreira and Azevedo 2002; Corrêa-Ferreira et al. 2009). The brown stinkbug, *E. heros*, considered of low occurrence in the 1970s, has adapted to the different Brazilian climatic conditions, becoming the most abundant bug in the soy crops of the country, and is found from south to north of Brazil (Panizzi et al. 2000; Silva et al. 2006).

In the course of the soybean harvest, the brown stinkbug lives through three generations, usually during the period of R5 (pod formation) to R7 (maturation) stages of the culture. They may be present during the vegetative phase, when there is no occurrence of plant damage, with up to eight stinkbugs per plant, not affecting the plant productivity, as little as the seed quality

**Table 21.1** Main insect pests of soybean in Brazil (Pedigo 2002)

| Insect pest | Features | Damage |
|---|---|---|
| Velvetbean caterpillar<br>*Anticarsia gemmatalis* (Hübner, 1818)<br>Lepidoptera: Noctuidae | The caterpillar is generally green with three light stripes arranged along the dorsum. Under conditions of high infestation, it becomes dark | Feeds on leaves |
| Southern green stinkbug<br>*Nezara viridula* (Linneus, 1758)<br>Hemiptera: Pentatomidae | The adult is green and lays eggs on the underside of leaves, arranged in the shape of hexagons. The nymphs are at first dark colored with white spots and later become green with yellow and red spots | The stinkbugs suck sap from plants, damaging the grain, and may cause physiological disorders called leaf retention or crazy-soy |
| Small green Stinkbug<br>*Piezodorus guildinii* (Westwood, 1837)<br>Hemiptera: Pentatomidae | The adult lays black colored eggs in double rows, preferably in the pods. The nymphs are green colored in the beginning with red and black spots on the back. The adult usually has bright green color, with a narrow spot at the base of the pro note | Sucks the sap, like the southern green stink bug |
| Brown stinkbug<br>*Euschistus heros* (Fabricius, 1798)<br>Hemiptera: Pentatomidae | The adult has a brown, almost triangular shape, with two lateral extensions in the upper part of the body, similar to spikes. Its postures are laid on the leaves and pods, with provision of two parallel lines. Nymphs are light colored soon after hatching, with light green abdomen and two dark spots on the dorsum | Sucks the sap similar to those described previously |
| Curculionid beetle<br>*Sternechus subsignatus* (Boheman, 1836)<br>Coleoptera: Curculionidae | The adult weevils are approximately 8 mm long, black colored with yellow bands on the dorsal thorax near the pro note and the elytra. These stripes may take the beige color, in situations of excessive moisture. Adults are found under the foliage of soybean during the day, moving up to the highest parts of the plants only overnight for mating | The adult scrapes and shred the tissues, while the larva feeds on the marrow of the main stem. This insect is more important in southern Brazil |
| Brown burrower bug<br>*Scaptocoris castanea* (Perty, 1830)<br>*Antarsocoris brachiariae* (Becher, 1996)<br>Hemiptera: Cydnidae | Adults are Coleoptera of approximately 8 mm long, with a bent down rostrum, black and bright yellow spots, and two yellow lines on the dorsal part of the elytra | Insects with burrowing habits which by continuous suction of sap through the roots take the plants to withering, drying, and death. Their proliferation has caused problems mainly in the center-south of Brazil |
| White grub<br>*Phyllophaga cuyabana* (Moser, 1918)<br>*Liogenys* sp.; *Plectros* sp.<br>Coleoptera: Melolonthidae | "C"-shaped body, generally white colored, with head and legs brown. The white rhizophagous (eat roots) grubs damage plant roots, having a life cycle that can last for 2 years | The white grubs cause damage during the third larval stage, consuming seeds and underground parts of plants. Cause considerable losses in Mato Grosso state |

(Corrêa-Ferreira and Azevedo 2002). The attack period of the stinkbugs determines the degree of damage to the crop. When it occurs during the period of pod forming, the losses can reach up to 30 %. If the attack occurs during the grain formation, which is the period of greatest occurrence, it may cause deformations, wilting, and stains on the grains. When the attack occurs after the formation of the grains, it may cause quality loss of the seeds and, thus, the reduction of the oil content in the grains and the leaf retention, which reduces the quality and commercial value of the grains (Corrêa-Ferreira et al. 2009; Schmidt et al. 2003).

The small green stinkbug, *P. guildinii*, is found in all regions of soybean cultivation of Brazil, predominates in many crops, and represents up to 85 % of the population of phytophagous stinkbugs (Silva et al. 2006). The damage caused by the nymphs increases the intensity with the insect development. Some recent studies suggest that the small green stinkbug has a major potential to cause quantitative and qualitative damages to the plants and causes more leaf retention than the other stinkbug species (Corrêa-Ferreira and Azevedo 2002). For the management of *P. guildinii*, the chemical control is the most utilized and, in some cases, may present low efficiency, becoming necessary the application of high doses of pesticides. The southern green stinkbug, *N. viridula*, is one of the most damaging species to soybean, causing high injuries. Besides the direct damage, they also cause indirect damage, injecting toxins to the sap, which, in turn, may cause physiological disturbances in the plant affecting its growth and the development. This specie is among the most frequent in the soybean-producing regions and is the most important in the southern states of Brazil (Hoffmann-Campo et al. 2000).

However, the knowledge of the insects' biology is not enough; data about the population during the time of the pesticide application may be very important during the choice of the pathogen agent to be chosen. Among the new technologies with potential to be used on the pest management of soybean, the precision farming uses advanced techniques of population. The practice of precision farming can bring many economic and environmental benefits, such as the localized application of the control agent, reducing up to 60 % of the pesticide use, minimizing the production costs and the environmental impact (Zambolin and Zambolin 2008).

### 21.3.1 Control Strategies

Favored by the strategies of the food production, many efforts have been performed to reduce the damage caused by insects in agriculture. The indiscriminate use of organosynthetic products, which originated from the 1940s for the control of agricultural pests, caused numerous problems, which worsened over the years. Several pests have become resistant to insecticides. New pests and with great intensity, due to destruction of their natural enemies (Batista Filho et al. 2003). With the discovery of dichlorodiphenyltrichloroethane (DDT) in 1939, which was widely used in chemical technologies for pest control, other technologies have been developed, emerging organophosphates and carbamates (Stoppelli and Magalhães 2005). These, despite having a lower environmental persistence and effective action to control pests, are toxic to mammals (Reigosa and Pedrol 2002). The accumulation of the application of these products in cultivated areas caused the degradation of the environment and undermined the viability of nontarget species as natural enemies, pollinator animals, and soil microorganisms (Carvalho and Barcellos 2012). In soybean culture, high concentrations of formulated chemicals are used annually in its production for the control of insect pests. This habit has led to the imbalance in soybean crops, causing serious problems such as the elimination of complex natural enemies and also the occurrence of resistant insect populations to chemical insecticides (Sosa-Gómez et al. 2009). However, new practices to manage the crops are of fundamental importance and, in this aspect, stand out the control of insect pests, which cause annual losses of millions worldwide.

In this sense, understanding the trophic relationships between plants and insects can

generate subsidies to create alternatives in the management and control of these insects more sustainably in order to generate less impact to the environment and reduction of chemical insecticide application, preventing the high rates on the reduction of productivity caused by the attack of insect pests (Aramideh et al. 2010). The control of major pests of soybean should be based on the principles of "Integrated Pest Management – IPM," which consist of decision-making control based on the level of attack and the number and size of insects and pests in stadium of plant development (Hoffmann-Campo et al. 2000). Several control methods used in IPM, among them, stand out biological control through the use of entomopathogenic microorganisms (Hoffmann-Campo et al. 2000; Corrêa-Ferreira et al. 2010). Biological control is a technique used to reduce the population of target specie that has the potential to cause economic damage and is recommended to reduce populations of insect pests, combat weeds, plant pathogens, and nematodes, among others (Melo and Azevedo 1998). It can be used as a tool in IPM or organic production and should never be considered singly or as a definitive technique in agroecological pest control (Gallo et al. 2002). The use of microorganisms as agents of pest control with entomopathogenic or insecticidal activity has become an alternative, as they are found naturally in the environment (Oliveira-Filho 2008).

## 21.4 Bacteria

Despite the importance and viability of microbial agents in pest control, only 2 % of the insecticides used worldwide are based on the application of biopesticides, which highlights *Bacillus thuringiensis* (*Bt*) (Berliner 1909), which represents about 95 % of total microorganisms used (Bravo et al. 2011). The Gram-positive bacterium *Bt* is characterized by the production of insecticidal crystal protein during sporulation. These proteins exhibit entomopathogenic properties to insects of the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera, Hemiptera, Dictyoptera, Orthoptera, and Mallophaga, besides nematodes (Strongylida, Tylenchida), protozoa (Diplomonadida), and mites (Acari) (Schnepf et al. 1998; Van Frankenhuyzen 2009). The relevance of the Cry proteins is due to its toxic properties produced after ingestion by the susceptible insects. In the case of susceptible insect larvae, after ingestion, the crystals are solubilized in the alkaline environment of the midgut and the protoxin liberated, which are activated by digestive enzymes, forming small protein fragments (Hofte and Whiteley 1989). These fragments cross the peritrophic membrane by binding to specific receptors of the apical membrane of intestinal cells, causing destruction to microvilli, followed by vacuolization of the cytoplasm and cell disruption, causing the insect to stop feeding, leading him to death (Fiuza and Berlitz 2009; Knaak and Fiuza 2010).

The *cry* genes encoding the Cry proteins are typically found in large conjugative plasmids or, more rarely in the chromosome. Different combinations of *cry* genes are found in several strains of *B. thuringiensis* strains including one, two, or even four different genes (Fiuza et al. 2012). The dispersion of these genes from strains may be due to the presence of transposable elements associated with the *cry* genes and the occurrence of the conjugation process. Thus, it is possible to explain why the same toxin can be found in isolates of *Bt* with different serotypes. The use of *Bt* products such as insecticides diluted and used in sprays, as well as its Cry toxins introduced into transgenic plants, has been providing a more targeted and effective way for the control of insect pests in agriculture. Biological control using *B. thuringiensis* agent is among the most popular alternative, more widely developed and secure compared to chemical pesticides to control insect pests (Jacobsen et al. 2004).

With the optimization of formulations, marketing intensified the emergence of several products, using different strains of *B. thuringiensis*. Since then, this microorganism has been the most important biological control agent marketed. Three hundred twenty-two products based on *B. thuringiensis* are responsible for 53 % of the

global market for biopesticides, generating annual revenues of $ 210 million (CAB International Centre 2010). The participation of biopesticides based on *B. thuringiensis* biopesticides market worldwide has been decreasing since 2000. During that year, it corresponded to 90 %, decreasing to 60 % in 2005 and 53 % in 2010. This reduction was due to the large increase in the use of entomopathogenic viruses (+100 %) and entomopathogenic fungi (+52 %) in the control of agricultural pests, while the market for Bt-based products increased by only 36 % (CAB International Centre 2010). There are various *B. thuringiensis* products developed to control insect pests. A large part of the spore-crystal formulations are obtained from different strains. It can be cited the strain HD1 *B. thuringiensis* var. *kurstaki* (*Btk*), strain HD73 *B. thuringiensis* var. *aizawai*, and strain HD137 *B. thuringiensis* var. *israelensis* are active against lepidopteran larvae (Soberón et al. 2009). The bio-insecticide mostly known and used by farmers is Dipel®, from Abbott Laboratories Company (Table 21.2). This product has the strain HD-1 as active ingredient and can be found in the form of vegetable powder and concentrated suspension.

## 21.5 Virus

Viruses are a potential source of biopesticides to control crop pests. Entomopathogenic viruses are found in many insect species, with members of the order Lepidoptera and Hymenoptera receiving considerable attention, because they include several important pests (Knaak and Fiuza 2005). Despite the existence of a large number of entomopathogenic viruses, the Baculoviridae family is the one with greater interest shown in applied entomology, as it encompasses several viruses already in use and others with great potential as biological control agents of insect pests in agroecosystems (Ribeiro et al. 1998; Moscardi 1999; Castro et al. 1999; Cory and Myers 2003). In addition, baculoviruses are widely used in biotechnological processes such as the expression of heterologous proteins and the development of vaccine vectors (Pushko 2010; Chen et al. 2010). The velvetbean caterpillar Baculovirus is a nuclear polyhedrosis virus (*Ag*NPV) belonging to the genus *Nucleopolyhedrovirus* of the Baculoviridae family. The virus particles are embedded in a protein mass (polyhedron). To become effective, it requires the insect to ingest the viral polyhedron, which is then dissolved in the midgut through the peritrophic membrane and fuse with the membrane of the microvilli of the epithelial cells of the midgut, initiating the replication cycle.

In the infection process, the caterpillar becomes weakened, losing the ability to feed (around the fourth day after infection) and mobility, and dies in about the seventh day after application. The viruses are specific and their protection in protein crystals allows the formulation of biopesticides with easy application technology, representing economy and biosecurity in relation to chemical insecticides. In several safety tests it was established that the virus is harmless to microorganisms, other invertebrates (except some insects), vertebrates, and plants (Groner 1989). The caterpillar's dead body, at the beginning, becomes whitish yellow and soft dimming with each passing day, breaking up and releasing large amounts of virus, which in turn will serve as the inoculum to infect new larvae (Moscardi and Souza 2002). The first commercial products to be registered in Brazil were nucleopolyhedroviruses of *Anticarsia gemmatalis* (*Ag*MNPV) and Argentina granuloviruses of *Cydia pomonella* (CpGV) (Sosa-Gómez et al. 2008). The number of viral insecticides currently registered comprises a relatively small number of entomopathogenic viruses, exclusively from the family Baculoviridae (Table 21.3).

Brazil, for its extensive area of soybean cultivation, has the largest program worldwide use of a virus against an agricultural pest. The use of the baculovirus *A. gemmatalis* (*Ag*MNPV) against the velvetbean caterpillar has been used in over 1.6 million hectares (about 10 % of the planted area of soybean in Brazil), currently marketed by different private companies in the alternative replacement of formulated chemicals to control the infestation levels of this pest (Silva and Moscardi 2002).

**Table 21.2** Commercial *Bacillus thuringiensis* products used against agricultural pests[a]

| Products | Companies | *B. thuringiensis* (*Bt*) | Target insects |
|---|---|---|---|
| Agree® | BioControle | *Bt kurstaki* + *Bt aizawai* | Lepidoptera |
| Bac-control® | Agricontrol | *Bt kurstaki* | Lepidoptera |
| Bactospeine® | Solvay | *Bt kurstaki* | Lepidoptera |
| Bactur® | Milenia Agrociências | *Bt kurstaki* | Lepidoptera |
| Biobit® | Novo-Nodisk | *Bt kurstaki* | Lepidoptera |
| Certan® | NovaArtis | *Bt aizawai* | Lepidoptera |
| Cutlass® | Ecogen | *Bt kurstaki* | Lepidoptera |
| Delfin® | Sandoz | *Bt kurstaki* | Lepidoptera |
| Dimy Pe®l | Dimy | *Bt kurstaki* | Lepidoptera |
| Dipel® | Abbott | *Bt kurstaki* | Lepidoptera |
| Di-Terra® | Abbott | *Bt san diego* | Coleoptera |
| | | *Bt tenebrionis* | |
| Ecotech Pro® | Bayer | *Bt kurstaki* | Lepidoptera |
| Florbac® | Abbott | *Bt aizawai* | Lepidoptera |
| Foil® | Ecogen | *Bt* (recombinant) | Coleoptera |
| Foil/Condor® | Ecogen | *Bt kurstaki* | Lepidoptera |
| Foray® | Novo-Nordisk | *Bt kurstaki* | Lepidoptera |
| Javelin® | Sandoz | *Bt kurstaki* | Lepidoptera |
| LarvoBt® | Fermone | *Bt kurstaki* | Lepidoptera |
| Lepinox® | DuPont | *Bt kurstaki* | Lepidoptera |
| M-One® | Mycogen | *Bt san diego* | Coleoptera |
| | | *Bt tenebrionis* | |
| M-One Plus® | Mycogen | *Bt san diego* | Coleoptera |
| | | *Bt tenebrionis* | |
| M-Trak® | Mycogen | *Bt tenebrionis* | Coleoptera |
| MVP® | Mycogen | *Bt kurstaki* | Lepidoptera |
| Novodo®r | Novo Nordisk | *Bt san diego* | Coleoptera |
| | | *Bt tenebrionis* | |
| Nubilacid® | Radonja | *Bt kurstaki* | Lepidoptera |
| Steward® | Thermo Trilogy Co | *Bt kurstaki* | Lepidoptera |
| Thuricide® | Sandoz | *Bt kurstaki* | Lepidoptera |
| Trident® | Sandoz | *Bt san diego* | Coleoptera |
| | | *Bt tenebrionis* | |
| XenTari® | Abbott | *Bt aizawai* | Lepidoptera |

[a]Table adapted by Fiuza and Berlitz (2009)

Unlike many chemical insecticides, the use of *Ag*NPV on pest control *A. gemmatalis* has several advantages. Among them we can highlight the production of the virus in the field on a large scale, reducing costs compared to chemicals and the nonsimultaneous occurrence of other important pests in the culture, which makes it very specific to the target insect (Moscardi 1998). In vast soybean plantations in central and southern regions of Brazil, *Ag*MNPV has proven safe and effective in controlling the target pest. Larval mortality has been above 80 %, and soybean defoliation has remained below the economic damage threshold, when the bio-insecticide is applied at the recommended conditions (Silva and Moscardi 2002). Furthermore, studies conducted in the laboratory and in the field with temporal variants of *Ag*MNPV showed that its virulence was unchanged after more than 15 years of use as a biological insecticide (Andrade et al. 2010). There was also no resistance in natural populations of insects in areas subjected

**Table 21.3** Commercial baculovirus products to control insect pests[a]

| Virus | Product name | Target pests | Crop |
|---|---|---|---|
| GV | Capex 2 | *Adoxophyes orana* | Apple |
| GV | Cryptex | *Cryptophlebia leucotreta* | Citrus |
| GV | Granupom | *Cydia pomonella* | Apples, walnuts, pears |
| | Madex | | |
| | Virin-GyAp | | |
| | Carpovirusine | | |
| | Carpovirus Plus | | |
| | Cyd-x | | |
| | Granusal | | |
| GV | PTM baculovirus | *Phthorimaea operculella* | Field and stored potatoes |
| NPV | Elcar | *Helicoverpa zea* | Cotton, vegetables |
| | Gemstar | | |
| NPV | Elcar | *Heliothis virescens* | Cotton |
| | Gemstar | | |
| NPV | Gypcheck | *Lymantria dispar* | Deciduous forests |
| | Disparvirus | | |
| | Virin-ENSH | | |
| NPV | Helicovex | *Helicoverpa armigera* | Cotton bollworm, corn earworm, tobacco |
| | Virin-HS | | |
| NPV | Leconteivirus Monisarmiovirus Virox | *Neodiprion sertifer* | Pine forests |
| NPV | Polygen, Multigen, Baculoviron, Baculovirus Nitral, Coopervirus, Protege | *Anticarsia gemmatalis* | Soybean |
| | VPN | | |
| NPV | Spodopterin | *Spodoptera littoralis* | Cotton |
| | Littovir | | |
| NPV | Spod-X | *Spodoptera exigua* | Vegetables flowers |
| | Spodopterin | | |
| NPV | TM Biocontrol-1 | *Orygia pseudotsugata* | Douglas-fir forests |
| NPV | Virin-ABB | *Hypantrea cunea* | Forest, mulberry |
| NPV | Virin-EKS | *Mamestra brassicae* | Cabbage |
| | Mamestrin | | |
| NPV | VPN 82 | *Spodoptera sunia* | Vegetables |
| NPV | VPN-80 | *Autographa californica* | Cabbage, cotton, vegetables |
| | Gusano | | |

[a]Table adapted by Knaak and Fiuza (2011)
*GV* granulovirus, *NPV* nuclear polyhedrosis virus

to various applications of virus when compared with insect populations in areas where the virus has never been applied (Abot et al. 1995). According to Fuxa et al. (1993), these agents probably are not being used in scale and are not frequency sufficient to allow the selection of virus-resistant populations. Thus, monitoring of field populations in terms of susceptibility to the virus, as well as evaluation under selection pressure in the laboratory, is extremely important since *Ag*MNPV has been extensively used as a pesticide in Brazil and occurs naturally (Knaak

and Fiuza 2011). According to Montor (2003), the program that is being developed in Brazil to control velvetbean caterpillar gives the country an estimated saving of 100 million dollars in pesticides, without considering the environmental benefits of not applying over 11 million liters of these products. Besides Brazil, other Latin American countries such as Argentina, Paraguay, and Bolivia have been using this microbial control agent of the velvetbean caterpillar.

## 21.6 Fungi

The entomopathogenic fungi are microorganisms that cause disease in insects, being primarily responsible for natural mortality of insect pests in agroecosystems. In Brazil there is a large number of species of entomopathogenic fungi causing epidemics that keep pests under control (Martins 2012). They cause 80 % of diseases responsible for outbreaks of epizootic ecosystems and agroecosystems and are easily spread, and some species have the ability to penetrate through the intact cuticle of arthropods and directly reach the hemocelio, even if provided with mealybugs' carapace (Martins 2012). Because of this mechanism of infection, the fungi were more advantageous than the other entomopathogens when plant-sucking insects become targets of microbial control (Lacey and Goettel 1995). Also, they have a high capacity to spread horizontally and can be taken by different agents and be spread to places far away. The genetic variability of these entomopathogens can be considered one of its main advantages in microbial control of insects (Alves 1998).

Among the most important species of fungi in biological pest control stand out *Metarhizium anisopliae*, *Beauveria bassiana*, and *Nomuraea rileyi*, which are the most common in agroecosystems. Historically the development of two mycopesticides spurred technological advances in the areas of production and formulation of entomopathogenic fungi on an industrial scale (Table 21.4) in the West. Boverin®, a mycoinsecticide based on Beauveria bassiana (Bals.) Vuill. to control *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) and *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) in the former Soviet Union, was developed in 1965 (Kendrick 2000). MyCar®, a mycoacaricide based on *Hirsutella thompsoni* Fisher, had granted registration by the Environmental Protection Agency of the United States in 1981 to control the citrus rust mite, *Phyllocoptruta oleivora* (Ashmead) (Acari: Eriophyidae) (McCoy et al. 1988). In the last four decades, more than 80 companies in the developed world and 171 mycoinsecticides and mycoacaricides (Faria and Wraight 2007).

Among the main advantages in the use and preservation of entomopathogenic fungi in crops, we can mention the following: it does not pollute the environment and nature, since it leaves no residues in food, water, and soil; it is selective, not affecting natural enemies, and is economically feasible. Thus, it is expected that the research will continue with entomopathogenic fungi, for a food production with technical, economic, social, and environmental sustainability – fundamental principles in Integrated Pest Management.

## 21.7 Parasitoids

In addition to microorganisms in soybean IPM, other control agents may be used for the control of pests mentioned. The hemipterans in particular are attacked by a number of natural enemies, including parasitoids and predator arthropods. Among the arthropods, the group of micro-Hymenoptera parasitoids of eggs is the most important. Among the various species of egg parasitoids of pentatomids in different Brazilian soybean fields mentioned, *Trissolcus basalis* and *Telenomus mormideae* are the most important. In the order Lepidoptera, the release of egg parasitoids, including wasps of the genus *Trichogramma* (Hymenoptera: Trichogrammatidae), has shown promising results (Bueno et al. 2009). The advantages are the easy creation of alternative hosts, which allow large scale rearing to be sent to control key pests of various crops, including soybeans (Parra and Zucchi 2004). The impact of these predators and parasites in pest populations in

**Table 21.4** Commercial entomopathogenic fungus products used against agricultural pests[a]

| Entomopathogenic fungus | Target pest | Commercial product | Industry |
|---|---|---|---|
| *Beauveria bassiana* | Coleoptera<br>Hemiptera | Bovenat® | Natural rural |
| | Coleoptera | Boveril ®ESALQ447 | Itaforte |
| | Coleoptera<br>Acaro | Boveril® WPPL63 | Itaforte |
| | Isoptera | Boveriol® | IPA |
| | Coleoptera | Bovemax® | Turfal |
| *Metarhizium anisopliae* | Hemiptera | BioCerto® | BioCerto |
| | Hemiptera | Biocerto® PM | BioCerto |
| | Hemiptera | BioControl® | Labormax |
| | Hemiptera | BioTech® | Biotech |
| | Hemiptera | Conbio® | Equilibrio |
| | Hemiptera | Metabiol® | Tecnicontrol |
| | Hemiptera | Metanat® | Natural rural |
| | Hemiptera | Metarril® WPE9 | Itaforte |
| | Hemiptera | Metarriz® | Biocontrol |

[a]Table adapted by Faria and Wraight (2007)

soybean is not well known. What is known, however, is that when eliminated by the use of broad spectrum insecticides, primary and secondary pest resurgences occur.

## 21.8 Insecticidal Plants

One alternative that has emerged alternatively to chemical control is the use of plants with insecticidal action, since they are readily degradable and contribute to reducing environmental impact, while maintaining the beneficial fauna (Estrela et al. 2006; Gonçalves-Gervásio and Vendramim 2004). Natural products obtained from vegetable raw material offer wide variety of molecules with great diversity in their structure and biological activity (Reigosa and Pedrol 2002). Besides these, phytotoxins present a wide range of new sites of action on target organisms, pointing the way to the synthesis of new products. This is important when considering the speed with which the insects and microorganisms have developed resistance to chemicals, commonly used as control agents target species (Knaak and Fiuza 2010). Plants are rich in substances that may have many modes of action, including toxicity, inhibition of feeding, development retardation, deterrence, and reduced fecundity and fertility, among others (Saito et al. 2004; Knaak and Fiuza 2010). The plant breeding has promoted the selection of different soybean cultivars with resistance against Lepidoptera and Hemiptera, such as *N. viridula* and *A. gemmatalis*. These express chemical characteristics that involve the production of toxins as isoflavonoids, which act as repellents, feeding and oviposition suppressors, and digestibility reducers of these insects (Chen et al. 2008). The use of these plants with other IPM techniques can keep the pest population levels below the levels of economic damages. In addition, plants are natural sources of active insecticides and antimicrobial agents, as they may be synthesized in the plant in response to attack by insects or microorganisms (Regnault-Roger 1997).

## 21.9 Transgenic Soybean

In 2009, over 40 million hectares of Bt cultures were grown throughout the world, resulting in a significant reduction in the use of chemical insecticides. Among the most important crops are soy, corn, cotton, and canola (James 2011). Bt soybean is the second largest group of Genetically Modified Plants (GMP) within this culture

(Crickmore 2006). The cultivars are characterized by expressing Cry toxins with insecticidal property to groups of insect pests of this crop. These cultivars, transformed with *cry* genes, also known as "Bt plants," present several advantages over the formulated *B. thuringiensis*, for needing foliar spray for the control of insects, since the toxin is expressed by the plant itself. Moreover, they decrease the amount of chemical insecticides released into the environment as well as the gases emitted by agricultural machinery used in its application (James 2011). The microorganism is characterized by the production of a crystal protein with insecticidal properties, formed during sporulation. These proteins have specific toxic activity of some insect orders, such as Lepidoptera (Hofte and Whiteley 1989; De Maagd et al. 2001). The first *Bt soybean*, which expresses the Cry1Ac protein, was recently released for sale in Brazil and should be planted from season 2012/13. This soybean has activity against *Anticarsia gemmatalis*, *Epinotia aporema*, *Pseudoplusia includens*, and *Heliothis virescens* and is an important alternative to reduce chemical insecticides.

Although all studies, for the release of transgenic cultivars, remaining question (Shelton and Sears 2001). In Brazil, the National Biosafety Technical Commission (CTNBio) establishes safety standards and technical advice to its release and production aiming the protection of human health and the environment. Several studies have been conducted in laboratory and field, in experimental and commercial scale to assess the impact of GMP on nontarget species has been performed. But no negative effect has been found. In different Bt crops in China, a reduction in insecticide use from 30 to 3 times per season was observed (Pray et al. 2002). The lowest number of applications of selective products like pesticides, according to the levels of action in pest management, can play an important role in the maintenance of natural enemies, predators, or parasitoids, which act regulating the populations not only of caterpillars but also of stinkbugs, currently primary pests in soybeans. The use of resistant plants can contribute to the reduction of insect pest population below the economic injury level, without causing imbalance in the agroecosystem. They have a cumulative effect and are persistent, do not promote increases in production costs, and are compatible with the other control tactics (Pitta et al. 2010).

In cases of GM plants with insecticidal toxins of *B. thuringiensis*, the transport of toxic proteins in body of the prey/host to predators and parasitoids has been investigated as a potential route of impact on nontarget insects Bt plants (Head et al. 2001,. Bernal et al. 2002), especially in the case of natural enemies, which are not exposed to this toxin presented in the plant, but are in contact with the toxin through their prey. Also according to Siqueira et al. 2004, the reduction in the use of pesticides on GM crops favors biodiversity, but the prolonged use of glyphosate or Bt crops may favor the evolution of resistance and environmental pollution, inherent risks to any kind of crop, not differing in the nature and magnitude of the conventional crops. The insecticidal effect of GM plants on natural enemies is a controversial issue. Even after rigorous risk assessment, the planting of these varieties has raised concerns about environmental impacts such as gene escape and effects on biodiversity. Considering soybeans, the risk of horizontal gene flow is the lack of remote compatible wild species. The gene escape to conventional crops can occur but can be avoided by isolation of crops (Siqueira et al. 2004). Monitoring conducted in fields of *Bt* crops in various countries like the United States, China, Argentina, and Brazil, since its commercial release, has shown that population density and biodiversity of insects in fields of Bt crops has been significantly higher than in conventional fields treated with insecticides (Siqueira et al. 2004).

There is great concern in making available the *Bt* toxin in large areas, as it may lead to selection of resistant phenotypes to this toxin. Susceptibility studies of toxic proteins to the main target pests are important for susceptibility levels and to determine doses for future diagnostic monitoring of possible changes in susceptibility. The development of resistance in target insect populations is becoming a major threat to the long term success of this technology (Bates

et al. 2005). These studies have been demonstrating the effectiveness and importance of new technologies being used to greater production efficiency, allowing more and more specificity in protection and reduction of implications and in the increase of the yield on a commercial scale.

## 21.10 Perspectives

We must invest in a precision agriculture, resulting in characteristics of social appeal in productivity, and conserve soil and water. Currently there are some indicators of sustainability and a concern to develop these environmental indicators. According to Marques et al. (2003), the sustainability of any activity should be evaluated considering the axes: economic, ecological, and social. The demand for healthier foods and the consumer concern in environmental problems tends to cause the consumer to become more aware of food production. As a result, the magnitude of the current requirements directly linked to changes in production systems in their local context and must meet the market demand for sustainability, responding to the concerns of global issues. In the 1990s, environmental concerns began to demand the creation of programs that start to enter any aspect of the evolution of the biosphere and not just limited to economic but also to social aspects.

Considering soybean an input in good results for agricultural development in the country, it is projected the culture production will jump over 40 % by 2020, while the largest seed producer country, the United States will reach, in the same period, 15 %. With this projection, Brazil reaches a production of more than 105 million tons, making it the largest producer alone of this commodity. Its national and global context justifies the need for researches and new technologies in order to improve the cultivation and to reduce the risk of yield loss, to enhance crop areas, and to increase their productivity (kg/ha). However, the development of sustainable agricultural systems is a global challenge to preserve the natural resources. The development of local and regional agriculture should address technologies that support and enable the management of all activities involved in farming.

## 21.11 Conclusions

In agriculture, activities handling plants can cause environmental impacts resulting from the replacement of native plants naturally adapted by another requiring containment of the natural process of succession, also involving the natural evolution of other organisms that occupy the natural space of the original environment. This modification causes the emergence of environmental problems such as the use of pesticides, related to the emergence of pests and diseases. Insect pests of soybean have been expressing great tolerance to the products used in their control in Brazilian agroecosystems. Pesticides have provided short-term solutions to this problem and, however, have caused the resistance of insects in biotech crops, requiring the development of strategies that avoid a dependence on synthetic chemistry. The occurrence of high population densities, the proven resistance of pest populations to some insecticides, and the applicability of active ingredients available and applied little in addition to the control failures and environmental imbalance are factors that boost the attack of insects, causing many concerns and serious damages to the grains and soybeans.

## 21.12 Recommendations and Future Focus

The integrated pest management has shown great effectiveness in the production of soybean in Brazil. Besides promoting positive activity in insect control, increases so does the resistance provided by genetic engineering, allowing the realization of a truly integrated pest management and environmentally sustainable production of these plants. The management also requires a constant analysis of the population of insect pests and their natural enemies during the soybean cycle. The integration of tools for pest management often depends on the availability of appropriate technology and its implementation, considering

the practical and economic issues. Knowing the complexity of possible interactions to control insects that cause economic damage in soybeans is still objects of studies that need to be understood and improved. Little is known about the harmonic combination of different tactics for different pest management systems in soybeans as well as their national situations and locations of production.

The improvement of techniques for the identification and application of phytochemicals involved in the resistance, as well as understanding the physiology of insects, especially their chemical receptors, may lead to the development of more efficient techniques of control and a better understanding of the mechanism of resistance. The development of effective biological control methods are also a great challenge and is contributing significantly to the success in controlling pests of soybean.

Plants genetically modified for insect resistance are a major challenge in the implementation of new genes encoding toxins and have a huge potential of providing a more effective method of control, especially when combined with other control practices. But care narrowing the genetic base of cultivars and high levels of antibiosis should be avoided as they can result in rapid selection of resistant insect pests.

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7, © Springer India 2014