Omkar *Editor*

Molecular Approaches for Sustainable Insect Pest Management

Springer

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ISBN 978-981-16-3590-8 ISBN 978-981-16-3591-5 (eBook)
https://doi.org/10.1007/978-981-16-3591-5

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

Insects comprise almost 80% of the entire world fauna (with almost 1 million species) and are present in all parts of the biosphere except the oceans. Many more genera and species of insects are still being reported, and these discoveries are not only bringing out new facts but also changing the very classification of insects. Despite being one of the most successful and diverse group of animals inhabiting planet Earth, they are poorly explored.

The word insect largely has strong negative connotations for most humans; though many of them are quite useful to human beings by yielding certain products directly used by the humans, others work as farmers' friends, being biocontrol agents, and still others are beneficial by providing various ecosystem services along with increasing crop productivity by facilitating and enhancing crop pollination.

Studies indicate that of the known 1 million species, hardly, 1% of them are harmful to human beings by the way of causing direct crop damages and lowering the yield, damaging the stored products and food produce, causing nuisance, or transferring disease-causing agents, besides causing nuisance and health hazards to our livestock. Such harmful insects are technically termed as pests and vectors. FAO estimates that annually between 20% and 40% of global crop production are lost to pests. It has been estimated that damages caused by these pests, vectors, and pathogens of crop plants are more than USD 13 billion per annum in India and around $250 billion globally. Possibly because of these facts, it has been emphasized that struggle between man and insects started long before the dawn of civilization, continued without break, and will probably continue as long as the human race exists.

It is these massive economic losses that are probably responsible for the global attention of entomologists towards curbing populations of harmful insects. This glaring monetary loss is probably the reason that most of the silently working beneficial insects providing ecosystem services are pushed to the back burner.

Through the ages, humans have been involved in finding ways and means to manage populations of insect pests. Cultural and chemical practices have been employed for the purpose since the tug of war between humans and insects started. Chemical practices have made their journey from initial crude options, such as ash, to more refined versions in the form of inorganic agrochemicals, synthetic organic

chemicals to plant products. In addition to the above practices, farmers across the globe have also employed various physical, mechanical, cultural, legal, genetic, and ecological approaches. Of all these, chemical approach has by far been the most successful one till date. However, the use of chemicals, termed as pesticides, while providing an immediate remedy to overcome insect pest problems has resulted in severe long-term consequences, such as disruption of interspecific competition resulting in damage to farmers' friends, the biocontrol agents of these pests, resistance in pest species, resurgence of new pest species, and damage to the environment and the biodiversity along with the human health hazards. This has gradually also changed the very concept from pest eradication to pest control to pest management, including the concept of integrated pest management, with the basic objective to integrate various ecofriendly tools and techniques, such as cultural practices, biocontrol using pathogens, parasitoids, and predators (natural enemies) for the pest management, and minimizing the use of synthetic chemicals in modern agriculture.

In the last few decades, the humans have witnessed major advancements in life sciences; as a result, several new and powerful tools and techniques have evolved. This has led to great advancements in microbial nutrition, genetics, and their application in different fields. In modern era of biotechnology, the microbes have provided solutions to many of the human problems and necessities and thus serve as human and farmers' friends. The microbes have proved to be successful tools for the pest management. Similarly, there has been much advancement in the field of molecular biology, where many more techniques have evolved, which can be helpful in the field of pest management too. Plant resistance, development of transgenic plants, and many more techniques are being considered the panacea to pest problems. On the other hand, there are widespread concerns of the safety of these microbial and biotechnological interventions with nontarget organisms, including humans. While the world stands divided on the ethical issues of these approaches and the many safety concerns, scientists believe that well thought of biotechnological interventions are probably the only safest ways possible for reducing pest attacks on crops.

Though several massive texts are available on insect pest management with exhaustive coverage of various means of insect pest management, my main objective to bring out a book entitled **Molecular Approaches for Sustainable Insect Pest Management** is to bring precise but specialized information covering modern aspects of pest management. Also, through this publication, my idea is to present the Indian perspectives on this discipline before international readership, involving various specialists from molecular biology.

I hope, the proposed book will not only present information on the modern and most effective means of pest management for postgraduate students and teachers and plant protection practitioners across the world but would also be quite useful to those involved in policy planning.

Lucknow, Uttar Pradesh, India Omkar
January 15, 2021

Acknowledgments

At the very outset, I express my gratitude to the contributors of different chapters contained in this book for sparing time from their routine to contribute their respective chapters to the level they could and I wanted.

I am thankful to Dr. Geetanjali Mishra, associate professor, Department of Zoology, University of Lucknow, Lucknow, for her intellectual inputs. I also extend my special thanks to Dr. Swati Saxena, M/S Priya Singh, Apoorva Shandilya, Chandni Verma, Tripti Yadav, Shriza Rai, Lata Verma, Dipali Gupta, and Gaurvanvita Singh for their assistance and support in various ways.

I am also grateful to my wife Mrs. Kusum Upadhyay and nephew Mr. Mridul Pandey for their encouragements and support by sparing me from household activities.

I extend my thanks to Ms. Aakanksha Tyagi, senior editor, Springer Nature India, New Delhi, and Mr. Lenold Esithor, project manager at Springer Nature, for taking keen interest in this project and for their support for publishing this work in time.

January 15, 2021 Omkar

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About the Editor

Prof. Omkar, FNASc, has been associated with teaching for about 35 years and research for more than 42 years. He is Former Head, Department of Zoology, University of Lucknow, Lucknow-226007,Former Coordinator, UGC-SAP (DRS-II), DST-FIST, DST-PURSE, Centre of Excellence, Govt. of UP, programs. He has worked on 10 more projects of state/central funding agencies. He specializes in Environmental Toxicology and Entomology, with particular reference to the Insect Pest Management .He is also an Associate Editor, International Journal of Tropical Insect Science, **Springer Nature** and Chief Editor, Journal of Applied Bioscience, besides being President of International Society of Applied Biology.

He is a receipient of several awards, like Saraswati Samman by the Department of Higher Education, Govt. of UP; Prof. T N Ananthakrishanan Foundation Award, Rescholar Award of Excellence in Agricultural Entomlogy by Association of ntomologists, Prof. G. S. Shukla Gold Medal by The Academy of Environmental Biology, Dr. S. Pradhan Memorial Lecture Award by The Devision of Entomology, IARI & The Entomological Society of India, ACCLAIM and Uddeepan awards from University of Lucknow. He is a Fellow of The Entomological Society of India, Zoological Society of India, Society for Biocontrol Advancement and Fellow of The National Academy of Sciences India (FNASc), besides many others. He has 16 books to his credit. He has published 235 Research papers, 10 Reviews, 40 Reviews as Book chapters, 17 Popular Science articles. Prof. Omkar has guided more than TWO dozen Ph. D. students. He has visited the School of Biological Sciences, University of East Anglia (2006) for three months and Department of Zoology, University of South Bohemia and Czech Academy of Sciences, Ceske Budejovice, Czech Republic (May 2016).

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Host Plant Resistance

1

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Omkar (ed.), *Molecular Approaches for Sustainable Insect Pest Management*,
https://doi.org/10.1007/978-981-16-3591-5_1

Abstract

In agricultural crop production, the yield loss due to insect pest infestations is causing a major concern. Though the chemical method of pest management is resorted by the farmers for quick relief from their infestation, there are several limitations, like the development of resistance to insecticides; resurgence of insect pests; pesticide residue problems; adverse effects on nontarget organisms,

like pollinators, natural enemies, etc.; and environmental pollution. These complications made the researchers to focus their attention on the development of resistant varieties against insect pests. Host plant resistance (HPR) to insect pests is an eco-friendly and economical way of pest management, and it is compatible with all the methods of insect pest management. It enables a plant to hinder the selection of host plant by the insect pests for their settling, oviposition and feeding, and even if they feed on the host plant, it interferes the biology of insect pests by affecting their growth and development and reducing their survival, or else they possess an ability to tolerate or recover from insect injury. Thereby, the resistant plants do not support the successful development of insect pests. This chapter covers the classification, types, mechanisms, nature/bases of resistance and genetics of resistance, and also the identification of sources of resistant genes from the germplasm. The development of resistant varieties utilizing conventional breeding methods and innovative approaches, viz. genetic engineering, marker-assisted selection, gene switches, altering metabolic pathways for secondary metabolites and genome editing are discussed in this chapter. The details on the selection of suitable screening methods along with rating scale for major crop pests are given. But the development of resistant varieties is a continuous process, as there is a constant arms race between host plants and insects due to coevolution. This chapter also covers information about the compatibility of HPR with other components of IPM.

Keywords

Insect pests · Mechanism of resistance · Factors affecting resistance · Sources of resistance genes · Screening techniques · Development of resistant varieties · Conventional and innovative approaches

Learning Objectives

1. Host plant resistance is one of the dominant factors in regulating the population of herbivorous insects, in addition to the natural enemies. The resistant plants are able to decrease the development of herbivores and/or significantly reduce the damage caused by them. Hence, host plant resistance is used as an important tool in integrated pest management by improving the plant germplasms.
2. This chapter deals with the classification of resistance from early period, types of resistance, different types of mechanisms of resistance, nature/basis of resistance and genetics of resistance.
3. The various available sources of resistant genes and the methods of transfer of resistant genes utilizing conventional and innovative approaches for the development of resistant varieties are discussed.

4. It also covers information about the compatibility of HPR with other components of IPM.

1.1 Introduction

The herbivorous insect pests are responsible for a considerable reduction in the yield potential of crops. Different methods of crop protection were adopted by the farmers to achieve maximum productivity. In nature, the host plant resistance is one of the dominant factors in regulating the population of herbivorous insects, in addition to the natural enemies. The resistant plants are able to decrease the development of herbivores and/or significantly reduce the damage caused by them (van Emden 1991; Francis et al. 2001; Sharma and Ortiz 2002). The resistant characteristics in plants enable them to hamper the process of host plant selection by insect pests and interfere in the biology of insect pests, or else they possess an ability to tolerate or recover from insect injury. Thereby, the resistant plants do not support the successful development of insect pests. It is one of the effective weapons against insect pests to minimize crop losses. Hence, breeding for host plant resistance is a key method advocated for regulation of insect pests infesting crop plants. There was a considerable progress made in the identification of source of resistant genes and development of resistant varieties through conventional and modern approaches.

In the United States, during the eighteenth and early nineteenth centuries, the insect-resistant wheat and apple cultivars were first developed and cultivated. In 1788, early maturing wheat cultivars were grown to avoid Hessian fly, *Mayetiola destructor*, infestation in the United States. During 1792, J.N. Havens identified the Hessian fly-resistant wheat cultivar 'Underhill' in New York. In 1831, Lindley recommended cultivation of the apple cultivars 'Winter Majetin' and 'Siberian Bitter Sweet', owing to their resistance against woolly apple aphid, *Eriosoma lanigerum*, infestation. An outstanding early success in utilizing host plant resistance in pest management was the control of the grape phylloxera, *Daktulosphaira vitifoliae* (Fitch), in France. Although host plant resistance to insect pests was documented in the nineteenth century, the breeding of insect-resistant cultivars was commenced only after the rediscovery of Mendel's law of heredity in 1900. In 1907, Biffen found that the yellow rust resistance is controlled by a single recessive gene in wheat, which kindled the interest of plant breeders and geneticists to search for resistant genes in crops. This started the modern era of plant breeding for insect resistance. In the twentieth century, the breeding for insect-resistant plants was a new phenomenon, which was developed based on the knowledge of basic genetics and the methodology of selecting, crossing and hybridizing plants. In the beginning of the early 1920s, R.H. Painter pioneered the modern work on plant resistance to insects at Kansas State University. Hence, he was recognized as the father of host plant resistance. In 1951, he published the first book on insect resistance, *Plant Resistance to Insect Pests*.

Until the mid-1960s, the potential of host plant resistance was not fully appreciated as an insect control method, because of overdependence on persistent

chemical insecticides against insect pests on high-yielding varieties. Later, there was crop failure due to epidemics of insect pests, which stirred entomologists to discover alternative approaches for pest control. At that time, the host plant resistance was formulated as a single means of control or with other management measures, such as cultural and biological methods and need-based use of pesticides. Later, intense research work was made on breeding for insect pest resistance and identified donors for resistance from wild germplasms. Worldwide a considerable progress was made in breeding plants for resistance to insects, and the use of insect-resistant cultivars has become significant in controlling insect pests and reducing the use of insecticides. With the advancement in biotechnology, especially tissue culture and molecular biology, new opportunities were made available for the development of host plant resistance. Through the use of embryo rescue and protoplast fusion techniques, it is now possible to produce distant hybrids and transfer genes for pest resistance from wild relatives to plants. Through genetic engineering techniques, novel genes for pest resistance can be introduced into the gene pools of crop species. With this historical background, the role of host plant resistance in the management of insect pests will be discussed here.

The plant species which are fed upon by an insect are called 'host plants', while those which are not fed at all are 'non-host plants'. The inability of insects to attack a non-host plant is termed 'immunity'. There is a functional relationship between insect pests and host plants. Every plant species shows diversity with respect to the extent of damage done by an insect. Insects select their host plants for oviposition, feeding and shelter. The selection of host plant is based on the physical features (colour, odour, texture, etc.) and biochemical characters (nutritional quality, presence/absence of toxic metabolites, etc.) of host plants. When the host plants harbour and sustain large population of insect pests and show more damage symptoms, those plants are called 'susceptible plants'. When the plants possess an innate ability to avoid or resist or tolerate the damage by insect pests, they are called as 'resistant plants'. The plants may possess two different types of resistance, viz. constitutive or induced. The constitutive resistance is always present in plants and expressed whenever the plants are infested by insects, whereas the induced resistance is triggered due to the external factors, like biotic or abiotic (Kogan 1994). These types of resistance affect the populations of insect pests through different mechanisms of resistance, viz. antixenosis, antibiosis or tolerance. The resistant plants could be able to avoid insect infestation simply either by using their external features, like presence of plant hairs/trichomes, thick leaf cuticle, stem hardness, waxy layer, etc., or by the constitutive or induced production of secondary plant metabolites. They could also tolerate the infestation by compensating the yield loss.

The host plant resistance interacts not only with insect pests (second trophic level) but also with third trophic level, viz. predators, parasitoids, etc. The resistance may have either positive or negative impact on entomophagous insects. For example, the populations of parasitoids were larger in aphid-susceptible barley cultivar, as they support large aphid populations. The density of parasitoids is not the only factor deciding the parasitism rate, host plant resistance also plays an indirect role, as the size or weight of host insect was maximum, when they grow on susceptible plants

(Brewer et al. 1998). With regard to predators, the susceptible and resistant host plants directly impact the development of predators by providing prey insect with different nutritional rates (Bommarco 1999). There are several benefits and drawbacks of adopting host plant resistance as a single pest management option. The advantages of plant resistance are species specific, having cumulative effect, compatible with other management methods, cost-effective, do not require special skill, eco-friendly and persistent. However, this tactic was also found to have a number of pitfalls, like the longer time requirement in the development of resistant varieties, genetic limitations and incompatible resistance characters. Still, the improvement of plant germplasm aiming at the development of resistant genotypes to insect pests may be an important tool in integrated pest management.

1.2 Classification of Resistance

1.2.1 Early Classification of Plant Resistance

Early classification of resistance to insects in plants has included terms such as 'physico-chemical' and 'physiological' resistance (Wardle and Buckle 1923). Physico-chemical resistance dealt with the integument of the plant, presence of hairs or presence of alkaloids, essential oils, etc. Physiological resistance was based on vigour, quick recovery or seasonal adaptation.

McColloch (1923) classified the plant resistance into two categories, viz. natural and artificial resistance. Natural resistance is exhibited by native plants or acquired by cultivated ones. Artificial resistance is developed through practical plant breeding.

Mumford (1931) classified resistance on the basis of 'epiphylaxis' or 'endophylaxis'. Epiphylaxis was related to external protection agencies. Endophylaxis was used for describing the internal protection afforded by biochemical qualities of the plant.

1.2.2 Based on Degree/Intensity of Resistance

Painter (1951) used the following scale to classify the degree of resistance based on intensity, such as immunity, high resistance, moderate resistance, low resistance, susceptibility and highly susceptibility. Intensity of resistance is a relative term and it is defined in relation to a susceptible cultivar of the same species. These terms are relevant to express levels of resistance while screening of varieties under field conditions but do not have any relationship with the mechanism of resistance.

1.2.2.1 Immunity

An immunity variety is one which will never be infested by a specific insect under any known condition. There are few cultivars immune to the attack of specific insect, which are otherwise known to attack cultivars of the same species.

1.2.2.2 High Resistance

This type of variety possesses qualities that result in small damage by a specific insect under a given set of conditions.

1.2.2.3 Moderate Resistance

Moderate or intermediate level of resistance results from any of at least three situations:

(a) A mixture of phenotypically high and low resistant plants
(b) Plants homozygous for genes, which under a given environmental condition produce an intermediate level of injury
(c) A single clone, which is heterozygous for incomplete dominance for high resistance

1.2.2.4 Low Resistance

This type of variety possesses qualities that result in lesser damage/infestation by an insect than the average damage caused by an insect.

1.2.2.5 Susceptibility

This type of variety exhibits average/more than average damage caused by an insect.

1.2.2.6 High Susceptibility

The high susceptible variety shows more than average damage by the insect under consideration.

1.2.3 Based on Plant-Insect Interactions

The pattern of constitutive and inducible resistance, at the plant or at the organ level, depends on the probability of the attack and the value of the organ (Zangerl and Rutledge 1996). The plants or organs that are regularly attacked by herbivores should have high levels of constitutive defences and low levels of induced defences. The resistance category can be divided into 'constitutive' or 'inducible' and 'direct' or 'indirect' subcategories (Chen 2008; Mithöfer and Boland 2012).

1.2.3.1 Constitutive Resistance

Constitutive plant resistance is resistance that is expressed regardless of the prior history of the plant. It is expressed by the plants always; it includes external mechanical defences and quantitative defences (Schoonhoven et al. 2005). Various physical and chemical attributes of plants, viz. trichome density, cell wall

lignification and silica deposition, serve as defence for the host plant (Kaplan et al. 2009). It is also called as direct defence. It is compatible with other management practices. Even partial resistance helps to increase the development time of insect pests, which makes them available for predators and parasitoids. The breakage of resistance is possible when insect pests evolve frequently (Teetes 2003). These characters of resistance interfere with the natural enemy activity (Bottrell and Barbosa 1998).

1.2.3.2 Induced Resistance

Inducible resistance is the resistance which is expressed only after the injury. It is referred to as direct/indirect defence. The plants possess a lot of chemical defence mechanisms utilizing different secondary metabolites, which exhibit a major barrier to herbivores. Some of them are constitutive defence resistance; other defence resistance mechanisms are induced after insect attack. The secondary metabolites are mostly induced/activated in response to insect attack. Most of the compounds affect the herbivores directly, while some are working indirectly through the attraction of natural enemies, thereby protecting the plant. The host plant responds to insect attack either through direct or indirect induced resistance. Synthesizing certain defensive compounds, viz. antifeeding proteins, insecticidal secondary metabolites, extrafloral nectars is called as Direct induced resistance. The production of volatile organic compounds (VOCs) to attract natural enemies of insects is known as Indirect induced resistance (Karban and Meyers 1989; Haukioja 1991; Karban et al. 2000).

1.2.4 Based on Evolutionary Concept

Resistance to an insect is evolved either due to long host plant and insect association at the gene centres (Leppik 1970) or due to pleiotropic effects of genes. In general, sympatric resistance is governed by major genes and allopatric resistance is polygenic in nature (Harris 1975). Based on these factors, host plant resistance to insects can be divided into sympatric and allopatric resistance.

1.2.4.1 Sympatric Resistance

It may be defined as those heritable qualities possessed by a host plant, which influence the ultimate degree of damage done by insect species having a prior, continuous, coevolutionary history with that host plant (Harris 1975). Association at the gene centres results in natural selection for resistance in plants. The resistance is evolved as a result of gene-for-gene nature of coevolution of plants and herbivores. This type of resistance evolves at original home of plants and insects. Hence, Leppik (1970) proposed that the search for source of insect resistance genes shall be conducted at the original home of the insect and plant.

1.2.4.2 Allopatric Resistance

It may be defined as those heritable qualities possessed by a host plant, which influence the ultimate degree of damage done by insect species having no prior continuous, coevolutionary history with that species or organism (Harris 1975). Allopatric resistance is not the result of coevolution, but rather due to fortuitous, pleiotropic effects of genes, which are present as a result of selective forces unrelated to the insect pest. The sources of allopatric insect resistance can be obtained outside of the geographic centre of origin of the pest. This type of resistance is often polygenic, more durable and offer defence against different biotypes of an insect.

1.2.5 Based on Trophic Level

Interaction among host plants, insect pests and their natural enemies leads to effective defence and attack at each level. On this basis, two types of plant resistance or plant defence have been recognized (Price 1986).

1.2.5.1 Intrinsic Resistance

When a host plant alone (first trophic level) produces defence through biophysical means (trichomes or toughness) or through production of biochemicals (toxins, digestibility reducers, nutrient imbalance) or both (glandular trichomes or resins), it is called as intrinsic resistance.

1.2.5.2 Extrinsic Resistance

When the natural enemies (third trophic level) of insect pests (second trophic level) benefit the host plants (first trophic level) by reducing the pest abundance, it is called as extrinsic resistance.

It has been established that intrinsic resistance of the host may affect positively or negatively the third trophic level and the factors associated with extrinsic resistance (Price 1986; Price et al. 1980; Shepard and Dahlman 1988).

1.2.6 Miscellaneous Categories

1.2.6.1 Based on Cross or Multiple Resistance

Cross Resistance

A variety resistant to primary pests confers resistance to another insect; also, it is referred to as cross-resistance. It is resistant for several species of insects; they may be closely related taxonomically. This type of resistance is attributed to physical properties of plants.

Multiple Resistance

A variety conferring resistance to biotic and abiotic stresses, viz. insects, diseases, drought, heat, cold, etc., is called as multiple resistance. It can be achieved by recombination breeding. The combination of productive genotypes with appropriate maturity, height, yield and multiple resistant factors is feasible.

1.2.6.2 Based on Crop Growth Stage

The resistance at different growth stages of the crop is classified as follows.

Seedling Resistance

This is also called as juvenile resistance. It is measured at the seedling stage of the crop.

Adult Plant Resistance

This is also called as mature plant resistance or aged resistance. This type of resistance is manifested only in older plants, which have been found to be susceptible at the seedling stage. Adult plant resistance is detected by sowing the plants at different dates. This type of resistance may involve horizontal resistance, but all types of horizontal resistance are not concerned with adult plant (Horber 1980).

1.2.6.3 Based on Screening Conditions

Greenhouse Resistance

This is the resistance detected under greenhouse conditions by artificially exposing the varieties to insect populations reared in laboratory. This may involve seedling as well as mature plant resistance.

Field Resistance

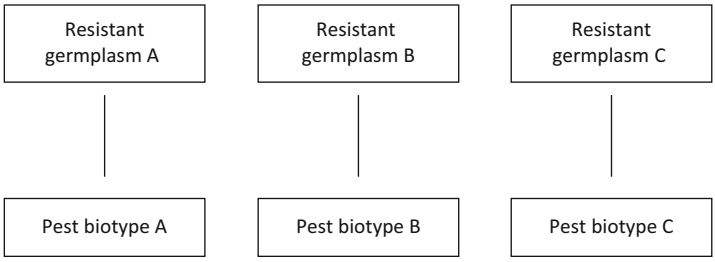
This is the resistance observed under field conditions due to the exposure of plants to natural populations of insects. It may also involve seedling resistance and adult plant resistance with respect to all locally occurring insect biotypes. Field resistance is also called as moderate resistance.

1.3 Types of Resistance

Based on genetic basis, Van der Plank (1963, 1968) proposed two types of resistance, viz. vertical and horizontal resistance, to explain plant-disease interactions. Gallun and Khush (1980) applied the same types for the study of plant-insect interactions.

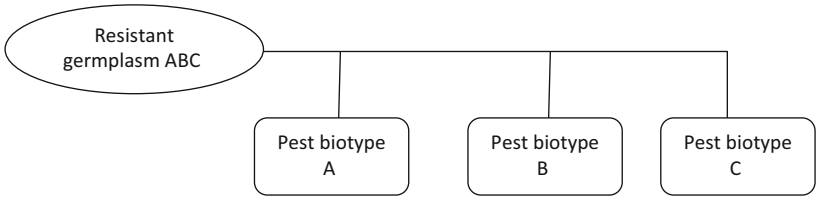
1.3.1 Vertical Resistance (VR)

This type of resistance is effective against certain specific biotypes of the insect, but not against others. It is generally determined by major genes or oligogenes and is characterized by biotype specificity. It is qualitative as the frequency distribution of resistant and susceptible plants is discontinuous. Hence, it is also known as biotype-specific resistance or qualitative resistance. The resistant gene may be 'dominant' if the F_1 progney of a resistant and susceptible parent is resistant or 'recessive' if the F_1 progeny is susceptible. In this type of resistance, some varieties show a resistant reaction, while other varieties show a susceptible reaction against the same biotype. Vertical resistance exerts a high selection pressure on the insect; hence, it is not long lasting, i.e. less durable or less stable than horizontal resistance (Gallun and Khush 1980).



1.3.2 Horizontal Resistance (HR)

It is effective against all the known biotypes of the insect. Horizontal resistance is quantitative as the degree of resistance depends on the number of minor genes or polygenes, each contributing a small cumulative effect. Hence, it is biotype non-specific or general resistance or quantitative resistance. In this type of resistance, different varieties of a crop show no differential reaction against different biotypes of the same insect. Horizontal resistance is more stable compared to vertical resistance because it does not exert a high selection pressure on the insect.



1.4 Mechanisms of Resistance

The true plant resistance is principally under the control of plant genetics. In other words, the mechanisms of resistance are derived from preadapted inherited characters. In addition, the expressions of these characters can also be mediated by environmental conditions. Hence, the mechanism of resistance is classified based on genetics and environment.

1.4.1 Genetic Resistance

The commonly recognized and widely adopted mechanisms in plant resistance to insect pest studies were proposed by Painter (1941), as preference, antibiosis and tolerance. In 1951, Painter added non-preference with preference as a mechanism of resistance, and in 1968, he started using non-preference alone as a resistance mechanism instead of preference, as it denotes susceptibility. The term 'non-preference' refers to a behavioural response of an insect to a plant, whereas 'antibiosis' and 'tolerance' refer to plant characteristics. This discrepancy was addressed by Kogan and Ortman (1978), who proposed the term 'antixenosis' to describe the plant properties responsible for non-preference, to replace Painter's term of non-preference. Both antibiosis and antixenosis mechanisms are related to insect pests' reaction to host plant characters, whereas the tolerant plants are responding to insect attack.

1.4.1.1 Antixenosis

The term 'non-preference' referred to the situation where herbivore (insect) behaviour was affected by certain plant traits, which led to reduced colonization or acceptance of a plant as a host (Painter 1951). 'Antixenosis' is the genetic character possessed by the host plant, which is perceived by the insect pest as an undesirable source for its food, oviposition or shelter (Painter 1958). Several authors defined the term antixenosis in different ways. In 1978, Kogan and Ortman stated that the insect pest selects an alternate host when the crop plants become a poor host. This type of resistance to insects is also known as 'non-acceptance'. It refers to various features of host plant that make it undesirable or unattractive to insects for food, shelter or reproduction. Plant physical and biochemical factors making the plant a refractory 'guest' (xenos in Greek) for the insect are the main causes of non-preference (Kogan 1994). Antixenosis is defined as the first stage in the encounter between the pest and the plant (van Emden 2002) or the first line of plant defence against insect pests, whereas Smith and Clement (2012) defined antixenosis as adverse effects on insect behaviour, which led to either delayed acceptance or possible outright rejection of a host plant.

1.4.1.2 Antibiosis

Antibiosis works only after colonization of insects and utilization of the plant by them. The term antibiosis refers to the adverse effects of resistant plants on the

physiology and biology of insect pests, i.e. survival, reduced growth, development and fecundity. The presence of plant biochemical compounds, toxins, antimetabolites, enzymes and growth inhibitors and the absence of nutritional compounds are associated with antibiosis. The insects feeding on resistant plants resulted in larval death in early instars, reduced size and weight of larva, prolonged larval period, failure to pupate, reduced adult longevity and fecundity and failure to hibernate.

1.4.1.3 Tolerance

The term tolerance is a basis of resistance in which the plant shows an ability to grow and reproduce itself or repair injury to a marked degree in spite of supporting a population approximately equal to that damaging a susceptible host. Horber (1980) defined tolerance as 'all plant responses resulting in the ability to withstand infestation and to support insect populations that would severely damage susceptible plants'. The plant may tolerate the damage without an economic loss in yield or quality by compensation and reduced symptom expression (van Emden 2002).

Coexistence/coevolution of insects and plants is the main basis for this mechanism. Several factors such as plant growth habit, wound healing, mechanical support in tissues and organs, early maturity and high flower production are the plant tolerance traits. Tolerant plants can be able to withstand heavy insect damage by regulating the uptake of water, nutrient and anchorage. Tolerance of the plant does not affect the rate of population increase of the target pest but raises the economic threshold level.

There are several examples for tolerance mechanism in plants against insect pests. Cowpea cultivars with less dense foliage and long peduncles holding the reproductive structures above the canopy had higher tolerance to *Maruca vitrata* (Usua and Singh 1979) and *M. testulalis* in cowpea (Oghiakhe and Jackai 1991). The cowpea variety IT91 K-180 tolerates a high population of thrips by producing more flowers and pods to compensate the pest damage (Alabi et al. 2003). The shoot damage caused by spotted bollworm, *Earias vittella*, in cotton; early shoot borer, *Chilo infuscatellus*, in sugarcane; and shoot and fruit borer, *Leucinodes orbonalis*, in brinjal is compensated by production of more side shoots.

Advantages of Tolerance

(a) The tolerant varieties have higher economic threshold level than the susceptible varieties.
(b) The use of tolerant cultivars would not adversely affect the biology of the insect, which would eliminate the selection pressure for the development of new biotypes.
(c) It increases the yield stability by providing at least moderate level of resistance.
(d) It maintains the population of predators and parasitoids, because it does not decrease their prey population.

1.4.2 Ecological Resistance

Sometimes, a plant or a variety may be classified as resistance due to unfavourable environmental conditions for the insect, and no heritable trait is involved. There may be differential impact of the environment on the host plant and on the insect, which affects the expression of resistance. Painter (1951) called this type of resistance as 'apparent resistance' or 'pseudoresistance'. It is not considered as 'true resistance'. This type of resistance mostly depends on the environmental conditions where the crops are cultivated than on the crop genetics. The characteristics are temporary, which is the result of transitory characters in the potentially susceptible cultivars. The characteristics are temporary and cultivars involved are potentially susceptible. It must be carefully synchronized with environmental conditions for its effectiveness. Pseudoresistance is generally classified into three broad categories (Painter 1951).

1.4.2.1 Host Evasion or Phenological Asynchrony

Under some situations, certain crop varieties can be able to avoid insect damage by passing the most susceptible stage of the crop rapidly. In this case, the use of early maturing crop varieties or fast fruiting varieties or short season varieties, to provide a long, host-free period, can be followed as an effective pest management strategy. Sometimes, the low infestation may be due to the less population of insect pests at that time. The plants that escape the insect infestation by this mechanism are likely to be infested due to early build-up of insect pest population, e.g. early sowing of paddy in *kharif* minimizes the stem borer, *Scirpophaga incertulas*, infestation. Early maturing varieties of paddy escape from brown planthopper (BPH). Sowing of sorghum soon after onset of monsoon in June helps to overcome shoot fly infestation. Short duration cotton varieties escape boll weevil and pink bollworm infestation.

1.4.2.2 Induced Resistance

It is a form of temporary resistance derived from condition of plant or environment, such as change in soil moisture levels or soil fertility. This type of resistance is also influenced by environmental factors, viz. temperature, photoperiod, production of secondary metabolites induced by the plants due to insect damage and nutrient balance of host plants. Once insect pests attack the plants, the production of phenolic compounds, such as phytoalexins, get enhanced; it facilitates the plants to resist further damage by the pests. The change in soil moisture levels may make the plants more tolerant to insect infestation than under other circumstances. Resistance is induced to aphids by providing a proper balance of nutrients in fertilizers. High nitrogen levels usually allow increased survival, but it is vice versa for high levels of potassium/silica. Potassium is a nutrient that plays an important role in the synthesis and allocation of primary metabolites in plants, and these physiological qualities influence metabolic, hormonal and signalling pathways in plants. These changes strongly influence the susceptibility or attractiveness of plants to insect pests (Amtmann et al. 2008). Some potassium-deficient plants accumulate the low-

molecular-weight organic compounds, like sugars, organic acids and nitrates, in leaves and roots, and these compounds make plants more susceptible and attractive to some insect pests (Marschner 1995; Amtmann et al. 2008). High phosphorus level increases root growth and induces resistance to root-feeding insects. Boron, zinc, copper and iron are also reported to induce resistance. When borax is sprayed at 0.5 ppm, it is able to reduce the stem borer and gall midge incidence in rice, and shoot and fruit borer damage in brinjal. Application of 500 g borax/tree induces resistance to coconut eriophyid mite.

1.4.2.3 Host Escape

It refers to the lack of infestation or injury on the susceptible host plants, because of transitory circumstances, such as incomplete infestation. If any uninfested plant is located in a susceptible population, it does not mean that it is resistant. Even under very heavy infestation, susceptible plants will occasionally escape. The reason is unknown (Painter 1951).

The terms host evasion and escape look like synonymous, but host evasion is related to the whole population of the plants under cultivation and absence or insignificant population of insects, while host escape relates to one or a few individuals of the host plant in the presence of insects causing damage to other plants.

1.5 Nature/Basis for Resistance

The plant traits and physiological processes that underlie resistance are called as basis for resistance. Antixenosis plays a major role in resistance, as it has a great impact on the selection of host plant itself. In the process of host plant selection, an insect detects a resource providing plant by a series of five steps, viz. host-habitat finding, host finding, host recognition, host acceptance and host suitability. The host-habitat finding by insect pests involves phototaxis, anemotaxis, geotaxis, temperature and humidity. It has nothing to do with host plant resistance. But the other four steps are influenced by the quality of host plants. Insect finds its host plant by distinguishing the physical features, viz. visual cues (colour), morphological (hairiness, surface wax, spines, thorns, etc.) and anatomical (tissue hardness, thickness of cell wall, gummosis, leaf angle, etc.) features. The morphological and anatomical basis of resistance intercepts in the host finding step. The biochemical basis of resistance interrupts in the host recognition (gustatory stimuli perception by test probe), host acceptance (perceiving chemical cues (odour), the presence of feeding or oviposition stimulant/attractants and absence of feeding or oviposition deterrent/arrestants) and host suitability (perception of adequate nutrients, absence of toxic chemicals). The mechanism of antibiosis is also associated with the presence of allelochemicals. The biochemical plant characters viz., secondary metabolites and deficiency in plant nutrients may cause antibiotic and antixenotic effects on insects.

1.5.1 Morphological Characters

The plant morphological characters accountable for insect resistance are trichomes on plant surface, surface waxes, hardness of plant tissues, thickening of cell walls and cuticle, anatomical modifications, silica content, colour, shape and size. This resistance mechanism interferes with insect movement, behaviour, feeding or oviposition and reproduction.

1.5.1.1 Trichomes

Trichomes are otherwise called plant hairs; they may be simple non-glandular or complex multicellular glandular structures. Their shape may be simple, branched, erect or hook-shaped. Plants belonging to Solanaceae have seven different types of trichomes. The trichomes act as a simple physical barrier for the movement of insects and interfere with the oviposition, fixation and feeding by insects. These effects are dependent on the length, density and orientation of the trichomes and on the insect's body size, mode of locomotion and type of mouthparts. In general, the longer, denser and/or more erect hairs provide a better barrier to insect herbivores than shorter, sparser or leaning hairs. Non-glandular simple trichomes act as physical barrier for insects, which prevents insects' feeding on the plant surface, while hooked and glandular trichomes either capture or pierce the soft-bodied insects, resulting in desiccation of body and death of insects. In addition, it affects the behaviour of insects, oviposition, growth and development of insect pests.

Trichome-Based Defence for Oviposition

Increased trichome density in plants is positively correlated with oviposition in many herbivorous species, as it offers protection for their eggs from egg parasitoids. The cotton varieties with high trichome density are more preferred by *Helicoverpa* spp. and *Earias vittella* for oviposition (Sharma and Agarwal 1983). The trichome density affected the oviposition behaviour of pink bollworm, *Pectinophora gossypiella*, in cotton, and the delayed development of pubescence in maize genotypes is less preferred by the corn earworm for oviposition (Chatzigeorgiou et al. 2010).

On the other hand, trichomes inhibit oviposition in some insect pests, especially soft-bodied insects. The soybean and tomato cultivars with pubescence were less preferred for oviposition by whitefly, *Bemisia tabaci* (Heinz and Zalom 1995; McAuslane 1996). The density of the non-glandular trichomes was negatively related with the number of whiteflies trapped, while it was positively correlated with oviposition per square centimetre per leaflet or leaf. Tomato cultivar, LA716, had high antixenosis level (ovipositional non-preference) towards *B. tabaci* B biotype related with type IV glandular trichome (Oriani and Vendramim 2010). Leaf area, lamina thickness and trichome length were significantly and positively correlated with whitefly eggs, nymphs and adults, whereas trichome density and angle were negatively correlated. Black gram genotypes with narrow, thin and highly pubescent leaves having short but erect trichomes should be selected for developing black gram varieties resistant to whitefly (Taggar and Gill 2012).

In addition, the glandular trichomes confer resistance to oviposition by insects, due to their toxic and deterrent nature of their exudates. Type VI glandular trichomes in tomato leaves exude a compound called 2-tridecanone, which interferes with the oviposition of whitefly (Williams et al. 1980). The trichomes in wild cowpea, *Vigna vexillata*, and cultivated cowpea, *V. unguiculata*, unfavourably affected the oviposition, movement of early stage pod borer larva, consumption and utilization of the legume by pod borer (Oghiakhe 1995).

Trichome-Based Defence for Feeding

The high density of non-glandular trichomes prevents the pod borer larvae from reaching the pod surface, and they starve or desiccate before feeding. The leafhopper fails to feed on cotton plants, when its epidermis is covered with a thick layer of cellulose hairs. Normal and dense pubescent types are highly resistant to cotton leafhopper. High densities of trichomes on the buds of cotton cultivars act as deterrent for feeding and oviposition by the boll weevil, *Anthonomus grandis* Boheman (Wessling 1958), and the trichomes in the lower leaf surfaces were more resistant to the cotton leafworm (Kamel 1965). Pubescent varieties of soybean and cotton are highly resistant to leaf hoppers (Kogan 1982; Khan and Agarwal 1984). For example, red plant body, smooth leaves, long pedicel, open canopy, frego bract, nectarilessness, thickness and hardness of boll rind of cotton plant are not preferred by bollworms, whereas hairiness of leaf and stem makes them non-preferable to jassids. The presence of dense covering of hairs on the leaves/pods confers resistance to many insect pests in grain legumes due to the presence of allomones, such as arcelins, L-canavanine, polyhydroxy alkaloids and saponins (Dilawari and Dhaliwal 1993).

Trichome-Based Defence for Mobility and Survival

Pubescence in tobacco leaves adversely affects the mobility and survival of young tobacco budworm, *Heliothis virescens*, larvae (Ramalho et al. 1984). The presence of glandular trichomes in the wild species of *Solanum berthaultii* and *S. polyadenium* reduces the mobility and increases the mortality of green peach aphid, *Myzus persicae*. The exudate produced by the trichomes accumulates on the insect's tarsi and labia, impeding movement and entrapping the insect on the foliage (Gibson and Turner 1977). The larval survival and development of Colorado potato beetle, *Leptinotarsa decemlineata*, are also affected (Casagrande 1982). Wild relative of cowpea pods, *Vigna vexillata*, is partly responsible for resistance to *Clavigralla tomentosicollis* Stal (Chiang and Singh 1988). The body parts of *Liriomyza trifolii* adults, like mouthparts, legs and ovipositor, are trapped by surface trichomes of *Phaseolus vulgaris* and, later, interfere with their ability to feed, walk and oviposit (Xing et al. 2017).

1.5.1.2 Colour and Shape

Colour-based insect resistance in plants does not exist, but genetic manipulation of plant colour usually has an effect on some fundamental physical plant processes (Norris and Kogan 1980). Some insects do not prefer certain colours; hence, those coloured plants are less attractive to them. Generally, red colour was not attractive to insects. For example, red coloured *Brassica* species (cabbages, broccoli and related species) are less attacked by imported cabbageworm, *Pieris rapae* and *P. brassicae*. Brassica genotypes with purple foliage and apetalous flowers were resistant to the development of *Lipaphis erysimi*. Red leaf colour in cotton has been reported to be developed by the plant as a defensive mechanism against aphids (Hamilton and Brown 2001). Reddish coloured leaf of lettuce varieties is less damaged by cucumber beetles. Red leaf colour in plants is due to the presence of anthocyanin pigments (Coley and Kursar 1996; Bohm 1998; Vargas et al. 2000). Brinjal varieties with light green coloured fruits were not preferred by the shoot and fruit borer (Jat and Pareek 2003). The oblong- and round-shaped fruits and high number of seeds were resistant to shoot and fruit borers (Prasad et al. 2014). Pea aphid prefers blue-green pea genotypes than yellow-green ones. Russian Red genotype displayed a medium level of resistance against whitefly in cotton (Alexander et al. 2004; Neto et al. 2008; Din et al. 2016).

1.5.1.3 Surface Wax

Cuticle is the barrier to insect pests for piercing into the plant system. Lignin, latex and wax deposits on leaves are other mechanical defences by the host plant to evade pest attack. Insects' feeding behaviour particularly the settling of probing insects, colonization and oviposition are affected by plant epicuticular waxes (EW). It may act as either feeding deterrents or phagostimulants. Plant waxes are esters formed by the linkage of a long-chain fatty acid and an aliphatic alcohol. Glossy, bloomless and glazed genotypes show different effects on arthropod behaviour and development. Wax blooms on the leaves of some cruciferous crops deter feeding of the cabbage flea beetle, *Phyllotreta albionica* (Anstey and Moore 1954). The lower populations of aphid, *Brevicoryne brassicae* L., in cabbage are associated with their glossiness (Way and Murdie 1965) and higher than normal populations of flea beetles (Dickson and Eckenrode 1980). Larvae of *Plutella xylostella* had non-preference for leaf wax in glossy-leaved resistant *Brassica oleracea* L. (Eigenbrode and Shelton 1990). The epicuticular wax from younger sorghum plants showed deterrent activity against *Locusta migratoria migratoroides* (Reiche and Fairmaire) (Atkin and Hamilton 1982). Wax content was the highest in the early stage in *Citrus maxima* leaves, and this internal hardness of leaf tissue could be an obstacle to feeding by citrus leaf miner larva. Wax extracts from the cabbage cultivars having glossy leaf deter the feeding of *Plutella xylostella* larvae (Eigenbrode and Pillai 1998). Glossy leaves of onion offer resistance to thrips. Bioassays with pure wax constituents showed that wax composition can significantly affect attachment by the predator *Hippodamia convergens* (Coccinellidae) (Eigenbrode and Jetter 2002). Agnieszka (2015) suggested that the surface waxes on triticale plants affected the probing behaviour of the grain aphid, *Sitobion avenae* F., and the waxy surface acted as an antifeedant.

In Sri Lanka, it was found that EW in sugarcane played a significant role in influencing the feeding of leafhopper, *Deltocephalus menoni*, vector of sugarcane white leaf disease (WLD). Therefore, sugarcane accessions having high level of EW could be incorporated into directional breeding of varieties to increase the resistance against WLD (Chanchala et al. 2020).

1.5.2 Anatomical Characters

1.5.2.1 Tissue Thickness/Toughness/Hardness

Thickening of cell wall affects the insects' feeding behaviour. In rice, the thicker hypodermal layers in stem offer resistance to stem borer. In wheat, the density of pith in stem and node tissues offers resistance to stem fly. In sorghum, the cell wall thickness offers resistance to shoot fly. In pulses, the pod wall strength and hardness are considered as important traits for resistance to pod borers (Rymal and Chambliss 1981; Oigiangbe et al. 2002). The feeding rate and larval growth of mustard beetle, *Phaedon cochleariae*, were retarded due to the toughness of turnip and Brussels sprout leaves (Tanton 1962). Thick sclerenchymatous layer of rice has been reported to associate with resistance to stem borer (Israel 1967). The hardness of rind, stem and fibre content of stalks in sugarcane confers resistance to the larvae of sugarcane borer, *Diatraea saccharalis*, *Chilo sacchariphagus indicus* and *Chilo infuscatellus* (Agarwal 1969). Brinjal varieties with thick-layered vascular bundles with lignified cells prevent the penetration into the apical shoot by shoot and fruit borer, *Leucinodes orbonalis* (Panda et al. 1971). Thick cortex in the stem of wild tomato prevents the stylet penetration into vascular tissues by the aphids, *Macrosiphum euphorbiae* (Quiras et al. 1977). Sorghum varieties with tightly wrapped leaves around the stem are resistant to shoot bug, *Peregrinus maidis* (Agarwal et al. 1978). Tight-husked ears of maize resist the corn earworm feeding and oviposition (Wiseman and Widstorm 1992). The inability of *Aphis craccivora* to colonize the plants was determined in the pods of the cowpea variety TVu-9930 which have a harsh surface texture (Ofuya 1993). Leaf thickness is an important criterion for citrus leaf miner attack (Alexander et al. 2015). There was negative correlation between percent leaf thickness and citrus leaf miner infestation (Mustafa et al. 2014).

1.5.3 Biochemical Characters

Biochemical characters play an important role in resistance to various insect pests. They are essentially phytochemical compounds, such as non-protein amino acids, cyanogenic glycosides, alkaloids, terpenoids, tannins, lignins, flavonoids and glucosinolates, that negatively affect the physiology or behaviour of the pest (Bennett and Wallsgrove 1994; Lattanzio et al. 2000; Dicke and Baldwin 2010). They are involved in both antibiosis and antixenotic mechanisms (Kogan 1994).

Due to nutritional factors, the cotton genotypes have been evolved with built-in resistance for insects, such as the leafhopper, *Amrasca biguttula* (Ishida); whitefly,

Bemisia tabaci (Gennadius); and thrips complex. The whitefly-resistant cotton genotypes showed higher contents of K, P and Mg and lower of N and Fe as compared to susceptible ones. But the other parameters, like sugars, proteins, Ca and Cu, did not show significant relationship with whitefly build-up. Total sugar content of cotton cultivars was positively correlated with whitefly incidence during the vegetative phase but negatively correlated with it during the reproductive phase of the crop.

Gossypol is known to adversely affect the nutritional quality of bolls by forming complexes with amino acids, proteins and enzymes. *Gossypium arboreum* L. genotypes with high gossypol-gland density on ovary surface showed low incidence of bollworms. The higher amount of gossypol present in the cotton genotypes restricted the development of pink bollworm, *Pectinophora gossypiella* (Saunders) larvae, increased the mortality rate, reduced the larval weight and adult fecundity (Agarwal et al. 1976). It also confers antibiotic resistance to *Heliothis zea* and *Heliothis virescens* (Kumar 1984). High percentage of cellulose, hemicelluloses and lignin in the pod wall inhibits pod damage by *Helicoverpa armigera* (Chhabra et al. 1990). Malic acid is highly resistant to *H. armigera* (Rembold 1981; Rembold et al. 1990). It acts as a deterrent. In pigeon pea, the amylase and proteinase inhibitors exhibited adverse effects on the growth and development of *H. armigera* larvae (Giri and Kachole 1998). The presence of sugar content and lower phenol content in the pod wall of cowpea varieties, TVNu 72 and TVNu 752, in cowpea affect the biology of *Maruca vitrata* (Oghiakhe et al. 1993). Cyanogenic heterosides, flavonoids, tannins and trypsin inhibitors were identified as antibiosis compounds in the cowpea variety IT86D-716 against the *Clavigralla tomentosicollis* (Dabire-Binso et al. 2010). The flavonoids present in chickpea viz., judaicin 7-O-glucoside, 2-methoxy judaicin, judaicin and maakiain possess antifeedant properties against *H. armigera* larvae (Simmonds and Stevenson 2001).

The silica content in the stem and leaf of maize is responsible for resistance to European corn borer (Rojanaaridpiched et al. 1984) and also the presence of chemical 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) due to leaf feeding (Klun and Brindley 1966), which has strong antixenotic and antibiotic properties (Robinson et al. 1982). The products, 6-methoxybenzoxalinone (MBOA) and DIMBOA, isolated from leaves of resistant maize plants were found to inhibit the growth of young larvae (Abel 1998). Deficiency in amino acid asparagine in rice causes reduced fecundity in brown planthopper, *Nilaparvata lugens*. In brinjal, the biochemical components like glycoalkaloid (solasodine), phenols, polyphenol oxidase and peroxidase expressed insect pest-resistant properties (Kalloo 1988; Doshi et al. 1998). Phenols are the important factors that confer non-preference and antibiosis mechanism in brinjal (Dar et al. 2017). Phenol content was higher in the shoots and fruits of *Solanum macrocarpon*, which exhibited a resistance to the shoot and fruit borer (Devarajaiah 1992). Different germplasm accessions of brinjal varieties, such as IC280954, IC099723, IC111013, IC111033 and EC038474, expressed resistance to shoot and fruit borers due to the presence of low amount of sugars and high concentration of phenol content

(Chandrashekhar et al. 2009; Prasad et al. 2014). The presence of starch and flavonols affects the biology and establishment of shoot and fruit borer.

Si absorbed by plants generally acts as direct and indirect plant resistance to insect pests through the deposition of SiO_2 as biogenic opals primarily in the epidermal cells of leaves, stems and roots (Liang et al. 2015). The accumulation of Si in the internodal epidermal tissue and root band of sugarcane enhanced the resistance; hence, the stalk penetration by *Eldana saccharina* larva was reduced (Kvedaras and Keeping 2007; Keeping et al. 2009) and also reduced the feeding and relative growth rate performance of root-feeding insect, the greyback canegrub, *Dermolepida albohirtum* (Frew et al. 2016, 2017). The larval survival and pupation rate of the rice leaf folder, *Cnaphalocrocis medinalis* Guenée (Lepidoptera: Pyralidae), was reduced by feeding on rice plants supplemented with Si (Han et al. 2015). Fecundity rate was affected in *Spodoptera frugiperda* female derived from caterpillars feeding on corn diet treated with Si (Alvarenga et al. 2017).

1.6 Genetics of Resistance

Development of resistant varieties relies on knowledge on the genetic background for the resistance. It provides a quantitative basis for designs to recombine genes and select for proper characters. It also allows the identification of stable resistance factors that are least likely to be overcome by a pest population.

1.6.1 Oligogenic Resistance

It is controlled by one or few major genes and each gene produces a large and distinct effect. It is also called as major gene resistance. It produces vertical resistance against insects and may be inherited through dominant or recessive genes. In many cases, resistance is controlled by single gene, which is referred to as monogenic resistance. In several cases of monogenic resistance, many different resistant genes for a particular insect are identified. Resistance to BPH in rice is a standard example of oligogenic resistance. More than 32 BPH resistance genes have been identified in indica rice cultivars and wild species. Most of the resistance genes are dominant, but a few are identified as recessive genes *viz., bph 2, bph4, bph5, bph7, bph8, bph19, bph25, and bph29*. In the case of resistance to Hessian fly in wheat, 26 genes have been identified, and all these genes are dominant except one. Modifying genes are known to affect the genes controlling the jassid resistance in cotton and green bug resistance in wheat.

Monogenic resistance is simply inherited, is easily transferrable, involves a single feature of host plant and is less stable due to the occurrence of resistance-breaking biotypes. But some examples of monogenic resistance, like jassid resistance in cotton, are highly durable as they have not been overcome by resistance-breaking

biotypes even after widespread cultivation of resistant varieties for a long period of time.

1.6.2 Polygenic Inheritance

It is governed by many genes, each gene producing a small and additive effect. It is biotype non-specific, is more durable and involves more features of the plant. This type of resistance shows continuous variation, hence difficult to transfer. The inheritance of polygenic traits is complex. The heritability is lower than monogenic resistance. The evolution of resistance-breaking biotype is rare as the insect has to adapt to more features of the plant. In certain crops, the cumulative effect of minor genes is expressed when the plants grow older, and this phenomenon is termed as 'adult resistance', 'mature resistance' or 'field resistance' (Russell 1978). Examples of polygenic inheritance are resistant to cereal leaf beetle in wheat, stem borer in rice, spotted aphid in alfalfa, earworm and leaf aphid in maize etc.,.

1.6.3 Cytoplasmic Inheritance

The genes (plasmagenes) present in the cytoplasmic organelles, viz. mitochondria and chloroplasts, control the resistance. There are only few cases have been reported to be controlled by plasmagenes. eg. resistance to European corn borer in maize, boll weevil and tobacco cut worm in cotton, root aphid in lettuce and potato aphid in tomato.

1.7 Identification of Sources for Resistant Genes from the Germplasm

The main aim of host plant resistance is to develop varieties resistant to insect pests to reduce the crop losses due to insect attack. To develop resistant varieties, germplasm lines with resistance genes are highly essential. Germplasm may be related to wild species, landraces, farmer's varieties and improved cultivars. These are screened to identify the donors for resistant genes. The identified donors will be used in hybridization programme to evolve resistant varieties. In addition to the available germplasm, unrelated organism also serves as a source of gene conferring insect resistance.

1.7.1 Wild Species

In many cases, the resistance genes may not be available in the cultivated crop species. In such condition, source of resistance should be searched in the related wild

species. The resistance genes have been successfully transferred from related wild species to cultivated species. In rice, grassy stunt virus resistance has been transferred from *Oryza nivara* to *O. sativa*. The wild species *O. eichengeri* is identified as a source of resistance genes for all planthoppers, while *O. brachyantha* carries resistance genes against stem borer and leaf folder. Resistance to shoot fly has been transferred from *Sorghum nitidum* to cultivated sorghum. Similarly, potato nematode resistant gene has been transferred from *Solanum vernei* to cultivated potato and jassid resistance genes from *Gossypium anomalum*, *G. tomentosum* and *G. armourianum* to cultivated cotton. In the case of sweet potato, resistance to nematodes has been transferred from hexaploid wild species *Ipomoea trifida*. Wild species identified in various crops acts as a source of resistance genes.

1.7.2 Landraces

The use of landraces as a source of resistance to biotic stresses is more practical than that of wild relatives because the introduction of resistant genes from landraces to improved cultivars is much easier than from wild relatives. Many rice landraces originated from South India and Sri Lanka possess resistance to BPH.

1.7.3 Cultivated Varieties

Resistance to insect pest can also be identified in the cultivated variety. For example rice variety TKM 6 has been identified as universal donor for stem borer resistance and it was utilized as one of the parents for the release of many rice varieties. High yielding varieties were developed with resistance to insect pests in major food crops.

1.7.4 Unrelated Organisms

Insect resistance gene may be transferred from unrelated organism into plants by recombinant technology. Plants carrying this transferred gene (transgene) are referred to as transgenic plants. The successful transgene is *cry* gene of *Bacillus thuringiensis*, which encodes a crystal protein. Development of *Bt* cotton through the transfer of cry gene is successful in maize, cotton and soybean. Proteinase inhibitor-encoding genes are the other important genes identified in many plants, e.g. the cowpea inhibitor (*CpTI*) gene.

1.8 Development of Resistant Varieties by Conventional Breeding Methods

The selection of breeding method depends on the mode of reproduction of a crop species. Most of the breeding methods are applicable to both self-pollinated and cross-pollinated crops with few exceptions. The pedigree method is mainly used for the improvement of self-pollinated crops. Recurrent selection is most commonly used for breeding cross-pollinated species (Mahabal 2014). The common breeding methods are discussed here.

1.8.1 Pure Line Selection

In pure line method, a large number of plants are selected from the base population and harvested individually. A part of seed of each plant is used for insect resistance screening in the laboratory condition. The remnant seeds of the identified resistant plants are raised in progeny rows in the field. The progeny rows are evaluated for insect/disease resistance, agronomic and grain quality characters. Only the desirable lines are harvested and inferior progeny rows are rejected. The promising lines are compared with the check varieties in replicated yield trials. The highest yielding line is released as a variety. This method was very popular in earlier days and is rarely used at present in the breeding programme.

1.8.2 Mass Selection

Mass selection involves selection of agronomically similar plants in each generation. Part of the seeds of each plant is used for resistance screening test. The remnant seeds of resistant plants are bulked to form uniformly resistant line. In this method, yield evaluation of bulked variety is not required. The variety developed includes few genotypes than the parental population. This method is seldom used in resistant breeding programmes and is widely used in programmes that are focused on purification of existing varieties (Panda and Khush 1995). Varieties resistant to potato leafhopper and spotted alfalfa aphid were developed through this method.

1.8.3 Pedigree Method

This method is widely used for the improvement of self-pollinated species. The main selection criterion is resistance to insect, and other desirable traits, viz. agronomic characters, disease resistance and quality, are also considered for selection. Selection of parents for hybridization is very important. One of the parents is a well-adapted high-yielding popular variety, and the other parent should be a resistant genotype. Individual plant selection is practiced from F_2 generation onwards. In the F_3 and subsequent generations, individual plant selection is made within and between

families till homozygosity is achieved. In each generation, selection is made based on the resistance to insects, agronomic traits as well as resistance to diseases. At the end of F_5 generation, most of the families reach homozygosity, and selection is mainly focused between the families. In F_6/F_7 generation, superior progeny are harvested as bulk and planted in multirow plots for yield evaluation and also tested for insect resistance. Superior progeny are evaluated in multilocation trial and released as variety (Khush 1977).

1.8.4 Single Seed Descent

In this method, single seed from each of the F_2 plants is harvested and bulked together to raise the F_3 population. This process is repeated till F_6 generation without artificial selection till homozygosity is achieved. At this stage, individual plant selection is made and individual families are raised. The families are evaluated for insect resistance to identify the resistant lines.

1.8.5 Backcross Method

This method is highly preferred for the transfer of resistance genes to a high-yielding popular susceptible variety. The variety which is used as donor for resistant gene is known as donor parent, and parent which is used in successive backcross is known as recurrent parent. Each of the backcross progeny (BC_1F_1) is evaluated for resistance if the gene controlling the resistance is dominant. Only the resistant plants are used for making next backcross. If the gene is recessive, the BC_1F_1 plants are selfed and only the homozygous recessive resistant plants are used for next backcross. In the backcross progeny, the transferred gene is in heterozygous condition. After the last backcross, the progeny are selfed and homozygous individuals are selected. They constitute a variety with the same yield, adaptation and grain quality, but are superior to the recurrent parent for resistance to the target insect. Unlike pedigree method, extensive yield trials are not required.

1.8.6 Recurrent Selection

This method is primarily used for the improvement of cross-pollinated crops. This method is highly suitable if the resistance is controlled by polygenes. In each cycle, two steps are followed: (1) selection of plants carrying polygenes for resistance and (2) intercross among the selected plants to obtain genetic recombination. This cycle of selection and intermating is repeated for four to five times, which results in the accumulation of polygenes for resistance from several parents. This method was successfully used by Widstrom et al. (1992) for the development of maize population resistant to fall armyworm by accumulating polygenes from 50 collections.

1.8.7 Wide Hybridization

Wild species are more resistant to insects and diseases. But many barriers are encountered in the transfer of useful genes from wild species to cultivated species. Among the barriers, abortion of hybrid embryos is a very important barrier. To overcome this barrier, embryo rescue technique is widely used. This method was successful in the transfer of genes for resistance to BPH and WBPH from *Oryza officinalis* to *O. sativa* (Jena and Khush 1990). Similarly, resistance to Hessian fly has been transferred from *Aegilops squarrosa* to bread wheat.

1.8.8 Mutation Breeding

Mutation breeding can be practiced to induce resistant mutants if the donors for insect resistance are not available in the germplasm of a particular crop. The mutants can be developed using any known physical or chemical mutagens. Generally, seeds of high-yielding adapted but susceptible variety are used for mutagen treatment. In some cases, the pollen grains are irradiated and used to pollinate the untreated plants of the same variety. The resultant progeny are evaluated for insect resistance. This method is very useful in creating change at single locus without disturbing other genes. Mutants resistant to brown planthopper were developed by gamma ray irradiation treatment of the rice variety Peltia 1/1 and released as Atomita 1 and Atomita 2 in Indonesia.

1.9 Development of Resistant Varieties by Innovative Approaches

1.9.1 Genetic Engineering

Genetic engineering is an innovative approach in plants, which comprises of incorporation and combination of single or multiple genes into a recipient plant, to create genome modification in the plants. This modified plant is called as transgenic plant or genetically modified plants. Normally, the traits responsible for insect pest resistance are transferred to crop varieties from non-cultivated plants or other organisms. With the advancement in new technologies, the identification of desirable genes and transfer into plants is possible without changing the quality characters. In addition, the genetic engineering has widened the genetic pool of genes, which has made it possible to introduce desirable genes from other plants, even from exotic source, viz. bacteria, snake venom, etc. This method takes less time compared to conventional breeding methods. The introduction of exotic insecticidal genes into plants has made significant progress in the development of insect-resistant varieties. Hence, the development of insect pest-tolerant plants through introgression of gene is a faster track towards improving crop varieties, not only in terms of offering insect pest resistance but also yield parameters.

1.9.1.1 Bt Genes for Insect Resistance

The bacterium *Bacillus thuringiensis* produces a number of insect toxins, and the most destructive one is protein crystal formed during sporulation. The *Bt* proteins are active against insects by binding with the specific receptors in the midgut cells of the target insect and form pores in the apical microvilli membrane of the cells. Genes coding for crystal (Cry) proteins have been isolated from *B. thuringiensis* and successfully used into crop plants through transformation techniques for the development of transgenic crops. More than 700 *cry* gene sequences that code for crystal (Cry) proteins have been identified so far. Most cry proteins, even within cry1A subfamily, have a distinctive insecticidal spectrum, which may be effective against insect species of Lepidoptera, Coleoptera, Diptera, etc. The crystal proteins, namely *Cry 1* are toxic against Lepidopterans, *Cry 2* against both Lepidopterans and Dipterans, *Cry 3* against Coleopterans and *Cry 4* is exclusively for Dipterans. Many of the identified cry genes (e.g. cry1Aa, cry1Ab, cry1Ac, cry1Ba, cry1Ca, cry1H, cry2Aa, cry3A, cry6A, cry9C, cry1F) have been engineered into plants against insect pests. Transgenic potato plants having *Cry1A (b)* showed high resistance to potato tuber moth. Perlak et al. (1990) introduced *Cry1A (b)* and *Cry1A (c)* genes into cotton plants. The transgenic plants showed resistance to the cotton bollworm. The *Bt* gene was inherited as a single dominant trait. Similarly, transgenic *Populus* plants with *Bt* gene registered resistance to forest tent caterpillar. The maize transgenic plants showed high level of resistance to European corn borer.

1.9.1.2 Proteinase Inhibitor Genes for Insect Resistance

Proteinase inhibitors (PIs) are ubiquitous in plant species. They are major components of both 'static' and 'active' defences in that they are accumulated in specific tissues ('static' defence) and are the major end product in the induced response to wounding ('active' defence). They are normally small proteins ranging from 4 to 25 kDa in size. They form tightly bound complexes with their target proteinases thereby inactivating the enzyme. Proteinase inhibitor from plants confers a natural defence system against insect attack. The storage tissues of many plants contain this inhibitor, which limit the consumption and digestion by insect pests. Some are protein inhibitors of insect digestive enzymes, and the responsible genes provide resistance to insects.

The first gene of plant origin successfully transferred to another plant was cowpea trypsin inhibitor (*CpTI*) gene. This inhibitor confers resistance to many insect pests belonging to Lepidoptera, Coleoptera and Orthoptera. Trypsin inhibitors reduce both survival and development of many insect pests. A serine proteinase inhibitor gene of cowpea was introduced into tobacco, and the transgenic plants showed a decrease in insect damage due to high level expression of *CpTI*. The cysteine proteinase inhibitor oryzacystatin has been isolated from seed. Oryzacystatin strongly inhibits gut proteases of rice weevil and red flour beetle. Additional inhibitors from other plants are mung bean trypsin inhibitor, potato proteinase inhibitors I and II and arrowhead proteinase inhibitor which are effective against rice insect pests (Chi 1990).

1.9.1.3 Amylase Inhibitors

These are small proteins resistant to proteolysis, ranging in size from 8 to 30 kDa, and they are active against insect amylases. Amylase inhibitors form tightly bound complexes with their target amylase. In the case of coleopteran herbivores, such as seed weevils (bruchids), amylase inhibitors from legume seeds are insecticidal (Suzuki et al. 1993) and also as causative factors in the resistance of specific varieties of legumes to bruchids (Ishimoto and Kitamura 1991). These proteins belong to a different sequence family and are similar to legume lectins in sequence.

The mechanism of toxicity involves inhibition of starch digestion, since bruchid larvae exposed to α-amylase inhibitor from French bean showed induction of amylase enzymes. High levels of toxicity towards insects have not been observed with amylase inhibitors. For example, α-amylase inhibitors are not strongly toxic to lepidopteran larvae, where the alkalinity of the gut interferes with the formation of inhibitor-enzyme complexes.

The best characterized α-amylase inhibitors are those from wheat (WAAI) and common bean (BAAI). A preliminary report suggested that the expression of WAAI in transgenic tobacco increased the mortality of lepidopteran larvae fed on it by 30–40% (Carbonero et al. 1993). The lectin like α-amylase inhibitor gene from common bean was isolated, and this gene was assembled into a construct with a strong seed-specific promoter (from the common bean seed lectin gene) and expressed in seeds of transgenic garden pea. The resulting seeds contained 3% of the foreign protein and were highly resistant to larvae of cowpea and azuki bean weevils (Shade et al. 1994).

1.9.1.4 Lectins

Lectins or carbohydrate-binding proteins occur in many plant species and get accumulated in seeds and other storage tissues as defensive proteins. They constitute about 1% or more of total protein. They are multimeric proteins containing polypeptides, which ranged from 10 to 35 kDa in size. The insecticidal activity of lectins was first observed in assays with larvae of coleopteran species. When lectins were incorporated into diets at 1–5% of total protein resulted in retardation of development and mortality. Lectins have relatively low antimetabolic effects on lepidopteran larvae due to high gut pH inactivating the carbohydrate-binding activity (Fitches et al. 1997).

The first demonstration of enhanced resistance of transgenic plants expressing a foreign lectin using the gene encoding the glucose/mannose-binding lectin from pea was proved by Boulter et al. 1990. Bioassays of transgenic tobacco expressing pea lectin against *H. virescens* showed significantly better performance than controls. Unlike many insecticidal lectins, such as wheatgerm agglutinin (WGA) and phytohaemagglutinin (PHA), pea lectin is of low mammalian toxicity. Unfortunately, it shows low insect toxicity.

1.9.1.5 Enzymes

Transgenic expression of various enzymes has been considered as crop protection agents. The most important enzyme is chitinase, since chitin is an important

structural component of insects. Expression of an insect chitinase in transgenic tobacco enhances resistance to some lepidopterans (Ding et al. 1998). A marginal protective effect from expression of bean chitinase in transgenic tobacco was observed.

Induction of polyphenol oxidase (PPO) synthesis is one of the end results of the plant wounding response. PPO activity leads to tissue browning that has been related with enhanced insect resistance. The oxidative cross-linking of tannins to proteins catalysed by PPO decreases protein digestibility and limits nitrogen availability.

Peroxidase activity is also induced when plants are stressed or attacked by pathogens as part of a lignification response. Several attempts have been made to over-express peroxidases in transgenic plants to enhance insect resistance (Felton et al. 1992).

1.9.2 Marker-Assisted Selection

Marker-assisted selection (MAS) refers to the use of DNA markers that are tightly linked to target loci as a substitute for or to assist phenotypic screening. The plants possessing specific genes or quantitative trait loci (QTLs) shall be identified based on their genotype rather than their phenotype by determining the allele of a DNA marker. Marker-assisted selection greatly increases the efficiency and effectiveness of breeding compared to conventional breeding.

1.9.2.1 Advantages of Marker-Assisted Selection

1. Simpler compared to phenotypic screening, when phenotypic screening is expensive, difficult or impossible.
2. MAS allows selection for all kinds of traits to be carried out at seedling stage.
3. To accumulate multiple genes for one or more traits into the same cultivar through gene pyramiding.
4. For incorporating genes for resistance to diseases or pests that cannot be easily screened.
5. Genotypic assays based on molecular markers may be faster, cheaper and more accurate than conventional phenotypic assays.
6. Higher effectiveness and efficiency in terms of time, resources and efforts.
7. More reliable.
8. The total number of lines that need to be tested may be reduced, since many lines can be discarded after MAS at an early generation.
9. Cost-effective for the traits which needs large-scale screening.
10. Accelerate the varietal development in breeding programmes.
11. Applicable for the traits with low heritability.

1.9.2.2 Application of MAS

Marker-Assisted Backcrossing (MAB)

Backcrossing is the most commonly used plant breeding method for incorporating one or a few genes into the elite or adapted variety. In most cases, the parent used for backcrossing has a large number of desirable attributes but is deficient in one or few characteristics (Allard 1999). The varieties carrying the genes for the trait of interest (donor parent) can be transferred into locally adapted high-yielding varieties that are lacking the trait of interest. The progeny with a gene of interest in subsequent generations can be selected using markers that are tightly linked to the gene of interest. The efficiency of selection of genes shall be enhanced by using DNA markers.

Marker-Assisted Pyramiding

This process helps in combining multiple genes/QTLs together into a single genotype simultaneously. It is possible through conventional breeding techniques, but it is tough or impossible to achieve at early generations. In the case of conventional phenotypic selection, the individual plants should be screened phenotypically for all the traits tested. Hence, it is a difficult process to evaluate all the plants in segregating generations (e.g. F_2) or for traits with destructive bioassays. The selection process shall be easily facilitated by the use of DNA markers, as the DNA marker assays are non-destructive. Moreover, using a single DNA sample, the markers for multiple specific genes/QTLs can be tested without phenotyping. Pyramiding has widespread application in combining multiple resistance genes to develop durable insect pest resistance.

1.9.3 Gene Switches

Chemically induced expression systems or gene switches facilitate temporal and spatial control of introduced genes or genes that are already present in the plants to impart resistance to insect pests. Many inducible genes have been identified in plants based on endogenous chemical signals, such as phytohormones, responses to insect attack or wounding. Effect of chemical injury inducer, Actigard, in providing resistance to many insects and pathogens in tomato has been confirmed by Inbar et al. (1998). Exogenous application of jasmonic acid and salicylic acid has also induced resistance to many insect pests. Proteinase inhibitors and oxidative enzymes persist for 21 days after induction in the affected tomato leaves. The best-studied system uses PR1-a promoter, which is induced in tomato during resistance reaction to pathogen infection (Uknes et al. 1993). Another system uses copper-dependent transcriptional activation, which includes *ace 1* gene controlling the constitutive expression of metalloresponsive factor in yeast. The gene *ace 1* gets activated in the presence of copper.

1.9.4 Altering Metabolic Pathways

Many of the most effective protective compounds in plants are small, non-protein secondary metabolites, like alkaloids, cyanogenic glycosides, glucosinolates, terpenoids, saponins, etc. These are usually the products of complex, multi-enzyme metabolic pathways.

These metabolic pathways can be effectively manipulated by the introduction (or elimination by anti-sense RNA technology) of enzyme-encoding sequences to increase the quantity of secondary metabolites. These metabolites play a major role in host plant resistance to pests and diseases, e.g. medicarpin and sativan in alfalfa, cajanol and stilbene in red gram, deoxyanthocyanidin flavonoids in sorghum and stilbene in Bengal gram (Sharma et al. 2002). Expression of bacterial cytokinin biosynthesis gene *P1-II-ipt* in *Nicotiana plumbaginifolia* has been correlated with increased resistance to green peach aphid (Smigocki et al. 2000). The role of phytoalexins in the activation of defence genes has been reported in a number of plant species.

1.9.5 Genome Editing

Genome editing has emerged as an innovative breeding approach for editing the genomes of plants, animals, microbes and human beings. Genome engineering refers to the process of bringing about 'precise heritable alterations' in the genomic DNA sequence of living organisms. This was made possible by the design (Bibikova et al. 2003) and use of synthetic nucleases, such as zinc finger nucleases (ZFNs) (Townsend et al. 2009) and TALENs (transcription activator-like effector nucleases) (Shan et al. 2015). To exploit these synthetic nucleases for making desired changes, they should be easy to develop and should be precise in targeting. But ZFNs and TALENs have some potential disadvantages, i.e. ZFNs are difficult to develop and TALENs have 'off target' mutagenic effects.

Recently, the discovery of the CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR-associated (Cas) proteins) system in bacteria has paved the way to initiate numerous genome engineering experiments across the world. This is because the Cas9 endonuclease brings about *precise* genetic changes in the DNA sequence depending upon two RNA molecules, namely the CRISPR RNA and tracrRNA. Both these RNA molecules help the Cas9 nuclease to bind to complementary genomic DNA sequence (target) and bring about 'desired mutations' in the target sequence (Tyagi et al. 2020).

1.9.5.1 Genome Editing in Insects to Modify and Mitigate Pest Population

Successful genome editing in *H. armigera* was reported through the knockdown of cadherin receptors that are genetically linked to Cry1Ac toxin resistance. Insects have specialized detoxification enzymes responsible to overcome chemical defence response in various plant species. An approach to target detoxification genes in

polyphagous pests can be a potential choice. Another way of insect control using genome editing is the ability to target genes that could interrupt chemical communication and mating partner identification. In insects, olfactory receptors (ORs) are important for the recognition of host plant and mating partner odorant. In *Spodoptera litura*, knockout of the Orco (olfactory receptor coreceptor) gene through CRISPR/Cas9 demonstrated distraction in the mating partner selection and loss of identity of host plants. Adoption of such technologies will be a potential choice to protect the crops and prevent insect damage.

In insects, female adults release pheromones and attract males. Males access the pheromone signals and select mature females. CRISPR/Cas9-based knockout of odorant receptor 16 (OR16) in *H. armigera* made males unable to receive pheromone signals from mature females. This resulted in mating of males with immature females, which subsequently led to the development of sterile eggs. Therefore, knockout of OR16 receptor in lepidopteran pests can be a new and effective strategy to regulate mating time for pest management in agricultural crops. Another approach for pest management is by knocking out developmental genes, such as abd-A (Abdominal-A) gene, a transcriptional factor involved in downstream regulation of various target genes that are extensively involved in development. Loss of function through CRISPR/Cas9 resulted in the generation of abd-A mutant phenotypes in *Spodoptera litura*, *S. frugiperda* and *Plutella xylostella*. Insects thus produced showed deformity in body segments, disarmed prolegs, anomalous gonads and embryonic lethality indicating the success of genome editing technologies.

1.9.5.2 Targeting Plant Genes Through Genome Editing for Insect Management

Genome editing in different agricultural crops has been successful against several fungal, bacterial and viral diseases. However, genome editing for insect pest management has been less exploited. Most polyphagous pests identify host plants using the plant's own volatile blends, visual appearance, oviposition sites and their interactions. Plant volatile blends are a mixture of volatiles, and out of which only a few are recognized by insects as sign for host selection and oviposition site. Studies have demonstrated that changes in volatile blends retract insects from host plants. Plants release sesquiterpene hydrocarbon (E)-β-farnesene (Eβf) when infested by aphid, which results in withdrawal of feeding by other host populations and also attracts a parasitic wasp. Alteration of plant volatile blends through genome editing is an alternate tactic in pest management. But this editing should not lead to any harmful effects on beneficial organisms.

Alteration in plant pigmentation has been found to change insect host preferences. This strategy has been successfully utilized in the area of genome editing for biotic stress resistance. This phenomenon was observed in transgenic red leaf tobacco that was developed by the modification of anthocyanin pathway. The overproduction of anthocyanin pigmentation resulted in the red coloration of leaves in the transgenic tobacco plant. This change in leaf colour proved to be acting as a control to *Spodoptera litura* and *Helicoverpa armigera* confirming the importance of leaf colour and appearance on host recognition in insects. These studies

demonstrated that engineering the anthocyanin pathway is a suitable approach for CRISPR-based editing for pest management.

Insects depend on chemical components from plants for their development, immunity and behaviour. This has been demonstrated in rice through the knockdown of CYP71A1 gene encoding tryptamine 5-hydroxylase using CRISPR/Cas9. CYP71A1 gene catalyses the conversion of tryptamine to serotonin in plants, resulting in reduced growth in planthopper. Serotonin, a neurotransmitter, is essential for larval immunity and behaviour.

1.10 Selection of Suitable Screening Methods Along with Rating Scale for Major Crop Pests

Development and standardization of screening methods for host plant resistance to insect is very important for screening and utilization of resistant sources of germplasms/cultivars/wild species/landraces in the resistance breeding programme. It requires adequate sources of germplasms, supply of test insects, efficient augmentation or artificial infestation techniques and efficient methods and techniques for evaluating the levels of resistance. It would be better to conduct the screening studies in hotspot areas at the susceptible stage of the crop without the interference by nontarget insect. The screening can be carried out under the laboratory, greenhouse and field conditions (Smith et al. 1994). The varieties developed through innovative approaches are also to be screened for verification of the transfer of resistant traits through screening techniques.

1.10.1 Screening Methods

1.10.1.1 Laboratory Screening

Laboratory screening is highly reliable than greenhouse or field screening as the environmental impact is largely avoided. However, it is practically impossible to maintain whole plants for resistance studies under laboratory conditions. Hence, the excised leaves or stems or roots or the fruiting structures are commonly used, which is the best way to evaluate the preference or non-preference of few accessions rather than screening a bulk germplasm. Olfactometer bioassays are done to check the settling preference. The ovipositional and feeding preference are assessed through no-choice or multiple-choice experiments. Feeding preference shall be assessed based on the area damaged or fed by the insect or by estimation of dry weight of check and test accessions after feeding.

1.10.1.2 Greenhouse Screening

In the greenhouse, the germplasms can be screened rapidly by infesting the plants artificially at the seedling stage. This technique is highly economic in space, time and labour. It can be used for screening the cultivars of cereals, millets, oilseeds, pulses

and forage crops. Greenhouse screening can also be used for confirming field response of different germplasm accessions to the pest.

1.10.1.3 Field Screening

Field screening must be conducted in an endemic/hotspot area, where the pest incidence is always occurring in large numbers. The germplasm to be screened should be planted at the proper season so that the evaluation can be done during the peak period of infestation by the target pest. The natural occurrence of the pest in endemic areas will envisage the evaluation process, and such evaluations are to be conducted during different seasons to ascertain the resistance levels of different genotypes at different population sizes of the infesting pest.

1.10.2 Screening Techniques for Sucking Pests

1.10.2.1 Rice Brown Planthopper

Screening methods for resistance against brown planthopper (BPH), *Nilaparvata lugens* (Stal), can be done under greenhouse or field conditions. There are two types of seed box techniques (Heinrichs et al. 1985).

Greenhouse Screening Techniques for BPH

Conventional Seed Box Screening Technique

It is a rapid method of qualitative resistance screening for large numbers of rice germplasms. About 25 seeds of each accession are to be sown in rows of 12 cm long along with a susceptible check in a standard seed box (60 × 40 × 40 cm). On the seventh day after sowing, the seedlings are at two-leaf stage, the seed boxes are placed in a water pan (with 5 cm water level) inside a room screened with wire mesh, and the seedlings are thinned to about 20 per row. After 10 days, 10 BPH nymphs cultured on susceptible variety are released onto each test seedling and evaluated as per the Standard Evaluation System for rice (SES) (IRRI 1996) when 90% of the susceptible check seedlings are wilted.

Modified Seed Box Screening Technique (MSST)

In this technique, the materials with moderate levels of resistance can be detected by infesting older seedlings. The hoppers are released at 10 DAS at 3–5 BPH per seedling and the entries are graded as per the IRRI SES score when susceptible check is rated at grade 7 (which generally occurs about 28 DAS).

Tillering Stage Screening Based on Days to Wilt Method (DW)

Days to wilt is a measure of tolerance to BPH infestation. After BPH infestation, the number of days required to kill the plants is counted on each accession, to assess the damage. The pregerminated seeds are sown on 15 cm diameter clay pots and emerging seedlings are caged with cylindrical Mylar sheet cage (14 × 110 cm). On each cage, 50 numbers of first to second instar nymphs are to be released on the

plants and allowed to feed after 45 days of sowing. The day on which the plant wilted completely is recorded (Timmanagouda and Maheswaran 2017).

Field Screening Technique for BPH

The varietal resistance has been challenged and well appreciated by evaluating their resistance in the field by three methods.

Resurgence Technique

Resurgence technique is followed when a population of BPH is too low for reliable field screening. Spraying of resurgence inducing insecticides viz. synthetic pyrethroids and quinalphos on the susceptible plants, planted along the border rows throughout the field, on 20 days after transplanting will induce BPH population. The accessions are graded for the damage score on a row basis, when the plants in the susceptible check start wilting as per the SES (IRRI 1996).

Polythene Barrier Technique

It is the modification of resurgence technique to prevent the movement of BPH nymphs outside the plot and to prevent predators from entering the plot by enclosing the test entries using 75 cm polythene sheets (top open) erected on 30 DAT and infested with BPH. Resurgence causing insecticides are sprayed over the entire plot on the tenth day of transplanting. The entries are graded for damage score by adopting the SES rating scale.

Microplot Techniques

Small plots of 1.5 × 1.5 × 1.0 m dimension are prepared in the experimental field. Seedlings of the test accessions along with a susceptible check are transplanted at 21 DAS with the spacing of 20 × 10 cm in the plots. Fibreglass mesh cages (1.5 × 1.5 × 1.0 m) are placed over these small plots. Natural enemies, if any, are killed by a spray of synthetic pyrethroids at 15 DAT and to induce resurgence of BPH. Subsequently, hoppers are released at two pairs/hill or 70 pairs/cage at 20 DAT. When 50% of the plants of the susceptible check show wilting or hopper burn, the entries are rated as per the SES scale (IRRI 1996).

1.10.2.2 Leafhopper

Cotton Leafhopper

Evaluation of cotton germplasm for resistance against leafhopper, *Amrasca biguttula biguttula*, is done by raising the test accessions in rows of 6 m length with the spacing of 75 × 30 cm. Okra is used as 'infestor' crop and raised at one row for every four rows of cotton. In each entry, ten plants are observed to record the population of nymphs and adults in three leaves per plant representing top, middle and bottom regions of the plant. The leafhopper population is counted on 30, 45 and 65 days after sowing (DAS), whereas the damage (hopper burn) is assessed on 45 and 65 DAS based on the grading suggested by Rao (1973).

The injury index is calculated by using the following formula:

$$\text{Injury index} = \frac{G1 \times P1 + G2 \times P2 + G3 \times P3 + G4 \times P4}{P1 + P2 + P3 + P4}$$

where G is the number of grades of injury and P is the population under that grade for each entry.

Based on the index, the cotton entries may be grouped into different categories of resistance based on the rating suggested by Rao (1973).

Okra Leafhopper (*A. Biguttula Biguttula*)

Sandhu et al. (1974) suggested the leafhopper injury grades based on the hopper burn symptoms to screen okra accessions. Later, Bindra and Mahal (1979) suggested the leafhopper injury grades for the screening of okra genotypes.

The leafhopper injury index for each genotype may be worked by using the following formula:

$$\text{Leafhopper injury index} = \frac{G_1L_a + G_2L_b + G_3L_c + G_4L_d + G_5L_e}{L_a + L_b + L_c + L_d + L_e}$$

where L_a to L_e are the number of leaves falling under the leafhopper injury and G_1 to G_5 are the leafhopper injury grades.

Groundnut Leafhopper

Feeding of leafhopper on groundnut causes folding of leaflets, followed by yellowing in a triangular fashion at the tip, and it has been used as a main criterion to assess the resistance levels of the groundnut entries. Test entries are raised in a row of 4 m length with a spacing of 60 × 15 cm and replicated thrice. Ten plants per row are observed for recording the damage. The leaflets showing yellow tip symptoms are counted from ten randomly selected leaflets in each plant and the injury rating is done (Anonymous 1986).

1.10.2.3 Cotton Whitefly

Evaluation of resistance in cotton germplasm against whitefly, *Bemisia tabaci* Genn., is done by growing the test entries in rows at 20 plants/row/entry. The population of whitefly nymphs is recorded on the third, fifth and seventh leaf from the terminal end of the main shoot in each plant from 55 to 110 DAS at weekly intervals. The test entries are graded by using the scale suggested by Vir (1989) and Saravanaraman and Prahalada (2019).

1.10.2.4 Sugarcane Scale

Evaluation of sugarcane germplasm for resistance against scale insect, *Melanaspis glomerata* Green, is done under natural conditions or by an artificial infestation technique. The leaves of the affected canes show signs of tip drying and unhealthy pale green colour and turn yellow under extreme infestations. Severely infested cane pieces are tied in the standing test canes when they are 6 months old and allow the scale insects to multiply on the canes. As per the following rating scale, the

sugarcane genotypes are evaluated based on cane drying and visual grading of pest infestation (David et al. 1986).

1.10.2.5 Aphids

Mustard Aphid

A screening technique for determining the resistance against the mustard aphid in terms of seedling survival was given by Jarvis (1970) using the optimum level of aphid population per plant under greenhouse conditions. The optimum levels of 10, 20 and 30 apterae forms and 1 and 3 ml aphids (1 ml = about 600 nymphs + apterae) per plant will be the optimal number for screening at the cotyledonary 2, 4 and 6 for leaf, flower bud initiation and flowering stages, respectively. Aphid injury symptoms expressed as injury graded (0–4) adopted by Pathak (1961) may be followed.

Safflower Aphid

The number of aphids is counted on 5 cm apical twigs from two randomly selected plants per entry. The aphid population is expressed as percentage of the aphid count on susceptible check to grade the relative response of different entries against the aphid. The drying of foliage due to aphid infestation can be recorded by visual scoring of the entries as indicated by Kavitha and Dharma Reddy (2012).

The Aphid Infestation Index (AII) is calculated by using the following formula:

$$\mathrm{AII} = \frac{1 \times a + 2 \times b + 3 \times c + 4 \times d + 5 \times e}{a + b + c + d + e}$$

where 1–5 are the different drying grades and *a*–*e* are the number of plants falling in each category.

Based on the AII, the safflower genotypes may be classified as per the scale given.

1.10.2.6 Onion Thrips

Seeds of the onion accessions are sown in flat beds (50 cm long, 20 cm wide, 6 cm high), which can accommodate four accessions of eight plants each. Plants are maintained in the greenhouse for 3 months at 27 ± 3 °C. At the end of 3 months, when the thrips damage is high, each accession is evaluated twice at an interval of 10 days for the intensity of thrips damage using the scale developed by AVRDC (1996).

1.10.2.7 Mites

Red Spider Mite

The methodology is similar to aphids, and the counting of mites and their eggs must be done under a dissecting microscope. The screening is done based on the rating of mite damage symptoms (Srinivasan and Rakha 2019).

Broad Mite in Pepper

Ten plants per accession are planted in a single row, and the plants are observed at weekly interval to assess the damage on a scale of 0–5 suggested by Srinivasan and Rakha (2019)

1.10.3 Screening Techniques for Borers

1.10.3.1 Rice Yellow Stem Borer

Screening of rice genotypes for resistance against yellow stem borer, *Scirpophaga incertulas*, is done in greenhouse or screen house. Resistance against stem borer is evaluated based on the percentage of 'dead heart' at vegetative phase and 'white ear' at reproductive phase.

Screening at Vegetative Phase

Fourteen days after sowing, the seedlings are transplanted in flat beds with a spacing of 20 × 20 cm. For every 20 rows of test entries, 1 row each of the susceptible (TN1) and the resistant checks (TKM6) is planted. On 30 DAT, newly hatched larvae are transferred using a fine camel-hair brush onto the youngest leaf or auricles at one larva per tiller. Dead hearts are counted for 4 weeks on 7, 14, 21 and 28 days after release. The percentage of 'dead heart' for each entry is computed as

$$\text{Dead heart } (\%) = \frac{\text{Number of dead hearts counted}}{\text{Total number of tillers observed } (\text{Healthy} + \text{Infected} + \text{Damaged})} \times 100$$

$$D(\text{Level of infestation}) = \frac{\%\ \text{dead hearts in test entry}}{\%\ \text{dead hearts in susceptible check}} \times 100$$

Screening at Reproductive Phase

Eight days after sowing, stem borer larvae are released at 1 larva/tiller at the topmost auricle, and the percentage of white ear is recorded 10 days after release. The test entries are evaluated based on the rating scale suggested by Kavitha and Dharma Reddy (2012).

1.10.3.2 Rice Gall Midge

Rice gall midge, *Orseolia oryzae* (Wood-Mason), infests the crop and causes 'silver shoot' or 'onion leaf'. Screening of rice germplasm for resistance to the gall midge is done in laboratory or field conditions.

Laboratory Screening

Seedlings of the test entries are raised in a box of 60 × 45 × 10 cm dimension with a row of susceptible check (TN1) at both sides. When the seedlings are 10 days old, the box is placed in a shallow iron tray containing water to provide better aeration

and the entire tray may be covered with nylon mesh. Around 30–50 gall midges are released using an aspirator for a box containing 20–30 entries at an age of 15–20 days. Water is sprayed onto the plants using hand atomizer after 2 days of infestation at 2–3 h intervals for 2 days to provide favourable condition for hatching. The percentage of plants damaged by gall midges is recorded after 4 weeks of release (Vreden and Arifin 1977):

$$\text{Damaged plants } (\%) = \frac{\text{Number of damaged plants}}{\text{Total number of plants}} \times 100$$

Then, the percentage of infested plants is converted to 0–9 scale using the SES for rice (IRRI 1996).

Field Screening

The test entries are planted in a field with a spacing of 25 × 20 cm between rows and plants. After every ten rows of test entries, a row of susceptible check (TN1) is planted. The method suggested by Prakasa Rao (1975) is followed for screening rice entries against gall midge. The silver shoots are recorded twice at 30 DAP and 50 DAP, and the data on the total number of hills and the number of infested and healthy hills and tillers are recorded to compute the percentage of hills and tillers damaged:

$$\text{Damaged plants } (\%) = \frac{\text{Number of infested plants}}{\text{Total number of plants}} \times 100$$

$$\text{Silver shoots } (\%) = \frac{\text{Number of infested tillers}}{\text{Total number of tillers}} \times 100$$

Then, the percentage of infested plants will be converted to 0–9 scale using the SES for rice (IRRI 1996).

1.10.3.3 Sorghum Shoot Fly

The screening of sorghum genotypes against shoot fly is done under field conditions with natural infestation through the calculation of percent infested seedlings. The number of dead hearts is counted after 25–30 days of planting to evaluate the level of resistance.

1.10.3.4 Sorghum Stem Borer

Sorghum stem borer, *Chilo partellus*, is screened by raising sorghum seedlings in microplots (3 × 1 m) with a spacing of 15 cm between rows and 10 cm between plants. Laboratory-reared larvae are dispensed onto the plants (9–10 days old) using bazooka larval inoculator at 3–4/stroke along with carrier into the leaf whorl of each plant. Larval feeding may be scored 7 days after infestation on a visual rating scale, and dead hearts are recorded 14 days after infestation (Sharma et al. 1992).

1.10.3.5 Sugarcane Early Shoot Borer

Evaluation of resistance in sugarcane genotypes against early shoot borer, *Chilo infuscatellus* Snell, is done by counting the total number of tillers and the infested tillers, and the percentage of incidence is worked out. The incidence level is assessed three times, viz. first, second and third months, after planting. The dead hearts are to be removed after each counting. The cumulative incidence by shoot borer is calculated by using the following formula:

$$\text{Cumulative incidence} = \frac{A1 + A2 + A3}{T3 + A1 + A2} \times 100$$

where $A1$, $A2$ and $A3$ are the number of affected tillers at first, second and third months, respectively, after planting and $T3$ is the total number of tillers at third month after planting. Based on the cumulative incidence, the genotypes are rated as suggested by Rajendran et al. (1998).

1.10.3.6 Sugarcane Internode Borer

Evaluation of resistance in sugarcane germplasm against internode borer, *Chilo sacchariphagus indicus* Kapur, is done by counting the total number of tillers or canes and the infested/affected tillers or canes in the test genotypes, and the percentage of incidence is computed. In the same sampled area, the total number of affected nodes in the canes is counted, and the percentage of intensity of infestation is calculated. The infestation index is calculated using the following formula:

$$\text{Infestation Index} = \frac{\text{Percent incidence (cane basis)} \times \text{Percent intensity (node basis)}}{100}$$

Based on the infestation index, the genotypes may be rated as suggested by Rajendran et al. (1998).

1.10.3.7 Sugarcane Top Borer

Evaluation of resistance in sugarcane germplasm against top borer, *Scirpophaga excerptalis* Walker, is done by counting the total number of tillers or canes and the infested/affected tillers or canes in the test genotypes, and the percentage of incidence is calculated. Based on the incidence percentage, the genotypes may be rated as suggested by Rajendran et al. (1998).

1.10.3.8 Cotton Bollworm

Evaluation of resistance in cotton germplasm against bollworms is done by growing the test entries in rows at 20 plants/row/entry. Ten plants per entry are observed to record the total number of fruiting bodies, viz. buds, flowers, squares, bolls, etc., as well as those infested by bollworms in each plant. The infestation levels are recorded from 35–40 to 110 DAS at weekly intervals to calculate the mean percent infestation for the entire season, and the resistance rating is done (Saravanaraman and Prahalada 2019).

1.10.3.9 Pod Borer of Chickpea/Pigeon Pea

Pod borer, *Helicoverpa armigera* Hubner, makes holes on tender chickpea pods and eats the internal contents. For screening chickpea germplasm, each test entry is raised in a row of 5 m length with a distance of 30 and 15 cm between rows and plants, respectively, preferably in the areas where pod borer incidence is predominant. Whenever the infestation is negligible, test entries are infested with pod borers reared on artificial semi-synthetic diets. The total number of pods and the pods damaged by the pest are counted from five randomly selected plants at the time of harvest to assess the pod damage. The damage caused by the pod borer is calculated and converted into percent damage by using the following equation:

$$\text{Pod damage } (\%) = \frac{\text{Number of damaged pods}}{\text{Total number of pods}} \times 100$$

The percent damage of the test entry may be compared with that of the check variety by using the following formula:

$$\text{Pest susceptibility percentage} = \frac{\%\ \text{P.D. of check} - \%\ \text{P.D. of test entry}}{\%\ \text{P.D. of check}} \times 100$$

where P.D. is the mean percentage of pods damaged. The pest susceptibility percentage is converted to a 1–9 rating scale described by Lateef and Sachan (1990) with slight modification.

1.10.3.10 Sesamum Shoot Webber Cum Capsule Borer

Screening of sesame germplasm for resistance to shoot webber cum capsule borer, *Antigastra catalaunalis* Dup., is done in both greenhouse and field conditions by observing leaf, flower buds and pod damage (Balaji and Selvanarayan 2009).

Greenhouse Screening

The seedlings of the test entries are raised in nursery bags and kept inside a screening cage (2 × 1 × 1 m) covered with nylon mesh all around. The 15 days old plants are exposed to infestation by releasing 10 pairs of adults per 50 accessions. After 15 days of release, the test entries are scored based on the intensity of damage and grouped into different resistant categories (Sridhar and Gopalan 2002).

Field Screening

Test entries are sown in the field with a spacing of 30 × 30 cm with a susceptible check, SVPR-1, at one row for every five rows of test entries. Two rows of the susceptible check may also be maintained around the screening field as infestor crop. The incidence of *A. catalaunalis* is recorded at weekly intervals by counting the numbers of infested leaves, flowers and capsules by the test insect and total number of respective plant parts, to arrive at mean per cent damage. Leaf damage, flower damage and capsule damage are recorded from 15 DAS, 36 DAS and 50 DAS

onwards, respectively. Based on the damage assessed during these stages, the entries are categorized by following the score chart formulated by Sridhar and Gopalan (2002) with little modifications. As the damage on the flowers and capsules affects the yield more than the leaf damage, it should be equated to a particular score as indicated below.

After the cumulative score is calculated based on percent damage on different parts of the plant, score (1–9) may be allotted by referring to the score chart, and the resistance rating may be given.

1.10.3.11 Groundnut Leaf Miner

Evaluation of leaf miner resistance in groundnut germplasm is done based on the area of leaflets dried due to mining (ICRISAT method) or based on percent leaflet damage (AICRP method) (Anonymous 1986).

ICRISAT Method

The test entries of groundnut are grown in rows, and ten plants are selected at random for each entry. In each selected entry, the area of leaf fed is estimated in the ten leaflets heavily damaged by the leaf miner and the per cent leaflet area destroyed is worked out to grade the test accessions using the rating scale.

AICRP Method

Test entries of groundnut are grown in a row of 4 m length with a spacing of 60 × 15 cm. Soybean is grown as 'infestor' crop for every five rows of test entries, and the following observations may be recorded. The total number of leaflets and the number of damaged leaflets from five plants/row are recorded to calculate the percentage of damaged leaflets, and the damage rating is done. Twenty leaflets are collected at random from each row to record the area of damage for assessing the percentage of leaflet area damaged, and the damage rating is done.

Further, the severity index can be calculated by using the following formula:

$$\text{Severity Index} = \frac{A \times B}{100}$$

where A is the mean rating for percent damaged leaflets and B is the mean rating for percent leaflet area damaged.

1.10.3.12 Fruit Borers

There are two stages in resistance screening techniques for borers, viz. tomato fruit borer (*H. armigera*), brinjal shoot and fruit borer (*Leucinodes orbonalis* Guenée), okra shoot and fruit borer (*Earias vittella* Fab.) and legume pod borer (*Maruca vitrata* Fab.), as suggested by Srinivasan and Rakha (2019).

In preliminary screening, ten plants are planted in a single row per accession along with standard known susceptible entry. As the damage scoring relies on the natural infestation, the susceptible accession is planted at one row for every ten rows, as well as all over the experimental field. Artificial infestation is required from the

laboratory cultures if there is less natural incidence. The total number and damaged number of shoots/fruits/pods/fruiting bodies are recorded on five plants from each accession at regular intervals (once in a week or 10 days). The data are recorded from the same plant (tagged) throughout the period of observation. The percentage damage for the whole plant is calculated, and the accessions are grouped for resistance using the following scale developed by Kashyap and Verma (1986).

1.10.3.13 Fruit Flies

Evaluation of resistance in cucurbitaceous vegetables against fruit fly, *Zeugodacus (Bactrocera) cucurbitae* Coq., is done by raising the test entries in rows with a spacing of 200 × 50 cm. The resistant and susceptible genotypes are raised on alternate hills. The number of fruit fly-infested fruits in each plant is recorded for 4 weeks from the initiation of fruit set. Laboratory-reared fruit fly adults are released if the natural incidence is less. Slurry of jaggery solution is sprayed to attract fruit flies. Resistance rating is made at the time of harvest based on fruit fly infestation (Chelliah 1972; Saravanaraman and Prahalada 2019).

1.10.4 Screening Technique for Defoliators

1.10.4.1 Rice Leaf Folder

Resistance screening for rice leaf folder, *Cnaphalocrocis medinalis* Guenée, is carried out in cages kept in the greenhouse. Earthen pots with five seedlings are placed in metal trays in flooded condition. At 14 DAS, around 120 such pots are enclosed in a large net cage, and 10 pairs of adult moths are released inside the cage. At 35 DAS, the damage evaluation is done when the susceptible check (TN1 or CR1009) shows 60% of symptoms on the leaf.

For field screening, five rows of the susceptible check are planted all around the screening field 1 month prior to planting of test entries. Twenty days after planting of susceptible check, phorate granules are applied at 1.0 kg a.i./ha to induce leaf folder resurgence. Each test entry is planted in four rows of five metre each with a spacing of 20 × 10 cm with a single row of susceptible check in between (Saravanaraman and Prahalada 2019).

The number of leaves in each grade is counted, and '*R*' (damage rating) is computed using the following formula:

$$R = \frac{\frac{(G1\times 100)\times 1}{\text{Total no. of leaves observed}} + \frac{(G2\times 100)\times 2}{\text{Total no. of leaves observed}} + \frac{(G3\times 100)\times 3}{\text{Total no. of leaves observed}}}{6}$$

where $G1$ is the total number of leaves with grade 1, $G2$ is the total number of leaves with grade 2 and $G3$ is the total number of leaves with grade 3.

Damage rating (R) for each test accession and the susceptible check (TN1 or CR1009) are calculated. Then the adjusted damage rating (D) for each test accession is determined based on the extent of damage in the susceptible check by using the following formula:

$$D = \frac{R \text{ in test accession}}{R \text{ in susceptible check}} \times 100$$

The overall damage rating (D) has to be fit into the 0–9 SES scale as suggested by Saravanaraman and Prahalada (2019).

1.10.4.2 Groundnut Defoliators

The methodology for the evaluation of resistance in groundnut for the defoliators, viz. tobacco caterpillar (*Spodoptera litura*), red hairy caterpillar (*Amsacta* spp.) and gram caterpillar (*H. armigera*), is similar for the field screening (Chhillar et al. 2004).

Groundnut accessions are raised in rows of 4 m length, and observations on the percent leaf area defoliated are recorded from five leaves per plant on ten randomly selected plants. If the natural infestation is insufficient, 10 egg masses of *S. litura* and *Amsacta* spp. are tagged per metre row length or 10 third instar larvae of all three species are released per metre row length from the laboratory culture on 45–60-day-old plants. The percent leaf area defoliated is recorded 15–20 days after the release of the larvae and 40 days after the tagging of the egg masses, and the accessions are rated using the 1–9 scale.

1.10.4.3 Pumpkin Beetle

Evaluation of resistance in cucurbits genotypes against pumpkin beetles is done by raising the genotypes in pots at ten seedlings/pot and screening under insect proof cages of 8 × 3 × 2 m dimension. Seedlings along with pots are kept inside the cage, and ten beetles are released at one beetle/plant when the cotyledonary leaves have fully expanded. The cucurbits genotypes are scored for resistance as per the scale when the susceptible check shows complete damage (Saravanaraman and Prahalada 2019).

1.10.4.4 Termites in Groundnut

Since the termites have aggregated distribution, the uniformity in pest distribution is a prerequisite for reliable screening. It can be achieved by alloting the land area for long term experiment by keeping it insecticide free for several years. In addition, the frequest use of raw farmyard manure in th experimental area and shallow plouging duing afternoon, as the termite colony workers come to soil surface for foraging during morning hours, also helps to attain termite infestation in the test area. The alate termites are caught during previous night using the light trap and released in the field during next day morning.

Odontotermes obesus kill the plants by boring into roots and stems, and other species of termite destroy pods by feeding on the pod shell (scarification). The observations for termite damage are recorded by counting the number of plants killed, the number of pods killed, the number of pods scarified and the extent of scarification on a 1–9 scale. The pods are uprooted one month after maturity for observing the pod scarification by termites or bury the pods 20–25 cm deep in soil for 3–4 weeks and record the number of pods scarified and extent of scarification and the germplasm may be rated as per the index suggested by Rohilla (2004).

1.11 Compatibility of HPR with Other Components of IPM

High levels of plant resistance are effective in providing optimum control of the target pests, which are available against a few insect and pathogen species. However, very high levels of resistance are not a prerequisite for use of HPR in pest management. Varieties with low to moderate levels of resistance or those which can avoid pest damage can be deployed for pest management in combination with other methods of pest management. Deployment of pest-resistant cultivars should be aimed at conservation of the natural enemies and minimizing the number of pesticide applications. The use of pest-resistant cultivars also improves the efficiency of other pest management practices, including the synthetic pesticides (Sharma 1993; Panda and Khush 1995).

Insect-resistant varieties in combination with early planting, early maturity, defoliation, destruction of stalks and deep ploughing can be used to control boll weevil and bollworms in cotton (Adkinson and Gaines 1960). This not only reduces the pest damage but will also decrease the overwintering population of the pests, which result in reduced crop loss in the following season. For example, late planting of sorghum varieties M 35-1 during the Rabi season can reduce the shoot fly damage substantially.

Generally, plant resistance to insects and pathogens is compatible with biocontrol agents. Varieties with moderate levels of resistance are best suited for use in pest management in combination with biocontrol agents. The natural enemies not only help to control the target pests but also reduce the population densities of other insect pests and pathogens within their host range. Pest-resistant varieties also increase the effectiveness of the natural enemies because of a favourable balance of population densities between the target pest and the natural enemies. Few studies have reported the effects of plant physiology and plant allelochemicals on the biology of parasitoids and predators (Boethel and Eikenbary 1986). For example, female parasitoid wasp, *Campoletis sonorensis*, responds to the volatiles of cotton over a short distance while searching for its prey, *Heliothis* spp. It is easier for the wasp to find the host habitat first and the prey itself within the vicinity of cotton plant (Williams et al. 1988).

Insects that feed on resistant plants will have retarded growth and extended development period. Such poorly developed insect herbivores are more vulnerable to natural enemies for a longer period, and their mortality rate is also higher. Insects that develop slowly on resistant varieties are more effectively regulated by predators than on susceptible varieties, because the predator has to consume more small-sized prey to become satiated (Price et al. 1980). The presence of secondary compounds in the plants that impart resistance is compatible with the natural enemies. For example, *Cotesia congregata*, a monophagous parasitoid of *Manduca sexta*, shows no detrimental effects on exposure to nicotine in tobacco (Barbosa et al. 1986).

Plant resistance also enhances the effectiveness of insecticides through better penetration of insecticides to target insects through modified plant morphology, e.g. loose panicle in sorghum or open canopy in cotton (Sharma et al. 1994). The imbalance nutrition of host plants adversely affects the growth and development of

insects, which may increase the insect susceptibility to insecticides and easy access to parasites and predators through change in plant canopy. The insecticide carbofuran in combination with plant resistance is effective in reducing the sorghum shoot fly, *Atherigona soccata* (Sharma et al. 1999).

Integration of the host plant resistance with selective insecticides and biocontrol agents leads to a reduction in the use of insecticides and effective management of *T. absoluta*. In which, the combinaiton of two components results in either synergism, antagonism or additive effects (Furlong and Groden, 2001). Peris et al. (2020) studied the combination of insecticide, chlorantraniliprole, with moderately resistant tomato variety, Rio Grande VF, which reduced the *Tuta absoluta* damage when compared to susceptible variety. This may be due to chemical compounds produced by the plants that affect the growth and development of insects, thereby increasing the susceptibility to insecticides. In contrast, susceptible tomato variety, Pesa F1, with biocontrol agent, *Macrolophus pygmaeus*, a zoophytophagous predator, significantly reduced the *T. absoluta* damage (Peris et al. 2020).

1.12 Conclusions

In agricultural crop production, the yield loss due to insect pest infestations is causing a major concern. Though the chemical method of pest management is resorted by the farmers for quick relief from their infestation, there are several limitations, like the development of resistance to insecticides; resurgence of insect pests; pesticide residue problems; adverse effects on nontarget organisms, like pollinators, natural enemies, etc.; and cause of environmental pollution. These complications made the researchers to focus their attention on the development of resistant varieties against insect pests. Through large-scale screening of germplasms/wild relatives, the source of resistant genes with reasonable level of resistance is being identified by entomologists, which is a basic requirement in the development of a resistant variety. Later, breeders/biotechnologists play a key role in the development of the resistant varieties by conventional breeding methods or modern biotechnological approaches for major insect pests in economically important crops. With the advent of various biotechnological tools, the transgenic plants are being developed by engineering with resistant genes, which is an alternative strategy in pest management. In addition to the transgenic plants, manipulation of plant secondary metabolism and plant-mediated RNAi strategy can also confer improved resistance to insect pests. Though several research findings are available in the transgenic plants, there is a huge social concern in the commercialization of the developed varieties. But the development of resistant varieties is a continuous process, as there is a constant arms race between host plants and insects due to coevolution.

Points to Remember

- Host plant resistance is a tremendously effective technique for suppressing the population of insect pests or their damage in plants. In addition, it is eco-friendly, economical and compatible with all the other components of integrated pest management and also a farmer-friendly technique.
- The development of resistant varieties involves the equal contribution of both the plant breeders and entomologists, which is a collaborative research. Importance is to be given for resistance characteristics of the germplasms in addition to yield potential. The resistant germplasm identified by the entomologists is to be utilized by the breeders while developing the resistant varieties.
- Enormous efforts have been made in the identification of resistant sources of germplasms, understanding the mechanism of resistance to major insect pests in greenhouse, field and laboratory.
- Earlier, the development of improved resistant varieties of crops through conventional breeding techniques required longer time, as the development of new varieties primarily depends on phenotypic selection and field evaluation. These processes require 10–12 years to release a new variety.
- With the advent of several modern approaches, viz. genetic engineering, marker-assisted selection, gene switches, altering metabolic pathways and whole-genome sequence-based approaches, it is possible to release new varieties in a short period of time.
- The latest advancement in genome editing technology using programmable nucleases, clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins, has paved the way in the new plant breeding era. Therefore, researchers have started using novel strategies for increasing the efficiency of crop breeding to evolve high-yielding resistant varieties.
- In this chapter, several aspects on types of resistance, mechanisms of resistance, genetics of resistance, identification of sources of resistance and screening methods, from traditional to modern breeding methods, including genome editing tools to evolve varieties with desirable resistance mechanism were reviewed as the host plant resistance plays a major role in pest management.

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Insecticide Resistance: Molecular Insight

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Omkar (ed.), *Molecular Approaches for Sustainable Insect Pest Management*,
https://doi.org/10.1007/978-981-16-3591-5_2

Abstract

Insecticide resistance is one of the major worldwide challenges in insect pest management. Conventional to molecular approaches have been used in identifying insecticide resistance aspects, i.e. behavioural, ecological, physiological and molecular. The molecular mechanisms of insecticide resistance detection are mainly determined by three factors, i.e. gene amplification, upregulation and structural changes in genes. Genome sequencing, DNA barcoding, genome editing, transcriptional control and epigenetic studies have helped in making tremendous progress in insecticide resistance research. The new era of molecular studies has opened more reliable, precise and appropriate options for insecticide resistance recognition and timely management of insect pests.

Keywords

Insecticide resistance · Molecular assay · Gene amplification · Gene upregulation · DNA barcoding · Transposable elements · Epigenetics · Detoxification enzymes

Learning Objectives

1. The molecular studies of insecticide resistance have opened a new era in the assay of insecticide resistance. The availability of insecticide resistance data on time and with high accuracy helps in making timely management strategies and reducing economical losses.
2. The molecular mechanisms of insecticide resistance are mainly determined by three factors, i.e. gene amplification, upregulation and structural changes in genes, and the sequencing of desired genes confirms the aforesaid studies.
3. This chapter has covered molecular aspects of insecticide resistance in addition to the novel epigenetic studies that throw a light on mysteries of recently discovered molecular studies.

2.1 Introduction

Agriculture is an important outcome of human civilization, and insect pests have always been its parallel associate. Synthetic chemical pesticides have protected the crop plants against harmful insects since long, but the recent problem of insecticide resistance has halted this progress. Insecticide resistance is one of the most nuisance and expanding problems, which has become a challenge for scientists working for the development of insect pest management strategies. Resistance is defined as 'the development of an ability in a strain of an organism to tolerate doses of a toxicant, which would prove lethal to the majority of individuals in a normal (susceptible) population of the species' (WHO 1957). However, the term insecticide resistance specifically deals with population of insects, which stops responding to application of recommended doses of insecticides (Javed et al. 2017). Pesticide-resistant insects are modified either by genetic or epigenetic changes, which ultimately leads to

biochemical, physiological and phenotypic differences among them (R4P Network 2016). In one of the recent newsletters of Insecticide Resistance Action Committee (IRAC), data of top 20 countries and top 20 arthropods showing resistance was released, which is very alarming. The crop losses caused by insect pests globally emphasize the threat of insecticide resistance in management practices, and this chapter will help understand the molecular studies associated with insecticide resistance to safeguard chemical management strategies, which is an integral component of integrated pest management (IPM) practices.

2.2 Assays for Detection of Insecticide Resistance (R4P Network 2016)

There are majorly three types of assays, i.e. bioassay, biochemical assay and molecular assay, which focus on the phenotypic, biochemical and genetic modifications.

2.2.1 Bioassay

The aim of bioassays is to determine the doses that affect insects as well as to test the level of resistance (Siqueira et al. 2000) by exposing live insects to determine doses and comparing them with sensitive population, popularly analysed by the dose-response curve. IRAC has formulated different methods of bioassays, but the standard method used is leaf immersion (Bacci et al. 2009). Although there are some limitations (time and space) associated with bioassays, the use of technologies, such as automated imaging platform (Stewart and McDonald 2014), could make a breakthrough by increasing the reliability of these tests.

2.2.2 Biochemical Assay

These assays are used to detect resistance regulated by target enzymes or metabolic enzymes. The measurement of specific activity of enzymes by absorbance or fluorescence reveals the variation in activity of pesticide detoxification enzymes (Reyes et al. 2012). The biochemical assay methodology of some important insecticide-degrading enzymes has been discussed by Kranthi (2005).

2.2.3 Molecular Assay

One of the major constraints of the above two assays was the requirement of live organisms, which in molecular assay is not a limitation. On the basis of technology used, the molecular assays are classified into two major types, i.e. (1) rugged or low-throughput assay and (2) hi-tech or high-throughput assay. Genotyping of

known mutations causing resistance and sequencing of full genotypes to know any level of variations are some examples of molecular assays. Very low detection threshold is the primary advantage of molecular diagnosis of insecticide resistance over all other types of assays (Black and Vontas 2007).

2.3 Molecular Mechanism of Insecticide Resistance

The genomic studies evolved from Mendelian genetics via phases, such as molecular genetics, genomics and most recently epigenetic studies. These studies have played an important role in insect pest management practices beginning from conventional breeding or selection strategies, such as sterile insect techniques (Haymer 2015), to novel techniques, like RNAi. A lot have already been studied about the conventional approaches of insecticide resistance paving a way towards advanced molecular studies. Since the studies up to the level of amino acid was very significant in insecticide resistance hence used the term landmark developments (Perry et al. 2011). There are mainly four aspects of insecticide resistance studies, viz. behavioural, ecological, physiological and molecular, which are further determined by several factors. Gene amplification, upregulation and structural changes in genes encoding detoxification enzymes (P450s, GSTs, esterases) are three factors responsible for molecular mechanism of insecticide resistance (Li et al. 2007) and thus emphasize the role of molecular biology, genomics, epigenetics and bioinformatics tools. Heckel (2003) in his review described the role of genomics in pure and applied biology and comprehensively covered all the fields of genomics, i.e. structural, functional and comparative genomics, and the importance of genomics in entomology.

2.3.1 Gene Amplification

Alteration in the copy number of genes determining the system responsible for detoxification of insecticide encountered by insects is gene amplification (Li et al. 2007), and the transcription and translation of the amplified gene lead to the production of functional proteins responsible for the expression of resistance traits (Feyereisen 1995). Out of the three major detoxification enzymes, the resistance mechanism of gene amplification has been observed in esterases and GSTs; however, more recently, it has been reported for P450s also (Bass and Field 2011). The evidences of gene amplification for insecticide resistance in *Myzus persicae* have been reported by Field et al. (1998); they found that it was due to amplification of gene esterase-4 (E4) or fast-E4 (FE4) (Field et al. 1998).

2.3.2 Upregulation/Altered Expression

Upregulation may be described as increased production of detoxification enzymes or proteins without showing any change in its genomic copy number like in gene amplification (Li et al. 2007), and the mutation in trans- and/or cis-acting regulatory loci has been documented as usual cause of upregulation (Bass and Field 2011). The first example of gene amplification of insecticide target site has been documented for AChE locus in two-spotted spider mite, *Tetranychus urticae* (Kwon et al. 2010). The Northern and Western blot analysis of PxGSTE1 gene in diamondback moth, *Plutella xylostella*, showed that resistance against OP insecticides is due to higher expression of the gene. The molecular reason behind was documented to be upregulation of the gene concerned since there was no evidence of gene amplification from Southern blot results (Sonoda and Tsumuki 2005).

2.3.3 Structural Change

Point mutations, like addition, deletion and substitution, may modify the sequence of DNA responsible for insecticide resistance (Feyereisen 1995). Substitution of one nucleotide with another nucleotide in the coding region may change three-dimensional structural change and may affect resistance against insecticide positively or negatively (Scott 1995).

2.4 Genome Sequencing, Genome Editing and Transcriptional Control

2.4.1 Genome Sequencing

Sequencing is a method for determining the position of nucleotide bases, and genome sequencing identifies every nucleotide in the genome. Early DNA sequencing technologies, also known as 'first-generation sequencing', include sequencing by synthesis (Sanger et al. 1977) and sequencing by cleavage (Gilbert and Maxam 1973), while second-generation sequencing or next-generation sequencing is the novel and highly efficient sequencing technology. Gene amplification and structural changes in the genome have been assayed using these sequencing technologies for both DNA and RNA (Leeuwen et al. 2020). Clarkson et al. (2018) described the role of whole genome sequencing in studying the molecular basis of insecticide resistance, and genomic studies of *Spodoptera litura* by genome sequencing, transcriptome analysis and physical mapping revealed adaptive changes, expansion of selected genes and ecological adaptations (Cheng et al. 2017).

2.4.2 DNA Barcoding

Like the barcodes used in package of any product, DNA barcoding is a system of biological identification by amplifying and sequencing a short reference region of the genome (Hanner et al. 2009). Gene region extensively used in the study of insects is mt-encoded cytochrome c oxidase subunit 1 (cox1, CO1) 648 bp region amplified by primer, and the most probable cause of its wide use is maternal inheritance and wide occurrence, making it suitable for examining population history and easy to isolate, respectively (Cameron 2014). DNA barcoding is applicable in taxonomic identification and early invasion of insects paving the way to apply management strategies on time (Hanner et al. 2009). It is also used in insecticide resistance studies and for the development of selective insecticides to protect natural enemies. In one of such studies on pond wolf spider *Pardosa pseudoannulata*, which is an important natural predatory enemy of rice planthoppers the molecular basis of selectivity of neonicotinoids was observed (Meng et al. 2015a) (Fig. 2.1). There are four major clades in the cytochrome P450 family, viz. CYP2, CYP3, CYP4 and CYPM, and in insects, CYP3 clade contains the majority of detoxifying P450 genes. In *P. pseudoannulata*, CYP2 clade was found to be superior, which is quite different from insects, and thus depicts the difference in resistance mechanism, which could be used for the formation of selective pesticides (Meng et al. 2015b) (Fig. 2.1).

2.4.3 Genome Editing or Genome Engineering

It allows the creation of double-stranded breaks (DSBs), followed by insertion or deletion of foreign DNA sequences. The methods for precise editing of a genome include (1) zinc finger nucleases (ZFNs) technology (Urnov et al. 2010), (2) transcription activator-like effector nucleases (TALENs) (Mussolino et al. 2014) and (3) clustered regularly interspaced short palindromic repeat (CRISPR) or CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9) system (Chylinski et al. 2014). CRISPR/Cas9 is the latest technology in genome editing and has been successfully employed in modification of the targeted insect. With the use of this technology, CYP6AE gene cluster was knocked down in *Helicoverpa armigera*, which was responsible for insecticide resistance, and the role of the concerned gene was proven (Wang et al. 2018).

2.4.4 Transcriptional Control

DNA is the carrier of biological information, and the information is transferred to RNA via transcription and is finally expressed by amino acids through the process of translation (Crick 1958). Regulation of genes of various functions occurs at the level of transcription in eukaryotes (Harshman and James 1998). Insecticide exposure induces transcriptional responses in insects that regulate the detoxification

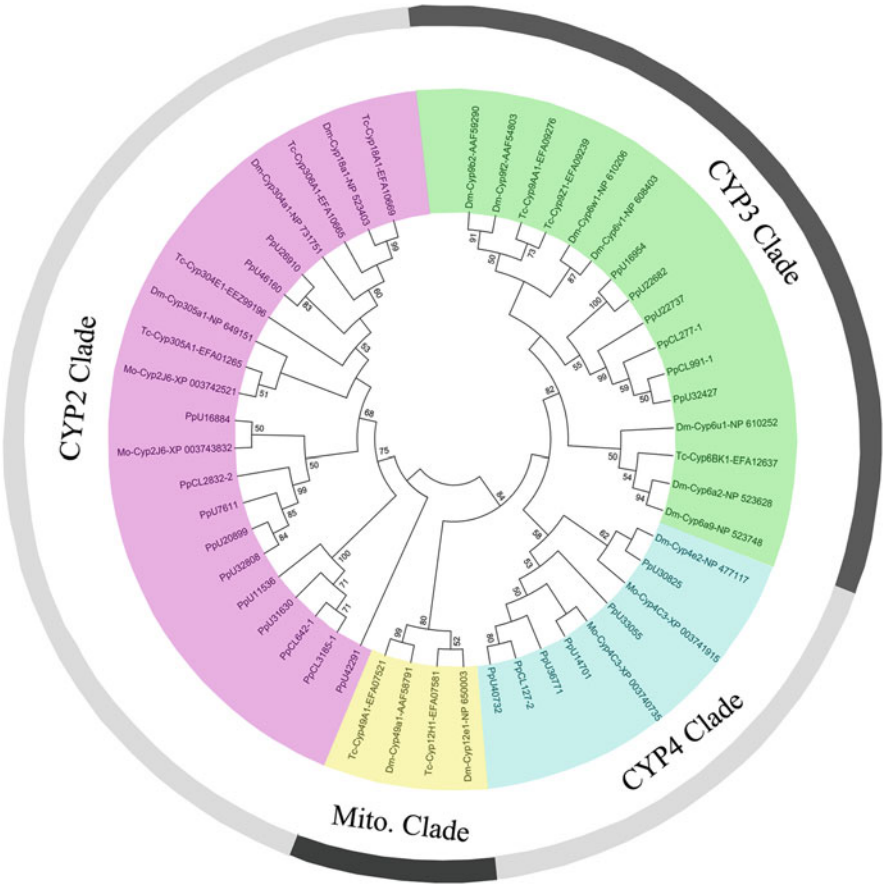

Fig. 2.1 Cytochrome P450 genes in *P. pseudoannulata*. (Source: Meng et al. 2015a)

mechanism (Misra et al. 2011). In peach potato aphid, *Myzus persicae*, insecticide detoxification by amplification of esterase is mainly determined by E4 and FE4 genes. In the absence of selection pressure among laboratory-selected populations, the aphids were reverted to susceptibility even after retaining amplified E4 genes; this was explained because of the decreased transcription in revertant aphids, leading to loss of detoxification enzyme production (Devonshire et al. 1998).

2.5 Transposable Elements (TEs)

Transposons, also known as 'mobile elements' or 'junk DNA' or 'selfish DNA' or 'jumping genes', are DNA sequences that are capable to transpose within the genome (Wilson 1993). It has been reported by Merrell and Underhill (1956) that insecticide resistance is an issue with the population showing more genetic

variability as compared to one with lower variability because more alleles will be available for selection in population with high genetic variability and transposable elements add to the genetic variability of the insect. In regulatory regions of the gene, TE insertion results in upregulation, which is caused because of built-in enhancer sequence in transposable elements (Zhang and Saier 2009). The studies of resistant genes have provided direct and indirect evidence supporting the role of TEs in the molecular resistance mechanism of insecticide (Rostant et al. 2012). For example, it was found in a study that xenobiotic-metabolizing P450 genes of both *Helicoverpa zea* and *Drosophila melanogaster* have TE insertion-rich regions (Chen and Li 2007).

2.6 Molecular Mechanism of Detoxification Enzymes

The FAO in its document on 'Guidelines on Prevention and Management of Pesticide Resistance' has described five categories of insecticide resistance mechanism, viz. (1) metabolic detoxification (enzymatic), (2) reduced sensitivity at target site, (3) reduced penetration, (4) sequestration and (5) behavioural resistance. Metabolic detoxification mechanism of enzymes, such as esterases, cytochrome P450 monooxygenases and glutathione *S*-transferases, is found to occur mainly in insects.

2.6.1 Cytochrome P450 Monooxygenases

This enzyme is a key component of the microsomal oxidase system and mitochondria in insects (Feyereisen 1999). These enzymes have also been mentioned as 'diversozymes' due to a diverse stoichiometry ranging from hydroxylation to epoxidation, *O*-, *N*- and *S*-dealkylations and *N*- and *S*-oxidations (Coon et al. 1996). P450 enzyme categorized into five insects specific six families, viz. CYP6, CYP9, CYP12, CYP18 and CYP28 and one family CYP4 from vertebrate (Feyereisen 1999). Studies on *Drosophila* revealed that when the flies were continuously selected with DDT, there was overexpression of *Cyp6g1* gene and the flies showing overexpression were also found to show cross-resistance with neonicotinoids, OP insecticides and growth regulators, such as lufenuron (Richard et al. 2004; Daborn et al. 2001, 2002).

2.6.2 Esterases

Two major enzymes belonging to the esterase family responsible for detoxification of insecticides are carboxylesterase and acetylcholinesterase (Kranthi 2005). Esterases regulate insecticide resistance by exhibiting insensitivity of the target enzyme (acetylcholinesterase) or by metabolic resistance mediated by carboxylesterase (Cui et al. 2015). Detoxification by esterase occurs via overexpression, which could be due to amplification or upregulation or both in combination (Panini

et al. 2016). Detoxification by amplification has been observed in *Myzus persicae*, *Culex* and *Nilaparvata lugens* (Bass et al. 2014; Hemingway et al. 2004; Small and Hemingway 2000) and by upregulation in *Aphis gossypii* and *Bemisia tabaci* (Cao et al. 2008; Alon et al. 2008).

2.6.3 Glutathione *S*-Transferase (GST)

GST-based insecticide resistance is mediated either directly by Phase I reactions or indirectly by Phase II reactions and ensures detoxification by neutralizing toxic chemicals to water-soluble compounds, finally leading to its excretion from the cells (Mannervik 1985; Habig et al. 1974). According to the location, insect GSTs are of two types, i.e. microsomal and cytosolic; however, it is the cytosolic GST that is vital for insecticide resistance (Panini et al. 2016). The genes related to insect GST can be divided into six families based on sequence similarity and substrate specificity, viz. delta, epsilon, omega, sigma, theta and zeta (Fang 2012). The modern approaches, like transcriptome analysis, forward and reverse genetics techniques and next-generation sequencing studies, have guided in-depth understanding of insecticide resistance mechanism facilitated by GSTs. In a recent study of gene knockdown by RNAi, Bt *GSTd7* gene was discovered to be responsible for imidacloprid resistance in *Bemisia tabaci* (He et al. 2018).

2.7 Epigenetics in Insecticide Resistance

Epigenetics may be described as changes in gene expression (but not gene sequence), ultimately leading to modified phenotype in response to intrinsic or environmental stimuli, which persist after cell division (Yan et al. 2015). There are three major epigenetic inheritance systems (Table 2.1).

Field et al. (1989) reported the first evidence of the role of epigenetics in insecticide resistance for peach potato aphid, *Myzus persicae*. Significance of epigenetics by modification of histone with acetyl group has been observed in honeybee, *Apis mellifera*, regulation of sodium butyrate, which acts as histone deacetylase inhibitor increase honeybee tolerance towards imidacloprid, which was otherwise found to be in low concentration in *A. mellifera* (Hu et al. 2017; Oppold and Muller 2017).

2.8 Genomic Studies of Insecticide Resistance in Some Important Insect Pests

2.8.1 Whitefly, *Bemisia tabaci* (Gennadius 1889)

Whitefly is an important invasive polyphagous pest infesting more than 500 crop plants (Cock 1993) and is a vector of one of the devastating yellow leaf curl and mosaic viral diseases in agronomically vital plants (Scholthof et al. 2011). On the

Table 2.1 Epigenetic inheritance systems (Glastad et al. 2019)

DNA methylation	Histone modification	Noncoding RNAs (ncRNAs)
Mediated by two classes of enzymes: 1. De novo DNA methyltransferase (DNMT3 protein) 2. Maintenance DNA methyltransferase (DNMT1 proteins) 3. DNA methylation occurs by addition of methyl group to cytosine residing in CpG Example: phenotypic plasticity in locusts and honeybees	The association between target histone and underlying DNA can be impacted by addition of acetyl, methyl or phosphorus groups Example: phase change in locusts (migratory and solitary)	These are a heterogeneous class of RNAs that are not translated into proteins: piRNA, microRNA, siRNA and long noncoding RNA Example: silk yield in *Bombyx mori* modulated by differentially expressed lncRNAs

basis of sequences of mitochondrial cytochrome oxidase I (MtCOI) gene, *B. tabaci* has been broadly classified into two globally important pest taxa: Middle East-Asia Minor 1 (MEAM1, formerly biotype B) and Mediterranean (MED, formerly biotype Q) (Liu et al. 2012). Whitefly genomic studies have explained the variability in the pests including the causes of invasiveness and insecticide resistance (Czosnek and Brown 2009). Chen et al. (2016) in their draft of whitefly genome have uncovered genomic mysteries of insecticide resistance in the pest and found that a total of 202 PEBPs are present in *B. tabaci* as compared to a maximum of 16 PEBPs reported in other 15 arthropods. The phosphatidylethanolamine-binding protein (PEBP) gene family has been found to occur in a wide range of organisms and is supposed to have a strong role in rapid evolution against insecticide resistance.

2.8.2 Tobacco Caterpillar, *Spodoptera litura* (Fabricius 1775)

S. litura is a highly polyphagous pest, which feeds on around 120 plant species (CABI Datasheet 2019). The pest has developed high resistance against insecticides and has been ranked at seventh position among the most resistant arthropods by IRAC (Sparks and Nauen 2015). The genomic information of *S. litura* provided an insight into the molecular mechanism of insecticide resistance of detoxification-related gene families. In a comparative study between highly polyphagous *S. litura* and almost monophagous *Bombyx mori*, expansion of chemosensory and detoxification-related gene families was observed in *S. litura* (Fig. 2.2) (Cheng et al. 2017).

Genomic annotation of the P450 genome in *S. litura* showed large expansions of P450 clan 3 and clan 4, and CYP9a especially was expanded greatly compared to other clans on exposure to insecticides (Cheng et al. 2017). It has also been confirmed in recent study the overexpression of *SlituCYP321b1* in the midgut of *S. litura* confirming its role in insecticide resistance (Wang et al. 2017).

	Family	Clan	*S.litura*	*B.mori*
Insecticide-tolerance gene families	**P450**		**138**	**83**
		Clan 3	61	32
		Clan 4	58	33
		Mitochondrial	11	11
		Clan 2	8	7
	GST		**47**	**26**
		ε	21	9
		δ	5	6
		ω	3	4
		σ	7	2
		θ	1	1
		ζ	5	2
		Microsomal	2	1
		Uncharacterized	3	1
	COE		**110**	**76**
		α-esterase	25	15
		Lepidopteran esterase	57	39
		JHE	8	7
		β-esterase	2	2
		Integument esterase	4	2
		Acetylcholinesterase	2	1
		Neuroligin	7	6
		Neurotactin	1	1
		Gliotactin	1	1
		Uncharacterized	3	2
		Clan 3	61	32
		Clan 4	58	33
		Mitochondrial	11	11
		Clan 2	8	7
	APN		**18**	**14**
	ABC		**54**	**51**
Chemosensory gene families	**CSP**		**23**	**21**
	OBP		**36**	**43**
	OR		**73**	**73**
	GR		**237**	**76**

Fig. 2.2 Comparison of detoxification and chemosensory gene families between the extremely polyphagous pest *S. litura* and the almost monophagous *B. mori*. (Source: Cheng et al. 2017)

2.9 Conclusions

Insecticide resistance management (IRM) has become an integral part of insect pest management and is a promising solution to challenging and widespread problem of insecticide resistance. Insecticide resistance gene database has become a boon to the researchers for conducting molecular studies on insect pests. Molecular mechanism

helps in the early detection of resistance in insects as compared to conventional methods and in increasing vigilance to avoid expression of resistance gene and in formulating timely management strategies and better IPM modules. The small sample size required in the molecular studies enhances the effectiveness of detecting resistant individuals, which is not possible with conventional methods and thus paving the way for the specific and accurate approach towards insecticide resistance management practices to overcome losses caused by insect pests and formation of selective solution for problem of insect pests.

Points to Remember

- Insecticide resistance is a new challenge in the management of insect pests and has become a global issue raising concern among the masses directly or indirectly related to its ill effects.
- Molecular assays by genotyping and sequencing have become a precise and timely assay methodology overcoming the limitations of conventional methods of insecticide resistance and described the resistance mechanisms via upregulation, amplification and structural changes.
- The application of first-generation and next-generation sequencing in addition to DNA barcoding opens vast possibilities of insecticide resistance observed in insects. Novel application of genome editing, like CRISPR/Cas9, has been successfully employed in the identification of modified target genes.
- Transposable elements and epigenetic studies comprehensively covered genomic studies of some major insect pests, like *S. litura* and *B. tabaci*, and thoroughly investigated and researched mechanism for insecticide resistance.

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Induced Resistance and Defense Primings

3

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Omkar (ed.), *Molecular Approaches for Sustainable Insect Pest Management*,
https://doi.org/10.1007/978-981-16-3591-5_3

Abstract

Priming is a phenomenon in which plants upon treatment with a resistance-inducing agent acquire an enhanced defensive capacity to respond faster and/or stronger at the moment that the plant is exposed to biotic or abiotic stresses. The priming can be found in different induced resistance systems to decrease lag time from the start of defense activation to the point when the defense is fully activated as well as to decrease the trade-off between induced resistance and the cost of defense activation. In addition, numerous chemical compounds, often of natural origin, have been found to act as priming stimuli. Priming also contributes in the existing relationship between members of a tritrophic system when plants upon damage by herbivorous arthropods release a mixture of HIPVs, green leaf volatiles (GLVs), terpenoids, and others to attract natural enemies of the

herbivores. Interestingly, when there is a strong selection pressure on plants, they can evolve mechanisms by which they pass the parental memory of herbivory to their progeny for enhanced defense, known as transgenerational priming. Heavy metals and some mineral elements like silicon can lead to priming in plants. Among different priming approaches, seed priming in which seeds expose to specific compounds to enhance seed germination was found to be a promising approach because it should enable seedlings to mount a robust immune response and thereby remain disease-free (or only moderately infected) for a long time with minimal labor and expense. However, although it has been reported that priming compared to elicitation generally results in low fitness costs for the plant, it could lead to the downregulation of some resistance pathways or could sensitize plants such that they respond to false alarm signals. Overall, new findings on priming and other upcoming techniques like symbiotic control and endophytes open a new era regarding biological control concepts in which not only natural enemies and pests are important, but also other factors like microorganisms that are in association with natural enemies (endosymbionts) and plants (endophytes) have a main important contribution.

Keywords

Induced resistance · Priming · Elicitor · Phytohormone · Crosstalk

Learning Objectives

1. For a long time, only synthetic pesticides have been used to control insect pests which has led to many problems in both human health and environmental pollution.
2. By recognizing inducible resistance mechanisms in plants and their role in induced resistance in plants, a new window was opened for scientists to use this potential against pests and diseases.
3. Synthetic analogs of endogenous phytohormones contributing in induced resistance were promising compounds to be practically involved in pest management.
4. Because the use of some resistance-induced compounds causes plants to cost part of their energy for resistance, scientists are looking for a way to do it with a lower cost.
5. With the discovery of priming and the possibility of minimizing plant costs, a new hope arises for pest and disease management.

3.1 Introduction

Protecting crops from insect pests and pathogens has become extremely important for food security worldwide. Pesticides are used by farmers globally to protect crops from pests and diseases, and they played important roles in “the green revolution” that brought huge benefits for agriculture and mankind. However, the excessive use of these compounds favors the development of resistant populations, rendering their

application counterproductive in the long term (Nombela and Muñiz 2009). Moreover, the conventional use of pesticides also has serious drawbacks as they contaminate the environment, cause fatalities, selection of pesticide-resistant strains and may foster a false sense of security regarding risks of pest outbreaks. Hence, both national and international authorities, e.g., FAO and EU, advocate development of alternative strategies (Song et al. 2017; Westman et al. 2019). Since plants lack an adaptive immune system, they have strong need for rapid detection of all kinds of pathogens. Therefore, plants have developed mechanisms to detect various forms of danger, including the attack by pathogens as well as tissue and cellular damage. The perception of defense-inducing molecular signatures like microbe-associated molecular patterns (MAMPs) and damage-/danger-associated molecular patterns (DAMPs) is viable for the fast initiation of defense responses. Regarding insect pests (Gully 2019), plants usually employ different defensive mechanisms against insects including constitutive or preformed factors such as physical barriers (cuticle, trichomes, spines, thorns, etc.) and stored insecticidal compounds. In addition, insect infestation also induces physical defenses in plants (Louis et al. 2015). Moreover, insect feeding on host plants activates different plant signal transduction pathways; therefore, the survival of plants depends on their ability to defend themselves through local and systemic responses with respect to an invasion or sensing of the presence of pathogens. Herbivory can induce both general and specific responses in plants that modify direct and indirect defenses against subsequent herbivory. The type of induction (local versus systemic induction, single versus multiple defense induction) may depend on both herbivore identity and relationships among different responses (Xiao et al. 2019). The defense signals triggered by pathogens and pests at the site of infection can lead to multiple protective responses against the invader and other unrelated pathogenic species (Pieterse et al. 2014).

Biotic stress induces the production of oxygen-derived radicals such as H_2O_2 (hydrogen peroxide), superoxide molecules, hydroxyl, and/or oxygen radicals which are the first lines of defense for a stressed plant (Nanda et al. 2010). Salicylic acid, jasmonic acid, and ethylene as well as substances like hydrogen peroxide and oxygen radicals are some certain plant hormones often implicated in the initiation and control of these phytodefense activities. They trigger the production of phytoalexins, callose depositions, cell wall thickening/strengthening, metabolite production, and pathogenesis-related protein synthesis. Together, these responses intercept and inhibit the action of the invading pathogens and pests (Vinale et al. 2008; Singh et al. 2016; Nie et al. 2017) generally known as induced resistance. Although induced resistance elicited by microorganisms in plants to other pathogenic microorganisms has been recognized for over 100 years (Chester 1933), knowledge of plant resistance induced by insect herbivores has had a much shorter history of $<$40 years (Green and Ryan 1972). However, the success of this defense response depends on the speed by which the plant recognizes the attacking pest or pathogen and the intensity by which the appropriate defense mechanism is activated. The effectiveness of this basal resistance can be enhanced by specific biotic or abiotic stimuli experienced by the plant before contact with the pest or pathogen (Pieterse et al. 1998; Zimmerli et al. 2000; Ton et al. 2002). In other words, plants

can acquire immunity upon perception of specific biotic and abiotic stimuli, a process mediated largely by priming of inducible defenses (Conrath et al. 2006). Immune priming enables faster and/or stronger induction of inducible defenses following subsequent pathogen or pest attack. The induced plants produce many compounds including alkaloids, phenolics, glucosinolates, betanins, terpenoids, cyanogenic glucosides, etc. that prevent further damage to them (Enebe and Babalola 2019).

In this chapter, we review the different types of inducible resistance, priming phenomenon, plant resistance inducers, and phytohormone interactions with emphasis on insect pest control, and finally, we provide a perspective on the future of these compounds if they want to be used in field conditions.

3.2 Type of Inducible Resistance

3.2.1 Systemic Acquired Resistance (SAR)

Plants have the ability to increase their level of basal resistance against future pest (Orlovskis and Reymond 2020) or pathogen attack upon appropriate stimulation. This phenomenon is known as induced resistance (Ton et al. 2009). Induced resistance is thought to be an adaptation to reduce the costs of expressing resistance-related traits in plants, particularly because threats from herbivores and pathogens can be highly variable in space and time (Karban 2011). However, the domestication has resulted in the loss of both basal and induced resistance in some crops. The effects of acibenzolar-*S*-methyl (ASM), as chemical plant resistance inducers, on wild and commercial accessions of common beans (*Phaseolus vulgaris*) against *Pseudomonas syringae* pv. *syringae* and *Enterobacter* sp. strain FCB1 showed that wild accessions had a higher basal defense and IR compared with commercial cultivars (Córdova-Campos et al. 2012). Inducible resistance is regulated primarily by three phytohormones, salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), which are interconnected by complex signaling networks and crosstalk phenomena (Pieterse et al. 2009). In addition, there are various types of chemical and biological compounds that can induce resistance in plants including β-aminobutyric acid (BABA), probenazole, saccharin, phosphite, biochar, mycorrhizal fungi, plant growth-promoting rhizobacteria (PGPR), plant growth-promoting fungi (PGPF), algal extracts, and elicitors from algae (Walters et al. 2013). Based on differences in signaling pathways and spectra of effectiveness, different types of induced resistance have been defined. The classic form of induced resistance is referred to as systemic acquired resistance (SAR) (the former type of IR has been called "localized acquired resistance" (LAR)). When a plant is infected by a pathogen or infested by an insect pest, it can develop resistance to a broad and distinctive spectrum of pathogens (Ryals et al. 1996; Orlovskis and Reymond 2020). The pathogen-induced resistance can be established in the tissue surrounding the site of initial infection and also in the distant, uninfected parts of the plants. Regarding insect pests, this resistance can develop to neighboring plants or may induce

intraplant SAR against the foliar pathogens, e.g., *Pseudomonas syringae* (Orlovskis and Reymond 2020). However, the identity of the long-distance signal(s) that travel (s) from the site of primary infection to the remote parts of the plant to induce pathogenesis-related gene expression and SAR is still unclear. In the early 1990s and by studies carried out on the transgenic tobacco and *Arabidopsis thaliana* plants that constitutively accumulate a salicylic acid (SA) hydroxylase of bacterial origin, it was clearly revealed that the plant hormone SA is required in the distal tissue for SAR to be expressed (Conrath 2009). This inference was confirmed by more recent work with *Arabidopsis* mutants affected either in the biosynthesis of SA or in SA signaling (Dong 2001). While the important role of SA in the development of SAR was without any controversy, it has remained unclear whether SA is the long-distance signal that travels from the site of primary pathogen infection or insect-infested leaf throughout the plant to induce SAR (Champigny and Cameron 2009). Some findings argued in favor of SA as a remote signal and others against it (Conrath 2009). Over the past few years, several other signaling molecules have been shown to be potential candidates for the endogenous long-distance signal for SAR including methyl salicylate (MeSA) (the methyl ester of SA) (Park et al. 2007); lipid-derived signaling molecules (Nandi et al. 2004), which include jasmonic acid (JA) (Truman et al. 2007) and azelaic acid (Jung et al. 2009); peptides (Xia et al. 2004); and reactive oxygen species (ROS) (Alvarez et al. 1998). Together, these findings argue that a complex and possibly variable combination of systemic signals may be required to fully induce the bona fide SAR response (Conrath 2009).

3.2.2 Induced Systemic Resistance (ISR)

Selected strains of nonpathogenic rhizobacteria such as *Pseudomonas*, *Bacillus*, or *Bradyrhizobium* can induce systemic resistance in both below- and aboveground parts of the plant. This form of induced resistance is often referred to as induced systemic resistance (ISR) (Pieterse et al. 1996; van Loon et al. 1998). In *Arabidopsis*, ISR triggered by *Pseudomonas fluorescens* WCS417r functions independently of SA, but requires an intact NPR1 protein and sensitivity to JA and ethylene (Pieterse et al. 1998). This form of ISR has a different spectrum of effectiveness than SAR and is predominantly effective against pathogens and insects that are sensitive to JA- and ET-dependent basal resistance (Ton et al. 2002). Unlike ISR against plant pathogens, in which more information has been presented over several decades, little information is available regarding the ISR activity against insect herbivores in comparison with the microbes in the soil (Rashid and Chung 2017). Herbivore-induced plant responses are generally organized via a complex network of interacting signaling pathways, orchestrated by several phytohormones, to activate attacker-specific defenses (Steenbergen et al. 2018). Based on available information, JA is the most important hormone in the regulation of plant defense against herbivores (Wasternack 2015). The activation of the JA signaling network leads to the production of various compounds that can serve as direct and/or indirect defenses (Howe and Jander 2008; Okada et al. 2015).

Salicylic acid, jasmonic acid, and ethylene are key plant hormones that regulate ISR during tritrophic interactions (Shavit et al. 2013). These hormone-dependent pathways can regulate defense responses in different ways against specific types of attacking insects (van Oosten et al. 2008). JA signaling is the main ISR pathway to be activated to defend plants against leaf-chewing insect pests and is triggered by root-associated microorganisms (van Oosten et al. 2008; Pineda et al. 2010). Below we can find a few examples of JA-mediated defenses against herbivorous insects. In one example, *Arabidopsis* roots treated with rhizobacteria induce resistance to chewing insects through the increased expression of the JA-dependent gene *LOX2*[1] and the JA- and ET-dependent genes *PDF1.2*[2] and *HEL* (Pangesti et al. 2015). The colonization of plant roots by rhizobacterium *P. simiae* WCS417r elicits higher expression of the JA-/ET-dependent ORA59 branch than the JA-dependent MYC2 branch and triggers ISR against leaf-chewing insects (Pangesti et al. 2016). Root colonization of cotton plants by plant growth-promoting rhizobacteria (PGPR) induces higher levels of JA, an octadecanoid-derived, defense-related phytohormone and JA-related genes, which may confer resistance against the leaf-chewing insect, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) (Zebelo et al. 2016). Using different mechanisms, *Bacillus subtilis* PGPR induces resistance against the *Bemisia tabaci* (Gennadius) (Hemiptera, Aleyrodidae) on tomato plants (*Solanum lycopersicum*), increased expression of both JA-independent genes (including photosynthetic genes, phenylpropanoid and terpenoid biosynthetic pathway genes) and JA-dependent genes including proteases and proteinase inhibitor coding genes (Valenzuela-Soto et al. 2010). However, all rhizobacteria do not behave in the same way, and some of them may cause their plant host to become susceptible to insects. For instance, Pineda et al. (2012) reported that *Arabidopsis* roots colonized by *P. fluorescens* WCS417r have enhanced susceptibility to the phloem-feeding aphid *Myzus persicae* (Sulzer) (Homoptera: Aphididae), although treated plants showed stronger expression of *LOX2* and *PDF1.2* gene following insect attack. Their outcomes showed that different rhizobacteria genera including *Bacillus* and *Pseudomonas* have different effects against phloem-feeding insects. In another instance, it has been revealed that thrips feeding activates the biosynthesis of JA (Abe et al. 2008, 2009) and the expression of JA-responsive genes (Abe et al. 2008, 2009; De Vos et al. 2005; Selig et al. 2016; Escobar-Bravo et al. 2017). From the total set of genes that are differentially expressed in *A. thaliana* during *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) feeding, 69% of the genes were JA responsive (De Vos et al. 2005). In Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) and tomato (*S. lycopersicum*), JA concentrations increased upon *F. occidentalis* infestation, corresponding with increased expression of JA-responsive marker genes (Li et al. 2002; Abe et al. 2009). The activation of the JA pathway most likely reinforces the plant's resistance to thrips, as exogenous application of JA reduces

[1]Lipoxygenase.

[2]Plant defensin 1.2.

plant susceptibility towards this herbivore (Abe et al. 2009), while plants insensitive to JA or deficient in JA accumulation are more susceptible to thrips (Abe et al. 2009).

3.2.3 Symbiotic Fungi

Associations of plants with some beneficial microorganisms other than those causing ISR can also result in systemic, broad-spectrum resistance. In an instance of this type of symbiosis-mediated inducible resistance created between barley roots and the endophytic basidiomycete *Piriformospora indica*, it could confer systemic resistance to insect pests (through promoting growth of the host plants and enhancing resistance to insects and tolerance to abiotic stress) (Gill et al. 2016) and various root and leaf pathogens (Waller et al. 2005) including the necrotrophic root-rot fungus *Fusarium culmorum* and the biotrophic fungus *Blumeria graminis* f. sp. *hordei* (Waller et al. 2005). The signaling mechanism by which *Pi. indica* induces resistance to these two pathogens in barley is unknown, but it seems to be independent of SA and JA while being associated with the activation of the glutathione–ascorbate cycle, indicating an increase in antioxidative capacity in *Pi. indica*-elicited IR (Waller et al. 2005). Systemic resistance induced by the endophytic fungus *Trichoderma asperellum* T34 conferred resistance to *Arabidopsis* against a wide range of pathogens through engagement of the same signaling components as used in *Pseudomonas fluorescens* (strain WCS417r)-mediated ISR (Segarra et al. 2009).

3.2.4 Chemicals

In addition to SAR, ISR, and symbiotic fungi-mediated induced resistance, some chemicals have an ability to induce resistance in plants. Some synthetic SA analogs, e.g., 2,6-dichloroisonicotinic acid and its methyl ester (both are referred to as INA), were the first synthetic compounds found to activate a phenocopy of bona fide SAR (Kessmann et al. 1994). Benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH; synonym: acibenzolar-*S*-methyl (ASM)) was a highly potent activator of SAR that was introduced a few years later (Conrath 2009). SA, INA (methyl ester), and BTH are assumed to activate SAR via a same signaling pathway (Ryals et al. 1996). Another type of induced resistance by applying chemicals has been reported through application of "β-aminobutyric acid (BABA)." The signaling pathway controlling BABA-induced resistance (BABA-IR) partially differs from that of SAR and ISR. Although BABA-IR against *P. syringae* pv. *tomato* depends solely on SA and NPR1 (Zimmerli et al. 2000), the BABA-IR against pathogenic fungi and oomycetes is controlled by a different defense pathway involving ABA- and phosphoinositide (PI)-dependent signaling (Ton and Mauch-Mani 2004; Ton et al. 2005). In *Arabidopsis* BABA-triggered resistance to pests, it is associated with a major metabolic shift that includes pipecolic acid (PA) accumulation. PA is considered a critical endogenous signal for priming (Conrath et al. 2015). BABA-IR is effective against both biotrophic and necrotrophic pathogens, as well as some

types of abiotic stress (Zimmerli et al. 2000, 2001; Cohen 2002; Ton and Mauch-Mani 2004; Jakab et al. 2005; Ton et al. 2005).

3.2.5 Resistance Induced by Wounding

It is generally assumed that physical injury can make living plant tissue prone to pathogen or pest invasion. However, over the past few years, it has become increasingly clear that wounding (whether caused by mechanical damage or feeding by herbivorous insects) can also serve as an effective stimulus for the induction of local and systemic resistance to microbial pathogens or herbivorous insects through direct activation of many genes, including those encoding protease inhibitors. These proteins can inactivate enzymes with important roles in either disease symptom development or digestion of plant tissue in the insect gut. Either the role of several compounds of the octadecanoid pathway (e.g., JA) or another JA-independent pathway can induce resistance by wounding indicate a complex nature of the wound response in plants (Conrath 2009).

3.2.6 Resistance Induced by Modifications of Primary Metabolism

Although it has been revealed that photosynthesis, partitioning of assimilates, and source–sink relationships can be affected in plants exposed to biotic or abiotic stresses (Schwachtje and Baldwin 2008), little is known about the impact of primary plant metabolism on IR in plants. One frequently reported resistance phenotype in plants is the so-called high-sugar resistance. This type of IR is associated with elevated levels of soluble carbohydrates which result from certain alterations in primary metabolism. The concept of "high-sugar resistance" has been supported by various studies demonstrating that application of sugar to various plant tissues, or provoking the accumulation of sugar in transgenic plants, can lead to activation of various PR genes (Conrath 2009). Similarly, there are other findings about resistance induced by modifications of primary metabolism, e.g., increased levels of soluble carbohydrates, resulting from certain alterations in primary metabolism an IR response to the soft-rot-causing bacterium *Pectobacterium atrosepticum* and the fungal pathogen *Alternaria solani* in the tubers, and with IR to the late blight-causing oomycete *Phytophthora infestans* in the leaves. Therefore, it is inferred that certain alterations in plant primary metabolism can cause tissue-specific resistance to a variety of biotic challenges (Conrath 2009).

3.2.7 Volatile Organic Compounds (VOCs)

Finally, there is increasing evidence that volatile organic compounds (VOCs) that are emitted by plants upon insect infestation have the ability to induce resistance in

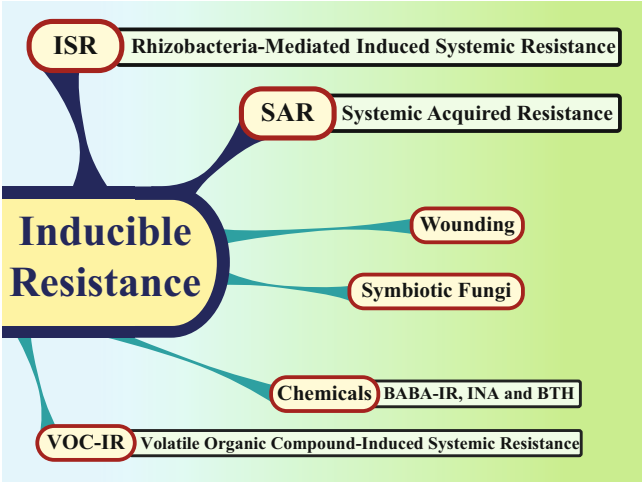

Fig. 3.1 Different types of inducible resistance including SAR (a pest-infested plant develops resistance to neighboring uninfested plants or other pathogens); ISR (induced resistance against the *Bemisia tabaci* on tomato plants using *Bacillus subtilis* PGPR); symbiotic fungi (associations of plants with some beneficial microorganisms, e.g., *Piriformospora indica*, confer systemic resistance to insect pests through promoting growth of the host plants); chemicals (induced resistance by applying some synthetic compounds); wounding (local and systemic resistance to microbial pathogens or herbivorous insects as a result of wounding); primary metabolism modification-mediated resistance (induced resistance as a result of modifications of primary metabolism, e.g., increased levels of soluble carbohydrates); VOCs (induced resistance in undamaged neighboring plants through damaged plants emitted volatile organic compounds caused by insect infestation)

neighboring plants against future attack by insects and pathogens (Kishimoto et al. 2005; Baldwin et al. 2006) (Fig. 3.1).

3.3 The Difference Between SAR and ISR

While ISR is elicited in response to plant growth-promoting rhizobacteria (PGPR) and is mediated primarily by JA and ET signaling pathways, SAR is elicited in response to chemical triggers, a wide range of necrotizing pathogens, and egg laying of insects (Ryals et al. 1996; Pieterse et al. 2009; Orlovskis and Reymond 2020), and it is mediated by SA signaling (van Loon et al. 2006; Hammerschmidt 1999). Generally, JA-mediated ISR responses are directed against herbivores and necrotrophic pathogens, whereas SA-mediated SAR responses are directed against biotrophic pathogens (Bostock 2005; Heil and Bostock 2002). SAR occurs after the hypersensitive response (HR), which is a highly specific interaction between a plant resistance protein and a pathogenic avirulent protein, leading to programmed cell death and pathogen growth arrest in infected plant tissue (Mysore and Ryu 2004; Jourdan et al. 2009; Zeidler et al. 2004). In contrast, ISR does not require HR.

Induced defense responses such as SAR are generally linked with allocation costs in the form of reduced growth and reproduction (Cipollini et al. 2003). For instance, benzothiadiazole (BTH) is a classic example of a chemical trigger used to elicit SAR that inflicts a growth penalty (Heil et al. 2000). This phenomenon is called "allocation fitness cost" or "trade-off" (Heil 2001). Growth reduction is attributed to the competing demands between metabolic biosynthetic pathways and the energy required for induced defense responses (Heil and Baldwin 2002). However, some elicitors used for other induced defense responses such as ISR are not associated with allocation fitness costs.

3.4 The Role of Secondary Metabolites Against Insect Pests

As a result of the invasion of herbivore attackers, various pathways are activated to induce resistance which one of them is the production of secondary metabolites. The biosynthesis of several secondary metabolites is constitutive, whereas in many plants it can be induced and enhanced by biological stress conditions such as wounding or infection (Pino et al. 2013). Secondary metabolites are low-molecular-weight (LMW) compounds involved in defense against insect pests classified according to their biosynthetic pathways and include terpenoids (>40,000 known structures from the isoprenoid pathway; Keeling and Bohlmann 2006; Mazid et al. 2011), phenolic compounds (>8000 known structures from the phenylpropanoid pathway; Bernards and Båstrup-Spohr 2008; Keeling and Bohlmann 2006; Mazid et al. 2011), and alkaloids (>12,000 known structures from the alkaloid pathway; Facchini 2001). Terpenes have 5-C isoterpenoid as their basic unit that are toxins and deters herbivores (Zaynab et al. 2018). In plant–insect pest interactions, LMW compounds that are synthesized de novo upon infestation are described as phytoalexins, while pre-existing LMW compounds are called phytoanticipins (Yactayo-Chang et al. 2020). Secondary metabolites do not reduce plant growth and development but affect the forage value of plant tissues where they are produced. These are either induced by microbes and insects (phytoalexins) or stored inactively (phytoanthines) (Table 3.1) (Zaynab et al. 2018).

The enhanced biosynthesis and accumulation of oleoresin as a complex mixture mainly consisted of monoterpenes and diterpenes and smaller amounts of sesquiterpenes and other compounds (e.g., phenolics) (Keeling and Bohlmann 2006), in many conifer species are integral components of the induced chemical defense system against pathogens (Zeneli et al. 2006) and insects (Franceschi et al. 2005; Keeling and Bohlmann 2006). Oleoresin is a viscous liquid produced in the resin ducts and related secretory structures of foliage, stems, and other organs. The induced oleoresin functions as a direct toxin by readily interacting with the cell membranes of the invasive organism, which can lead to uncontrolled cell leakage, finally resulting in cell death. Invaders are also expelled from the tree in the flow of the oleoresin or trapped within the exudate as the wound is sealed by crystallization.

Table 3.1 List of plant secondary metabolites, their categories, and the insect pest species targeted (Zaynab et al. 2018)

Secondary metabolites	Plants	Categories	Induce resistance against
Terpenoids	Citrus	Terpenoid, limonene	*Atta cephalotes*
Steroids	Common fern	Phytoecdysones	*Insect*
Terpenoids	Tobacco	Trans-anethole and thymol, citronellal	*Spodoptera litura*
Phenolics	Wheat	Phenolics	*Rhopalosiphum padi*
Phenolics	Willow plant	Phenolics	*Galerucella lineola*
Benzoic acid	Salix	Benzoic acid	*Operophtera brumata*
Phenolics	Strawberry	Phenolics	*Tetranychus urticae*
Phenolics	Cotton	Gossypol	*Heliothis virescens*, *Heliothis zea*
Alkaloids	Nightshade potato	Alkaloid demissine	*Leptinotarsa decemlineata*
Benzoxazinoids	Gramineae	DIMBOA	*Ostrinia nubilalis*
Cyanogenic glucosides	Cassava	CNglcs	*Cyrtomenus bergi*
Cyanogenic glucosides	Bitter almond plants	Amygdalin and prunasin	*Capnodis tenebrionis*
Cyanogenic glucosides	*Trifolium repens*	Amygdalin and prunasin	*Hypera postica*
Cyanogenic glucosides	Lotus	Cyanogenic glucosides	*Zygaena filipendulae*
Cyanogenic glucosides	*P. lunatus*	CNglcs	*Spodoptera eridania*

3.5 History of Priming

Plants possess a remarkable capacity to perceive numerous environmental signals that allow them to respond to their surroundings. Stimuli from pathogens, beneficial microbes, or arthropods, as well as chemicals and abiotic cues, can trigger the establishment of priming by acting as warning signals (Mauch-Mani et al. 2017). For many years, IR in plants has been suggested to be on the basis of the direct activation of defense responses in the systemic tissue of pathogen-infected or pest-infested plants. In the case of SAR, these directly induced responses in the systemic tissue include the accumulation of PR proteins (Ryals et al. 1996). However, when it was determined that the expression of cloned genes for PR proteins in transgenic plants does not generally lead to enhanced resistance against diverse pathogens, the actual contribution of PR proteins to IR appears to be minor (van Loon 2000). In addition, as research on IR had focused primarily on the role of PR proteins and other directly induced defense-related compounds, it has not been widely appreciated that the enhanced defensive capacity characteristic of IR is also associated with a

sensitized state in which the plant responds more rapidly and/or more robustly with the activation of defense responses after exposure to a biotic or abiotic stressor (Conrath 2009). The state of enhanced capacity to activate stress-induced defense responses has been called the "primed" (or "sensitized") state of the plant. Kuć (1987) was the first person who argued that priming would be an important component of SAR. Yet, although priming could be a unifying mechanism for the different types of IR in plants, the phenomenon did not attract much attention at the time (van Loon 2000). In the 1990s, an important role of priming in SAR was supported by the finding that showed a close correlation between the capability of various chemicals to activate resistance against *tobacco mosaic virus* (TMV) in tobacco (Conrath et al. 1995) and their capacity to prime for enhanced phenylalanine ammonia lyase (PAL) gene expression induced by microbe-associated molecular pattern (MAMP) elicitor treatment in cultured parsley cells (Thulke and Conrath 1998), or upon infection of *Arabidopsis* plants with *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst*) (Kohler et al. 2002). Since the first systematic review of priming in plant cell suspension culture by Kauss et al. (1992), various examples of priming have been reported in plants against pathogens, insects, and abiotic stress (van Hulten et al. 2006). Hence, priming appears to be a common feature of a plant's immune system that offers protection against a wide spectrum of environmental stresses (Conrath et al. 2006). Mur et al. (1996) provided the first in-depth analysis of the priming phenomenon in intact plants by soil drench pre-application of SA to transgenic tobacco plants expressing chimeric *PR-1::GUS or PAL-3::GUS* defense genes and declared that it did not cause significant gene activation. Although priming of defense against biotic stresses has a longer history in the plant–pathogen interactions (Conrath 2009), in the plant–insect interactions, just in the last two decades valuable information has been provided in this regard including information regarding priming of defense mediated by herbivore-inducible plant volatiles (HIPVs) that are produced and released by the neighboring plants (or plant parts) under herbivore attack; priming of defenses across generations (Rasmann et al. 2012); insect oviposition; seed treatment with plant defense elicitors (Worrall et al. 2012), and by heavy metal stress, etc. In addition to recording numerous cases of priming in various plants, recently, worth information is available on the molecular mechanisms underlying the priming of defense in the plant–insect interactions (Conrath 2011; Jaskiewicz et al. 2011; Rasmann et al. 2012).

3.6 What Is Priming

Plants have evolved a variety of antiherbivore defenses through the interactions with herbivorous insects over evolutionary time. Main plant defensive mechanisms can be categorized into either constitutive or induced defense by whether a given environmental stress elevates the basal level of resistance to the stress (Kim and Felton 2013). With induced defenses, plants are allowed to manage resources flexibly between defense and growth by eliciting antiherbivore defense only when necessary, although following initial damage there is a lag time from the start of

defense activation to the point when the defense is fully activated (Karban 2011). Priming is a phenomenon in which plants upon treatment with a resistance-inducing agent acquire an enhanced defensive capacity, resulting in a faster and/or stronger defense reaction at the moment the plant is exposed to biotic or abiotic stresses. In other words, priming is a process of sensitizing and preparing the plant's defense responses to be faster and stronger to future herbivorous insect threats (Pappas et al. 2017; Ye et al. 2013). Regarding insect pests, when plants anticipate herbivory in the future through the perception of indicative signal cues or the experience of herbivory at their parental generation, plants are physiologically prepared and induce stronger and faster defenses upon the anticipated herbivory. In this situation, plants are better defended against the insect herbivores with enhanced resistance and/or with reduced lag time (Karban 2011). It works in favor of the plant because when the expected herbivory does not ensue, plants would not waste resources because the cost of priming itself is considered moderate (van Hulten et al. 2006) and may allow the primed state to remain efficient for a longer time (Kim and Felton 2013). Increased plasticity of induced defense by priming has another advantage because it may also reduce the possibility of development of counteradaptive strategies by insect herbivores (Kim and Felton 2013). As mentioned, priming of defenses can occur after exposure to induced plant volatiles from adjacent plants through exposure to other (synthetic) elicitors such as beta-aminobutyric acid (BABA) or through the addition of rhizobacteria, indicating priming can occur in all forms of inducible resistance (Engelberth et al. 2004; Heil and Bueno 2007; Ton and Mauch-Mani 2004) and it can be applied to various tissues and at diverse developmental stages (e.g., to foliage or roots of mature plants or to seeds) (Westman et al. 2019). Induction of the primed state may be mediated by an enhanced accumulation of signaling compounds, such as transcription factors (TFs), that remain inactive until the plant is exposed to stress (Ton et al. 2009). These properties could make priming a promising add-on for fine-tuning the application of induced defenses in horticulture or agriculture without compromising crop production (Heil and Kost 2006; Martinez-Medina et al. 2016; Pappas et al. 2017). Although priming has been known to occur in plants for decades, most progress in the understanding of this phenomenon has been made over the past few years. Recent insights in the mechanisms behind systemic acquired resistance (SAR), β-aminobutyric acid-induced resistance (BABA-IR), rhizobacteria-mediated induced systemic resistance (ISR), and volatile organic compound-induced resistance (VOC-IR) against insects have revealed various priming mechanisms that protect against different stresses which are described below (Fig. 3.2).

3.6.1 Priming in SAR

It has been demonstrated that a soil drench pretreatment with SA of transgenic tobacco plants did not significantly induce gene activation, while after infection with *Ps. syringae* pv. *syringae* or after wounding, activation of the reporter gene was much stronger in the SA-pretreated plants than that of the plants not pretreated with

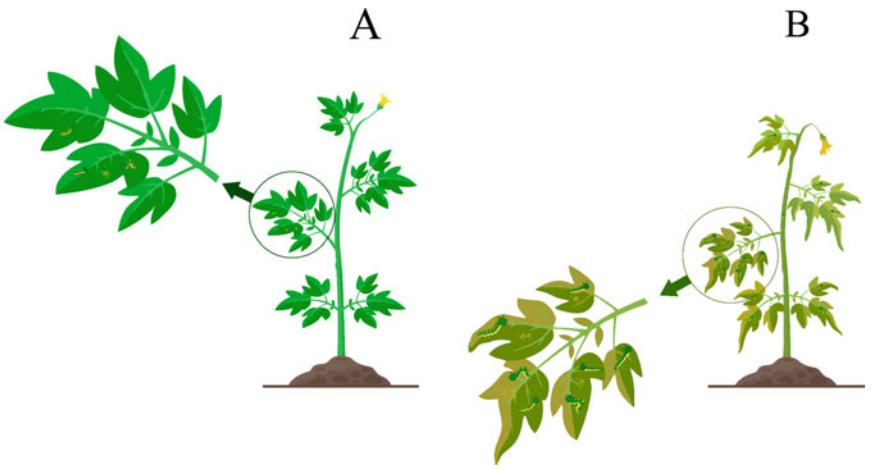

Fig. 3.2 Primed plants (**a**) vs. unprimed plants (**b**). The primed plants have the ability to decrease pest damage via different pathways including produced secondary metabolites, etc. The pests fed on primed plants have lower weight and lay fewer eggs. In contrast, unprimed plants become weak and wither as a result of pest attack. The pests fed on these plants gain more weight in the absence of antibiosis and produce more eggs

SA (Mur et al. 1996). In *Arabidopsis*, van Wees et al. (1999) demonstrated that induction of SAR by infection of *Arabidopsis* leaves with avirulent $Pst_{avrRpt2}$ led to priming of the systemic tissue, exhibited as elevated expression levels of PR genes. Pretreatment with BTH likewise primed *Arabidopsis* for more robust induction of the PAL gene by *Pst* (Kohler et al. 2002). In other species, observations were similar to those made with the parsley cell culture and tobacco and *Arabidopsis*. For instance, pretreatment with physiological concentrations of SA had negligible effects on soybean cell suspension cultures. However, when the SA-pretreated cells were challenged with an avirulent strain of *Ps. syringae* pv. *glycinea*, the activation of defense genes, the oxidative burst, and cell death were markedly enhanced (Conrath 2009).

Regarding insect pests, it has been revealed that insect eggs are recognized by plants and induce direct and indirect defenses. For insects, the site of oviposition is determinant for the hatching progeny, and any mechanism enhancing larval survival may be favored. Some studies have shown that previous oviposition affects the performance of hatching larvae, although the effect is variable across plant species. In *Arabidopsis*, insect eggs provoke cellular and molecular changes that are observed during infection with biotrophic pathogens. Indeed, oviposition by *Pieris brassicae* (L.) (Lepidoptera: Pieridae) triggers localized necrosis, accumulation of reactive oxygen species, and expression of hundreds of genes that are drastically distinct from those differentially regulated after larval feeding. Strikingly, egg-induced transcriptional profile is enriched with genes regulated by the salicylic acid (SA) signaling pathway. Accordingly, oviposition by *P. brassicae* leads to SA accumulation in local and systemic leaves, and crude egg extract (EE) activates

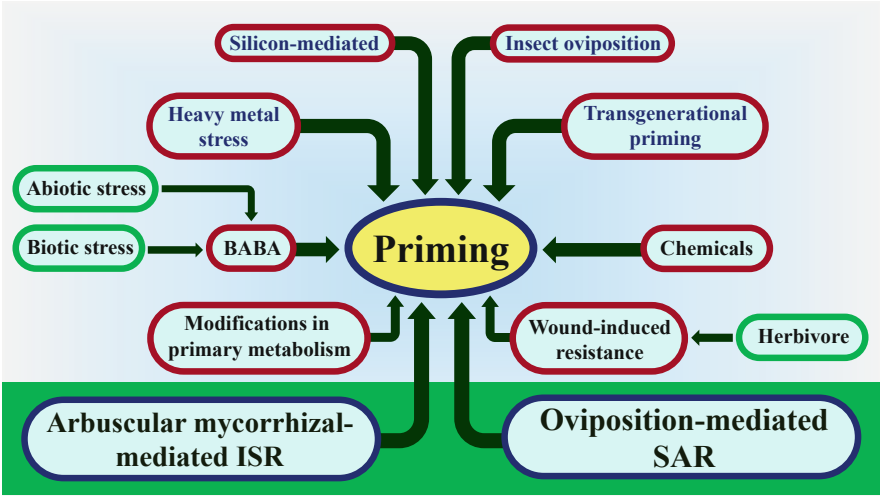

Fig. 3.3 Priming in the different inducible resistance mechanisms (SAR and ISR). Plants can also be primed by beneficial microorganisms; chemicals, nutrient elements, e.g., silicon; heavy metals; insect oviposition and transfer of the parental immunological experience to its progeny

expression of SA- and innate immunity-dependent genes. EE application enhances further larval performance of the generalist *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) by suppressing expression of jasmonic acid (JA)-dependent genes. This effect is lost in the SA biosynthesis-deficient *sid2-1* mutant, illustrating the known antagonistic interaction between SA and JA pathways, and suggests that insect eggs may in some cases use the SA pathway to dampen defenses against generalist larvae (Orlovskis and Reymond 2020).

Recently, it has been revealed that oviposition induces a SAR in *Arabidopsis*. When plants were pretreated with intact eggs or EE, the growth of the bacterial pathogen *P. syringae* pv. *tomato* DC3000 (*Pst*) was significantly inhibited in local and distal leaves. By reducing bacterial infection on the plant, this egg-induced SAR may prove beneficial for hatching larvae. It was indeed shown that *P. brassicae* larval performance was reduced on *Arabidopsis* plants infected with *Pst* and that this effect was less pronounced when plants were pretreated with EE (Orlovskis and Reymond 2020) (Fig. 3.3).

3.6.2 Priming Induced by Beneficial Microorganisms (Priming in ISR)

Beneficial microbes including plant growth-promoting rhizobacteria (PGPRs) and plant growth-promoting fungi (PGPFs), both of which can induce systemic resistance, can also trigger defense priming. The subtle costs associated with these interactions are insignificant under pathogenic pressure, and many studies have

provided evidence that the induced resistance they trigger is based on priming (Mauch-Mani et al. 2017). The genera *Pseudomona*s, *Serratia*, and *Bacillus* are the most studied PGPRs, and *Trichoderma* spp., nonpathogenic strains of *Fusarium* spp., *Piriformospora indica*, and arbuscular mycorrhizal fungi (AMFs) from the genus *Glomeromycota* are the most studied PGPFs. The goal of the initial chemical interplay between microbe and plant is the establishment of symbiosis. However, the involved signals can also serve as stimuli for defense priming (Mauch-Mani et al. 2017). The first evidence that priming of plant defense responses is involved in ISR came from experiments with carnation (*Dianthus caryophyllus*) in which inoculation with *F. oxysporum* f. sp. *dianthi* of carnation plants displaying ISR led to a faster rise in phytoalexin levels than in noninduced control plants. Similarly, *Bacillus pumilus* (strain SE34) induced systemic resistance against the root-rot fungus *F. oxysporum* f. sp. *pisi* in bean (Conrath 2009). There are beneficial microorganisms other than PGPR which can trigger priming phenomenon. For instance, colonization of tomato roots by mycorrhizal fungi protected the plant systemically against *Phytophthora parasitica* with no detectable accumulation of PR proteins before pathogen assault. Only after *Ph. parasitica* attack, mycorrhizal plants accumulated significantly more PR-1a and basic β-glucanases than nonmycorrhizal plants (Conrath 2009).

In this study, the tripartite interaction between potato, the arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis* (Glomerales: Glomeraceae), and cabbage looper (*Trichoplusia ni* Hübner) (Lepidoptera: Noctuidae) was examined to determine whether potato exhibits mycorrhiza-induced resistance against this insect. Plant growth, insect fitness, AM fungal colonization of roots, and transcript levels of defense-related genes were measured in shoots and roots after 5 and 8 days of herbivory on mycorrhizal and nonmycorrhizal plants. AM fungal colonization of roots did not have an effect on potato growth, but root colonization levels increased by herbivory. Larval weight gain was reduced after 8 days of feeding on mycorrhizal plants compared with nonmycorrhizal plants. Systemic upregulation of *Allene Oxide Synthase 1 (AOS1)*, *12-Oxo-Phytodienoate Reductase 3 (OPR3)* (jasmonic acid pathway), *Protease Inhibitor Type I (PI-I)* (antiherbivore defense), and *Phenylalanine Ammonia Lyase (PAL)* transcripts (phenylpropanoid pathway) was found during the tripartite interaction (Schoenherr et al. 2019). In another study, Serteyn et al. (2020) showed that induced systemic resistance by a PGPR impacts the development and feeding behavior of *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae) (Fig. 3.3).

3.6.3 Priming in BABA-IR

3.6.3.1 Priming Against Biotic Stress

Research on the mechanism(s) of BABA-IR in different plant species has shown that this type of IR is frequently associated with priming for various pest- or pathogen-induced defense responses. For example, BABA application improves soybean resistance to aphid through activation of phenylpropanoid metabolism and callose deposition (Yao et al. 2020). They found that the application of BABA effectively

enhanced soybean resistance against *Aphis glycines* Matsumura (Hemiptera: Aphididae), the soybean aphid. Consistent with significantly increased content of isoflavones, especially genistein, the related biosynthetic genes were upregulated by the use of BABA. Lignin, another important defense component against arthropods, accumulated at a high level, and four lignin biosynthesis-related genes were also activated. Additionally, BABA application augmented the expression of callose synthase genes and increased callose deposition in the soybean aphid (SBA)-infested seedlings. In non-caged and caged tests, SBA numbers were significantly reduced in BABA-treated seedlings.

In another study, Hodge et al. (2005) applied BABA as a soil drench to legumes and monitored its effects on the pea aphid *A. pisum* and found on tic bean (*Vicia faba* var. *minor*), BABA increased aphid mortality, caused a reduction in the mean relative growth rate of individual insects, and lessened the intrinsic rate of population increase (*r*). BABA also caused significant reductions in the growth rate of *A. pisum* on pea (*Pisum sativa*), broad bean (*Vicia faba* var. *major*), runner bean (*Phaseolus coccineus*), red clover (*Trifolium pratense*), and alfalfa (*Medicago sativa*). No direct toxic effects of BABA against *A. pisum* were found, and no phytotoxic effects that may have caused a reduction in aphid performance were detected.

In the plant–pathogen interactions, in *Arabidopsis*, BABA-IR to *Hyaloperonospora arabidopsidis* coincided with fast and robust deposition of callose-containing papillae (Zimmerli et al. 2000). This correlation between BABA-IR and augmented papillae formation was intensively studied using the interaction of *Arabidopsis* with the two necrotrophic fungi *Alternaria brassicicola* and *Plectosphaerella cucumerina*. The use of various *Arabidopsis* mutants indicated that neither the phytoalexin camalexin nor SA-, JA-, or ET-dependent defense responses seem to play a critical role in BABA-IR to these two necrotrophic pathogens (Ton and Mauch-Mani 2004). Cytological investigations at sites of attempted penetration by *A. brassicicola* and *Pl. cucumerina* demonstrated that the formation of callose-rich papillae was increased in attacked epidermal cells of BABA-pretreated plants (Ton and Mauch-Mani 2004) (Fig. 3.3).

3.6.3.2 Abiotic Stress and Abiotic Stimuli

It is known that SA and its derivative acetyl-SA (aspirin) can protect various plants from abiotic stresses, such as chilling, heat, drought, and wounding (Kohler et al. 2002). However, much more information is available for the BABA-induced protection from abiotic stress. For instance, BABA is known to protect *Arabidopsis* from heat (Zimmerli et al. 2008), drought, and salt stress (Jakab et al. 2005). The BABA-induced tolerance to the latter two abiotic stresses correlated with primed expression of SA- and ABA-responsive genes upon exposure of the BABA-pretreated plants to drought or salt (Jakab et al. 2005). In another point of view, abiotic stresses themselves can trigger priming. In a study carried out on *Arabidopsis*, it was demonstrated that repetitive exposure of a plant to mild abiotic cues, such as heat, cold, or salt, can enhance resistance against virulent *P. syringae* pv. *tomato* (*Pst*) DC3000 by acting at the epigenetic level. Interestingly, when plants were subjected to long-term exposure or high salt concentrations, priming did not occur. Different

forms of abiotic stimulation can also induce resistance in plants including mechanical stimulation by repetitive leaf rubbing or bending and wounding. In addition, submergence and exposure to ultraviolet light or ozone can induce protection against pathogens, although the role of defense priming is not clear (Mauch-Mani et al. 2017) (Fig. 3.3).

3.6.4 Priming in Wound-Induced Resistance

3.6.4.1 Priming in IR to Herbivores and Arthropod-Derived Stimuli

Plants upon damage by herbivorous arthropods release a mixture of HIPVs, green leaf volatiles (GLVs), terpenoids, and others to attract natural enemies of the herbivores (McCormick et al. 2012). HIPVs also contribute as between- and within-plant signaling cues and induce or prime defensive responses in neighboring intact plants or intact plant parts on the same plant (Engelberth et al. 2004). Volatiles produced upon mechanical damage and insect feeding and several GLVs among them are capable of priming defenses. Plants prime a variety of defensive responses to HIPVs including accumulation of JA, linolenic acid (precursor of JA and GLVs), plant secondary metabolites, increased protease inhibitor activity, enhanced transcription of antiherbivore defense genes, emission of HIPVs, secretion of extrafloral nectar (EFN; extra sugar source to attract general predators such as ants on plants), reduced herbivore performance, and attraction of natural enemies of herbivores (Kim and Felton 2013). HIPV-mediated priming was found in various types of plants (not yet in flowerless plants). HIPV-based signal cues were effective in the laboratory, growth chamber, greenhouse, and natural environment (Heil and Kost 2006). Green and Ryan (1972) found that insect feeding on potato and tomato plants activates local and systemic accumulation of proteinase inhibitors that hinder the activity of digestive proteases in the insect gut. Pechan et al. (2002) showed the enhanced resistance to caterpillars in the insect-resistant Mp708 due to Mir1-CP accumulation at the site of insect feeding. Mir1-CP attacks the lepidopteran peritrophic matrix (PM) that protects the caterpillar midgut (Pechan et al. 2002). Constitutive low levels of Mir1-CP and *mir1* transcripts were detected in Mp708 plants prior to insect attack (Harfouche et al. 2006). After caterpillar feeding, Mir1-CP accumulates at elevated levels at the site of insect infestation within 1 h and also in the vascular tissues (Lopez et al. 2007). Accumulation of Mir1-CP in the vascular tissues showed that Mir1-CP can also potentially function as a phloem-mobile protein.

Chassot et al. (2008) illustrated wound-induced local and systemic resistance to pathogenic microorganisms or herbivorous insects and demonstrated that wounding leaves either by squeezing with a pair of forceps or by puncturing holes with a needle induces resistance to the gray mold fungus *B. cinerea* in *Arabidopsis*. They showed that this wound-induced resistance (WIR) appears to require neither SA nor JA or ET, and it rather needs glutathione and the phytoalexin camalexin (Chassot et al. 2008). In response to wounding or herbivore attack, plants often release extrafloral nectar or VOCs. Some of these serve to attract parasitic or predatory natural enemies of the herbivores, and others have a role in the activation of resistance in the same

(Heil and Bueno 2007) or even nearby, unharmed plants (Conrath 2009). During the past years, there was strong evidence priming derived VOC-mediated IR. In a study, Engelberth et al. (2004) showed that when maize seedlings were exposed to certain volatiles from neighboring plants and subsequently challenged by a combination of mechanical damage and exposure to regurgitant of caterpillars of the beet armyworm (*S. exigua*), they had higher production of volatile sesquiterpenes and JA compared with triggered plants not exposed to the volatiles before. In a follow-up study, it was shown that the VOC-induced priming for augmented induction of defense genes and emission of aromatic and terpenoid volatiles in maize correlates with reduced caterpillar feeding and enhanced attraction of the parasitoid wasp *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) (Conrath 2009).

Herbivore-associated stimuli can be of biological or physical origin. Biological stimuli include oral secretions, insect-associated microbes, insect-associated molecular patterns (IAMPs), and oviposition signals, and physical signals consist of spatiotemporal repeated patterns and trichome sensing of insects walking on leaf surfaces. Moreover, herbivore-induced plant volatiles have been described as elicitors of priming because they act as stimuli to neighboring plants. All of these stimuli are produced during challenge with an arthropod, which obviously triggers direct defenses in the plant, but when these physical or biological stimuli are used experimentally, they can induce a faster and/or stronger defensive behavior in the attacked plants (Mauch-Mani et al. 2017).

In oral secretions, plants will be able to distinguish mechanical wounding from insect herbivory. Among the many components found in caterpillar secretions, fatty acid–amino acid conjugates are responsible for triggering specific responses in attacked plants. Volicitin was one of the first fatty acid–amino acid conjugates reported in lepidopteran larvae. In addition, insect oral peptides and sulfated fatty acids act as primary stimuli in insect–plant interactions. Although oviposition signals are considered as herbivore-associated stimuli to trigger priming, it may play a dual role depending on the challenge that follows the stimulus (i.e., they can induce priming or the suppression of host defenses according to the subsequent challenge) (Mauch-Mani et al. 2017).

In addition to the stimuli described above, trichomes can perceive insect contact and prepare the plant to defend against herbivore attack. Moreover, certain entomophytophagous beneficial insects can act as stimulants by injecting stylets into the plant's stem and activating indirect defense mechanisms and antixenosis. In the manner of volatile organic compounds, arthropods can trigger the release of plant volatile organic compounds (VOCs). VOCs can prime distal plant parts and neighboring plants (Mauch-Mani et al. 2017). VOCs can serve as priming-inducing signals even between plant individuals of different species. Kessler et al. (2006) reported that VOCs from clipped sagebrush (*Artemisia tridentata*) prime nearby wild tobacco (*Nicotiana attenuata*) plants for quicker production of trypsin inhibitors, and this was associated with lower herbivore damage and higher mortality rate of young tobacco hornworm (*Manduca sexta*) caterpillars (Kessler et al. 2006). Currently, it has been found that VOCs can also trigger the resistance to pathogens, an effect that might be due to different mechanisms: the priming of an induced expression of

resistance genes in the receiver or direct inhibitory effects on microbial pathogens that cause a passive "associational" resistance in the VOC-exposed plant (Quintana-Rodriguez et al. 2015).

A relevant subset of priming stimuli within VOCs induced by insects is the herbivore-induced plant volatiles (Kim and Felton 2013). Engelberth et al. (2004) illustrated how *Arabidopsis* plants exposed to several green leaf volatiles (small aliphatic alcohols and aldehydes) displayed primed jasmonate (JA)-dependent signals that were enhanced only following infestation with a caterpillar. Among the relevant set of VOCs that induce priming, terpenoids are the main priming stimuli against *S. littoralis* in maize (Mauch-Mani et al. 2017). Recently, a more detailed study of herbivore-induced plant volatiles has revealed that indole is present in the blend of volatiles released by infested leaves and that it triggers priming by enhancing the terpene levels in systemic leaves and neighboring plants (Erb et al. 2015). In an experiment, Maurya et al. (2019) studied the effect of seed exposure to common HIPVs on growth, reproduction, and defense characteristics of *A. thaliana* and *M. truncatula*. Among HIPVs tested, it was revealed that indole specifically reduced both beet armyworm growth on *A. thaliana* and pea aphid fecundity on *M. truncatula*. They demonstrated that induction of defense genes was not affected by seed exposure to indole in both plant species and concluded that seed priming operates independently of induced resistance. Moreover, the vegetative and reproductive growth of any species was not affected negatively by seed exposure to HIPVs.

It is now well established that VOCs emitted by the roots in the plant rhizosphere also play important ecological roles in the soil ecosystem, notably in plant defense because they are involved in interactions between plants, phytophagous pests, and organisms of the third trophic level. The roles played by root-emitted VOCs in between- and within-plant signaling, however, are still poorly documented in the scientific literature (Delory et al. 2016). Following attack, primed plants show a range of amplified defense responses. Increased expression of defense-related genes in *N. attenuata* (wild tobacco) is a good instance in this context (Karban et al. 2000). Rhoades (1983) reported that undamaged *Salix sitchensis* Sanson ex Bong (Sitka willow) trees growing close to herbivore-infested conspecifics mounted a higher chemical defense to *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae) (fall webworm) larvae than controls from a more distant site. Field studies found that herbivory rates on *Alnus glutinosa* (L.) trees were lower when growing close to damaged conspecifics (Dolch and Tscharntke 2000). Finally, induced VOCs contribute to indirect defenses by attracting natural enemies such as predators (Shepherd et al. 2005) and parasitoids (Hilker et al. 2002). An example of a belowground interaction is the release of VOCs from the roots of *Thuja occidentalis* (L.) when attacked by *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae) (black vine weevil) larvae. These VOCs have been shown to attract *Heterorhabditis megidis* Poinar, Jackson, and Klein (Rhabditida: Heterorhabditidae) (entomopathogenic nematodes) which are predators of *O. sulcatus* (van Tol et al. 2001). In aboveground interaction, VOCs were released from the needles of *Pinus sylvestris* (L.) (Scots pine) following egg deposition by *Diprion pini*

L. (Hymenoptera: Diprionidae) (pine sawfly) (Hilker et al. 2002). These oviposition-induced VOCs, characterized by larger quantities of the sesquiterpene (*E*)-β-farnesene than their controls, have shown to attract egg parasitoids (Mumm et al. 2003) (Fig. 3.3).

3.6.5 Chemical Stimuli

Numerous chemical compounds, often of natural origin, have been found to act as priming stimuli. As this group normally induces a much more reproducible response, investigators frequently prefer to focus on them to carry out molecular and genetic studies on priming. β-Aminobutyric acid (BABA), probenazole, benzothiadiazole (BTH), and salicylic acid (SA), all of which can induce resistance in plants by protecting against a broad range of pathogens. SA is a hormone that triggers several direct responses in plants, but at low doses, it has been reported to enhance flg22-induced MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3) and MPK6 activation. BTH and BABA have been thoroughly studied as priming agents against pathogens and insects. Likewise SA, both of these chemicals may directly induce defenses when applied at high doses (Mauch-Mani et al. 2017). Dempsey and Klessig (2012) presented valuable information regarding natural secondary metabolites found to mediate systemic acquired resistance, including JA, azelaic acid, dehydroabietinal, glycerol-3-phosphate, methyl salicylate, and pipecolic acid. These compounds, however, are likely to trigger priming, as has been confirmed, for example, for azelaic acid and pipecolic acid. Because the molecular mechanisms behind the induced resistance by chemicals are not fully understood, it is not always easy to classify them as priming stimuli (Mauch-Mani et al. 2017).

In one case, it was found that Colorado potato beetle (CPB; *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae)) susceptible to an entomopathogenic nematodes (EPNs) would avoid chemical cues from EPNs and plants associated with these natural enemies. Helms et al. (2019) showed that some plants, including potato, respond to EPNs or their chemical cues by activating or enhancing their defenses. Their findings indicated that plants and insect herbivores responded to belowground chemical cues from organisms at the third trophic level, thereby the potato plants induced or primed their defenses following exposure to EPNs or EPN cues, respectively, the performance of CPB larvae decreased. In addition, female CPBs avoided EPN cues and laid fewer eggs on plants with EPNs. They concluded that plants benefited from responding to chemical cues from a herbivore natural enemy, as enhanced plant defenses led to reduced herbivore performance and damage. They expressed several potential ecological explanations to this issue. One possibility was that this response originated as a case of mistaken identity and overlapping cues, where plants detected EPN cues as a direct threat from pathogens or herbivores. EPNs rely on symbiotic bacteria to infect, kill, and prevent putrefaction in their hosts (Lewis et al. 2006). Helms et al. (2019) declared that following exposure to EPNs or their chemical cues, plants elevated defenses typically associated with pathogens, plant-parasitic nematodes, or phloem-feeding

herbivores (Conrath et al. 2006; Manosalva et al. 2015). It is likely that compounds identified from EPN-infected cadavers are produced by EPN symbionts and plants might associate these cues with a microbial threat (Tomberlin et al. 2017). Indole was one of the compounds emitted by EPN cadavers, and it is also produced by some plant species after herbivore damage and has been identified as a key defense priming signal in maize (Erb et al. 2015). Helms et al. (2019) expressed another possibility that plants respond to cues from these herbivore natural enemies because they perceive the presence of EPNs as an indication that herbivores are also present and pose a threat. It is possible that plants detect the physical presence of live EPNs as an indication of immediate danger and respond with direct induction of defense, possibly due to mistaken identity or correctly identifying EPN and preparing for future herbivore damage. EPN chemical cues, on the other hand, could represent a potential, though less urgent threat, leading to defense priming (Helms et al. 2019). These findings indicate that plants can modify their responses in a context-dependent manner, responding differently to physical or chemical cues (Fig. 3.3).

3.6.6 Priming by Modifications in Primary Metabolism

Although it has been shown that application of sugar to various plant tissues, or provoking the accumulation of sugar in transgenic plants, can lead to activation of various PR genes (Johnson and Ryan 1990), the "high-sugar concept" of resistance (Horsfall and Dimond 1957) has been called into question by recent work, showing the expression of a yeast invertase in the cytoplasm of potato tuber cells leads to decreased levels of starch and enhanced levels of glucose, yet to drastic susceptibility to the soft-rot pathogen *Pe. atrosepticum* (Conrath et al. 2003). In addition, no association was found between the enhanced resistance to *Ph. infestans* in leaves of transgenic potato plants and decreased activity of the plastid ATP/ADP transporter with obvious changes in carbohydrate accumulation, in contrast to the enhanced disease resistance seen in the tubers (Conrath et al. 2003). This issue shows that the resistance of plant tissue with elevated levels of carbohydrates is not due to enhanced sugar levels, and it was supported by findings demonstrating that increased glucose levels are not associated with constitutive expression of *PR* genes in potato tubers with reduced activity of the plastid ATP/ADP transporter (Conrath et al. 2003). Detailed analyses on the timing and extent of defense responses in these same plants provided an alternative explanation for the IR phenotype observed. Upon exposure of leaf or tuber tissue to culture supernatants of *Pe. atrosepticum* or pep13, a 13-amino acid MAMP elicitor from oomycete cell walls, there was enhanced activation of defense responses, including defense gene activation and the oxidative burst (Linke et al. 2002). Thus, the IR of transgenic plants with reduced ATP/ADP transporter activity seems to be mediated by metabolic priming for enhanced induction of defense responses rather than by the associated elevation in carbohydrate levels in these plants. A correlation between elevated sucrose levels and priming of defense responses was reported recently also for rice overexpressing the *PRms* gene from maize, which encodes a PR-1-type protein. In these plants, elevated levels of

sucrose were associated with quicker and more robust induction of defense responses during pathogen infection and broad-spectrum disease resistance (Conrath 2009) (Fig. 3.3).

3.6.7 Other Forms of Defense Priming in Plants

Different mechanisms of defense priming have been documented other than what mentioned above, including transgenerational priming of defense (Rasmann et al. 2012), priming of defense by insect oviposition (Kim and Felton 2013), priming of defense by seed treatment with defense elicitors (Worrall et al. 2012), and priming of defense by heavy metal stress that in follow they are described briefly.

3.6.7.1 Transgenerational Priming

In the natural environment, some plants may have sustained feeding by the same insect species over generations. Therefore, in these conditions, there may be a strong selection pressure on plants to evolve mechanisms by which they pass the parental memory of herbivory to their progeny for enhanced defense. A few reports are available on the enhanced antiherbivore resistance of plants whose parents experienced herbivores in wild radish (*Raphanus raphanistrum*), yellow monkeyflower (*Mimulus guttatus*), and apomictic dandelion (*Taraxacum officinale*) (Agrawal et al. 1999; Holeski et al. 2012). Priming appears a good strategy to express inherited defense traits in the progeny plants when there is a "good" probability of herbivory in the progeny by the same insect species that exists and a small chance that the expected herbivores do not occur. Rasmann et al. (2012) found that *Arabidopsis* and tomato plants whose parents sustained feeding damage exhibited significantly enhanced resistance against herbivory by the same insect species. Transgenerationally primed responses of *Arabidopsis* included JA accumulation, JA-dependent antiherbivore defense genes, and production of leaf glucosinolates. Primed defenses showed specificity; on the plants whose parents were fed by *Pieris rapae* L. (Lepidoptera: Pieridae) and *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), larvae of *P. rapae* and *S. exigua* showed reduced performance, whereas larval growth of *P. xylostell*a and *Trichoplusia ni* was not influenced by the parental experience.

More interestingly, *Arabidopsis* mutants defective in the RNA-directed DNA methylation (RdDM) pathway could not inherit the resistance, indicating involvement of DNA methylation in transgenerational priming of defense (Fig. 3.3).

3.6.7.2 Priming of Defense by Insect Oviposition

Oviposition by herbivorous insects on the host plant results in herbivory by the hatchlings; therefore, each induced defense response resulting in displacing or killing the eggs before hatching should be advantageous because plants will not then sustain herbivore damage. A variety of egg-induced defenses that dislodge or remove the eggs from host plants have been reported; some plants directly kill eggs by producing ovicidal substances or by changing the physical structure or

physiological conditions at the oviposition site, and some plants release airborne signals to announce the existence of insect eggs to egg parasitoids and predators. The first report of priming of defense by insect oviposition was presented from the system of tomato (*S. lycopersicum*) and its fruitworm moth *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae). Tomato plants oviposited by *H. zea* adults showed stronger induction of *Protease inhibitor2* (PIN2) gene expression and higher accumulation of JA upon subsequent mechanical wounding and application of *H. zea* larval oral secretion (OS; regurgitant + saliva). Protease inhibitor2 (PIN2) inhibits protein digestion in the insect gut after ingestion; the Pin2 should target larvae, not eggs (Kim and Felton 2013) (Fig. 3.3).

3.6.7.3 Priming of Defense by Heavy Metal Stress

Heavy metals are usually found at low concentrations in nature. Some heavy metals (Fe, Zn, Cu, Ni) are important constituents of several key enzymes and play important roles in various oxidative-reduction reactions (Baghazadeh Daryaii et al. 2021). Although many of them are necessary for plant growth, they can be toxic above certain concentrations. Priming of antiherbivore defensive responses in plants under copper (Cu) stress has been demonstrated previously. Heavy metal stress caused by copper primes (80μmol/L of Cu) for enhanced VOC and JA production in response to feeding by *S. frugiperda* larvae in maize plants. Priming of antiherbivore defenses by Cu treatment was accompanied with H_2O_2 accumulation in roots. However, treatment with another toxic heavy metal, cadmium (Cd), did not result in defense priming and H_2O_2 accumulation in roots (Kim and Felton 2013) (Fig. 3.3).

3.6.7.4 Silicon-Mediated Induced Resistance to Insects

Silicon (Si) is an important element in plant nutrition, and it is the most common element, after oxygen, on earth. Silicic acid, $Si(OH)_4$, is the bioavailable form of silicon in soil solution that is taken up by plant roots (Exley 1998; Epstein 2009). It is translocated through the xylem to the shoots where it condenses into polymerized silica gel (Ma and Yamaji 2006). According to the ability of plants in accumulating Si, the plants have been classified to high (10–15%) (wetland grasses, e.g., rice, bamboo, and sugarcane), medium (1–3%) (terrestrial grasses, e.g., wheat), and non-(<1% Si dry mass, dm) (common eudicots) Si accumulators (Ma and Takahashi 2002). Besides its role in plant mineral nutrition, Si has been shown to modulate stress tolerance and confer pest and pathogen resistance. It is now well established that Si enhances plant resistance and reduces plant damage caused by pathogens, insect pests, and non-insect pests through the mediation and upregulation of both resistance mechanisms that are constitutive (i.e., irrespective of insect presence) and induced (i.e., in response to insect attack) (Ma 2004; Liang et al. 2015). Many good instances are available on the ability of Si in increasing the resistance of both monocotyledonous crops and numerous dicot plant species to insect pests of diverse feeding guilds belonging to Lepidoptera, Hemiptera, Diptera, Thysanoptera, and Coleoptera (Alhousari and Greger 2018) as well as to non-insect pests (Laing et al. 2006; Nikpay and Nejadian 2014). Si deposition patterns within plant tissues lead to

the hypothesis of mechanical or physical barriers to insect feeding, as silica makes plant tissues difficult for insects to efficiently chew, penetrate, and digest. In addition, silica's beneficial roles in plant physiology, regulation of defense-related enzymes, plant hormone signaling, and alteration of plant volatile blends elucidate the association of Si with biochemical/molecular defense mechanisms (Kvedaras and Keeping 2007; Reynolds et al. 2009; Fauteux et al. 2005; Ye et al. 2013; Nazaralian et al. 2017).

In addition to a mechanical barrier, Si can reduce pest damage by enhancing the induced chemical defenses of plants following insect attack. Silicon behaves as an abiotic elicitor of systemic stress signals, mediated by phytohormone pathways, leading to the efficient synthesis of defensive compounds (Fauteux et al. 2005). Defense against phloem-feeding insects is regulated by both SA and JA signals (Moran and Thompson 2001). Interestingly, there are valuable evidences regarding the strong interaction between Si and JA against insects (Ye et al. 2013; Liu et al. 2017). Si may trigger different plant species to emit, amplify, and/or alter HIPVs. It has been revealed that in response to feeding by the rice leaf folder (*Cnaphalocrocis medinalis* Guenée) (Lepidoptera: Crambidae), a wild-type rice plant supplied with Si mounts a strong indirect defense based on HIPV production. Among which are 2-ethylhexanal, α-bergamotene, β-sesquiphellandrene, and cedrol, produced in significantly smaller amounts in infested Si-treated plants (Liu et al. 2017). These changed HIPV profiles then significantly enhanced the attraction of adult females of the parasitoids *Trathala flavoorbitalis* (Cam.) (Hymenoptera: Ichneumonidae) and *Microplitis mediator* Haliday (Hymenoptera: Braconidae) to the Si-treated plants attacked by *C. medinalis*. The signaling pathways that allow rice plants to mount resistance against the chewing insect *C. medinalis* are JA dependent (Liu et al. 2017). To elaborate, Si and JA linked strongly to different components of the rice defensive system. This can be expressed in increasing the levels of transcripts encoding defense genes, the activities of defense-related enzymes (polyphenol oxidase, peroxidase, and trypsin protease inhibitor), in addition to HIPVs alteration (Ye et al. 2013). Under both laboratory and semi-field conditions, Si-treated plants attracted significantly more of the predator *Dicranolaius bellulus* (Guérin-Méneville) (Coleoptera: Melyridae) to cucumber plants (a medium Si-accumulator dicot) infested with *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) (Kvedaras et al. 2010).

Another well-established example of this phenomenon is in *Vitis vinifera* L., a dicot and Si non-accumulator. A positive correlation was observed between plant tissue Si content and attraction of the predator *D. bellulus* to grapevines infested with *Epiphyas postvittana* (Walker) (Insecta: Lepidoptera: Tortricidae). Moreover, seven volatile compounds emitted in *Phalaenoides glycinae*-infested grapevines were identified. One of them, *n*-heptadecane, was released in significant amounts only by Si-fertilized grapevines (Connick 2011). Similarly, treating wheat plants with silicon could negatively affect the feeding behavior and population growth rate of the greenbug *Schizaphis graminum* Rondani (Hemiptera: Aphididae). Suppressing the percentage of *S. graminum* reached the phloem ingestion phase indicates that Si-induced resistance possibly localized at the phloem level. The Si-induced

mechanism in wheat plants could be explained by increasing the activities of peroxidase, polyphenol oxidase, and phenylalanine ammonia lyase. The peroxidase is involved in plant defense via lignification, suberization, and production of ROS and quinones, which exhibit antibiotic properties (Gomes et al. 2005; Costa et al. 2011).

Regarding Si-mediated resistance, it has been revealed that the attack of aboveground plant shoots by insects can also result in root responses defending against root feeders. Induced defenses mediated by JA signaling have been found to improve rice resistance to the rice water weevil (*Lissorhoptrus oryzophilus* Kuschel (Coleoptera: Curculionidae)), whose larvae feed on rice roots under flooded conditions (Lu et al. 2015). Accordingly, the interaction between both constitutive and Si-induced resistance could strongly enhance plant resistance and reduce damage caused by root-feeding insects. In another example, Assis et al. (2012) showed that the damage of larvae of *Diabrotica speciosa* (Germar) (Coleoptera: Chrysomelidae) was reduced by foliar applications of silicic acid. The larvae of this pest damage plant roots and create holes in the tubers of the potato (*Solanum tuberosum* L.), whereas the adults consume the leaves. By foliar applications of silicic acid, the number of holes in the tubers of treated plants was reduced (Assis et al. 2012).

Si induces lignin accumulation in the roots of both sugarcane (a monocot) (Frew et al. 2017) and oilseed rape (a eudicot) (Tissier 2012), increasing toughness and, eventually, resistance to insect attack (Johnson et al. 2010). Though the accumulation of Si differs among plant species, they likely display similar Si defense mechanisms against insects. Similarly, monocot and eudicot species seem to respond similarly to insect attack through similar Si-mediated mechanical and biochemical mechanisms. Generally, chewing insects and phloem-feeding insects (e.g., whitefly and aphids) induce distinct plant responses to attack. Chewing herbivores have stronger inductive effects than do sucking ones (Rodriguez-Saona et al. 2010; Li et al. 2016). For example, compared with the chewing caterpillar *S. exigua*, the phloem feeder *Bemisia tabaci* (Gennadius) (Hemiptera, Aleyrodidae) did not induce the emission of HIPVs in *Gossypium hirsutum* L. (Rodriguez-Saona et al. 2003, 2010). Similarly, *S. littoralis* induced HIPV emissions, whereas the aphid *Rhopalosiphum maidis* (Fitch) (Hemiptera: Aphididae) induced no measurable emissions even after heavy infestations in the monocot *Zea mays* L. (Turlings et al. 1998). Regardless of the effect magnitudes, Si affects both direct and indirect plant defenses against both chewing and sucking insects, leading to similar impacts on biological parameters such as development time, immature survival, and rate of population increase. Taken together, plants employ both Si-based resistance mechanisms synergistically rather than singly, relying on combined physical, chemical, and biochemical mechanisms to reduce damage by insect pests (Fig. 3.3).

3.6.7.5 Priming of Defense by Seed Treatment

Seeds remain in the soil for a certain period of time to absorb water and some essential nutrients to grow. Seed priming is a technique to reduce this time and enables the germination quickly and uniformly. In addition to hydration, priming also reduces the sensitivity of seed to external environmental factors (Afzal et al.

2016). Priming promotes seed germination under three stages such as imbibition, germination, and growth (Waqas et al. 2019). In fact, "seed priming refers to the use of natural or synthetic compounds to induce a particular physiological state in seedlings before germination" (Parera and Cantliffe 1994). Hydropriming, osmopriming, chemical priming, hormonal priming, biological priming, redox priming, and solid matrix priming are common seed priming methods widely used (Neamatollahi and Souhani-Darban 2010; Sun et al. 2011). In seed priming, seeds expose to specific compounds to enhance seed germination. Seed treatment is a promising approach because it should enable seedlings to mount a robust immune response and thereby remain disease-free (or only moderately infected) for a long time with minimal labor and expense. Another benefit of seed treatment with biopesticides is that it synchronizes seed priming with seed germination (Taylor and Harman 1990). Worrall et al. (2012) reported priming of defenses by tomato seed treatment with JA and β-aminobutyric acid (BABA) and showed enhanced defensive gene responsiveness and increased resistance against tobacco hornworm (*M. sexta*), green peach aphids (*M. persicae*), and spider mites (*Tetranychus urticae* Koch (Acari: Tetranychidae)). When tomato seeds were treated with BABA, the resulting plants showed improved resistance against biotrophic fungal pathogen powdery mildew (*Oidium neolycopersici*). BABA is a nonprotein amino acid which is rarely found in nature and has been known to induce or prime plant defenses against a wide range of tomato biotic stresses including microbial pathogens, nematodes, and aphids and against abiotic stresses as well (Pieterse et al. 2006). It is generally recognized that induction of plant defense against arthropod herbivores and necrotrophic pathogens is dependent on JA biosynthesis pathway, and defense against biotrophic pathogens is regulated by salicylic acid (SA)-dependent defense pathway, and JA and SA often act antagonistically (Pieterse et al. 2009). Intriguingly, when seeds were treated with both JA and BABA, the antagonistic effects between JA- and SA-regulated plant defense pathways were not significant (Worrall et al. 2012). Maintenance of primed defensive state in the crop field could be a promising strategy for integrated pest management with enhanced defense activation and efficient resource management. Priming of plant defense by seed treatment with defense elicitors (e.g., JA, BA) is of the greatest importance among priming mechanisms from the perspective of agricultural applications with the following reasons. First, the method (i.e., dipping seeds in the elicitor solutions) is exceptionally easy and industrially applicable (Worrall et al. 2012). Second, the antagonistic effects are frequently found between JA-dependent plant defenses against insect herbivores and SA-dependent plant defenses against biotrophic plant (Worrall et al. 2012). Last, JA-dependent plant defenses are well characterized from the molecular to ecological levels, and any toxicological or environmental problems have not been reported yet. Maurya et al. (2019) demonstrated that the pregermination exposure of seeds to indole enhances resistance against herbivores of two feeding guilds in two different plant species (*A. thaliana* on the performance of *S. exigua* and *Medicago truncatula* Gaertn. on the performance of *A. pisum*) without any apparent effects on plant growth or fitness (Maurya et al. 2019). They showed that seed exposure to HIPVs had no adverse effect on seed germination, vegetative growth, and

reproductive output of the primed mature plants (Maurya et al. 2019). It has been revealed that treatment of seeds by phytohormones can be effective in changing the volatile composition of mature plants to attract mite predators in the later stages of the plant. Seed treatment with JA changed the volatile composition of mature plants, making their blends more attractive to predatory mites (Smart et al. 2013). Similarly, seed treatment with salicylic acid (SA) enhanced the expression of SA-related genes and the endogenous SA level against root holoparasite (*Orobanche cumana*) (Yang et al. 2016).

Despite the examples mentioned above, there are few studies on the plant immune activation through seed priming. In some cases, an emerging technology, "seed defense biopriming" (SDB), has been applied that combines seed priming with elicitation of plant immunity using biologically active compounds (Song et al. 2017). Biopriming is a process by which seeds or seedlings are hydrated in a spore suspension of beneficial biological organisms. It has been found to promote rapid early seedling establishment as well as offer protection against pathogens and pests (Huong et al. 2009; Begum et al. 2010; Pill et al. 2011). There are many good instances regarding biopriming in different plant species. Begum et al. (2010), for instance, showed that biopriming soybean seeds with *Pseudomonas aureofaciens* enhanced seed germination by 32.4–60% and promoted vigorous seedling stand by 56–73.9%. Also, application of microbial inoculants to seed and/or transplants was reported to enhance plant tolerance to soilborne pests (Sikora et al. 2008; Huong et al. 2009) and plant pathogens (Mathre et al. 1994; Pill et al. 2011; El-Bab and El-Mohamedy 2013). Biopriming faba beans (*Vicia faba*) with endophytic fungal isolates belonging to the genera *Beauveria*, *Trichoderma*, and *Gibberella*, for example, was reported to reduce aphid fecundity, lengthen nymph developmental time, as well as protect bean seedlings from aphid attack (Akello and Sikora 2012).

In another research, Song et al. (2017) prepared heat-stable metabolites from 1825 root-associated *Bacillus* spp. isolated from diverse plant rhizosphere in South Korea and tested their ability to induce SDB in cucumber and pepper seeds and trigger plant immunity. They found that SDB with heat-stable metabolites of the selected *Bacillus gaemokensis* strain PB69 significantly reduced subsequent bacterial diseases under in vitro and field conditions and increased fruit yield. Transcriptional analysis of induced resistance marker genes confirmed the upregulation of salicylic acid, ethylene, and jasmonic acid signaling. Mortality of the insect pest *S. litura* increased when larvae fed on SDB-treated cucumber tissues. Analysis of the causative bacterial metabolites identified a leucine–proline cyclodipeptide and a commercially obtained leucine–proline cyclodipeptide induced similar results as treatment with the bacterial preparation. Similarly, Akello et al. (2017) assessed the ability of *Trichoderma asperellum* Samuels, Lieckf, and Nirenberg, *Beauveria bassiana* (Bals.) Vuil., and *Metarhizium anisopliae* (Metsch.) strains M2RT4, S4SU1, and S4ST7, respectively, in the production of extracellular enzymes, as well as determine their impact on French beans seedling emergence and growth, and leaf miner fecundity and pupation. Pathogenicity assessment revealed that the screened bioinoculants were highly toxic to leaf miner larvae following the submersion of larval-infested leaves in spore suspension, reaffirming the insecticidal

properties of the two entomopathogenic isolates, *B. bassiana* and *M. anisopliae*. They showed the ability of the endophytic strains M2RT4 and S4SU1 to enhance the chlorophyll content of leaves sampled from treated bean seedlings, as well as promote better nodulation in primed seedlings. However, they did not find any detrimental effects of these bioinoculants on deterring adult flies feeding and oviposition on treated seedlings because *Liriomyza sativae* Blanchard (Diptera: Agromyzidae) and *L. trifolii* (Burgess) females could successfully lay the eggs in all plants irrespective of the treatment. The inability of insects to detect endophyte-infected and non-infected plants for egg laying has been confirmed by Faeth and Hammon (1996). They found that females of the horse chestnut leaf miner (*Cameraria* sp.) are unable to discriminate between endophyte-infected and non-infected Emory oak leaves. Nonetheless, there is a report on the systemic effects of endophytic fungi on the oviposition of other insect species that have been demonstrated before by Jallow et al. (2008). Apart from insecticidal properties, seed coating with plant growth-promoting rhizobacteria (PGPR) and plant growth-promoting fungus (PGPF) enhanced its germination and establishment and boosts induced defenses in future plants in SA-, ET-, and JA-dependent manners (Ryu et al. 2004; Rudrappa et al. 2010; Sharifi and Ryu 2016).

3.7 Molecular Mechanisms of Defense Priming

The molecular mechanisms behind priming are largely unclear. Priming has been associated with the accumulation of posttranslational modification of cellular compounds. These compounds have important roles in signal transduction and/or amplification. In general, an accumulation or modification of these compounds does not activate a broad panel of plant's defense responses (Conrath et al. 2006). Epigenetic regulation of gene expression is another widely discussed mechanism involved in defense priming. It has been shown that histone modifications at promoters of defense-related transcription factors such as WRKY6, WRKY53, and WRKY29 contribute to priming of gene expression by benzothiadiazole (BTH) (Jaskiewicz et al. 2011). An additional epigenetic regulation mechanism is DNA methylation. In plants, DNA methylation is present in all three possible sequence contexts (CG, CHG, CHH, whereas H is A, T, or C) and has been shown to influence defense responses (Gully 2019). DNA methylation in the CG context can be maintained by DNA METHYLTRANSFERASE 1 (MET1), and in all sequence contexts, it can be triggered by the RNA-directed DNA methylation (RdDM) pathway. In RdDM, one of the main players is NUCLEAR RNA POLYMERASE D1 (NRPD1), the largest subunit of RNA Polymerase IV (Pol IV), which plays a key role in the initiation of siRNA production (Gully 2019). Another emerging regulation of gene expression involves antisense transcripts. It has been previously reported that genes transcribed in sense orientation can also be transcribed in antisense orientation. Antisense transcripts include partial or complete sequences complementary to other transcripts and are endogenous RNA molecules (Gully 2019). They play an important role in various processes, including the adaptation

to biotic and abiotic stresses. Antisense transcripts are widespread in both prokaryotes and eukaryotes (Gully 2019). In the following, we explain the molecular mechanisms of defense priming in detail.

3.7.1 The Priming Phase: Changes Following Stimulation

The priming phase refers to the biological process of acquiring priming, which takes place from the initial stimulation through the exposure to a challenging stress and includes all changes that occur in the plant after the perception of a stimulus and prepare the plant for enhanced responsiveness when a challenge occurs. These changes can take place at the physiological, molecular, and epigenetic levels; can occur within seconds or hours after stimulation; can be transient or maintained throughout the lifetime of a plant; and can even be inherited by subsequent generations. Different priming stimuli may cause similar changes as well as specific ones. Stimulus specificity may reside, for example, in the activation of only some of the responses described below (Mauch-Mani et al. 2017).

3.7.1.1 Physiological and Transcriptional Changes

Transient changes in the level of intracellular calcium occur within a few seconds or minutes and are among the best-known early responses to stimulation. Cytosolic calcium rapidly increases, for instance, in cells neighboring a wound site or after leaf rubbing, and the calcium increase is crucial for local priming by wounding. Both pathogen-associated molecular patterns (PAMPs) and insect feeding (but not BABA) have been reported to transiently impact calcium levels during the priming phase. Calcium fluxes could also play a role during root colonization by arbuscular mycorrhizal fungi (AMFs) (Mauch-Mani et al. 2017).

The increase in intracellular calcium can precede the generation of reactive oxygen species (ROS), the so-called ROS burst. Alvarez et al. (1998) demonstrated that, after inoculation with avirulent *P. syringae*, both the localized oxidative burst and the subsequent secondary microbursts in distal leaves were necessary to establish systemic acquired resistance. A fine-tuning of ROS homeostasis seems to also be crucial for priming, as reported in *Arabidopsis* after treatment with BABA (Mauch-Mani et al. 2017). Stimulus perception and downstream cellular immune responses are rapidly linked by sequential phosphorylation events (Mauch-Mani et al. 2017). It is widely accepted that local and systemic transcriptional reprogramming may occur during the priming phase. For example, quantitative polymerase chain reaction analysis revealed that application of BABA or inoculation with *Pseudomonas fluorescens* WCS417r in *Arabidopsis* induced the expression of transcription factors associated with defense response mechanisms. Importantly, transcriptional changes induced by different priming stimuli are partially specific (Mauch-Mani et al. 2017).

Massive transcriptomic changes have been also reported following mycorrhizal colonization of maize and tomato plants by the AMF *Rhizophagus irregularis*. In maize, one group of the induced genes was related to anthocyanin and lipid metabolism, most likely dependent on the improved phosphorus status of mycorrhizal

plants. Interestingly, leaf analysis also revealed a systemic induction of defense-related genes and a concomitant induction of secondary metabolites in addition to changes in genes involved in primary metabolism, such as the metabolism of carbohydrates, organic acids, and amino acids. In a parallel study performed in tomato plants, *R. irregularis* inoculation caused changes in systemic leaves for 742 out of 21,113 genes analyzed by RNA sequencing (RNA-seq) and induced resistance against *Xanthomonas campestris* pv. *vesicatoria*. Changes in gene expression affected hormone metabolism, biotic and abiotic stress responses, signaling, and transport, suggesting that this transcriptional reprogramming may facilitate defense responses to subsequent infection with *X. campestris*. Some studies have investigated changes at the protein level induced by priming-inducing chemicals during the priming phase. BABA, for example, may in some cases directly induce pathogenesis-related (PR) proteins, and lipopolysaccharides can transiently increase the enzymatic activity of a tyrosine decarboxylase. Importantly, protein levels corresponding to pattern recognition receptors and coreceptors increase after treatment with BTH (124), suggesting that following stimulation, plants prepare their defensive system for an enhanced sensitivity against potential attackers (Mauch-Mani et al. 2017).

3.7.1.2 Metabolic Changes

The accumulation of inactive forms of defense-related hormones seems to be implied in the sensitization of defenses. For instance, the constitutively primed *Arabidopsis* mutant nitrate transporter 2.1 (nrt2.1) has low basal levels of free SA that rapidly increase after challenge with *Pst*. Similar mechanisms seem to occur with hormone conjugates, phytoanticipins, and indolic glucosinolates. In addition, priming activators can increase the levels of compounds involved in primary metabolism, such as amino acids, tricarboxylic acids, glycerol-3-phosphate, myoinositol, and xylitol, as well as the levels of methyl salicylate and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, as found in tomato after seed treatment with JA. Importantly, treatment with BABA also induces the accumulation of aspartic acid as a direct consequence of the blockage of the enzymatic activity of IMPAIRED IN BABA-INDUCED IMMUNITY 1 (IBI1), an aspartyl-tRNA synthetase that functions as the BABA receptor. In addition, treatment with BABA or infection with avirulent *Pst* AvrRpt2 causes similar metabolomic changes in *Arabidopsis*. On the basis of studies that have analyzed different priming stimuli, a common subset of shared compounds can be identified that are then referred to as the priming fingerprint. These compounds undergo a slight induction after stimulation, but their accumulation following challenge is faster and/or stronger in challenged plants than it is in unstimulated controls (Mauch-Mani et al. 2017).

Beneficial microorganisms can induce metabolic changes in colonized plants that can be helpful for the plant to enhance responsiveness upon subsequent challenge. For instance, maize roots colonized by PGPRs of the genus *Azospirillum* significantly affect the benzoxazinoid profile in a strain- and cultivar-dependent manner. The metabolic fraction analyzed by liquid chromatography–mass spectrometry showed no overlap in a principal component analysis among different *Azospirillum*

strains, either in the root extracts or in the shoot. Some benzoxazinoids, such as 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one or its glucoside, were detectable only with specific strains. This hints at specific mechanisms of interaction and recognition between the host plant and PGPRs (Mauch-Mani et al. 2017).

3.7.1.3 Epigenetic Changes

Epigenetic changes include priming smells of epigenetics and chromatin modifications. Epigenetic modification of chromatin is currently the most promising candidate of molecular mechanism of defense priming. DNA methylation and histone posttranslational modifications induce changes in chromatin structure, which alters gene transcription. DNA methylation occurs at cytosine residues at the CG or CHG sites or asymmetrically at CHH sites (H = A, C, or T). Two copies of each of histone proteins, H2A, H2B, H3, and H4, agglomerate into the core of the nucleosome, a unit of chromatin, with 147 bp genomic DNA strands wrapped around the core. The structure of histone proteins is posttranslationally modified at lysine (K), arginine (R), proline (P), and serine (S) residues of the N- and C-terminal tails by methylation (mono-, di-, or trimethylation), acetylation, phosphorylation, ubiquitylation, and SUMOylation (SUMO, or small ubiquitin-like modifier) (Kim and Felton 2013) (Fig. 3.4).

Recent papers provide evidence indicating that priming of defense against pathogens and herbivores accompanies epigenetic modifications to promote transcription efficiency of defense genes upon subsequent stimulus (Jaskiewicz et al. 2011; Rasmann et al. 2012). According to Jaskiewicz et al. (2011), in *Arabidopsis* plants primed by BTH treatment at moderate concentrations, several types of histone modifications were abundant on the promoter regions of SA-dependent transcription factors *WRKY6*, *WRKY29*, and *WRKY53*. Trimethylation and dimethylation at lysine 4 of H3 (H3K4me3 and H3K4me2, respectively) were abundant on the promoter regions of *WRKY6* and *WRKY53*, and the promoter region of *WRKY29* was labeled with acetylations at H3K9, H4K5, H4K8, and H4K12 as well as H3K4me3 and H3K4me2 (Jaskiewicz et al. 2011). Most of histone modifications found in BTH-primed plants are known to favor gene activation (Berger 2007). Rasmann et al. (2012) reported transgenerational priming of antiherbivore defenses in *Arabidopsis* and tomato.

One of the functions of siRNA is to induce de novo DNA methylation of cytosines in the DNA region whose sequence is homologous to small interference RNA (siRNA) (Matzke et al. 2007). *Arabidopsis* mutants defective in the production of siRNA failed to exhibit transgenerational priming, implying that RNA-directed DNA methylation (RdDM) is critical in conveying environmental memories of parents to their progeny for augmented resistance (Rasmann et al. 2012). DNA methylation generally inhibits transcription (Berger 2007). The reason that DNA methylation in the transgenerational priming of defense is specifically important is that DNA methylation is epigenetically inherent, but histone modifications are lost during the meiosis (Rasmann et al. 2012). Increasing evidence indicates that DNA methylation and histone modification may be complementary so that DNA methylation might guide restoration of histone marks after lost during meiosis (Cedar and

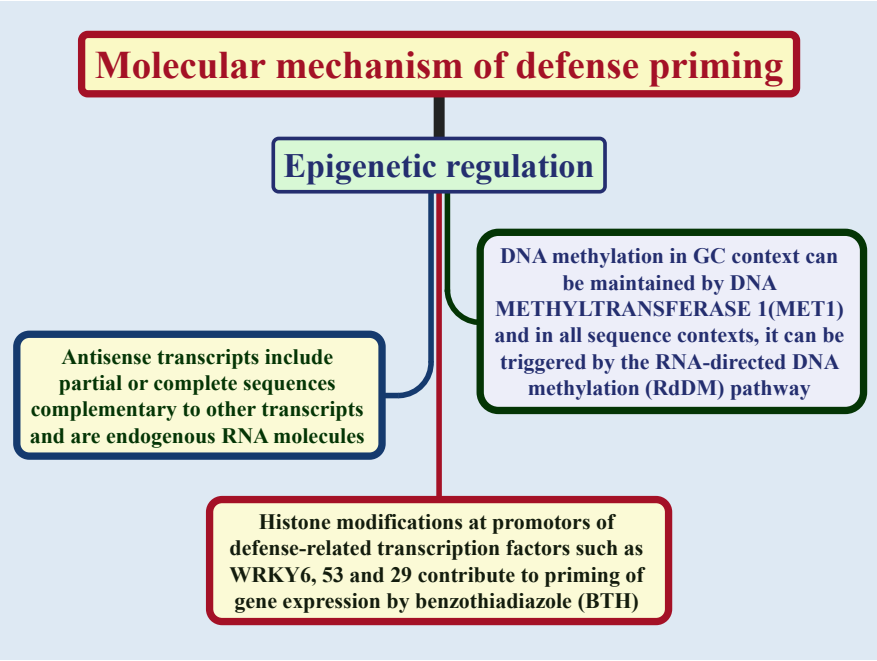

Fig. 3.4 DNA methylation and histone posttranslational modifications induce changes in chromatin structure that alters gene transcription. DNA methylation occurs at cytosine residues at the CG or CHG sites or asymmetrically at CHH sites (H = A, C, or T). Two copies of each of histone proteins, H2A, H2B, H3, and H4, agglomerate into the core of the nucleosome, a unit of chromatin, with 147 bp genomic DNA strands wrapped around the core. The structure of histone proteins is posttranslationally modified at lysine (K), arginine (R), proline (P), and serine (S) residues of the N- and C-terminal tails by methylation (mono-, di-, or trimethylation), acetylation, phosphorylation, ubiquitylation, and SUMOylation (SUMO, or small ubiquitin-like modifier)

Bergman 2009). More reports support the role of DNA methylation in defense priming. *Arabidopsis* plants exposed to virulent *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) at the parental generation displayed enhanced resistance in the offspring (Luna et al. 2012). The plants whose parents were infected with *Pst* DC3000 were more resistant against the conspecific pathogen and (hemi) biotrophic pathogen *H. arabidopsidis* than control plants. In addition, in the primed progeny, the promoter regions of SA-inducible defense gene *PATHOGENESIS-RELATED GENE 1 (PR1)* and transcription factors *WRKY6* and *WRKY53* were marked with H3K9ac, whereas the promoter of JA-inducible promoter *PLANT DEFENSIN 1.2* (PD1.2) with H3K27me3 (Luna et al. 2012). H3K9ac generally instructs gene activation, and H3K27me3 acts against transcription (Berger 2007). Probably, as a result, upon subsequent stimulus, *PR1*, *WRKY6*, and *WRKY53* in the primed plants exhibited enhanced transcription levels, but transcription of JA-dependent PD1.2 was even more reduced in *Pst* DC3000-primed plants than nonprimed plants (Luna

et al. 2012). Furthermore, the *drm1drm2cmt3* triple mutant impaired in DNA methylation on CHG and CHH sites failed to show transgenerational priming of defense (Luna et al. 2012), meaning hypomethylation at CHG and CHH sites is critical in transgenerational defense priming. In the follow-up study, another RdDM pathway mutant, drm1drm2, which is impaired in DNA methylation on CHH sites, showed no impairment in transgenerational priming defense, meaning hypomethylation at CHH sites is not required in defense priming across generations. Therefore, the authors reached the conclusion that hypomethylation on CHG sites is critical in transgenerational defense priming (Luna and Ton 2012).

3.8 Duration of the Primed State

Unlike mammals, plants have a nonadaptive immune system that relies on biochemical changes. Nevertheless, priming of induced resistance influences responses after an initial stimulus, and it therefore represents a type of immunological memory that allows plants to remember stressful situations. Epigenetic modifications are one of the mechanisms that enable plants to acquire memory and can cause long-term alterations to gene responsiveness (Mauch-Mani et al. 2017).

3.8.1 Long-Term Responses Within the Same Generation

Initial epigenetic changes in chromatin structure via DNA methylation and post-translational modifications provide long-term memory within a generation that allows the plant to keep defense mechanisms primed for future attacks. A recent study has reported that DNA methylation and demethylation do not play a role in systemic acquired resistance 4 weeks after the initial stimulus. Other studies have reported long-lasting induced resistance to pests and pathogens. For instance, it is possible to achieve durable induced resistance in tomato after seed treatment with BABA or JA. This long-lasting resistance was based on priming of gene expression and did not cause any reduction in growth (Mauch-Mani et al. 2017).

3.8.2 Transgenerational Immune Resistance

The finding that certain DNA methylation patterns are inheritable paved the way to the hypothesis that some traits that are regulated by DNA methylation could be passed on to subsequent generations. Several studies showed an effect in the progeny of plants infected with *tobacco mosaic virus* or exposure to UV light or flg22 treatment. Progeny of plants infected with *tobacco mosaic virus* showed greater resistance, while plants of which parents were exposed to UV or flg22 resulted in a greater homologous recombination frequency (Gully 2019). Interestingly, also the chemical SAR activator BABA was shown to induce resistance in the progeny (Gully 2019). After comparison of transgenerational resistance in RNA-directed

DNA methylation (RdDM) mutants with wild-type plants, it was suggested that transgenerational SAR is achieved through induced hypomethylation at non-CG DNA sites (Gully 2019). Next to the possibility of inherited DNA methylation marks, an alternative is that histone modifications are inherited through nucleosome recycling or the copying of modifications onto newly incorporated histones. This hypothesis is based on findings on the widely studied gene FLOWERING LOCUS C (FLC). This transcription factor acts as repressor of floral transition and is regulated by the histone mark H3K27me3. During embryogenesis, the vernalized state of FLC is reset by the activity of an H3K27 demethylase (Gully 2019). Mutants lacking the demethylase inherit the vernalization state to their offspring. Intergenerational stress memory was confirmed in a study on hyperosmotic stress priming. Plants which were stressed during their vegetative development passed on the stress memory for at least two generations. However, this stress memory was reset after one stress-free generation (Gully 2019). Transgenerational epigenetic stress memory is meiotically stable and extends for at least one stress-free generation. One study showed that stress-dependent mobilization of retrotransposons and their directed integration in the genome can be stably inherited. This stable integration could possibly lead to a more stress-resistant progeny (Gully 2019).

3.8.3 Memory Is Not for Free: Costs and Omission of Stress Priming

Induced transgenerational resistance could possibly result in a cost for the plant. On the level of hormonal regulation of plant defense, it was shown that the progeny of plants primed with a SA pathway-inducing pathogen downregulated JA-dependent defenses. This results in an increased susceptibility in these plants against JA pathway-dependent bacterial infections (Gully 2019). Overall, defense priming is assumed to be beneficial for the plants with a generally positive cost–benefit balance in times of stresses. However, the advantage of a primed "ready state" becomes only obvious upon a subsequent exposure to a second stress, whereas a primed plant can outperform an unprimed plant. If this second stress is not accruing, only the costs of priming influence the plants' fitness. The activation and maintenance of the prime state of enhanced defense in the form of the deposition of dormant signaling enzymes as well as the storage in the form of epigenetic marks on defense gene promoters could result in fitness consequences (Gully 2019). However, defense priming has lower costs than the direct activation of defense.

3.8.4 Plants Can Also Forget

Priming generally results in low fitness costs for the plant. However, it could lead to the downregulation of some resistance pathways or could sensitize plants such that they respond to false alarm signals. For these reasons, Crisp et al. (2016) recently hypothesized that plants might be better at forgetting previous stresses in order to avoid compromising development, yield, and ultimately survival. This is in

accordance with the hypothesis that the durability of transgenerational defense priming over stress-free generations may be linked to the level of the stress originally encountered. For example, infections with virulent *Pst* and herbivory attack were able to induce a long-lasting resistance that was maintained over at least one stress-free generation, whereas resistance after infection with avirulent *Pst* was lost at this stage. Transgenerational resistance can be sustained through more generations when the initial stress is repeatedly applied, thus warning the progeny of a persistent stress. These differences hint at a dependent relationship between the intensity of the stimulation and the durability of the transgenerational resistance. Accordingly, owing to the fast and reversible nature of epigenetic modifications, it is likely that transgenerational immune priming is erased after certain stress-free generations, thus removing the plausible costs (Mauch-Mani et al. 2017).

3.8.5 Transgenerational Resistance in Crops

Since the discovery that defense priming can be transmitted to subsequent generations, several publications have described similar findings in crops. For instance, Rasmann et al. (2012) demonstrated that transgenerational resistance to herbivory attack can be achieved in *S. lycopersicum*. However, transgenerational resistance can also be obtained in legumes. Therefore, transgenerational immune priming does not seem to be limited to short-life model species, such as *Arabidopsis*, and is achievable in economically relevant crops with longer life spans. Studying the mechanisms behind this phenomenon will open opportunities to optimize resistance in cultivars via epigenetic exploitation (Mauch-Mani et al. 2017).

3.9 Specificity of Primed Defenses

Each defense trait of a plant impacts a specific spectrum of target herbivores. As a plant sustains damage by several insect herbivores, identification of the given insect species is critical for the induction of a selective set of defenses effective on the performance of the given insect herbivores. Specificity of defense is parsed into "specificity of elicitation" and "specificity of effect." How distinct defenses are induced upon different insect species defines "specificity of elicitation." Accumulated results indicate that plant induced defense in some cases is specific enough to show distinct responses to feeding by two closely related species of whitefly. "Specificity of effect" is defined by whether the induced defenses are effective on the performance of subsequent herbivores. Plant defenses induced by one insect species could be effective, neutral, or countereffective on the other, and the generalized pattern of specificity of effect has not been established yet.

Specificity is also found in the primed defenses. Between two GLVs, (*Z*)-3-hexenol and (*E*)-2-hexenal, reported to prime plant defensive responses in maize and native tobacco, respectively, only (*E*)-2-hexenal primed defensive response of *Arabidopsis* for subsequent MJ treatment, implying plant's perception of specific

volatiles and specificity of elicitation of primed defense. Among HIPVs of native tobacco, (*E*)-2-hexenal and methacrolein primed defense, whereas MJ did not. Specificity of effect of primed defense is also described in the report of transgenerational priming of defense of *Arabidopsis*. Larvae of *P. rapae* and *S. exigua* showed reduced performance on the plants whose parents suffered herbivory by *P. rapae* and *Plutella xylostella*, whereas larval herbivory by *P. xylostella* and *Trichoplusia ni* on the parent plants did not influence on the performance of *P. rape* on the progeny. Primed defense has one more dimension of specificity because, in response to a given stimulus, plants may prime some defense traits and induce others. In response to HIPVs from the herbivore-damaged neighboring plants, the receiver hybrid aspen induced extrafloral nectar (EFN) secretion but primed HIPV emission for the secondary herbivory. More specificity of primed defenses is found at the epigenetic level (Kim and Felton 2013).

3.10 The Role of Endosymbionts in Breaking Down Resistance in Primed Plants

Several groups of elicitors have been found in oral secretions of lepidopteran larvae, such as lytic enzymes like b-glucosidase, fatty acid–amino conjugates, for example, volicitin (*N*-(17-hydroxylinolenoyl)-L-glutamine), and chloroplastic peptide fragments called inceptins. However, another lytic enzyme, glucose oxidase, found in high concentrations in the oral secretions of *Helicoverpa* spp., may function in defense suppression as a counter-defense strategy (Eyles et al. 2010). Currently, it has been found that insect-associated microbes play important roles in the detoxification of plant toxins and defenses (Shikano et al. 2017). For instance, the gut bacterial community of the cabbage root fly was a source of isothiocyanate-degrading enzymes, which aid in the detoxification of these toxic metabolites produced by the host plant (Welte et al. 2016). In some studies on the role of PGPRs on insect pests' suppression, it has been demonstrated that PGPR-mediated resistance has not had a negative effect on pests. For instance, Hackett et al. (2013) stated that the allocation of biomass to roots was reduced in potatoes colonized by the aphid *Macrosiphum euphorbiae* (Thomas) (Hemiptera: Aphididae) harboring *Hamiltonella defensa*, compared to plants attacked by *H. defensa*-free aphids (Hackett et al. 2013). Serteyn et al. (2020) in comparing two clones of *A. pisum* (one only harbored the primary endosymbiont *Buchnera aphidicola*, while another harbored *B. aphidicola* and the facultative endosymbiont *Hamiltonella defensa*) found that the PGPR treatment of broad bean plants could not reduce the reproduction of a clone, harboring both of two endosymbionts. Serteyn et al. (2020) in a study on whether PGPR-induced defenses in broad bean plants impact the pea aphids, depending on their genotype and the presence of endosymbionts, found the phenomenon of PGPR-induced plant defense priming, but no noticeable plant growth promotion. They suggested that the endosymbiont *Hamiltonella defensa* played a key role in plant–insect interactions, possibly helping aphids to counteract plant-induced resistance and allowing them to develop normally on PGPR-treated plants.

Their results implied that plant- and aphid-associated microorganisms add greater complexity to the outcomes of aphid–plant interactions (Serteyn et al. 2020). They concluded that *H. defensa* imposed higher nutritional demands on its host, resulting in a higher phloem uptake by aphids, which would provoke a higher compensatory photosynthetic activity, eventually resulting in resource allocation to the stem and leaves, instead of roots. *Wolbachia* is a naturally occurring endophytic intracellular bacterium and may infect a broad array of herbivorous insects. Western corn rootworms (WCRs; *Diabrotica virgifera virgifera*) infected with *Wolbachia* suppressed defense-related genes in maize roots compared to uninfected rootworms (Shikano et al. 2017). *Wolbachia* secretes small noncoding RNAs that can regulate host and bacterial genes (Mayoral et al. 2014) and thus could indirectly influence herbivore-associated molecular patterns (HAMPs) found in rootworms. Other insect species may also secrete bacteria during feeding; many of the bacterial genera found in Hessian fly (*Mayetiola destructor*) larvae are also found in fly-infested wheat, suggesting that bacteria are secreted into the host plant during feeding (Bansal et al. 2011). Approximately 70% of bacterial genera detected in larvae through culturing were also found in the infested wheat, showing the bacteria may be secreted into host plants as part of Hessian fly oral secretions and could influence plant defenses. The rootworm oral secretions or regurgitant (REG) of the Colorado potato beetle, which contains large amounts of bacteria, suppresses jasmonic acid (JA)-regulated defense transcripts (e.g., proteinase inhibitors, polyphenol oxidase (PPO), arginase, etc.) in tomato (Chung et al. 2013). It has been revealed that CPB larvae secrete bacteria during feeding that suppress antiherbivore defenses in tomato (Shikano et al. 2017). Plants fed on by larvae that were not treated with antibiotics showed decreased production of JA and JA-responsive antiherbivore defenses but increased salicylic acid (SA) accumulation and SA-responsive gene expression. The beetles benefited from the downregulation of JA-regulated plant defenses by exhibiting enhanced larval growth. In SA-deficient plants, suppression was not observed, indicating that suppression of JA-regulated defenses depends on the SA signaling pathway. Applying bacteria isolated from larval REG to wounded plants confirmed that at least three bacteria belonging to the genera *Stenotrophomonas*, *Pseudomonas*, and *Enterobacter* are responsible for defense suppression (Chung et al. 2013).

3.11 Relationship Between Aboveground and Belowground Parts of Plants in Induced Resistance

Induction of resistance in belowground herbivores can affect aboveground herbivores and vice versa. For instance, in maize, it is well known that the western corn rootworm *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) feeding increases JA levels both locally and systemically, which results in the activation of a suite of herbivore defense-related genes especially those encoding enzymes in the JA biosynthetic pathway. Belowground herbivory by *D. virgifera virgifera* induced aboveground resistance against the generalist herbivore *S. littoralis* and the necrotrophic pathogen *Setosphaeria turcica*. Furthermore,

downstream genes that encode direct defense proteins such as proteinase inhibitor, chitinase, and Ribosome-Inactivating Protein 2 are induced by caterpillar feeding (Louis et al. 2015). The role of aboveground to belowground communication and vice versa is one of the emerging areas of research in the field of plant–insect interactions (Nalam et al. 2013; Soler et al. 2013). For instance, it has been revealed that upon foliar insect infestation in tobacco (*Nicotiana* spp.), the insecticidal compound nicotine synthesized in the roots transported to the shoot through the vascular tissues, providing defense to subsequent insect attack (Louis et al. 2015). There are other instances regarding translocation of insecticidal compound in plants. In *Arabidopsis* (*A. thaliana*), aphid infestation on the foliage induces the activation of lipoxygenase 5 (LOX5)-derived oxylipins in the roots (Nalam et al. 2012). The LOX5-derived oxylipins (e.g., 9-hydroxy-10*E*,12*Z*-octadecadienoic acid) are translocated from the roots to the shoots, where it activates the different defense signaling genes against aphids (Nalam et al. 2012; Louis and Shah 2015). Similarly, fall armyworm (*S. frugiperda*) infestation in the whorl regions of maize resulted in the accumulation of Mir1-CP in the roots (Lopez et al. 2007). Louis et al. (2015) showed that corn leaf aphid (CLA), *R. maidis*, feeding-induced expression of *maize insect resistance1* (*mir1*) contributes to enhanced defense in the Mp708 maize inbred line. They also suggested that aboveground feeding by *R. maidis* transduced yet-to-be discovered signal(s) to the roots that triggered belowground accumulation of *mir1* (Louis et al. 2015). Overall, plant roots in addition to the food/nutrient storage and resource acquisition may also act as a site for toxin synthesis in response to aboveground herbivory (Nalam et al. 2013).

3.12 Elicitors

Elicitors (also called plant resistance inducers) are considered to be biocontrol products in agriculture as they induce plant resistance to various diseases and pests. Treatment of plants with various agents, including cell wall fragments, plant extracts, and synthetic chemicals, can induce resistance to subsequent pathogen and pest attack both locally and systemically (Walters and Fountaine 2009). In fact, elicitors are compounds that induce accumulation of antimicrobial phytoalexins and any type of defense response (Keen and Bruegger 1977). Elicitors have been isolated from bacteria, fungi, oomycetes (Nürnberger 1999), sea algae (Arman and Qader 2012), and plants or even chemically synthesized (Walters and Fountaine 2009) and can be proteins, peptides, fatty acids, glycoproteins, lipids, oligosaccharides, and polysaccharides.

Kobayashi et al. (1993) showed that cell wall components of marine brown algae induce the formation of antifungal compounds in alfalfa cotyledons. They stimulated several resistance reactions in tobacco suspended cells and consistently induce both local resistance and systemic resistance to *tobacco mosaic virus* (TMV) (Klarzynski et al. 2003). Conrath et al. (2002) tested the direct antifungal activity of algal product (AP) against *Phytophthora infestans* and *Botrytis cinerea* using disk diffusion method and found no growth inhibition activity of AP against the studied pathogens.

Afterwards, they studied the ability of AP in inducing two resistance reactions of tomato plants and found when tomato leaves were infiltrated with AP, a fast and significant induction of superoxide (O_2^-) occurred. The superoxide is of the early plant resistance reactions induced following pathogen infection or elicitor application, which is involved in direct pathogen control and the pathways of other resistance reactions (Conrath et al. 2002). All elicitors are not equally successful in inducing the resistance in plants, and some may not be as effective, which requires the most proper compounds to be determined by using laboratory and field tests. For instance, among several elicitors reported to induce resistance reactions in tomatoes, only salicylic acid (SA) and chitosan induced the tomato's resistance to pathogens (Thakur and Sohal 2013). Sbaihat et al. (2015) investigated the ability of a novel elicitor extracted from the brown sea algae (*Sargassum fusiforme*) to elicit induced resistance in tomato and showed the studied elicitor induced hypersensitive cell death and O_2^- production in tomato tissues. They found that the elicitor significantly reduced severities of late blight, grey mold, and powdery mildew of tomato.

Elicitors can also be used to increase the production of secondary metabolites in vitro cultures (Namdeo et al. 2002). In this context, Sohrabi et al. (2019) tested the effect of a biological elicitor consisted of *Alternaria solani*, *Fusarium* sp., and *Setosphaeria rostrata* in increased the amount of secondary metabolites by adding it into the culture medium containing callus of *Citrullus colocynthis* (L), Schrad. (a well-known medicinal plant). The findings showed that by adding the elicitors to the culture medium, the cell growth increased. In addition, the highest antioxidant activity was observed in culture medium when a mix of the three fungi was used as an elicitor.

Some elicitors can trigger priming in plants and prepare the plant for a faster and stronger resistance only when a subsequent pathogen or pest attack occurs (Mire et al. 2016). Priming is more cost-effective than elicitation because the energy cost of induced resistance in the plant is optimized (Beckers and Conrath 2007). Although the molecular mechanisms behind priming remain poorly understood, some natural and synthetic compounds have demonstrated good priming-inducing activity in laboratory and field conditions, such as the nonprotein β-aminobutyric acid (BABA) (Mire et al. 2016).

Pest monitoring programs can be used not only for deciding when to apply pesticides but also to optimize timing of defense elicitor applications. Treating plants with such elicitors basically mimics the "natural" initiation of systemic resistance due to insect feeding albeit much faster and possibly stronger. Although several of such products have been identified, e.g., jasmonate, benzothiadiazole (BTH), and BABA, many of these compounds have not yet been widely used. In principle, applying elicitors could also allow the use of mutant crop plants that do not accumulate insect-induced defense hormones upon insect feeding, thus fine-tuning the trade-off between resistance and yield and/or flavor. Complete control of pest and pathogen damage is rarely proven to occur by elicitor-induced resistance but often results in reducing lesion size and/or number. However, due to the multitude of plant traits affected by such elicitors, including plant growth and reproduction

parameters, uncoupling defense elicitation from herbivory bears risks for crop yield and product suitability (Pappas et al. 2017).

Lipopeptides (fengycin and surfactin) are elicitors produced by the PGPR genus *Bacillus*, involving in the induction of plant resistance (Ongena and Jacques 2008). After the perception of these elicitors by root cells, pathways regulated by jasmonic acid (JA) and ethylene are activated, resulting in the chemical priming of the plant (Walters and Heil 2007; Ongena and Jacques 2008; Conrath et al. 2002; Choudhary and Johri 2009). Therefore, the capacity of primed plants to mobilize defense responses is lastingly augmented, and defense responses only occur once the pathogen or the pest attacks (van Peer et al. 1991; Pastor et al. 2013). The negative impacts of PGPR-based elicitors on insect development (lepidopteran pests and aphids) have been documented in different research activities, sometimes accompanied by a promotion of plant growth, which balances the effect of pest invasion (Fahimi et al. 2014; Disi et al. 2018).

3.12.1 Commercial Elicitor Products

3.12.1.1 PRIs and Primings

Plant resistance inducers (PRIs) are agents that lead to improved protection against pathogens and pests by inducing the plant's own defense mechanisms, so-called induced resistance (IR). They are also referred to as plant resistance activators, plant defense activators and elicitors. PRIs are known to be effective against various pathogens and pests. PRIs can be chemical compounds as well as microbial or plant extracts (Walters et al. 2013; Alexandersson et al. 2016). Depending on their very nature, they either behave as non-self determinants (mimicking MAMP (microbe-associated molecular patterns) or DAMP (damage-associated molecular patterns) general elicitors) perceived by pattern recognition receptors (PRR) present at the cell surface (Henry et al. 2012) or mimic plant downstream signaling molecules such as phytohormone analogs or derivates (Derksen et al. 2013). Exogenous application of PRIs aims at leading the plant defense system into an induced or primed state. The induced resistance generated by PRIs is intended to be broad spectrum and long-term efficient, but the PRIs seldom lead to full pathogen control. Several factors influence its success such as plant genotype, developmental stage, environment, as well as timing and the manner of their application. Importantly, all PRI strategies need to be tested in an agricultural setting as many treatments have only been shown to be successful in more controlled conditions. The effects of PRIs can be both local and systemic (Sandroni et al. 2020). However, PRIs are seen as a hopeful strategy in light of the current awareness of the need to reduce the use of pesticides; nevertheless, the extent of the fitness penalty differs largely between PRIs and is also dependent on the growth environment. By identifying SA as an essential endogenous signal for the SAR response, an intensive search was initiated in order to identify synthetic chemicals able to mimic SA in SAR induction and many different organic and inorganic compounds identified to activate IR in plants (Goellner and Conrath 2008). The compounds 2,6-dichloroisonicotinic acid and its methyl ester

(INA) were the first synthetic compounds reported to activate the bona fide SAR response in plants (Kessmann et al. 1994). Later, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) became an attractive synthetic SAR activator. SA, INA, and BTH are assumed to activate SAR via the same signaling pathway (Goellner and Conrath 2008). One of the most studied PRIs is acibenzolar-*S*-methyl (ASM), a salicylic acid functional analog belonging to the benzothiadiazole (BTH) family. It is a synthetic SAR inducer for crop protection that it has been registered under different trade names. ASM efficiency has been reported in many crop species for its performance in controlling a large number of pathogens and/or in inducing or priming multiple immune responses (Marolleau et al. 2017).

The commercial products that currently exist in the marketplace are used mainly in integrated pest management (IPM) strategies as complementary tools to help reduce chemical inputs. By now and depending on their efficiency, elicitors are usually applied alone or in combination with other fungicides, once or several times in a crop cycle (Walters et al. 2013). Among chemical PRIs, potassium phosphite (Phi) has been widely studied in controlled environments and, in fewer cases, in the field conditions. In the study on the effects of azelaic acid (AA), benzothiadiazole (BTH), gibberellic acid (GA), harpin, and jasmonic acid (JA) on the fall armyworm (FAW), *S. frugiperda* (Lepidoptera: Noctuidae), reared on four crop plants, cotton, corn, rice, and soybean, under greenhouse conditions, it was revealed that treatment with JA consistently reduced the growth of FAW reared on treated cotton and soybean. In contrast, FAW fed BTH- and harpin-treated cotton and soybean tissue gained more weight than those fed control leaf tissue, showing there was a negative crosstalk between the salicylic acid and JA signaling pathways. No induction or inconsistent induction of resistance was observed in corn and rice (Gordy et al. 2015). The co-application of adjuvants with JA could not increase the effectiveness of induction by JA, and the effect of JA may be species specific because the soybean looper (*Chrysodeixis includens* (Walker)), a relative specialist on legumes, was less affected by JA-induced responses in soybean compared with FAW (Gordy et al. 2015). They concluded that the effectiveness of elicitors as a management tactic will depend strongly on the identities of the crop, the pest, and the elicitor involved (Gordy et al. 2015). Moreover, the response of dicotyledonous and monocotyledonous plants to the exogenous JA application may be different. For instance, the statistically significant reductions in FAW growth were found only in the treated cotton and soybean and not in treated corn and rice.

In laboratory trials, BABA-induced priming for enhanced stress responses was associated with augmented resistance not only to biotic but also to abiotic challenges such as drought and salt stress (Jakab et al. 2005). Moreover, BABA and some other priming-inducing compounds were also shown to be potent inducers of stress tolerance in the greenhouse and field conditions (Cohen 2002). However, due to the general lack of consumer acceptance of some priming agents like BTH, it became opportune to identify plant-protecting compounds teaming both direct action on the pathogen and priming-inducing activity in the plant. Some strobilurin fungicides seem to combine both these activities (Goellner and Conrath 2008). Similar observations with pyraclostrobin have recently been made in laboratory

and field experiments with the insecticide imidacloprid. One of its major degradation products, 6-chloronicotinic acid, has a structure very similar to INA and is suspected of producing a so-called stress-protective effect on products by preparing them to increase the expression of defense genes and increase their tolerance to biotic and abiotic stresses and increased plant growth and yield (Thielert 2007).

3.12.2 Elicitors in IPM

Integrated pest management (IPM) is a multifaceted approach to mitigate damage by herbivorous insect pests which unfortunately still relies too heavily on broad-spectrum synthetic insecticides for many crop–pest systems. The use of elicitors for the induction of plant defenses that result in decreased herbivore fitness may be an additional tactic in IPM programs. Gordy et al. (2015) believe that the effect of elicitors as a management tactic will depend strongly on the identities of the crop, the pest, and the elicitor used. To enhance the performance of a biological control program, it is therefore important to identify and apply biological control agents that not only can cope with the induction of defenses by pests but also can manipulate these in favor of plant productivity. In this context, there may be opportunities for enhancing the synergistic effects or attenuating the negative interactions between these organisms. For example, infesting plants with beneficial microbes to combat a foliar pathogen may variably affect induced plant susceptibility to the phytophagy of zoophytophagous predators or result in increased predation against a herbivorous prey. On the other hand, applying defense elicitors to enhance plant resistance against a single herbivore may provide empty niches for secondary pests such as other herbivores or plant pathogens and may also affect other plant traits in an unwanted way (Pappas et al. 2017).

Although natural plant defenses clearly can be put to work for crop protection, simply stacking defenses, green chemistry, and biological control in IPM may do more harm than good. Plant defenses may interfere directly by negatively affecting predator performance and indirectly by affecting prey quality and eventually crop yield. This may not always clearly reveal itself since natural enemies may still be effective albeit less efficiently than they could be. Taking a community perspective, biological control is important to reveal opportunities for combining induced plant defense with biological control using natural enemies. Carefully exploring the net benefits of combining these different approaches may prevent this multipurpose tool from turning into a double-edged sword (Pappas et al. 2017).

3.13 Increase Plant Resistance to Pests and Diseases by Stimulating Plant Growth Using Biostimulant Products

In recent years, eco-friendly approaches are increasingly considered to support agricultural sustainability. Biostimulants are organic molecules or plant extracts known (usually consisting of various substances and microorganisms including

microbial inoculants, humic and fulvic acids, seaweed extracts, protein hydrolysates, amino acids) to stimulate plant growth (du Jardin 2012; Calvo et al. 2014). Biostimulant products can be based on a single PGPR strain, a PGPR mix, or a mix of PGPR and PGPF, but when they are used in consortia, they can reach most of the empty niches because of their increased genetic diversity and they colonize the root zone much faster than single strains (Reddy 2014). Products with a mix of PGPR strains can therefore compete spatially with a broader range of potential pathogens under different plant growth and environmental conditions (Reddy 2014). In addition, recent studies have shown that PGPR used to complement mineral fertilization can reduce conventional fertilizer rates. Adesemoye et al. (2009) showed that a combined inoculation of the two PGPR strains *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4 with a strain of the arbuscular mycorrhizal fungi *Glomus intraradices* reduced fertilizer use by 25%. They showed that this combination was as efficient as a 100% fertilizer application in terms of plant growth, yield, and nutrient uptake. In addition to increased crop quality, they can also prime a plant to deploy its defense machinery in a faster, stronger, and/or more sustained manner while under herbivore attack (Hodge et al. 2005; Pangesti et al. 2013; Du Jardin 2015), including colonization of *A. thaliana* roots by the plant growth-promoting rhizobacterium *P. simiae* WCS417r and induced systemic resistance to the generalist caterpillar *S. exigua* by activating the JA pathway (van Oosten et al. 2008); colonized onion plants (*Allium cepa* L.) with the endophytic fungi *Clonostachys rosea* ICIPE 707, *Trichoderma asperellum* M2RT4, *Trichoderma atroviride* ICIPE 710, or *Hypocrea lixii* F3ST1; and reduced feeding of *Thrips tabaci* (Muvea et al. 2014). The abovementioned examples illustrate that priming is a common phenomenon that takes place during most types of induced resistance.

3.14 Formulation and Application Methods

The formulation and application methods are probably among the most important parameters that determine the efficiency of a biocontrol product. The formulation should be easy to use and preserve the effectiveness of an effective plant growth-promoting agent or a biocontrol product (Bashan et al. 2014; Mire et al. 2016). Currently, microbial-based bioproducts employed under the different trade names including biostimulants, bioinoculants, biofertilizer, and biopesticides type have been considered as essential components of ecological sustainability and improved the crop productivity to a greater extent in an eco-friendly manner. Bioformulations are defined as any biologically active substances derived from microbial biomass or product containing microbes and their metabolites that could be used in plant growth promotion, nutrient acquisition, and pest and disease suppression in an eco-friendly manner (Aamir et al. 2020). Bashan et al. (2014) summarized various formulation methods, from the choice of carriers (peat, coir dust, charcoal, sawdust, clay, perlite, vermiculite, polymer-like alginate) to the formulation process. They also summarized various practical techniques for inoculant application and production achievement. Seed treatment has attracted main attention as a simple and

economically viable technique, being convenient for both farmers and industry (Bashan et al. 2014). The seeds are usually coated with a carrier and PGPR, with or without adhesives (carboxymethylcellulose, sucrose, vegetable oil, Arabic gum). This is currently the method most often used to apply PGPR inoculants as it ensures an optimal threshold number of PGPR cells per seed needed to cover the seedling roots. Although the cell threshold differs among strains, the common concentration is 10^8 cells per plant (Bhattacharyya and Jha 2012). Soil applications of PGPR is another approach used when a large population of rhizobacteria is needed at a specific and crucial plant growth stage (e.g., tillering or flowering stages) (Bashan et al. 2014). However, soil and open-air conditions (humidity, temperature) can affect the success of the soil application. Extreme temperatures can cause a decline in the PGPR survival rate, and soil humidity determines the effective mobility of the inoculated bacteria in the rhizosphere (Bashan et al. 2014). Using enough water (e.g., at least 100 L/ha) in the mixture with liquid or powder-based inoculants also ensures that the bacteria are positioned near the root system. Additional PGPR inoculations could be needed to maintain a minimal bacterial population in the case of stressful conditions such as winter and drought (Bashan et al. 2014).

3.15 The Trade-Off Between Induced Resistance and the Cost of Defense Activation

One of the questions in inducing resistance in plants is the trade-off between disease or pest resistance and the high costs of defense activation and whether this energy is reversible or not (Björkman et al. 2008). For instance, exogenous applications of MeJA on *Pinus sylvestris* seedlings and *P. abies* trees resulted in 30% less radial sapwood growth than in control trees (Heijari et al. 2005; Krokene et al. 2008). Elevated resistance of *Pinus radiata* to *Diplodia pinea* induced by foliar applications of MeJA was accompanied by a reduction in seedling growth rate in the second week following treatment (Gould et al. 2008), although the seedlings recovered and eventually their growth rate exceeded that of control seedlings. Costs can arise from the allocation of resources to defense and away from plant growth and development, and there are also ecological costs which result from trade-offs between induced resistance and the plant's interaction with beneficial organisms, e.g., mycorrhizal fungi (Walters and Heil 2007). The activation of direct defense reactions by exogenous application of high doses of SA and jasmonic acid or by the action of resistance (R) genes has been found to reduce plant fitness traits such as growth and fruit or seed set under pathogen-free conditions (van Dam and Baldwin 2001). Furthermore, plants transformed with genes encoding SA biosynthesis enzymes (Mauch et al. 2001) or gain-of-resistance mutations in *Arabidopsis* such as *cpr1*, *cpr5*, and *cpr6*, which all contain constitutively high levels of SA, permanent expression of defense-related PR genes, and a dwarf phenotype, have been associated with reduced fitness (Bowling et al. 1997). These observations were also confirmed in the field when Heidel et al. (2004) showed that *Arabidopsis* mutants blocked in SA-inducible defense, as well as mutants showing constitutive expression

of these defenses, were affected in growth and seed set. Similar conclusions were drawn from studies on the costs of jasmonic acid-inducible defenses, which seem to be affordable only when the plant is actually exposed to herbivore attack (Agrawal et al. 1999). The trade-off dilemma between disease resistance and costs of defense activation can probably be overcome by priming. In a study carried out by van Hulten et al. (2006), the costs and benefits of priming in *Arabidopsis* were determined and compared to those of the direct induction of defense (application of low doses of the nonprotein amino acid β-aminobutyric acid (BABA) induced a primed state, resulted in only minor reductions in growth, and had no obvious effect on seed production). In contrast, direct induction of defense responses by high doses of either BABA or BTH strongly reduced both these fitness traits. This issue shows that priming has a smaller effect on fitness than directly induced defense (van Hulten et al. 2006) and the observed reduction in growth is likely to be a transient effect and will probably have little impact on long-term tree growth, but recovery may be linked directly to the duration of the heightened IR state. Transgenerational resistance can also incur associated costs. For instance, crosstalk between SA- and JA-dependent resistances has been demonstrated in the progeny of virulent *Pst*-infected plants. SA-primed progeny downregulated JA-dependent defenses, resulting in plants that were more susceptible to the necrotrophic fungus *Alternaria brassicicola*. Lopez et al. (2011) also demonstrated epigenetic regulation of SA–JA crosstalk using mutants impaired in enzymes that mediate RNA-directed DNA methylation, which were more resistant to biotrophic pathogens but more susceptible to necrotrophs. Thus, different immune responses can achieve transgenerational priming with a certain level of specificity to the parental stimulus, resulting in costs associated with the inheritance of defense-hormone crosstalk (Mauch-Mani et al. 2017).

3.16 Phytohormones

Phytohormones are a group of naturally occurring organic substances which influence physiological processes at low concentrations. The processes influenced consist mainly of growth, differentiation, and development, though other processes, such as stomatal movement, may also be affected (Davies 2010).

3.16.1 Interaction Between Phytohormones

The systemic and specific plant responses to herbivore feeding are governed by networks of hormones and other signals (Campos et al. 2014). As mentioned before, the plant hormones most commonly associated with inducible resistance are jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) (Pieterse et al. 2014) in which JA is a key regulator of responses to chewing herbivores and necrotrophic pathogens in plants. It is also involving in the inhibition of seed germination and plant growth and promotes leaf senescence, fruit abscission, tuber formation, flower

and fruit development, pigment formation, and tendril coiling (Davies 2010). Following attack by a herbivore, levels of endogenous JA increase and, in response, secondary metabolites are produced in vivo. These metabolites deter insect feeding, toxify or interfere with acquisition of nutrients by insects, or attract natural enemies (Smith et al. 2009). SA, on the other hand, is a key regulator of responses to biotrophic pathogens and piercing-sucking insects (Smith et al. 2009; Glazebrook 2005; Goggin 2007). Likewise JA, levels of SA increase following attack by biotrophic pathogens and piercing-sucking herbivores, increase and lead to the production of responses associated with resistance to pathogens such as pathogenesis-related proteins (Goggin 2007). Interactions among these hormones appear to be important, with one of the best-studied interactions being the negative crosstalk that exists among JA- and SA-mediated responses (Pieterse et al. 2014). Other hormones, such as gibberellins and abscisic acid, also play roles in induced resistance, for example, as modulators of JA, SA, and ET (Karban 2011). Historically, the most important models for the study of the hormonal control of induced responses have been dicots, and it is unclear whether the roles of JA and SA in monocots are identical to their roles in dicots (Karban 2011; Tamaoki et al. 2013). Although signaling networks among multiple phytohormones fine-tune plant defense responses to insect herbivore attack, in some cases, some phytohormones have been shown to work independently. For instance, although it has been reported that the synergistic combination of ethylene (ET) and jasmonic acid (JA) was required for accumulation of the *maize insect resistance1* (*mir1*) gene product as a cysteine (Cys) proteinase that is a key defensive protein against chewing insect pests in maize (*Zea mays*), Louis et al. (2015) showed that *mir1*-mediated resistance to corn leaf aphid (CLA; *Rhopalosiphum maidis*), a phloem sap-sucking insect pest, is independent of JA and regulated by the ET signaling pathway. In addition, they underscored the significance of ET acting as a central node in regulating *mir1* expression to different feeding guilds of insect herbivores.

3.16.2 Crosstalk Between Phytohormones

Although hormonal crosstalk between different plant defense pathways has often been hypothesized to be a cost-saving strategy that has evolved as a means of the plant to reduce allocation costs by repression of unnecessary defenses, thereby minimizing trade-offs between plant defense and growth (Vos et al. 2015), some pests have shown to be able to change this approach in their favor and to destroy the plant's immune system. For instance, it has been revealed that in tomato, the polyphagous *T. urticae* Koch and the Solanaceae specialist *T. evansi* Baker and Pritchard can suppress SA- and JA-dependent responses, although these mechanisms seem to be time dependent (Agut et al. 2018). In fact, the mite *T. evansi* can suppress JA-dependent responses by stimulating the SA pathway, activating negative crosstalk between these hormones (Pieterse et al. 2009). Non-adapted strains of *T. urticae* induce both JA- and SA-dependent defenses, whereas the specialist *T. evansi* suppresses a larger subset of genes activated by

T. urticae (Alba et al. 2015). It has been shown that salivary secreted proteins can suppress responses downstream of SA and JA pathways. The transient expression of three proteins discovered in the secretome of *T. urticae* in *Nicotiana benthamiana* improved the performance of the mite. Despite the fact that some proteins, such as TE8, may act as elicitors to recognize an attack, other proteins such as TU28, TU84, and TC84 may function as effector proteins, suppressing plant response (Agut et al. 2018). As *T. urticae* avoids epidermal damage, this may contribute to minimizing detection of the attack by the leaf surface, therefore delaying the plant response. Although it is almost impossible to find a single herbivore attack in real field conditions, little is known about plant responses to multiple herbivore attacks. In this context, Glas et al. (2014) demonstrated that *T. urticae* colonizes plants already infested with *Aculops lycopersici* (Massee) (Acari: Eriophyidae) with greater intensity because *A. lycopersici* induced SA responses in tomato plants that suppress the JA pathway. On the contrary, there are examples in which *T. urticae* did not benefit from an interspecific infestation. For example, when *Macrolophus pygmaeus* (Rambur), a zoophytophagous biological control agent that is used against whiteflies, aphids, and spider mites, feeds on plants, triggering increases in the levels of proteinase inhibitors in local and systemic tissues, which negatively impact the *T. urticae* performance (Agut et al. 2018).

Likewise mites, generalized plant responses to aphid feeding (e.g., cell damage by aphids) are mediated by phytohormonal signaling that induce jasmonic acid (JA) and ethylene production across a broad swathe of plant species (Ferry et al. 2011). Aphids are commonly susceptible to externally induced JA-mediated defenses. The exogenous application of JA to tomato plants and previous damage by leaf-chewing herbivores that induces JA was shown to impair aphid population growth and have a negative effect on aphid performance; in contrast, SA-mediated defenses have less-consistent effects on aphid performance. For example, induction of the SA pathway by a pathogen on tobacco did not impact subsequent feeding by the aphid *M. nicotianae* Blackman (Homoptera: Aphididae), but SA signaling and exogenous application of SA analogs did reduce the performance of *Myzus persicae* on *A. thaliana* and *M. euphorbiae* on tomato. Phytohormonal signaling is evolutionarily highly conserved, and the two hormones SA and JA are natural antagonists, most likely as part of the plant's strategy to fine-tune its defense (Thaler et al. 2012). By inducing the plant's SA pathway, aphids may be able to use this hormonal "crosstalk" to suppress a potentially more detrimental JA response. In support of this hypothesis, mutant *Arabidopsis* plants that are deficient in SA signaling (and thus unaffected by such manipulation) are more resistant to aphids than wild-type plants (Mewis et al. 2005). It is also important to note that activation of the SA pathway by aphids commonly induces unique plant responses compared to exogenous SA application (Ferry et al. 2011), suggestive of a finely tuned manipulation of plant responses by the aphid (Fig. 3.5).

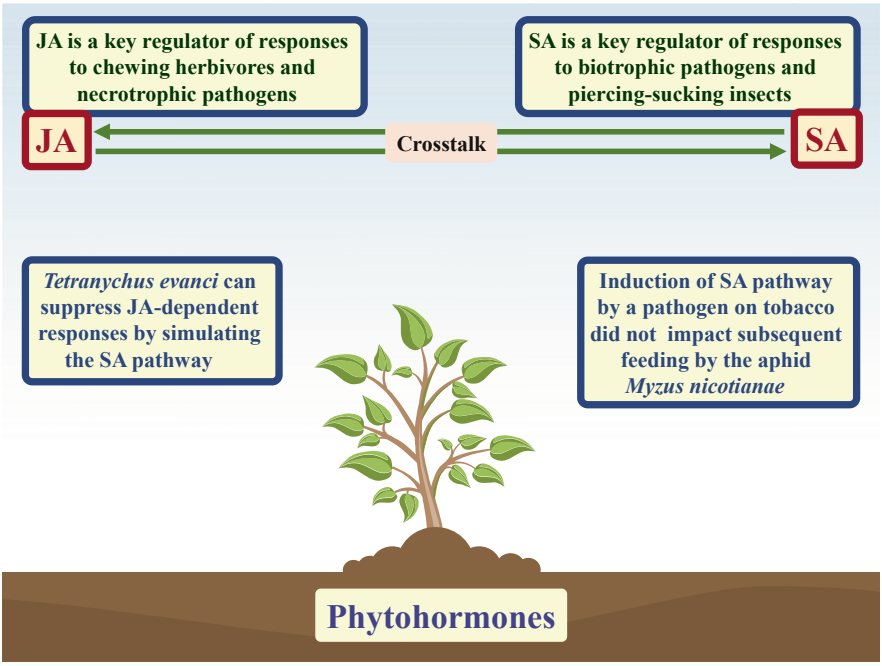

Fig. 3.5 Negative interaction between phytohormones makes the plant more vulnerable to the attacks of pathogens or insect pests

3.17 How Can We Use PGPR, Elicitors, and Semiochemicals?

Westman et al. (2019) showed that defense priming effects on *Arabidopsis* plants depend on both the priming agent and the antagonist. The screening of suitable PGPR inoculants, elicitors, and semiochemicals for specific crops, growth conditions, and pathogens is critical if the efficacy of these products in the field is to be guaranteed. However, a common method for screening an effective PGPR inoculant is to isolate strains from plant growth-promoting soil or from pathogen-suppressive soil (Mendes et al. 2011). Screening failures can occur, as some PGPR strains which show limited ability to promote plant growth during screening trials under controlled conditions can be among the most effective strains in the field (da Silva Araújo et al. 2013). In the case of PGPR, bacterial concentrations in commercialized products can fall below the desired threshold (usual concentration: 10^8–10^{11} cells/mL), especially under long-term or inadequate storage (Bashan et al. 2014), and these problems may result in failure of our control program. A less effective interaction can also occur when the PGPR inoculant is not adapted to the host plant or the local environmental conditions (climate, soil characteristics, and agronomic practices). For instance, modern rice varieties selected to use N fertilizers

effectively are less interactive with native N-fixing bacteria than that of traditional varieties (da Silva Araújo et al. 2013). Similarly, the performance of elicitors depends greatly on field environmental conditions (temperature, relative humidity, and disease pressure), crop systems (plant genotype, nutritional requirements, physiological state), and the formulation (Mire et al. 2016). The effectiveness of the abovementioned compounds may also vary based on different populations of a pest or disease, as geographically isolated or close populations of a pest may be different in terms of genetic pattern (Bagheri et al. 2018) which requires balancing the management approach. It is clear now that responsiveness to PRIs is dependent on many factors and varies according to the plant genotype. In a 3-year study using a combination of acibenzolar-*S*-methyl (ASM), BABA, and *Cis*-jasmonate, barley cultivars showed differences in IR against *Rhynchosporium secalis* and *Blumeria graminis* f. sp. *hordei* in controlled environment and field conditions (Walters et al. 2011). Similar to what said for pests, inducibility may also depend on the pathogen strain. By applying BABA on different tomato accessions, inducibility varied significantly not only among genotypes but also depended on the isolate of *Ph. infestans* tested (Sharma et al. 2010) which adds to the complexity of conducting these studies, that will be a definite challenge if IR will be included as a future target in breeding programs. The availability of mineral nutrients in soils can also affect the plant's degree of JA-dependent resistance. It has been revealed that *A. thaliana* plants grown under potassium-deficient conditions are less susceptible to thrips attack. This effect is most likely mediated by enhanced JA-associated responses, as some responses to potassium deficiency were dependent on coronatine insensitive1 (COI1), an essential regulator of JA signaling (Armengaud et al. 2010).

3.18 PRIs and Primings and Their Future Perspectives

Recently, the impact of climate change on plant protection strategies has received much attention because the relative increase in temperature, limited water resources, and also increase in CO_2 levels will affect both the geographical distribution of pests and pathogens. These environmental factors also affect the physiology of plants, including the innate immune system (Sandroni et al. 2020). Therefore, research on plant immunity inducers needs to focus on finding new biological pesticides and on working with industry to develop such compounds and organisms into safe, inexpensive products for commercial release (Dewen et al. 2017). Although the excessive use of chemical toxins is destroying our ecosystem, in developing countries, farmers are less likely to use these methods instead of using chemical pesticides. Farmers are not always enthusiastic about offering alternative methods, especially on small-scale farms or in developing countries (Gozzo and Faoro 2013; Bashan et al. 2014). They do not tend to adopt biostimulant products or innovative crop protection strategies unless their success is guaranteed. The highest number of farmers currently using bio-based products (including plant biostimulants and biopesticides) is in North America, representing 40% of the biocontrol market, compared with 25% in Europe, 20% in Asia, 10% in South America, and 5% in the rest of the world (Cox

and Wong 2013). The main reason for farmers' skepticism about these alternative methods is their variable effect in the field compared to conventional chemical inputs (Arora et al. 2010; Walters et al. 2013). In addition, many studies have shown that these products usually have a variable performance in the field conditions, compared with the promising results obtained in the laboratory or in greenhouse conditions (Gozzo and Faoro 2013).

Farmers' decisions on whether or not to adopt new methods often depend on how much they want to change their agricultural practices. Total reliance on new strategies can be challenging. The benefits of these strategies have to be clearly demonstrated through educational programs that focus on field data (e.g., pest/disease identification, timing of infestation, crops) (Maurya et al. 2019). This includes detailed knowledge about agronomic parameters and designing adapted crop management techniques, with the appropriate biostimulant or biocontrol product applied at the right time and frequency, in combination with other control methods and on responsive cultivars (Walters et al. 2013; Bashan et al. 2014).

3.19 Disadvantages of PRIs and Primings in Plants

Although it is generally believed that inducible defenses have evolved to save energy under enemy-free conditions, costs still arise upon activation of these defenses under hostile conditions. These costs can stem from allocation of limited resources to defensive compounds or toxicity of the defense to the plant's own metabolism. In this context, Yip et al. (2019) in a study on goldenrod (*Solidago altissima*) found that defense priming influenced growth and reproduction under seminatural field conditions by manipulating exposure to priming cues (volatile emissions of a specialist herbivore, *Eurosta solidaginis*), competition between neighboring plants, and herbivory (via insecticide application). Although primed plants grew faster than unprimed plants, they produced fewer rhizomes, suggesting reduced capacity for clonal reproduction (Yip et al. 2019). In addition, costs can arise from external factors, when the defensive trait affects a beneficial interaction with another organism in the environment. It is therefore reasonable to assume that plants express their inducible defenses only if the benefits (i.e., protection against the attackers) outweigh the costs of the resistance (van Hulten et al. 2006). Other various studies have demonstrated costs related to jasmonic acid (JA)-inducible defense against herbivory. These costs can affect plant growth and reproductive traits (van Hulten et al. 2006). In wheat, Heil et al. (2000) demonstrated costs of SA-inducible defenses on growth and seed set. In *Arabidopsis*, Cipollini (2002) showed that exogenously applied SA reduced seed production. Also though it has been reported that priming compared to elicitation generally results in low fitness costs for the plant, it could lead to the downregulation of some resistance pathways or could sensitize plants such that they respond to false alarm signals. For these reasons, Crisp et al. (2016) recently hypothesized that plants might be better at forgetting previous stresses in order to avoid compromising development, yield, and ultimately survival (Mauch-Mani et al. 2017).

3.20 Conclusions

Priming is a phenomenon that augments multigenic basal resistance; therefore, the induced resistance can be more durable than race-specific resistance, which is based on single resistance genes (Ahmad et al. 2010). In addition, despite the fact that priming rarely provides complete protection (Walters et al. 2013), application of priming-inducing agents is increasingly considered for exploitation in integrated pest and disease management (Beckers and Conrath 2007; Conrath et al. 2015). However, like any other control method, this method also requires a number of prerequisites. For instance, an accurate weather forecast and readiness to apply priming agents as environmental conditions change would be crucial for the exploitation of their full potential. The possibility to predict the magnitude of the upcoming stress and, hence, adapt the dosage would similarly be important for the chemical priming toolbox (Kerchev et al. 2020). In addition, defense priming agents should be carefully validated before using under field conditions, particularly with respect to their effects on allocation fitness cost, environmental impacts, effects of light and heat, and economic cost.

Although it has been said that plants have different mechanisms to cope with pests and diseases, sometimes plants experience severe pest damages under natural conditions. This issue can be due to the antagonistic relationship between phytohormones when several pests attack a plant. In these situations, although the plant has stopped some of these pests using induced resistance or priming defense, some of them may take advantage of the antagonistic relationships between phytohormones to establish themselves on the plant. Of course, this issue needs to be further studied. Overall, new findings on the priming agents, endophytes, and endosymbionts have opened a new era regarding biological control concepts. In the new approach, not only natural enemies and pests are important, but also other factors, e.g., the microorganisms that are in association with natural enemies (endosymbionts) and plants (endophytes), have a main important contribution. In fact, it should be a positive interaction between each of these agents to ensure the success of biological control. In the new approach, before any action, the compatibility of these factors with each other should be guaranteed.

Points to Remember

- Although the history of understanding the inducible resistance is strongly in association with plant diseases, it is now clear that they can be used practically against pests in all forms.
- It is widely agreed that PGPR biostimulants, elicitors, and semiochemicals should not be used as stand-alone methods in agroecological management.
- Determining the best phenological stage of plants for primings has an important role in increasing their efficiency; the seed stage has been shown to be a very promising stage for priming in many plant species.
- Utilizing the potential of some mineral elements such as silicon is a great opportunity both to improve plant growth and to increase the physical and biochemical resistance of plants to pests.

- Integration of primings with other pest and disease control approaches helps us to reduce the amount of chemicals through reducing the dosage and application frequency.
- Determining the relationships between primed plants and the third trophic members in the integrated pest management programs is critical to increase priming efficiency.
- By accurately identifying resistance pathways, the possibility of crosstalk for pests is minimized.

Acknowledgments The authors are grateful to Mousa Abdollahipour, PhD student of Entomology at Tarbiat Modares University, for his great assistance in preparing the figures.

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Transgenic Plant Technology: An Insight into Insect Resistance

4

Amit Choudhary, Rashmi Srivastava, Rakesh Srivastava, and Praveen Chandra Verma

Contents

Abstract

The production of the transgenic plant is an important tool in plant and agricultural biotechnology, which alters the plant genetic characters for improving the species-specific traits or for adding any novel or a beneficial trait that usually remains absent naturally in economical crops. The introduction of genetic

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Omkar (ed.), *Molecular Approaches for Sustainable Insect Pest Management*,
https://doi.org/10.1007/978-981-16-3591-5_4

transformation conquered the major constraint of conventional plant breeding. As a result, transgenic plant technology has been shown to enhance crop yield, reduce the use of insecticides and pesticides, and reduce crop production costs. Notably, crop yield loss due to insects is a leading threat to economic loss and food security worldwide. Insects cause two main classes of damage to growing crops—one is direct contact and the other is indirect damage through infection. One of the accomplishments of the transgenic plant has been the establishment and commercial cultivation of insect-resistant plants against different insect pests. This chapter sheds light on an important aspect of the different transgenic plants used in the development of insect resistance and their future impact on their ecological and economic perspectives.

Keywords

Transgenic plants · Insect pests · Resistance · Tolerance · Human health · Environmental safety

Learning Objectives

1. The introduction of genetic transformation conquered the major constraint of conventional plant breeding. Particularly, crop yield loss due to insects is a leading threat to economic loss and food security worldwide.
2. The production of the transgenic plant is an important tool in plant and agricultural biotechnology, which alters the plant genetic characters for improving the species-specific traits or for adding any novel or a beneficial trait that usually remains absent naturally in economically important crops.
3. Consequently, the development and deployment of transgenic plant technology have been revealed to enhance crop yields, decrease insecticide and pesticide usage, and reduce crop production costs.
4. This chapter provides an insight into the formulation of strategies of the different transgenic plants used in the development of insect resistance and their future impact on their ecological and economic prospects and in-hand societal awareness.

4.1 Introduction

The World population may cross the numbers 9 billion in recent times, there will be tremendous rise food of requirement in the future. To suffice the needs of such an enormous amount of food, crop productivity must increase at the same rate or even more with the increase in population. Agriculture is the most important socioeconomic practice in the entire world, and sustained agricultural growth is a necessity, not an option, for all the developing countries. Agriculture has always been the most important economic sector, which is strongly impacted by complex biotic stresses, like pathogens and insect pests. Insects are the most flourishing organism on the planet in terms of habitat and adaptation. Interestingly, insect pests have been a

threat to crop cultivation ever since man has started growing crops. Severe losses in crop yields are caused due to the concerning blooms of insect pests. Among these, 9000 species are insects and mites, which are responsible for major yield losses in several of our important crops, particularly the tropical crops. As a result of the rigorous plant-pathogen interaction for several hundred million years, plants have developed some defense features against various insects as revealed by a plethora of key stress-inducible genes being identified, which are associated with defense response (Ferry et al. 2006; Lodhi et al. 2008; Srivastava et al. 2014, 2018; Ali et al. 2018; Pandey et al. 2019; Agarwal et al. 2020). However, these defense strategies remain insufficient to combat the major crop insect pests due to the experimental limitations involving these studies. One of the major problems in insect pest management using an insecticide is their broad-spectrum aspects, which makes them more vulnerable to kill several insect species including beneficial ones. This is itself a serious issue because we are losing the beneficial ones too, besides several other problems. Additionally, the development of insecticide resistance within 2–4 years of heavy use and the emergence of secondary insect pests due to loss of parasitoids and predators are other ancillary problems.

Applications of transgene technology in agriculture have clearly defined benefits, providing greater sustainability in terms of improved levels of crop protection, resulting in higher yields and reduced pesticide application (Tabashnik 2010). Some potential transgenic have been developed out of so many plant species against various insects. These transgenic plants are performing well in terms of pathogen resistance/tolerance as well as crop production (Babu et al. 2003). More resistance towards insects and diseases will allow plants to last longer and more crop productions. The need to feed the growing population with more desirable products will be solved by natural plant variety, breeding, or genome-edited plants (Rai et al. 2019; Dixit et al. 2020). Therefore, it is a primary requisite to use genetic modifications for the improvement of crops, which leads to a promising increase in yield, with desired traits and pest/pathogen tolerance. The concept of utilizing a transgenic approach to host plant resistance was realized in the mid-1990s with the commercial introduction of transgenic maize, potato, and cotton plants expressing genes encoding the insecticidal δ-endotoxin from *Bacillus thuringiensis*.

There has been an increase in the yield due to the introduction of insect resistance or tolerance in the transgenic crops. However, a major challenge in front of this new industry is the proper identification of suitable genes that are more specific to the target keeping in mind its benefits. In terms of insect resistance, several different classes of bacterial-, plant-, and animal-derived proteins have been reported to be insecticidal towards a wide range of economically important insect pests from different orders of the taxonomic hierarchy. With several advantages as well as disadvantages, the future of transgenic plant remains a subject of debate and examination for its future use and associated applications. There are two most important views for transgenic crop regulation (Dale 1995). In the first opinion, transgenic crops are improved versions of conventional crops and have been generated responsibly following the guidelines by researchers and plant breeders. The second point

suggests that there is a need to develop more detailed and stringent regulations to govern genetic modification technology.

4.2 Transgenic Crops for Insect Pest Management: Advantages and Disadvantages

Insect tolerance in crops has been a key objective in agricultural and plant breeding applications. Almost billion dollars are spent on synthetic pesticides per year; for example, 15% and 23% of these insecticides are used to protect rice and cotton, respectively (Krattiger 1997). Pesticides worth billions of dollars are required annually for the production of economical crops, such as corn, tomato, wheat, cotton, or rice, to prevent different pathogens. However, pesticides have a significant role in the sustainable development of human society by increasing the quality and quantity of plant production. In contrast, unavoidable fears are also arising regarding their regular and continuously increasing use. The WHO's evaluation suggests that poisoning by pesticides causes 3 million cases per year, which further accounts for 250,000 deaths per year generally, because of unprofessional management and treatment (Stoytcheva 2011).

Application of insect-/pathogen-resistant crop varieties should be economically, environmentally, and ecologically beneficial. It is reported that the total cultivated area for genetically modified (GM) crops has reached 185.1 million ha till 2016 (Briefs 2016; Brookes and Barfoot 2017, 2018). GM crops mostly include crops such as corn, canola, rice, wheat, tomato, soybean, sugar beet, and cotton. These crops are mainly resistant to biotic stresses, such as insects, herbicides, and other abiotic stresses (Brookes and Barfoot 2017, 2018). For more than two decades, crops compassing toxin genes for insects have become commonly used in agriculture, which has brought about the reduction in pesticide application but also reduced the cost of production (Toenniessen et al. 2003; Gatehouse 2013). The first report on transgenic plants is comprised of gene encoding *Bacillus thuringiensis* (Bt) toxin that exhibited increased resistance to insect herbivores (Barton et al. 1987; Fischhoff et al. 1987; Vaeck et al. 1987; Gatehouse 2013). Reports suggest that a large reduction of insecticide usage occurred due to insect-resistant cotton (Naranjo 2009; Romeis et al. 2019). Due to the Bt cotton effectiveness, the utilization of synthetic insecticides has gone down (Bakhsh et al. 2009). It is also revealed that countries, such as Argentina, Mexico, India, China, and South Africa, lowered their insecticide practice by approximately 33–77% (Qaim 2009). After several studies encompassing the concepts of insect resistance, a series of effective researches on transgenic plants were recognized, the examples of which are listed in Table 4.1.

In addition to Bt genes, several additional genes of microorganisms, plants, and other origins depicting resistance for insect pests are used in crops (Table 4.1) (Kereša et al. 2008; Schuler et al. 1998; Gatehouse 2008). The proteinase inhibitors play a significant function in insect resistance and cause inhibitory activity in insect digestive enzymes. The genes for potato proteinase inhibitor II have been inserted in rice, cotton, and other economical crops (Gatehouse 2008; Duan et al. 1996). The

Table 4.1 Some genes used for the development of insect pest-resistant transgenic plants

Pathogen	Gene	Plants	Reference
BPH	*GNA*	Rice	Rao et al. (1998)
BPH	*ASAL*	Rice	Chandrasekhar et al. (2014)
Coleoptera	*cry3A(a)*	Potato	Adang et al. (1993), Perlak et al. (1993), Morán et al. (1998)
Coleoptera	*cry3A*	Alfalfa	Tohidfar et al. (2013)
Corn leaf aphid	*GNA*	Maize	Wang et al. (2005)
Cotton aphid	*ACA*	Cotton	Wu et al. (2006)
Cowpea aphid	*ASAL*	Chickpea	Chakraborti et al. (2009)
Grain aphid	*GNA*	Wheat	Stoger et al. (1999)
Jassid and whitefly	*ASAL*	Cotton	Vajhala et al. (2013)
Lepidoptera	*cryIA(b)*, *crylA(c)*	Cotton	Perlak et al. (1990)
Lepidoptera	*cryIA(b)*	Cotton	Tohidfar et al. (2005, 2008), Khan et al. (2011)
Lepidoptera	*crylA(c)*	Cotton	Bakhsh et al. (2012)
Lepidoptera	*cry1EC*	Cotton	Pushpa et al. (2013)
Lepidoptera	*cry1IA1*	Potato	Veale et al. (2012)
Lepidoptera	*cry1Ac9*	Potato	Davidson et al. (2004)
Lepidoptera	*Cowpea trypsin inhibitor*	Potato	Newell et al. (1995)
Lepidoptera	*cry1A(b)*	Soybean	Parrott et al. (1994), Dufourmantel et al. (2005)
Lepidoptera	*cry1A(c)*	Soybean	Dang and Wei (2007)
Lepidoptera	*cry1A(b)*	Rice	Fujimoto et al. (1993), Wünn et al. (1996)
Lepidoptera	*cry1A(b)*, *cry1A(c)*	Rice	Cheng et al. (1998)
Lepidoptera	*cry1A(c)*, *cry2A*	Rice	Bashir et al. (2005)
Lepidoptera	*cry1C*	Rice	Tang et al. (2006)
Lepidoptera	*sbk and sck*	Rice	Zhang et al. (2013)
Lepidoptera	*cry1A(b)*	Maize	Koziel et al. (1993)
Lepidoptera	*cry3Bb1*	Maize	Vaughn et al. (2005)
Lepidoptera	*cry3Bb1*, *cry34/35Ab1*	Maize	Gassmann et al. (2011)
Lepidoptera	*cry1A(c)*	Canola	Tabashnik et al. (1993), Stewart et al. (1996), Halfhill et al. (2001), Ramachandran et al. (1998)
Lepidoptera	*cry1A(c)*	Chickpea	Sanyal et al. (2005), Indurker et al. (2007)
Lepidoptera	*cry2A(a)*	Chickpea	Acharjee et al. (2010)
Lepidoptera	*cry1A(b)*, *cry1A(c)*	Chickpea	Mehrotra et al. (2011)
Lepidoptera	*cry1A(b)*	Tomato	Kumar and Kumar (2004), Koul et al. (2014)
Lepidoptera	*cry1A(c)*	Tomato	Mandaokar et al. (2000)
Mustard aphid	*ASAL*	Indian mustard	Dutta et al. (2005)

(continued)

Table 4.1 (continued)

Pathogen	Gene	Plants	Reference
Mustard aphid	*ASAL*	Indian mustard	Bala et al. (2013)
Mustard aphid	*ACA (Amaranthus caudatus agglutinin)*, *ACA-ASAL*	Indian mustard	Hossain et al. (2006)
Mustard aphid	*WGA-B*	Indian mustard	Kanrar et al. (2002)
Peach-potato aphid	*ConA*	Potato	Gatehouse et al. (1999)
Sap-sucking insects including BPH	*GNA*	Rice	Tang et al. (1999)
Sap-sucking insects including BPH	*DB1/G95A-mALS*	Rice	Yoshimura et al. (2012)
Sap-sucking insects including BPH and GLH	*GNA*	Rice	Foissac et al. (2000)
Sap-sucking insects including BPH and GLH	*GNA*	Rice	Nagadhara et al. (2003)
Sap-sucking insects including BPH and GLH	*ASAL*	Rice	Saha et al. (2006), Sengupta et al. (2010)
Sap-sucking insects including SBPH	*GNA*	Rice	Wu et al. (2002)
Sap-sucking insects including BPH, GLH, and WBPH	*GNA*	Rice	Ramesh et al. (2004)
Sap-sucking insects including BPH, GLH, and WBPH	*ASAL*	Rice	Yarasi et al. (2008)

BPH brown plant hopper, *WBPH* white-backed plant hopper, *SBPH* small brown plant hopper, *GLH* green leafhopper

lectins have also been effectively used against insect pests for crop protection (Goldstein and Hayes 1978). Several plant lectins have been shown to be lethal to various species of the orders Coleoptera, Diptera, and Lepidoptera (Czapla and Lang 1990; Eisemann et al. 1994).

It is gradually clear that consistent strong insect control approaches are required; the next generation of insect-resistant crops has the potential to accomplish this objective. Besides, the approaches (for instance, applying toxic proteins from other organisms, inhibitors, or lectins) of accomplishing insect resistance, plant-mediated RNAi machinery, and genome editing have emerged to fight insect infestations, particularly to address the development of resistance against the targeted insect pests (Rai et al. 2019; Tyagi et al. 2020; Price and Gatehouse 2008; Bisht et al. 2019). RNAi has a huge possibility to develop an effective method for insect pest

management. The dsRNA comprising transgenic plants could be cost-effective due to the constant delivery of RNAi inducers throughout the whole plant life cycle. The knockdown of the specific gene has succeeded via orally served dsRNA in the different insect orders, such as Coleoptera, Diptera, Hymenoptera, and Lepidoptera (Terenius et al. 2011; Lynch and Desplan 2006; Dzitoyeva et al. 2001; Tomoyasu et al. 2008; Bakhsh et al. 2015). Accumulating studies suggest that many encouraging effects of plant-mediated RNAi technology have been used for knockdown of genes, such as cytochrome P_{450}, ecdysone receptor, and hunchback to give resistance or tolerance against insect infestations, like *Helicoverpa armigera*, *Spodoptera exigua*, and *Myzus persicae*, respectively (Mao and Zeng 2014; Mao et al. 2011; Zhu et al. 2012).

Genome editing in insects can be effectively used in different applications that interrupt chemical communication, chemical defense, and breeding companion identification (Tyagi et al. 2020). For instance, the olfactory receptor co-receptor gene knockout in *Spodoptera litura* by the CRISPR/Cas9 system leads to interruption in the breeding companion choice and impairment of insect infestation to host plants (Koutroumpa et al. 2016). The odorant receptor-16 gene knockout through CRISPR/Cas9-based techniques in *H. armigera* causes the males incapable of accepting pheromone signals from the mature females, thus succeeding in mating with undeveloped females that consequently headed to sterile eggs dumping, which is a very effective approach to control mating period for insect pest management in crops (Sun et al. 2017). The knockdown of the CYP6AE enzyme by CRISPR/Cas9 in the *H. armigera* verified the function in the purification of several toxic phytochemicals (Wang et al. 2018). Implementation of these technologies will be a probable choice to stop insect infestation in crops.

The use of transgenic crops has always been a subject of concern associated with human health and environmental safety. Due to some uncertain reasons, it has been found that some people are allergic to transgenic crops (Ferber 1999). Transgenic crops also comprise antibiotic resistance genes, which probably lead to superbug formation, and therefore, that microorganism becomes resistant to the particular antibiotic and eventually cannot be killed and hence the remnants are harmful to human society and other organisms (Losey et al. 1999). The natural environment also gets damaged by transgenic crops; for example, monarch butterfly larvae are being killed by transgenic corn pollen because it contains a bacterial toxin (Losey et al. 1999). Toxin containing corn pollen can be dispersed over 60 meters by wind flow and ingested by monarch butterfly, which is a nontarget organism and becomes dead. In this way, one of the beautiful examples of genetic polymorphisms as in the case of the monarch butterfly may face the challenges of negative evolutionary selection. Another reason for the disadvantage of the transgenic plant is the uncertainty in the authoritative regulation through government organizations, specifically for the approval of the use of specific proteins required for human drug use (Doran 2000; Shih and Doran 2009).

4.3 Limitations of Translation Regarding Transgenic Plants

Despite all the complications that GM crops have brought forth in many nations of the world, the use of transgenic technology to overcome insect pests has had a progressive impact on worldwide cultivation. While considering long-term effects, it is very challenging to take responsibility for the severe influences of transgenic plants on the surrounding environment. Transgenic plants in the field turn out to be the major component of several ecological pathways, like pollination and herbivory, hence affects insects and other plant species in various ways including the soil ecology after decomposition of the dead plant. Allergenicity, toxicity, and genetic hazards are three key threats to health that probably are associated with transgenic foods.

4.3.1 Impacts on Human Health and Animals

Allergens are not formed by genetic modification in any plant itself. If some gene is responsible for causing allergy and this gene is introduced in the plant, then only it can cause allergic reactions directly (e.g., by consuming the plant or its products) or indirectly (e.g., by inhaling pollens). Allergies for nuts are very common symptoms in human inhabitants. For example, Pioneer Hi-Bred developed a maize transgenic plant that causes allergy (Goodman et al. 2008). Another good example is transgenic soyabean plant containing a gene from Brazil nut induces the methionine level increase in the soybean increasing its nutrient value. As this transgenic soybean plant also caused an allergy, it became a serious concern against transgenic plant products. Nordlee et al. (1996) tested transgenic soybean and found that some people were allergic to nuts of the transgenic soybean and concluded that the Brazil nut gene responsible for increased nutritional value was accountable for producing allergic reactions. So, the transgenic plant regulation must be examined adequately to regulate the commercial use of transgenic plants (Nordlee et al. 1996).

Losey et al. (1999) reported that a monarch butterfly species showed harmful effects on its larvae due to the formation of insecticidal Bt toxin in the plant by entirely feeding on the pollen of Bt maize (Losey et al. 1999). Later on, many other studies established that the presence of Bt toxin in transgenic maize plant, which is consumed by monarch butterfly larvae, is sufficient enough to cause damage and mortality (Sears et al. 2001; Stanley-Horn et al. 2001). Carman et al. (2013) showed a significant increase in the weight of the uterus and severe stomach inflammation in transgenic maize plant feeding pigs. They took one herbicide-tolerant and two insect-resistant protein-coding transgenic maize plants as feeding material (Carman et al. 2013). Another study has been executed in poultry with Bt maize, and a significant difference between animals feeding on Bt maize and wild-type maize was observed. Czerwiński et al. (2015) also showed that two cultivars (Bacilla and PR39F56) of Bt maize feeding animals revealed an enlarged weight in the spleen, as well as a lower proportion of T-helper and T-cytotoxic cells in comparison with wild-type maize (Czerwiński et al. 2015).

4.3.2 Ecological Impacts

Transgenic plants, by sexual hybridization with related weeds, probably give rise to weeds that can be resistant to insect pests or herbicides due to acquired traits. These resistant weeds with acquired traits venture into the environment for ages and could compete with the transgenic plants or other crops for selective breeding. Insect pest and herbicide resistant weeds can take over massive space that can or be problematic for crop fields (Liang et al. 2018). The development of transgenic plants requires the introduction of antibiotic-resistant DNA into the genome. Although antibiotic-resistant DNA marker has no functional aspects outside the laboratory, still it is an integral part of plant genome and should be explored in future. It raises concerns about soil microorganisms, by acquiring antibiotic-resistant genes from transgenic plants through decomposition, leading towards the resistance of antibiotics in microbial organisms, consequently causing an alarming increase in antibiotic resistance levels in the natural environment (Tarafdar et al. 2014). With the growing cultivation of insect pest resistant/tolerant transgenic plants, the occurrence of nontargeted insect pests is highly increased that promises an alarming situation vis-à-vis ecological stability. As targeted insect pests could not depend on their preferred target plant, which has been genetically engineered, insects, therefore, can move to other plant species and this alteration, in turn, can affect the interruption of the regular flow of food chain in the ecosystem because this shift might bring new insect predators leading to an increase in competition for these genetically engineered plants (Bawa and Anilakumar 2013).

4.4 Horizontal Gene Transfer

Horizontal gene transfer (HGT) is the process of genetic material transfer to a living cell or organism, which is independent of sexual reproduction; however, it is expressed only after it enters into the cell. HGT has been acknowledged within and between diverse life forms ranging from lower to higher organisms such as the Bacteria, Archaea, Viruses, and Eukarya in the hierarchy of life (Dunning Hotopp 2011). HGT can happen in the human and animal gastrointestinal tract. The constitutive CaMV35S promoter is a highly used promoter that overexpresses the desired proteins in plants (Pandey et al. 2019; Srivastava et al. 2018). Conversely, through HGT, it is possible that in the gastrointestinal tract, the constitutive CaMV35S promoter becomes inserted in the human genome and causes some genes to express severely, affecting serious problems to human health. Besides the CaMV35S promoter, there is the likelihood of insertion of a gene that has been transformed in the plants, and toxic nature for insecticidal activity, like Bt transgenics, which form mycotoxins, can harm humans or animals significantly.

4.5 Imminent Scenarios for Transgenic Plants in Insect Pest Management

Pests and diseases cause severe loss to economically important crops, and reduction in such losses through the proper harnessing of molecular biology and biotechnology studies may increase crop yield and productivity. In this light, plant protection depends heavily on chemical pesticides, which is certainly not a sustainable approach as revealed by recent failures against cotton bollworms and several other major crop pests (Carrière et al. 2014). In this regard, integrated pest management (IPM) with a major focus on biological control and other nonchemical methods is strongly recommended by the central and state governments (Kos et al. 2009). However, biological control and use of other nonchemical pesticides remain doubtful among the plant protection practitioners and farmers due to a lack of competent strategies to cover up the efficacy of chemical pesticides. Hence, to overcome the loopholes of pest management, insect-resistant transgenic plants appear to provide the much-needed strength and stability to IPM.

Biosafety concerns, like toxicity, allergenicity, cross-pollination, effects on nontarget organisms including biological control agents, insect resistance, etc., should be thoroughly investigated and justified before the technology is commercialized through the regulatory protocols. The major concern about the possibility of the target pests developing resistance to Bt protein can be overcome by adopting certain insect resistance management (IRM) strategies, like gene pyramiding, optimum dosage, monitoring for resistance, deploying IPM strategies, growing non-Bt crop as refugia, etc. (Anderson et al. 2019; Alemu 2020; Huseth et al. 2020; Zafar et al. 2020).

Transgenic technology can be easily integrated with other control methods, like biological, cultural, mechanical, pheromones, and even chemical pesticides. In consequence, agricultural crop production throughout the world is poised to realize the benefits of transgenics for pest management and quality improvement. Concerns regarding transgenics should be addressed scientifically and uncover the aspects of cost-effectiveness, greater public awareness, and farmer education, which would make this technology more acceptable. The effective dissemination of correct information and proper guidance is a prerequisite to removing any misconception or apprehension about this remarkable new technology (Karthikeyan et al. 2012).

Transgenic plants incorporated with insecticidal genes are set to feature prominently in pest management in both developed and developing countries. Entomologists, breeders, and molecular biologists need to determine how to deploy this technology for pest management and at the same time reduce possible environmental hazards. To achieve these objectives, we need to have a proper understanding of the insect biology, behavior, its response to the insecticidal proteins, temporal and spatial expression of insecticidal proteins in plants, strategy for resistance management, the impact of insecticidal proteins on natural enemies and nontarget organisms, and a mechanism to deliver the technology to the resource-poor farmers. Several such genes are presently being evaluated for their biological efficacy against sorghum shoot fly, *Atherigona soccata*; spotted stem borer, *Chilo partellus*; tobacco

caterpillar, *Spodoptera litura*; and cotton bollworm/legume pod borer, *Helicoverpa armigera* (Sharma and Ortiz 2000).

The transmission of zoonotic diseases to humans underlines the biological interaction of living things and could inspire us to grapple with the complexity and uncertainty involved in the conservation of life forms effectively and building social ecological systems that are both resilient and adaptable. Land degradation is extensive in many countries, brought about by heavy grazing, invasion by non-native plants, and unsustainable agricultural and forestry practices. Habitat degradation shrinks the resilience of ecosystems, reducing population sizes, and restricting gene flow; also, many emerging infectious diseases arise from human encroachment into wildlife habitats that activate transmission of diseases from animal populations to humans more likely (Allen et al. 2017; Rohr et al. 2019). Furthermore, the use of GM crops with inbuilt herbicide tolerance (Woodbury et al. 2017) leads to increased herbicide use and associated loss of weeds that support pollinator species (Benbrook 2012). Wildlife-friendly, locally appropriate means of securing food and diversifying livelihoods are needed that support human and ecological health at the same time as conserving the genetic heritage that is in danger of being lost due to agricultural intensification and homogenization (Isbell et al. 2017).

Certain issues, like the development of resistance, performance limitations, insect sensitivity, gene escape into the environment, secondary pest problems, search for new genes, environmental influence on gene expression, and anthropogenic activities, should be addressed well before introduction of transgenic plants into the environment (Sharma and Ortiz 2000). Apart from these aspects, challenges regarding plant conservation are also surfacing, which should be taken into consideration during the application of transgenic plants or the management of insect pests (Le Hesran et al. 2019; Gillson et al. 2020).

4.6 Conclusions

Considering the increasing human population, the rapid change in climatic conditions, and the shrinking arable land area, there is an urgent need for the development of high-yielding crop varieties, which are equipped with nutritional contents and also tolerant/resistant to various biotic and abiotic stresses. The transgenic plant development explains two key groups of discussion, encircling the ethical issues and scientific values. The scientific approach towards the direct solution of the problems that human beings face in the present time duration or the upcoming years is reminiscent of food scarcity. To achieve and fulfill the demands of the huge human population, the transgenic approach in various ways has become a direct solution. But with so many pros, there are some serious cons, which are of course preventable by following some stringent regulations that will protect them from the harsh impacts of transgenic use, and finally their commercialization can be made safer. Further, there is a need to encourage the research and development of plant transformation methods for eliminating the use of selective markers. Another concern of antibiotic resistance genes used in transgenic development may cause a

highly negative impact on the environment by increasing antibiotic-resistant microorganisms. To reduce this risk, the FDA (Food and Drug Administration) recommends transgenic plant developers not to employ commonly used antibiotics for disease treatment in humans. Numerous threats of transgenic crops are under examinations scientifically, because ignoring them in the excitement of instantaneous advantages is equally unscientific. Therefore, with the help of a holistic approach, the use of transgenics in crop improvement may be highly recommended for mankind.

Points to Remember

- Insect-resistant transgenic plants offer protection from various insect pest infestations.
- Insect-resistant transgenic plants contribute to high-yield crop production, which is essential for the nutrient needs of the growing human population.
- Despite several advantages of insect-resistant transgenic plants, there is an urgent need to balance the trade-off between the scientific approach and environmental safety issues.

Acknowledgment This work was supported by the SERB, New Delhi, Government of India (Project No. GAP-3401). Institute's Manuscript Number is CSIR-NBRI_MS/2020.

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Molecular Approaches

5

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Omkar (ed.), *Molecular Approaches for Sustainable Insect Pest Management*,
https://doi.org/10.1007/978-981-16-3591-5_5

Abstract

The threats posed by deadly biological weapons, viz. insects and pests, are now easily combated by the use of latest technologies available in the field. Besides conventional methods, genetic manipulation of lethal genes to create sterile insect is one of the popular methods used in controlling insects and pests.

One of the recent techniques of gene editing has been proved to be very effective in this context. The CRISPR-Cas system was used in gene editing of different types of flies, mosquitoes, moths, butterflies and other non-insect arthropods to disrupt key genes that control female viability and male sterility.

In silico studies play an important role by integrating with core data analysis and research and development section of pest management. The designing, screening, prediction of toxicity and optimization process of pesticide production are few areas where this science plays a vital role. Many specialized databases have been developed in the field of pest management.

This chapter covers all major aspects of molecular approaches of insect pathogens, viz. genetically modified organisms to endogenous to indirect defences.

Keywords

Molecular approaches · Insect sterility · Insect-pest control · CRISPR/Cas9 · RNAi

Learning Objectives

1. Huge economic losses have been encountered due to poor heavy pest infestations. Recent advancements in molecular technology enable human beings to combat the threats posed by insect pests.
2. Genetic manipulation causing sterility in insects by creating homozygosity of dominant lethal gene is one of the important strategies to control insect pests. Gender-specific lethality, targeting female insects is another effective strategy. Development of transgenic crops with insecticidal toxins has also been exploited. With the advances in techniques, there had been a clear understanding of genetic structures and newer technologies; consequently, the control of insect pests is receiving newer dimensions.
3. Gene-editing tools have been proved to be very effective in understanding the functions of target genes. The CRISPR-Cas system is used in gene editing of different types of flies, mosquitoes, moths, butterflies and other non-insect arthropods.
4. Using the CRISPR-Cas9 gene-editing methods gives a ray of hope. CRISPR-Cas9 tool empowered scientists to suppress populations of insect pests. Disruption of key genes that control female viability and male sterility has been achieved using CRISPR.

5. Solitary development of new pesticides will not aid in the fight against these insects. Bioinformatics and computational biology are getting integrated with core data analysis and research and development for pest management.
6. The scenario is changing, and we need to have an integrated approach of interdisciplinary areas for handling the pest problem in near future, where we can happily share a negligible amount of food with pests.

5.1 Introduction

Insect pests are a huge threat to the crop productivity, as almost 18% of the yield is lost annually. Conventional use of synthetic pesticides has environmental concerns and the genetic methods of development of sterile insects are very time-consuming techniques. Recent advances in molecular biology have provided several modern tools, like molecular markers, gene-editing technology and RNAi technology. RNAi provides an environment-friendly and economic alternative to the chemical pesticides. Commercially many genetically modified plants have been developed that express dsRNA that in turn can silence essential genes in insect pests and phytoparasitic nematodes with substantial application in insect pest management. There has been significant breakthrough research in utilizing RNAi technique in curbing the pest menace. For instance, silencing the V-type ATPase A gene in the midgut cells of western corn rootworm led to reduced growth and mortality of the larvae (Baum et al. 2007). CYP6AE14 (cytochrome P450 monooxygenase) gene expressed in midgut cells of cotton bollworm larvae has correlation to gossypol tolerance. Constructed GM plants producing CYP6AE14 dsRNA when fed to the larvae successfully decreased endogenous CYP6AE14 mRNA in insects severely affecting their growth and increasing sensitivity to gossypol (Mao et al. 2007). Gene editing (GE) is the process of substitution of the base in the target sequence by insertion and deletion, and it is one of the advanced plant breeding techniques.

CRISPR/Cas9 is a prokaryotic immune system that is now becoming popular as a genome engineering tool for biotechnological product development and disease protection (Gantz and Akbari 2018; Zhang et al. 2019; Karimian et al. 2019). CRISPR/Cas9 is the most common technique used for gene editing (Xu et al. 2019c). Cas9 referred to endonucleases that recognize CRISPR sequences and specifically cleave complementary DNA strand to CRISPR. CRISPR/Cas9 is widely expanding the information for gene disruption and is used in biological research. This tool is widely applicable to disease models, gene mapping and functional genetics (Hannon 2002; Sadhu et al. 2016).

DNA-based markers are also used for pest management. These molecular markers could be utilized by population geneticists and pest control workers in the identification and tracing of the geographical origins of colonist pest populations and assessment of their risk and invasive potentials, thereby assisting regulatory authorities in implementing quarantine restrictions and other pest control measures. Besides that, designing, screening, prediction of toxicity and optimization process of pesticide production are few areas where this science plays a vital role. Many

specialized databases have been developed in the field of pest management. Spatio-temporal modelling for various pests and fast-track application of RNAi for controlling pest as well as developing effective methods to save beneficial insects and arthropods from viral and parasitic diseases are some of the important applications of computational biology in pest management.

In this chapter, comprehensive information of all the available molecular tools for insect pest management is compiled in detail. This would provide an understanding of opportunities in genetic-based insect control with the help of the leads achieved so far.

5.2 Classical Genetics Methods

Classical genetics focuses on studies of inheritance of characters from one generation to the next. It also studies inheritance of mutation, especially economically important mutation. Bifurcation of field of genetics into biochemistry and molecular biology has enhanced the fundamental understanding of the nature of genes and inheritance. Focus of modern genetics has changed from individual gene to whole genome level (Dale et al. 2012).

Genetic tools employed for pest management were initially based on breeding and inheritance studies. Some of the methods focused on the development of sterile insects and other development of transgenics. The target varied form single gene to whole genome. Thus, ideas were taken from contemporary and molecular genetics in pest management.

5.2.1 Inheritance of Sterility

Delayed sterility is one of the important genetic approaches, where sterile insect progeny were raised from fertile parents. This is also called F1 sterility or partial sterility commonly reported in the orders Lepidoptera and Hemiptera.

There are some cytological methods for introduction of sterility:

1. Interspecific hybridization
2. Cytoplasmic incompatibility
3. Multiple ploidy

5.2.2 Genetics in Pest Management Using SIT

Sterile insect technique (SIT) is a very common and old technique for pest management (Klassen and Curtis 2005). Here, males are sterilized during rearing and later released to mate with wild female pests; thus, wild females were eliminated from breeding pest population because of lack of reproductive success; thus, elimination of target pest population is achieved (Klassen 2005). This is successful only in cases

of those insects where the females mate only once in a lifetime. SIT had been used across the world in pest management, particularly some species. However, this is not very much effective for other species where females mate multiply. In such cases, there was a need for introduction of new strains that could improve the efficiency of this method (Condona et al. 2007). Genetic manipulation could be done in SIT to make it more effective for the development of new species. The new strains were given term 'genetic sexing' strains, and to develop these, there was a need for basic molecular genetic information on species where this approach could be applied (Robinson and Hendrichs 2005).

Chromosomal translocation: Identification of sexes is important for implementation of SIT. Chromosomal translocation is one of the important cytological techniques. This has been feasibly applied in tephritid species by translocating portion of the Y chromosome for identification of males. The presence of at least part of the Y chromosome was sufficient for male sex determination (Lifschitz and Cladera 1988; Anleitner and Haymer 1992). The identified male would be reared and sterilized before release in the environment.

5.2.3 Conditional Mutations

Mutations that are dependent on environmental conditions for their expression are called conditional mutation. Under this technique, genetically modified insects were developed with sensitivity towards environmental conditions, particularly temperature sensitivity. In this technique, strains of insects are produced by genetic manipulation so that they carry traits that are detrimental to the species in the native environment. The most desired condition is temperature, particularly for mass rearing situations; conditional mutations exhibiting sensitivity to temperature were in fact the most highly desired (Schetelig et al. 2009). Exposure to higher temperature, i.e. about 33 °C or higher, is lethal for female larvae as it activates temperature-sensitive lethal gene (tsl). This has provided a system where females could be selectively eliminated at any time during the rearing phase simply by exposing the larvae to an elevated temperature.

5.2.4 Transposable Elements and SIT

The use of jumping genes or transposable elements is a little advanced technology over the traditional one of chromosomal arrangements. Using these elements, genes could be directly introduced into strain, without any involvement of chromosomes. However, there is still requirement of some sex-specific markers or identification of gene-specific promoters or other regulatory systems where expression is controllable or limited to only one sex. A number of genes involved in the sex determination pathway of *Drosophila* had been identified, and complete DNA sequences are available for many of them (Saccone et al. 2011).

5.3 RNAi Technology

RNA interference (RNAi) is a biological phenomenon during which selective mRNA molecules are degraded, thus blocking the expression of respective genes. This regulatory process naturally evolves in organisms as a defence against molecular parasites or viral invasion. When a dsRNA is introduced after sequential selection of a target, an RNase-III processing enzyme called dicer gets activated that cuts the dsRNA into fragments of 20–25 bp, called small interfering RNA (siRNA). Eventually, there is formation of RNA-induced silencing complex (RISC) that destroys the sense strand (matches with the target gene) of siRNA, while the antisense strand gets integrated to RISC and leads to the specific mRNA degradation, thus preventing protein synthesis (Baum et al. 2007). The phenomenon of RNAi was first discovered in nematode worm, *Caenorhabditis elegans*, by Andrew Fire and Craig Mello in 1998, for which they received Nobel Prize in the year 2006. This post-transcriptional gene silencing technique has found application in several aspects of science, especially crop improvement and pest management, for instance, biotic/abiotic stress tolerance, nutritional improvement, deletion of allergens, prolongation of shelf life, seedless fruit development and engineering of secondary metabolites, among others (Ferry et al. 2004).

There have been studies on GM potatoes targeting β-actin gene of Colorado potato beetle, *Leptinotarsa decemlineata*, and GM *Nicotiana benthamiana* targeting the acetylcholine esterase 2 gene of *Helicoverpa armigera* that suggest the absence of RNAi machinery in the chloroplast; as a result, dsRNA produced in the chloroplast does not enter the cytoplasm; however, it can be absorbed by the insect midgut cells triggering RNAi pathway, thus making the chloroplast a good way of dsRNA expression (Bally et al. 2016). There could be several ways to deliver RNAi construct to the target plant, such as **foliar spray** that could be directly sprayed on the foliage to induce lethal effects on feeding pests. dsRNA can also be delivered via crop roots by doing **irrigation** using RNAi products. Successful application of **microinjection** has also been reported in *Bombyx mori* (Linnaeus), *Manduca sexta* (Johannsen) and *Apis mellifera* (Linnaeus) (Aronstein et al. 2012). In insect cell lines (S2, Sf21, CiE1), dsRNA has been introduced effectively using **soaking and transfection** method (Johnson et al. 2010). In order to silent the midgut genes of *M. sexta*, *CYP6B46* dsRNA was generated using **viral vectors** and introduced in the host plant, *Nicotiana attenuata* (Kumar et al. 2012). Another way of improving the RNAi efficiency by increasing the uptake of dsRNA is by using **nanoparticle** gene carriers. It has been demonstrated recently that application of nanocarrier/dsRNA combination leads to around 80% aphid suppression and significant gene knockdown impact in *Aphis glycines* (Shi et al. 2020). Reproduction in pests can be severely hindered using RNAi; for instance, blocking the expression of Bicaudal-C (Bic-C) gene in *Nilaparvata lugens* leads to undeveloped ovaries and no oocyte growth, thus disrupting oogenesis altogether (Zhang et al. 2015). Researchers have used RNAi successfully in several insect species. Table 5.1 below summarizes some of the significant ones.

Table 5.1 Insect species, where post-transcriptional gene silencing technique has been implemented successfully. The target gene and mode of delivery of dsRNA are also given

S. no.	Insect species	Target gene	Mode of delivery
1.	*Anopheles gambiae*	AgCHS1, AgCHS2	Nanoparticles
2.	*Aedes aegypti*	Sema1a, Sim, Vg	Nanoparticle (chitosan)
3.	*Diabrotica virgifera*	Vacuolar ATPase subunit A, DvSnf7	Artificial diet
4.	*Glossina morsitans*	Midgut protein TsetseEP	Feeding
5.	*Helicoverpa armigera*	Acetylcholine esterase, CYP6AE14	Feeding/transgenic plant
6.	*Plutella xylostella*	CYPBG1	Droplet
7.	*Spodoptera frugiperda*	Allatostatin C, allatotropin 2	Droplet
8.	*Apis mellifera*	Toll-related receptor vitellogenin	Natural diet and soaking
9.	*Phyllotreta striolata*	PsOr1, arginine kinase	Injection, feeding
10.	*Reticulitermes flavipes*	Cellulase	Artificial diet
11.	*Epiphyas postvittana*	Gut carboxylase	Droplet feeding
12.	*Drosophila melanogaster*	Gamma tubulin	Liposomes

5.4 CRISPR/Cas9

Insects constitute the most economically important species causing huge losses in various sectors. *Drosophila* is used as model organisms for basic biological research. Food production has been mostly affected by insect pests of food grains, pulses, oilseeds, forages, cash crops, livestock and agriculture products. The total $470 billion yearly global losses are caused by arthropod crop pests across the globe (Culliney 2014). There are plenty of agricultural pests affecting crop yields, and some of them can have crop-devastating effects. Furthermore, the frequent and incorrect use of traditional chemical pesticides has led to the development of resistance (Le Goff and Giraudo 2019) in many destructive insect pests, including *Locusta migratoria*, *Plutella xylostella*, *Agrotis ipsilon*, *Bactrocera dorsalis*, *Helicoverpa armigera* and *Spodoptera litura*. CRISPR/Cas9-based gene-editing studies indicated its potential in insect control as well as in reducing the development of pesticide resistance. Gene knockout (KO), knock-in (KI) and knockdown are the new techniques for editing genome in non-model organisms. CRISPR/Cas9 editing tool is generally applied for it, which helps to control the pest species and increase the crop production, as the gene drive-based strategies should be receiving more attention from policymakers and public at large for environmental concern. CRISPR/Cas9 is chiefly used for functional genomics studies and for designing new pest management strategies. A lot more work has been done in the functional genomics of insects and much more is to be done in translating the understanding of these functional genomics studies into *on-field* pest management (Fig. 5.1).

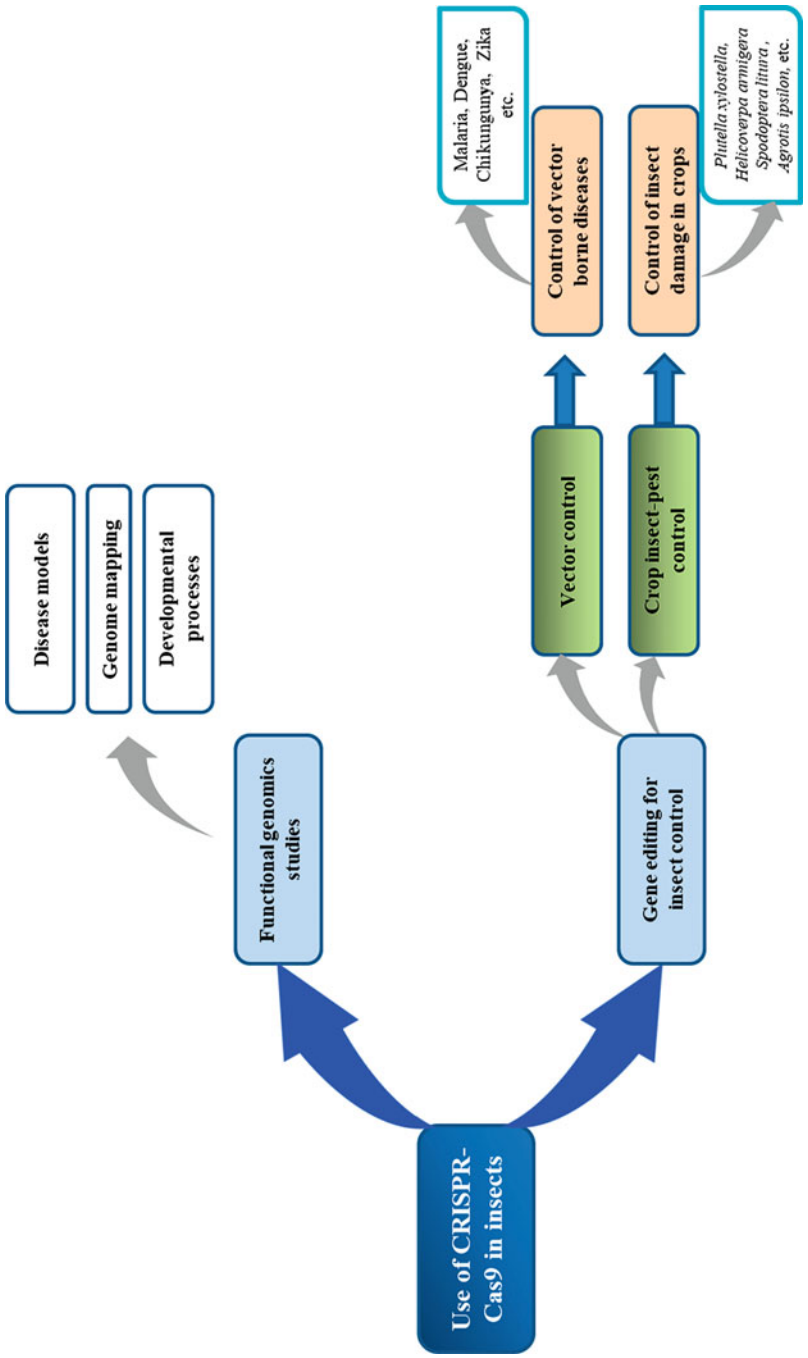

Fig. 5.1 CRISPR/Cas9-based gene editing and its applications in insects

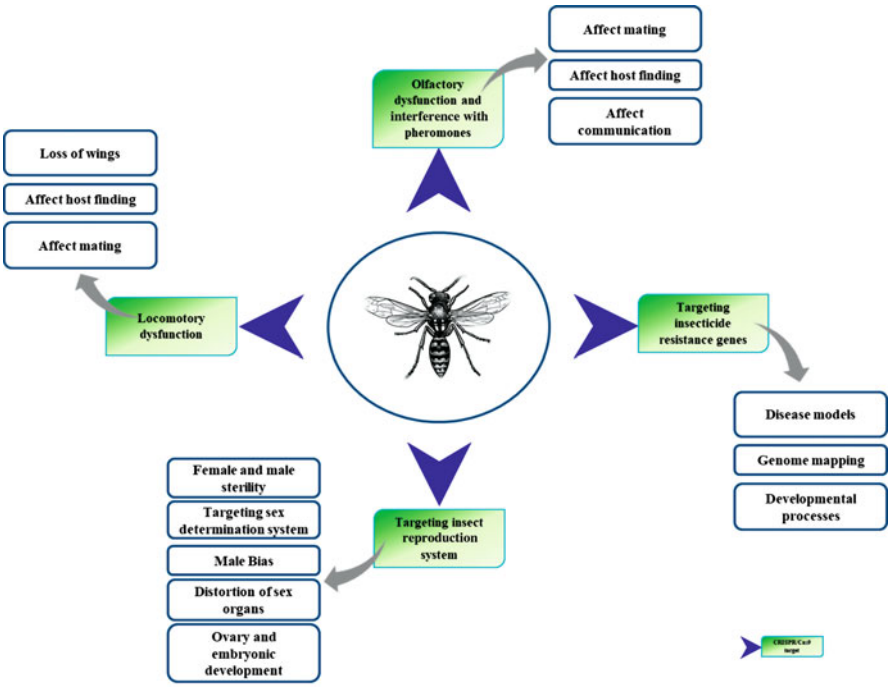

Fig. 5.2 Different frontiers of CRISPR/Cas9-based insect control

In eukaryotic organisms including insects of the orders Diptera (Galizi et al. 2016; Zhao et al. 2019), Lepidoptera (Xu et al. 2019a; Peng et al. 2020), Hemiptera (Li et al. 2016) and Coleoptera (Gui et al. 2020), CRISPR/Cas9-based knockout is successfully achieved for insect control mechanisms. It is detailed in lepidopteran insects, such as *Plutella xylostella*, *Spodoptera litura*, *Agrotis ipsilon* and S*podoptera litura*, for frontier work in insect control (Chen et al. 2019; Xu et al. 2019a, b; Zhu et al. 2019; Wang et al. 2019; Peng et al. 2020). Results on CRISPR/Cas9-based disruption of insect digestive proteinases along with their key regulators are also promising (Singh et al. 2020). In this way, the gene editing could result in insect-specific control measures. Pest control can be done by choosing a particular target gene. In this way, it induces critical phenotypes.

5.4.1 Potential CRISPR/Cas9-Based Insect Control Strategies

Some of the excellent leads were obtained in CRISPR/Cas9-based insect control, typically in generating male-biased population, male sterility, female sterility, disrupting olfactory and locomotory functions and many more. However, they are still in the laboratory-based studies and need further confirmation and improvements before it can be used for field-level control. The available literature on CRISPR/Cas9-based insect control could be grouped in the following classes (Fig. 5.2; Table 5.2) which are later discussed in detail:

Table 5.2 Use of the CRISPR/Cas9 system in insect control strategies

S. no.	Insect	Order	Purpose	CRISPR/Cas9 intervention	References
1.	*Anopheles gambiae*	Diptera	Selectively destroying the X chromosome by CRISPR/Cas9 gene editing	Producing extreme male bias in the population to reduce reproduction rate	Galizi et al. (2016)
2.	*Agrotis ipsilon*	Lepidoptera	*Aidsx* was disrupted using a CRISPR/Cas9 system targeting female- and male-specific *Aidsx* exons	Sex-specific, sexually dimorphic defects in external genitals, gonads and antennae	Chen et al. (2019)
3.	*Spodoptera litura*	Lepidoptera	To obtain *Osp* mutants in the model lepidopteran insect	Deletion of *Osp* resulted in female sterility	Xu et al. (2019a)
4.	*Plutella xylostella*	Lepidoptera	*Ser2* gene is evolutionarily conservative and can provide new strategies for biological pest control	Disrupt *Ser2*, which encodes a seminal fluid protein	Xu et al. (2019b)
5.	*Plutella xylostella*	Lepidoptera	*VgR* disruption using CRISPR/Cas9 technology affects Vg transport, ovary development and oviposition of *P. xylostella*	Affect oviposition and embryonic development	Peng et al. (2020)
6.	*Plutella xylostella*	Lepidoptera	Mutations in the male-specific isoform, the female-specific isoform and common regions of *Pxdsx*	Caused sex-specific defects in external genitals and partial sexual reversal	Wang et al. (2019)
7.	Migratory locust, *Locusta migratoria*	Orthoptera	CRISPR/Cas9 sex distortion system that targets ribosomal sequences	Disrupt the gene encoding the odorant receptor co-receptor (*Orco*), affect	Li et al. (2016)

(continued)

Table 5.2 (continued)

S. no.	Insect	Order	Purpose	CRISPR/Cas9 intervention	References
				olfactory response	
8.	*Laodelphax striatellus*, small brown planthopper (SBPH)	Hemiptera	CRISPR/Cas9-based determination of rice stripe virus (RSV) infection and expression of *LstrOrco* of olfactory signalling	Disruption of olfactory signalling of SBPH which is enhanced	Li et al. (2016)
9.	S*podoptera litura*	Lepidoptera	*SlitPBP1* is more important in the sex pheromone perception	Knockout of either *SlitPBP1* or *SlitPBP2* in males decreased response to sex pheromone	Zhu et al. (2019)
10.	Colorado potato beetle (CPB), *Leptinotarsa decemlineata*	Coleoptera	Deformed wings by CRISPR/Cas9	Mutation in *vest* resulted in adults with no hindwing and elytron formed	Gui et al. (2020)
11.	Mediterranean fruit fly, *Ceratitis capitata* (medfly)	Diptera	CRISPR/Cas9-mediated disruption of segmentation paired gene (Ccprd) caused segmental malformations	Cas9 RNP-based gene editing to introduce mutations in *C. capitata* caused segmental malformations	Meccariello et al. (2017)
12.	*Bactrocera dorsalis*	Diptera	Co-injection of the *white* and *tra* by CRISPR/Cas9 mRNA into *B. dorsalis* embryos caused eye colour and other changes	KO of *transformer* (*tra*) in *B. dorsalis* caused male-biased sex ratio and abnormal outer and interior reproductive organs	Zhao et al. (2019)
13.	*Cochliomyia hominivorax* and *Lucilia cuprina*	Diptera	CRISPR/Cas9 method used for the generation of directed and inheritable modifications in	Mutations in *Chtra* locus developed mosaic phenotypes, and females showed	Paulo et al. (2019)

(continued)

Table 5.2 (continued)

S. no.	Insect	Order	Purpose	CRISPR/Cas9 intervention	References
			the genome of the flies and disrupt the *C. hominivorax transformer* gene (*Chtra*)	transformed ovipositors with abnormal reproductive tissues	
14.	*Anopheles gambiae*	Diptera	X chromosome-shredding I-PpoI nuclease by coupling this to a CRISPR-based gene drive inserted into a conserved sequence of the *doublesex* (*dsx*) gene	The gene drive in *dsx* locus led to a male-only population which collapsed in 10–14 generations	Simoni et al. (2020)
15.	Oriental fruit fly, *Bactrocera dorsalis*	Diptera	Knockout of *Bdpaired* led to lack of segment boundaries, cuticular deficiency and embryonic lethality	Affect embryonic development	Wang et al. (2020)
16.	*Aedes aegypti*	Diptera	The CRISPR/Cas9 system was performed to genes hypothesized to control flight in mosquitoes	KO affect flight muscle, affect female flight	O'Leary and Adelman (2019)

1. Olfactory dysfunction and interference with pheromones
2. Targeting insect reproduction system
   (a) Female sterility
   (b) Male sterility
   (c) Distortion in sex organs
   (d) Ovary and embryonic development
3. Locomotory dysfunction
4. Targeting insecticide resistance genes

5.4.1.1 Olfactory Dysfunction and Interference with Pheromones

Olfactory, the power 'to smell' in insects, provides an understanding of the surroundings and thus their responses towards it. It help insects in several important physiological responses, such as finding the mating partners, food, alarming against

enemies or toxic chemicals, etc. Disruption of olfactory function by the CRISPR/Cas9 system could revolutionize the management of pests of global threats (Soroker et al. 2019), e.g. migratory locust by disruption of communication. Locust, an orthopteran insect, causes huge loss of crops and pasturelands across the countries as seen in the year 2020 (FAO Locust Watch 2020). The CRISPR/Cas9-mediated olfactory deficiency heritable mutagenesis in locust was developed by targeting odorant receptor co-receptor '*Orco*'. Results convinced that the electrophysiological behaviour and olfactory responses are severely impaired. Disrupting the *Orco* gene using Cas9-mRNA and *Orco*-gRNA resulted in highly efficient (71.7%) gene editing in G0. Further, these mutant lines were tested to be stable by crossing. Loss of attraction towards aggregation pheromone was observed, while the locomotory activity was unaffected (Li et al. 2016). These impaired mutants could be effective in advanced pest management strategies of migratory locusts. The major loss from migratory locust is caused by 'mass migration', and CRISPR/Cas9-based disruption of *Orco* could directly inhibit the movement towards migration. It could be a breakthrough in locust control as it is a worldwide problem.

In a similar approach against the small brown planthopper (SBPH), *Laodelphax striatellus*, CRISPR/Cas9-mediated disruption of *LstrOrco* gene resulted in no response or slower response of the olfaction in the nymphs and seeking behaviour for rice seedlings. The SBPH also transmits rice stripe virus (RSV), and it was found that the *LstrOrco* expression enhances due to RSV infection in the SBPH. Probably, it is an evolutionary adaptation possessed by RSV for its spread on host plants. CRISPR/Cas9-based gene editing in *LstrOrco* gene would not only reduce the *Laodelphax striatellus* infestation in rice fields but also lower the chances of RSV spread. This could be a potential tool in vector control in other viral diseases (Li et al. 2019).

In Europe, Asia and Africa, the *Spodoptera littoralis* is devastating pest of cotton and vegetable crops. In this pest, CRISPR/Cas9 is used to disrupt the odorant co-receptor gene *Orco* to stop the signalling between species (Larsson et al. 2004; Benton et al. 2006). In rice and wheat, it is demonstrated by CRISPR/Cas9 based gene editing (Shan et al. 2013).

Sex attractants are the pheromones produced for attracting insects of the opposite sex of the same species for mating. Pheromone perception is a complex biochemical behaviour for the very specific attraction of the opposite sex of the same species. The concept of pheromones has long been utilized in insect control, e.g. 'helilure' are preparations available as commercial products containing sex pheromones of *Helicoverpa armigera* for the false attraction of the males in the surrounding area to minimize the mating and number of offspring in the next generation (Rajnish 2014). CRISPR/Cas9-based editing in the key genes responsible for pheromone perception can be done for reduced mating for keeping the insect population below the economic threshold level (ETL).

In tobacco cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae), pheromone-binding proteins (PBPs) are found in the sensilla lymph of the antennae to sense the pheromones. They are a sub-family of odorant-binding proteins. CRISPR/Cas9-based knockout (KO) of *SlitPBP1* or *SlitPBP2* in newly laid eggs of *S. litura*

generated targeted mutagenesis. The homozygous KO males showed reduced electrophysiological and behavioural response towards three pheromones by an extent of 40–60%, and KO of *SlitPBP1* was more effective (Zhu et al. 2019). The reduction in the perception was similar in all three pheromone components used (Z9, E11-14:Ac, Z9,E12-14:Ac, and Z9-14:Ac). The study indicated that CRISPR/Cas9-based KO can be critical in developing genetic-based insect control strategies, particularly to bring down the population below the economic threshold level (ETL). The third PBP gene of *Spodoptera litura SlitPBP3* was also studied for its effect on pheromone perception by other workers and found to be less effective than *SlitPBP1* (Liu et al. 2013; Zhu et al. 2016).

5.4.1.2 Targeting Insect Reproduction System

The fecundity (reproduction rate) is one of the most crucial factors in insect ecology (Leather 1995); thus, targeting the reproduction system could be of much greater significance in the genetic-based insect pest control. CRISPR/Cas9-mediated disruption of the gene responsible for insect reproduction could, therefore, be very successful for *on-field* pest control. Although gene-editing-based pest control is in initial phase, several insect behaviour genes were tested for the potential to be used as gene-editing-based pest control measures.

The CRISPR/Cas9-based attempts of targeting insect reproduction system could be divided into strategies, like production of male-biased population, male sterility, female sterility and physiological defects in sexual organs. These are being tested in different insects of economic importance apart from conducting functional genomics studies. The details of these methods are as follows.

Targeting Sex Determination System

CRISPR/Cas9-based disruption of critical genes in the insect sex determination pathway could cause heritable mutations in sexual differentiation and further development. Doublesex (*dsx*) is a gene of the sex determination pathway in the insects. In diamondback moth (DBM; *Plutella xylostella*), male- and female-specific genes were identified. CRISPR/Cas9-based disruption of *Pxdsx* resulted in specific defects in genitals along with causing partial sexual reversal; it also affected the expression of sex-biased genes proving that *Pxdsx* is crucial in sex determination and sterility (Wang et al. 2019). The defects caused by deletion of *Pxdsx* can be used as genetic-based control strategy of DBM.

In the case of *dsx* gene of *Agrotis ipsilon* (black cutworm), *Aidsx* is also reported to be involved in sex determination, and CRISPR/Cas9-based deletion of *Aidsx* causes sex-specific defects in sexual organs and antennae in both of the sexes (Chen et al. 2019).

Male Bias

In malaria vector control, CRISPR/Cas9-based X chromosome shredding was reported to produce extreme male bias in the population without affecting the fertility. Release of gene-edited malaria vector *Anopheles gambiae* could bias the sex ratio and reduce the population size further due to the lack of females in the

population (Galizi et al. 2016). CRISPR/Cas9-based shredding during spermatogenesis is reported to produce male bias and could be a potential tool in malaria control programmes.

A similar approach was used in the control of malaria vector *Anopheles gambiae*, which selectively produced male bias. The unisex population resulted in a reduction in the population further. Invasion dynamics modelling of CRISPR/Cas9-based gene drive of doublesex (*dsx*) gene indicated a quicker impact than female sterility (Simoni et al. 2020). Since diseases like malaria, dengue, chikungunya and Zika are transmitted through female vectors, CRISPR/Cas9-based male-biased sex-distorter gene drive (SDGD) could cause severely bias male offspring and reduce the chances of disease spread. The male population collapsed after 10–14 generations, giving no space for selection of resistance among the populations. Therefore, this could be a good malaria control strategy.

The problem of development of resistance against control strategies in insects is a common phenomenon; thus, the probability of developing resistance against gene drive was also assessed. Development of resistance in driving Y chromosome was found to be affected by several factors (Beaghton et al. 2017). Based on the prediction of population modelling, Beaghton et al. (2017) have assessed the possible factors that could be affecting the likelihood of resistance development. It was found that chances of resistance development increase with an increase in mutation rate along with an increase in population, whereas probability decreases with drive strength and pleiotropic fitness costs associated with the resistant allele. This is further affected by the time of release into the environment. The probability could be reduced by selecting target sequences, which have a fitness cost on an individual upon mutation (indicated in Fig. 5.3).

Distortion of Sex Organs

Several workers are reporting genome manipulation tools based on CRISPR/Cas9 for creating targeted inheritable mutagenesis for pest control. A similar strategy was tested on major livestock pests, *Cochliomyia hominivorax* and *Lucilia cuprina*, by targeting yellow genes (*ChY* and *LcY*, respectively). Mutants of *ChY* and *LcY* produced disruption in pigmentation. Further, CRISPR/Cas9-based disruption of transformer (*Tra*) gene resulted in mosaic phenotype with defective reproductive tissue having transformed ovipositors, which suggest that *Chtra* and *Lctra* could be effective in curbing livestock pests (Paulo et al. 2019).

Targeted gene disruption in the pest *Bactrocera dorsalis* was reported by Zhao et al. (2019) using white and transformer genes. The white gene caused change in the eye colour (helping in detecting the mutants while injected with *tra* gene). Knockout of *tra* gene (along with white) resulted in male-biased sex ratio in the offspring having defective external and internal sex organs, opening a window of population control using gene drive.

Male Sterility

Sterile insect technique (SIT), i.e. releasing sterile insects in the environment to reduce the reproduction and thus population growth, is a strategy of pest control. In

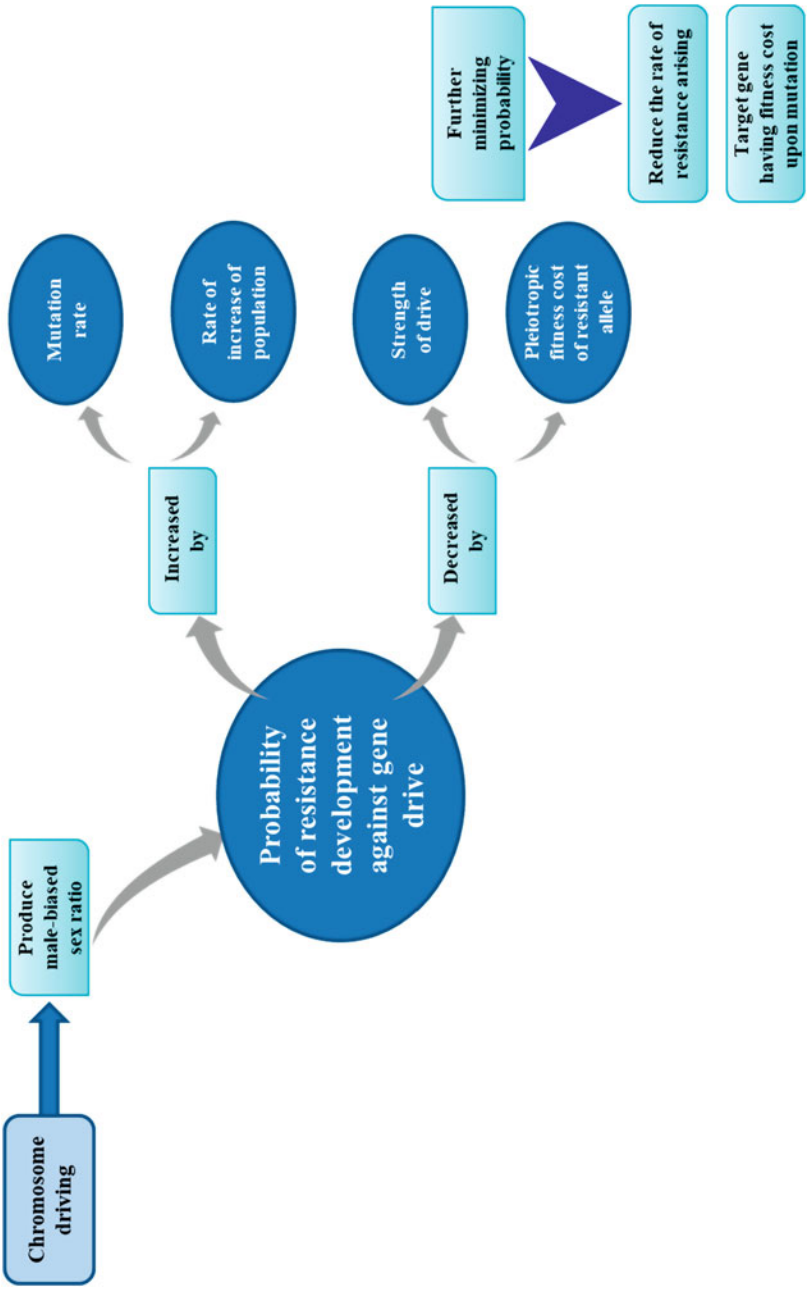

Fig. 5.3 Probability of developing resistance against gene drive and ways of further minimizing

the genetic-based gene drive system, it is a prerequisite that it should create male sterility without affecting the mating competence and survivability. The CRISPR/Cas9-based approach for creating specific mutations for male sterility is thus a viable option with practical utility. An example of CRISPR/Cas9-mediated male sterility is *Ser2* gene knockout in *Plutella xylostella*. *P. xylostella* is a devastating pest of cruciferous plants, and CRISPR/Cas9-based disruption in *Ser2* (seminal fluid protein) has shown to produce heritable male sterility. In this gene editing, the mating is normal but the eggs thus produced did not hatch. No other differences except sterility were obtained in the males emphasizing the potential of mating competence (Xu et al. 2019b).

Female Sterility

Similar to insect control using male sterility, female sterility could also be a potential tool in insect control. Mutation in *ovarian serine protease* (*Osp*) caused an array of deletions in *Osp* loci in *Bombyx mori* and *Spodoptera litura*. Since *Osp* is involved in oogenesis, its deletion resulted in female sterility. In these female mutants, mating was normal but fewer eggs were laid, which did not hatch (Xu et al. 2019a). This study has proven that *Osp* is very important for reproductive success in two lepidopteran insects. Since *Osp* is a highly conserved gene among insects, a successful CRISPR/Cas9-based deletion could be highly useful.

Ovary and Embryonic Development

The vitellogenin receptor (VgR) is responsible for Vg transport, oocyte development and yolk deposition. Thus, it is a promising target for genetic-based pest control. In diamondback moth (*Plutella xylostella*), *PxVgR* is expressed in female adults. CRISPR/Cas9-based *PxVgR* knockout resulted in *VgR* mutant. In these mutants, ovarioles were shorter in females. Numbers of eggs laid were not affected, but it was smaller with reduced hatching rates. Targeting *P. xylostella* ovary development could be employed in reducing the population growth of pest species and thus preventing the crop losses (Peng et al. 2020).

A CRISPR/Cas9 target on embryogenesis (*paired* gene) was performed in oriental fruit fly, *Bactrocera dorsalis*. Knockout of paired gene (*Bdpaired*) resulted in embryogenic lethality apart from affecting segmental boundaries and circular deficiency. It has caused different indels in *Bdpaired* locus (Wang et al. 2020). Since it is crucial for embryogenesis, this is a suitable target for pest management. Similar segmental malformations were observed in Mediterranean fruit fly (*Ceratitis capitata*) by CRISPR/Cas9-based disruption of *white eye* (*we*) gene (as a marker of mutagenesis) along with paired gene (*Ccprd*), which could be used to control fruit fly (Meccariello et al. 2017).

5.4.1.3 Locomotory Dysfunction

In *Aedes aegypti*, females rely on the flight for mating and finding hosts. Therefore, KO in the gene responsible for flight could cause flightlessness to be an important breakthrough in insect control. CRISPR/Cas9-based KO of several genes of flight muscle development by embryonic microinjection resulted in flightlessness in

females, whereas males were having normal flight (O'Leary and Adelman 2019). Although this concept is in the initial phase of testing, this could be a very potential strategy for futuristic pest control.

Similarly in Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, the CRISPR/Cas9-based mutagenesis of *vest* gene (involved in the wing development) resulted in adults with no hindwings and elytron (Gui et al. 2020). This study showed the utility of CRISPR/Cas9-based gene editing in pest control directly.

5.4.1.4 Targeting Insecticide Resistance Genes

CRISPR/Cas9 could also be utilized in targeting the genes responsible for the development of insecticide resistance. In *Drosophila* G275E, a site-specific CRISPR/Cas9 mutation into the nicotinic acetylcholine receptor (nAChR) resulted in the loss of resistance to spinosad insecticides. Mutation in G275E was found to be related to spinosad resistance in *Drosophila* (Zimmer et al. 2016). This could be another dimension of CRISPR/Cas9-based gene editing in maintaining the efficiency of pesticides by targeting genes responsible for the evolution of pesticide resistance. In *Drosophila*, mutation in succinyl-CoA synthetase/ligase (SCS) alpha subunit (*Scsα*) using the CRISPR/Cas9 system was attempted since it is important for proper energy metabolism. The deficiency caused by disrupting *Scsα* resulted in altering TCA cycle metabolites and developmental delays in the individuals, locomotor dysfunction and higher mortality under starvation (Quan et al. 2017).

5.4.2 CRISPR/Cas9 in Stored Grain Pest Management

CRISPR/Cas9 is also having use in pest management (PM) in food storage needs (Baum et al. 2007; Huvenne and Smagghe 2010; Noh et al. 2012). Insect destroys the storage products, like grains and milling facilities, warehouses and consumer pantry (Hagstrum et al. 2012). This technology is utilized in model organisms, such as *Tribolium castaneum* (Gilles and Averof 2014). It has been focused on the red flour beetle, *T. castaneum*, for coleopteran agricultural pests (Consortium 2008). Since the genetic information is available on *T. castaneum*, it is used as genetic model for molecular-based pest control.

5.4.3 Biosafety Regulations for Genome Engineering by CRISPR/Cas9

CRISPR/Cas9 is an advanced technology used in gene editing of insects; foreign gene is not used in the gene editing. Since the CRISPR/Cas9 based gene editing causes gene drive, this is having potential to alter the entire population and even ecosystem (Oye et al. 2014; Champer et al. 2016). Since it is an evolving technology, several changes are required in the regulations. The release of risk assessment of nontarget effects to avoid inadvertent ecological consequences is required by the CRISPR/Cas9-edited insects carrying gene drive. Traditional control cautionary

applicable method has been used to assess the technology to avoid any error or failure. In 1989, Environmental (Protection) Act came into picture for regulating genetic engineering for crop improvement. Although it is an old act and gene editing is a new technique, it governs regulation on biosafety issues. Therefore, in 2017, new biosafety regulations on recombinant DNA research biocontainment were released by the DBT (2017). This has marked SDN1 in biosafety level 1, and the researcher need not take permission for working with SDN1; they just need to inform the IPAC. In January 2020, the DBT released a draft document on genome-edited organisms, and unfortunately, one guideline is given for all humans, animals and plants (DBT 2020). Similarly, the USDA also released new guidelines on biotechnological regulations (Barrangou 2020). Since plants do not have much problem with off-targets, they should not be governed along with humans and animals. This document is, however, in the initial drafting phase, and comments from different stakeholders are invited; there would be appropriate changes to better suit the needs of present biotechnological advancements keeping all the biosafety issues on top priority.

5.5 DNA-Based Markers and In Silico Approach

DNA-based methods are used to manage and identify natural enemies of pests where morphological differentiation is challenging. Molecular procedures also offer a considerable advantage over traditional morphological methods of fruit fly and parasitoid discernment within-host parasitoid identification. This relies on division of immature parasitoids from the host, or lengthy and labour-intensive background methods. Some of the recent research concentrating on the use of molecular approaches for fruit fly and parasitoid for their effective management.

There has been an advent of protease inhibitor (PI)-based strategies for insect pest control. In silico analysis, docking and dynamic studies have proved that there is an interaction between insect gut protease and PIs (Ware et al. 2018). This development can lead to the identification of novel promising PI contender for transgenic studies with reduced cost and time span for virtual screening.

There is a lot that could be done in silico to develop plant extracts as biocides with no side effects on the useful co-inhabitants. Docking analysis, dynamic studies, proteomic analysis and structural studies of these compounds can pave a new direction for pest control (Rinkevich and Bourgeois 2020).

5.6 Conclusions

Most of the works done on insect pest management are on gene editing or on functional genomics to establish gene functions and on vector control for human diseases. Molecular markers also play a vital role in the authentication of insect pests, they are rapid, and simple procedure has been developed effectively to identify the control of insect pests.

A rather lesser number of studies are targeting the phenomenon that could be a crop pest control strategy. A huge potential relies on CRISPR/Cas9-based gene editing for sustainable pest management and reducing pesticide resistance. The leads discussed in this chapter could be a potential control measure of insect pests upon further extensive research on their stability and environmental competence. Further improvements in biosafety regulations associated with the environmental release of gene-edited individuals could open new opportunity for field-based success of CRISPR/Cas9-based pest control techniques. Insect pests are a huge threat to the crop productivity as almost 18% of the yield is lost annually. RNAi provides an environment-friendly and economic alternative to the chemical pesticides. Commercially many genetically modified plants have been developed that express dsRNA that in turn can silence essential genes in insect pests and parasitic nematodes with substantial application in insect pest management. In silico studies on protease and protease inhibitor are a novel approach in insect management.

Points to Remember

- Environmental concerns regarding excessive pesticide use and the development of pesticide resistance among the destructive insect pests have driven the pest management strategies to newer horizons.
- Sterile insect technique is one of the important tools in classical genetics that is used in insect/pest management.
- Molecular markers are more advanced tools for diagnostics and ecological studies, particularly for insects where morphological identification is difficult as well as time-consuming.
- RNAi technique has provided a unique and effective platform in controlling the pest infestation in crops; however, there are several aspects that need to be studied in detail like target gene identification, designing the dsRNA, uptake of genes, level of expression of genes, signal amplification, insect gut pH and many more. It is an amazing example of applying scientific feat from laboratory to fields.
- Gene editing is a novel frontier in pest management research that could form the basis of next-generation insect control. CRISPR/Cas9 is a fast and effective gene-editing tool.
- In silico approach like docking analysis, dynamic studies, proteomic analysis and structural studies could also be used for managing pests.

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RNA Interference Technology

6

S. N. Nagesha

Contents

Abstract

RNA interference (RNAi) is a biological process in which RNA molecules inhibit gene expression or translation by neutralizing targeted mRNA molecules. Historically, RNAi was also known by other names, including co-suppression, post-transcriptional gene silencing (PTGS) and quelling. Two types of small ribonucleic acid (RNA) molecules, microRNA (miRNA) and small interfering

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Omkar (ed.), *Molecular Approaches for Sustainable Insect Pest Management*,
https://doi.org/10.1007/978-981-16-3591-5_6

RNA (siRNA), are central to RNA interference. RNAs are the direct products of genes, and these small RNAs can direct enzyme complexes to degrade messenger RNA (mRNA) molecules and decrease their activity by preventing translation, via post-transcriptional gene silencing. Functional stages of gene silencing with double-stranded RNA (dsRNA) in cells of insects involve in local and systemic gene silencing. Exogenous dsRNA is imported into cells, processed by dicer into small interfering RNA (siRNA; 21 bp + 2-base 30 extensions on each strand) and assembled with the argonaute protein into the RNA-induced silencing complex (RISC). The RISC complex targets and degrades specific mRNAs based on the siRNA sequence. Systemic RNAi effects are mediated through the production of new dsRNAs by RNA-dependent RNA polymerase (RdRP), which uses the target RNA as a template and is primed by siRNA strands. RNAi is applied in various fields, like RNAi-based insecticides and transgenic plants against pests and diseases.

Keywords

RNAi · siRNA · miRNA · dsRNA · Insects

Learning Objectives

1. **RNA interference** (**RNAi**) is a biological process in which RNA molecules inhibit gene expression or translation by neutralizing targeted mRNA molecules.
2. Historically, RNAi was known by other names, including *co-suppression*, *post-transcriptional gene silencing* (PTGS) and *quelling*.
3. Andrew Fire and Craig C. Mello shared the Nobel Prize in Physiology or Medicine (2006) for their work on RNA interference in the nematode worm *Caenorhabditis elegans*, which they published in 1998.
4. Two types of small ribonucleic acid (RNA) molecules—microRNA (miRNA) and small interfering RNA (siRNA)—are central to RNA interference. RNAs are the direct products of genes, and these small RNAs can direct enzyme complexes to degrade messenger RNA (mRNA) molecules and thus decrease their activity by preventing translation, via post-transcriptional gene silencing.
5. Functional stages of gene silencing with double-stranded RNA (dsRNA) in cells of insects involve in local and systemic gene silencing. Exogenous dsRNA is imported into cells, processed by dicer into small interfering RNA (siRNA; 21 bp + 2-base 30 extensions on each strand) and assembled with the argonaute protein into the RNA-induced silencing complex (RISC).
6. The RISC complex targets and degrades specific mRNAs based on the siRNA sequence. Systemic RNAi effects are mediated through the production of new dsRNAs by RNA-dependent RNA polymerase (RdRP), which uses the target RNA as a template and is primed by siRNA strands.
7. RNAi is applied in various fields, like RNAi-based insecticides and transgenic plants against pests and diseases.

6.1 Introduction

RNA interference (**RNAi**) is a biological process in which RNA molecules inhibit gene expression or translation by neutralizing targeted mRNA molecules. Historically, RNAi was known by other names, including *co-suppression*, *post-transcriptional gene silencing* (PTGS) and *quelling*. The detailed study of each of these seemingly different processes elucidated that the identity of these phenomena were all actually RNAi. Andrew Fire and Craig C. Mello shared the Nobel Prize in Physiology or Medicine (2006) for their work on RNA interference in the nematode worm *Caenorhabditis elegans*, which they published in 1998. Since the discovery of RNAi and its regulatory potentials, it has become evident that RNAi has immense potential in suppression of desired genes. RNAi is now known as precise, efficient, stable and better than antisense therapy for gene suppression (Saurabh et al. 2014). However, antisense RNA produced intracellularly by an expression vector may be developed and find utility as novel therapeutic agents (Weiss et al. 1999).

6.2 Discovery of RNA Interference and Brief History

The process of RNAi was referred to as 'co-suppression' and 'quelling' when observed prior to the knowledge of an RNA-related mechanism. The discovery of RNAi was preceded first by observations of transcriptional inhibition by antisense RNA expressed in transgenic plants (Ecker and Davis 1986) and more directly by reports of unexpected outcomes in experiments performed by plant scientists in the United States and the Netherlands in the early 1990s (Napoli et al. 1990). In an attempt to alter flower colours in petunias, researchers introduced additional copies of a gene encoding chalcone synthase, a key enzyme for flower pigmentation into petunia plants of normally pink or violet flower colour. The overexpressed gene was expected to result in darker flowers, but instead caused some flowers to have less visible purple pigment, sometimes in variegated patterns, indicating that the activity of chalcone synthase had been substantially decreased or became suppressed in a context-specific manner. This would later be explained as the result of the transgene being inserted adjacent to promoters in the opposite direction in various positions throughout the genomes of some transformants, thus leading to expression of antisense transcripts and gene silencing when these promoters are active. Another early observation of RNAi came from a study of the fungus *Neurospora crassa* (Romano and Macino 1992), although it was not immediately recognized as related. Further investigation of the phenomenon in plants indicated that the downregulation was due to post-transcriptional inhibition of gene expression via an increased rate of mRNA degradation (Van Blokland et al. 1994). This phenomenon was called *co-suppression of gene expression*; not long after, plant virologists working on improving plant resistance to viral diseases observed a similar unexpected phenomenon. While it was known that plants expressing virus-specific proteins showed enhanced tolerance or resistance to viral infection, it was not expected that plants

carrying only short, noncoding regions of viral RNA sequences would show similar levels of protection. Researchers believed that viral RNA produced by transgenes could also inhibit viral replication. The reverse experiment, in which short sequences of plant genes were introduced into viruses, showed that the targeted gene was suppressed in an infected plant. This phenomenon was labelled 'virus-induced gene silencing' (VIGS), and the set of such phenomena were collectively called *post-transcriptional gene silencing* (Ratcliff et al. 1997).

After these initial observations in plants, laboratories searched for this phenomenon in other organisms. Craig C. Mello and Andrew Fire's 1998 *Nature* paper reported a potent gene silencing effect after injecting double-stranded RNA into *C. elegans* (Fire et al. 1998). In investigating the regulation of muscle protein production, they observed that neither mRNA nor antisense RNA injections had an effect on protein production, but double-stranded RNA successfully silenced the targeted gene. As a result of this work, they coined the term *RNAi*. This discovery represented the first identification of the causative agent for the phenomenon. Fire and Mello were awarded the Nobel Prize in Physiology or Medicine in 2006 (Daneholt 2006).

6.3 Mechanisms of RNA Interference: Components and Mode of Action

Two types of small ribonucleic acid (RNA) molecules, microRNA (miRNA) and small interfering RNA (siRNA), are central to RNA interference. RNAs are the direct products of genes, and these small RNAs can direct enzyme complexes to degrade messenger RNA (mRNA) molecules and thus decrease their activity by preventing translation, via post-transcriptional gene silencing. Moreover, transcription can be inhibited via the pre-transcriptional silencing mechanism of RNA interference, through which an enzyme complex catalyses DNA methylation at genomic positions complementary to complexed siRNA or miRNA. RNA interference has an important role in defending cells against parasitic nucleotide sequences—viruses and transposons (Fig. 6.1).

The RNAi pathway is found in many eukaryotes, including animals, and is initiated by the enzyme dicer, which cleaves long double-stranded RNA (dsRNA) molecules into short double-stranded fragments of ~21 nucleotide siRNAs. Each siRNA is unwound into two single-stranded RNAs (ssRNAs), the passenger strand and the guide strand. The passenger strand is degraded and the guide strand is incorporated into the RNA-induced silencing complex (RISC). The most well-studied outcome is post-transcriptional gene silencing, which occurs when the guide strand pairs with a complementary sequence in a messenger RNA molecule and induces cleavage by Argonaute 2 (Ago2), which contains an RNase H-like domain responsible for target degradation (Martinez et al. 2002). The process is closely related to post-transcriptional gene regulation by microRNAs (miRNAs), where the end result is inhibition of translation initiation and shares many of the same components. In plants and nematodes, RNAi can have systemic effects on gene

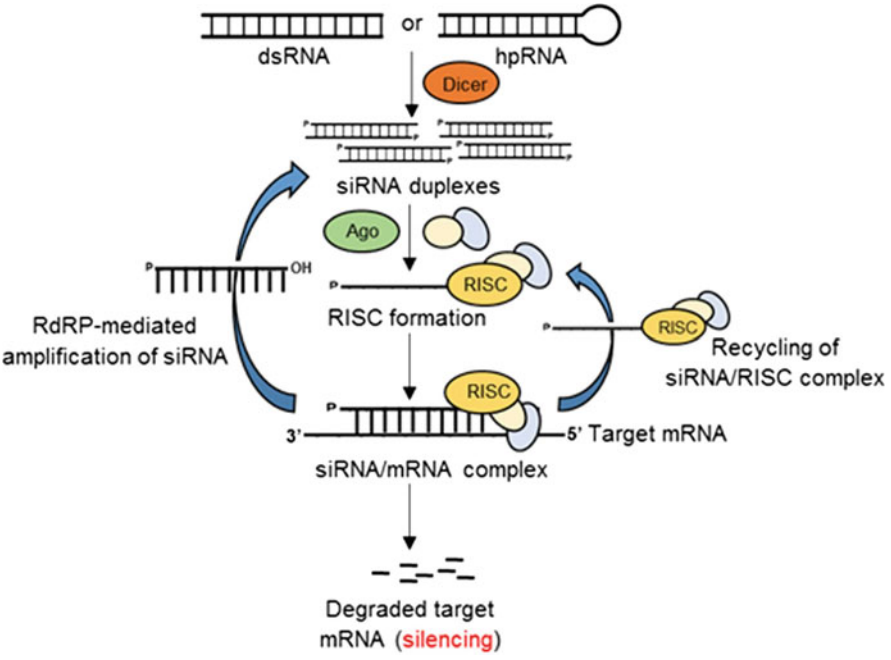

Fig. 6.1 Mechanisms of RNA interference—components and mode of action

expression, so that gene knockout spreads throughout the organism and persists over development. The basis of this effect is thought to lie in the presence of an RNA-dependent RNA polymerase (RdRP) that is able to interact with the RISC complex and generate new dsRNA based on the partially degraded target template by using the hybridized siRNA strands as primers. The synthesized dsRNA is then acted on by the dicer enzymes to generate new siRNAs (secondary siRNAs), thus acting as an amplification step. In this way, once a dsRNA is introduced into a cell, its effect can persist over development; in addition, the dsRNAs can be exported to neighbouring cells and thus spread the gene knockout effect through the organism (Fig. 6.2).

6.4 RNAi Techniques and Their Applications in Pest Control

A decade has passed since the initial discovery of RNA interference (RNAi) in the nematode *Caenorhabditis elegans* (Fire et al. 1998), and it is now clear that double-stranded RNA (dsRNA)-mediated gene silencing is a conserved mechanism in many eukaryotes (Geley and Muller 2004; Hannon 2002). Since its initial description, the technique has become a valuable tool for functional genomics in insects, particularly in studying gene function in the model insect *Drosophila melanogaster* (Kennerdell and Carthew 1998; Kennerdell et al. 2000; Misquitta and Paterson 1999). The

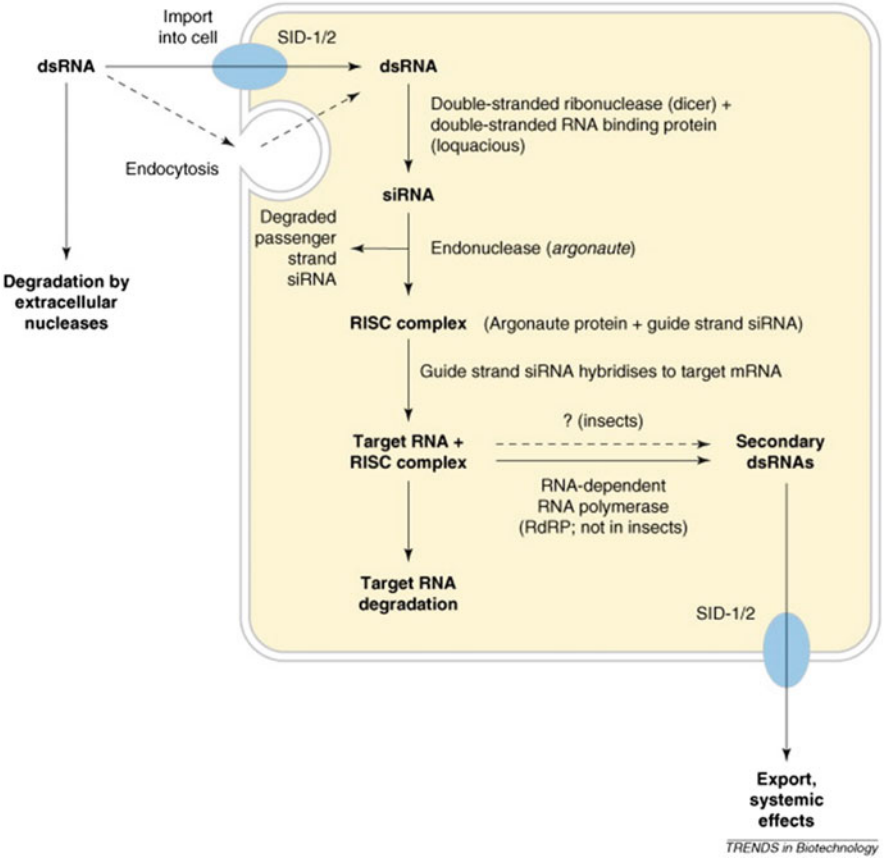

Fig. 6.2 Functional stages of gene silencing with double-stranded RNA (dsRNA) in cells of lower animals. The figure shows steps involved in local and systemic gene silencing. Exogenous dsRNA is imported into cells, processed by dicer into small interfering RNA (siRNA; 21 bp + 2-base 30 extensions on each strand) and assembled with the argonaute protein into the RNA-induced silencing complex (RISC). The RISC complex targets and degrades specific mRNAs based on the siRNA sequence. Systemic RNAi effects are mediated through the production of new dsRNAs by RNA-dependent RNA polymerase (RdRP), which uses the target RNA as a template and is primed by siRNA strands. The secondary dsRNAs can be exported from the cell to spread the RNAi effect to other cells. Gene names in italics have been identified in *Drosophila melanogaster*. The transport proteins SID-1 and SID-2 have been identified in *Caenorhabditis elegans*, as has the RdRP enzyme. Transport mechanisms might differ between different organisms

preferred delivery methodology in the majority of insect studies has been microinjection of nanogram amounts of long dsRNA, synthesized in vitro, into the insect haemocoel (Dzitoyeva et al. 2001). This method of delivery contrasts with the situation in *C. elegans*, where RNAi effects can be produced by feeding bacteria expressing dsRNA (Timmons and Fire 1998; Timmons et al. 2001) or even by soaking nematodes in dsRNA solution (Tabara et al. 1998). Microinjection of

dsRNA in insects was considered to be necessary to produce RNAi effects because the complete genome sequence for *D. melanogaster* (and, subsequently, for other insects) has shown that they lack genes encoding RNA-dependent RNA polymerase (RdRP). RdRP is the enzyme necessary for the siRNA amplification step that leads to persistent and systemic RNAi effects (Sijen et al. 2001). The RdRP function is defined by a characteristic domain, designated PF05183 in the PFAM database (http://pfam.sanger.ac.uk), that has been identified in gene products of eukaryotic microorganisms, fungi, plants, nematodes and a primitive vertebrate (*Branchiostoma floridae*—a cephalochordate) but not in insects, molluscs or other vertebrates. The absence of RdRP in insects predicts that any effects of RNAi will be limited to cells that have taken up dsRNA and will require continuous input of dsRNA to persist. Injection of dsRNA into the body cavity, where it can circulate through the haemolymph, allows short-term effects on gene expression in most cells to be assessed. The possibility of using RNAi effects to protect plants against insects by downregulating essential gene functions in the herbivores, thus resulting in its death, has been recognized for many years, but the method was considered unfeasible. The absence of dsRNA amplification implies that gene knockdown effects produced by feeding RNAi to insects would be limited. Effects would only be expected in cells exposed to the nucleic acid; these cells would be those of the midgut and associated structures because these are the only regions of the insect not covered by the chitin exoskeleton. Degradation of dsRNA in the gut would require continuous administration of high levels of dsRNA; production of sufficient dsRNA in a transgenic plant and its delivery in a sufficiently undegraded state to the insect would provide another significant technical problem, if a role in defence against insect pests was required. However, recent results have shown that many of these preconceptions were unduly pessimistic and that viable levels of insect resistance can be achieved by producing dsRNAs in plants (Baum et al. 2007; Mao et al. 2007).

6.5 RNAi in Insects; Cellular dsRNA Uptake and Export

RNAi-mediated gene knockdown in *Drosophila* is localized to the site of dsRNA delivery, and effects are temporally limited; indeed, a systemic long-lasting RNAi response has never been observed in *Drosophila*, in contrast to *C. elegans* (Fire et al. 1998). The systemic RNAi effect in *C. elegans* is a multistep process that requires the amplification and spread of the silencing signal (Sijen et al. 2001; May and Plasterk 2005). If a similar system was present in insect pests, it would enable targets to be selected from the whole insect (not just gut-specific targets). In addition, the RNAi amplification step would negate the need for a continuous supply of high levels of dsRNA and thus could avoid many of the problems associated with the instability of dsRNA in the insect gut. What lessons can be learned from the use of RNAi in model organisms in relation to a 'real-life' biological problem, such as crop protection against insect pests? Uptake of dsRNA in *C. elegans* has been studied by genetic analysis. A mutant has been identified that is impaired in its ability to mediate a systemic RNAi response when dsRNA is delivered orally (Feinberg and

Hunter 2003). The gene identified, systemic RNA interference deficient-1 (sid-1), is essential and sufficient to mediate systemic RNAi effect in *C. elegans*. When expressed in *Drosophila* S2 cells, sid-1 enhanced the ability of S2 cells to uptake dsRNA at suboptimal dsRNA concentrations. The gene is predicted to encode an 11-helix transmembrane channel protein that is expressed on the cell surface and enables uptake of dsRNAs, thereby mediating a systemic RNAi effect. Further potential mechanisms for RNA transport have been suggested by the recent identification of a further *C. elegans* dsRNA uptake mutant, sid-2 (Winston et al. 2007). The sid-2 mutants are unable to mediate an RNAi response when fed bacteria expressing specific dsRNAs. The sid-2 gene product has been identified as a gut-specific transmembrane protein with a single transmembrane region. To demonstrate functionality, a related nematode, *Caenorhabditis briggsae*, which is defective in uptake of dsRNA from the gut lumen, was transformed with *C. elegans* sid-2, and a systemic RNAi phenotype was restored (Winston et al. 2007). This demonstration of the complexity of RNAi uptake mechanisms and the systemic spread of an RNAi signal in a single organism needs to be borne in mind when considering RNAi in insects. Could the absence of RNA transport mechanisms explain why *Drosophila* cannot manifest a systemic RNAi response? Homologues of the *C. elegans* sid-1 gene have been identified in insects such as *Tribolium castaneum*, *Bombyx mori* and *Apis mellifera* but not in the *Drosophila* genome. Sid-2 homologues have only been detected in nematodes closely related to *C. elegans*. A sid-1 homologue has also recently been identified in aphids (Xu and Han 2008). However, recent evidence suggests that dsRNA uptake into cultured *Drosophila* S2 cells does not involve a sid-1-based mechanism but takes place by receptor-mediated endocytosis because pharmacological inhibition of endocytosis also inhibited RNAi effects. Endocytosis of dsRNA also seems to occur in *C. elegans* because knockdown of components of the endocytotic pathway by RNAi results in worms with a 'loss-of-RNAi-function' phenotype (Saleh et al. 2006). These results suggest that receptor-mediated endocytosis is a widespread mechanism for dsRNA uptake and might well occur across different insect orders. If this is the case, herbivorous insect pests from different orders can be effectively targeted by oral delivery of dsRNA. Further understanding of the complexities of insect dsRNA uptake mechanisms might facilitate the targeting of specific insect pests.

6.6 Systemic RNAi in Insects

To evaluate the potential for systemic RNAi effects in insects, an experimental approach using species other than *Drosophila* has been pursued. Insect systemic RNAi was first documented in the coleopteran *Tribolium castaneum* (flour beetle) by two independent studies. In the first, a homologue of the *Drosophila* sensory bristle-forming gene Tc-achaete-scute (Tc-ASH) was identified and targeted. Injection of Tc-ASH dsRNA into larvae at a single discrete site resulted in a 'loss-of-bristle' phenotype over the entire epidermis of adult insects (Tomoyasu and Denell 2004). In the second study, a parental RNAi effect transmissible between generations was

demonstrated by identifying and targeting developmental genes. Injection of dsRNA specific to (i) Distalless (leg development gene), (ii) maxillopedia (homeotic gene) and (iii) proboscipedia (encoding a homeotic protein required for the formation of labial and maxillary palps) was used to produce an RNAi effect in both mother insects (injected) and developing progeny embryos after egg hatch (Bucher et al. 2002). Thanks to its well-documented, robust systemic RNAi response and the recent completion of its genome sequence, *Tribolium* is becoming an accepted model for the study of systemic RNAi in insects. Intriguingly, a recent genome comparison of *C. elegans* and *Tribolium* revealed a lack of conservation of a systemic RNAi mechanism (Tomoyasu et al. 2008). For example, *Tribolium* lacks a *C. elegans*-like RdRP, so the signal amplification observed in *Tribolium* must be based on a different gene with a similar activity, or possibly even a different mechanism. RdRP-like activity has been demonstrated in cell-free extracts from *Drosophila* embryos, even though the RdRP gene is not present in insects (Lipardi et al. 2001). Future research aimed at elucidating the mechanism of systemic RNAi in insects is likely to broaden the range of insects amenable to systemic RNAi and of genes that can be regarded as targets for a knockdown effect on expression. RNAi-mediated gene knockdown has been reported in several insect orders, including Diptera, Coleoptera, Hymenoptera, Orthoptera, Blattodea, Lepidoptera and Hemiptera (Niu et al. 2006), although most of these studies have used injected dsRNA.

6.7 dsRNA Feeding in Insects

Development of a robust dsRNA feeding methodology in insects that mimics the results obtainable with *C. elegans* (where efficient suppression of gene expression by orally delivered dsRNA is routine) is a prerequisite for utilization of RNAi for crop protection against insect pests (Turner et al. 2006). Turner et al. (2006) provided a convincing demonstration of RNAi effects after dsRNA feeding in larvae of the light brown apple moth (*Epiphyas postvittana*). dsRNAs directed against carboxyesterases were incorporated into an artificial diet. Gene repression was observed after 2 days of feeding, and maximal repression occurred after 7 days. These genes are thought to be gut-expressed, and thus only a local RNAi effect was required for repression. However, in the same investigation, knockdown of a gene expressed in the adult antennae could be achieved through feeding dsRNA to larvae, demonstrating a persistence of the RNAi signal throughout the larval and adult stages and a systemic spread of RNAi signal from the gut to the antennae. In contrast to these positive results, an earlier report showed that midgut aminopeptidase-N gene in larvae of the lepidopteran *Spodoptera litura* was efficiently downregulated by microinjection of dsRNA into the insect haemocoel but stated that attempts to feed dsRNA were unsuccessful in generating an RNAi response (Rajagopal et al. 2002), although no details of methodology were given. An RNAi response after feeding dsRNA has also been reported in the bug *Rhodnius prolixus* (Hemiptera), where a salivary gland transcript encoding nitroporin 2 (NP2) was targeted both by oral

delivery of dsRNA and by microinjection (Araujo et al. 2006). Both treatments produced downregulation of NP2 expression; however, microinjection was more effective (75% reduction in gene expression) than dsRNA feeding (42% reduction). Variation in the midgut environment between different species might dictate whether a feeding approach will be successful. However, comparisons based on existing data are difficult because the susceptibilities of different targets to RNAi effects show considerable variation in model species. Some targets have proved to be completely refractory to suppression, for example, most of the neuronally expressed genes of *C. elegans* (Kennedy et al. 2004).

6.8 Lessons Learned from Development of RNAi for Plant Parasitic Nematodes

Plant expression of dsRNAs directed against genes in pathogens has become an established technique, and plants that show increased resistance to a plant virus (Pooggin and Hohn 2003) and bacteria (Escobar et al. 2001) through RNAi effect have been described. The use of dsRNA approaches for the control of plant parasitic nematodes has been recently reviewed in detail (Lilley et al. 2007); however, it is worth highlighting some of the key developments in the application of this technology. Transgenic plants expressing dsRNAs specific to genes encoding a root knot nematode (*Meloidogyne* spp.) splicing factor and integrase (a chromatin remodelling protein) successfully knocked down transcripts in the pest, resulting in almost complete resistance (Yadav et al. 2006). In another study, a nematode secretory peptide (16D10) that stimulates root growth was successfully downregulated in four closely related species of root knot nematode by transgenic plants expressing dsRNAs, resulting in levels of resistance that varied between 63% and 90% (Huang et al. 2006). A further study demonstrated the feasibility of downregulating a root knot nematode transcription factor with plant-expressed dsRNAs; however, in this case, loss of function did not result in a deleterious phenotype (Fairbairn et al. 2007). To date, there is only one report of the successful downregulation of a cyst nematode transcript via similar approaches (Steeves et al. 2006); this might reflect the poor uptake of dsRNAs by cyst nematodes, in which the feeding tube has a lower exclusion limit than in root knot nematodes (Lilley et al. 2007). Although the nematode system clearly differs from insects, it highlights several important points that must be considered in developing an RNAi approach in insect pest species. RNAi effects are species-specific because knockdown experiments and identification of lethal phenotypes in *C. elegans* have not resulted in a universal set of 'nematode target genes' that are useful for protection against plant parasitic nematodes. Therefore, the success of the RNAi approach is dependent on careful target selection (which takes into account differences in specificity between different species) and the ability of the target nematode to mount a systemic RNAi response.

6.9 Using RNAi to Produce Insect Pest-Resistant Plants

Despite having been considered for many years, application of RNAi technology to give resistance to herbivorous insects has only just been realized. The recent experiments have described transgenic plants producing dsRNAs directed against insect genes. These plants showed enhanced resistance to the economically important agricultural pests, like cotton bollworm (*Helicoverpa armigera*; Lepidoptera) and western corn rootworm (WCR; *Diabrotica virgifera virgifera* LeConte; Coleoptera). The key to the success of this approach is (i) identification of a suitable insect target, which includes off-target minimized siRNA production (Asokan et al. 2012), and (ii) dsRNA delivery, which includes in planta expression of dsRNA and delivery of sufficient amounts of intact dsRNA for uptake by the insect. Although different approaches were used for the generation of insect-resistant plants, careful target selection was common to both. Baum et al. (2007) utilized a screening approach where genes from WCR were identified in cDNA libraries and genes encoding polypeptides predicted to provide an essential biological function were classified as 'targets'. A total of 290 potential targets were identified, and corresponding dsRNAs were synthesized in vitro; their effects on larval performance were determined by delivery in artificial diet feeding trials. Using this approach, a total of 14 genes from the initial list demonstrated specific downregulation of target sequences at low dsRNA concentrations and resulted in insect stunting and mortality. The most effective dsRNA, directed against a gene encoding V-type ATPase A, demonstrated rapid knockdown of endogenous mRNA within 24 h of ingestion and triggered a specific RNAi response with low concentrations of dsRNA. The orally delivered dsRNA could produce systemic silencing of genes (encoding both V-type ATPase subunits and β-tubulin) throughout the insect. The specificity of RNAi-mediated insecticidal effects is an important consideration for the use of this technology in a practical application; effects on nontarget insects should be minimized. dsRNAs directed against three target genes (β-tubulin, V-ATPase A and V-ATPase E) demonstrated an effective RNAi response in WCR that resulted in high larval mortality. These dsRNAs were also delivered to three other coleopteran plant pests: southern corn rootworm (SCR; *Diabrotica undecimpunctata howardi*), Colorado potato beetle (CPB; *Leptinotarsa decemlineata*) and cotton boll weevil (*Anthonomus grandis* Boheman). The dsRNAs demonstrated significant larval mortality in SCR and CPB, although only at higher concentrations than those used for WCR. The sequence identities between WCR and CPB were only 83% and 79% for V-ATPase A and V-ATPase E, respectively. As expected, synthesis of gene-specific dsRNAs for CPB V-ATPase A and V-ATPase E showed increased effectiveness in feeding trials compared with the WCR orthologues. Cotton boll weevil was not only completely insensitive to the three WCR-directed dsRNAs, but was also insensitive to dsRNAs directed against orthologous boll weevil genes, emphasizing the differences between insect species in susceptibility to orally delivered RNAi strategies. To demonstrate the practical application of this technology, transgenic corn was engineered to express dsRNA directed against WCR V-ATPase A. The plants were subjected to WCR infestation and demonstrated a significant level of

protection compared to controls; that is, they showed reduced damage from WCR feeding. A different approach was used by Mao et al. (2007). By studying the interaction between cotton bollworm and cotton, they identified a cytochrome P450 gene, CYP6AE14, which is highly expressed in the insect midgut and whose expression is correlated with larval growth when gossypol, a cotton secondary metabolite, is added to artificial diets. The authors concluded that expression of CYP6AE14 is causally related to gossypol tolerance, presumably via detoxification of this compound, and that suppression of the expression of this gene could increase the sensitivity of the insect larvae to the plant's endogenous defence. Tobacco and *Arabidopsis* plants were engineered to produce dsRNAs directed against the bollworm CYP6AE14 gene. When plant material of both species was fed to larvae, effective repression of the endogenous CYP6AE14 transcript was observed, and the insects showed increased sensitivity to gossypol when transferred to artificial diets. Interestingly, expression of CYP6AE14-directed dsRNA in an *Arabidopsis* dicer mutant (knockout of *Arabidopsis* dicer genes DCL2, DCL3 and DCL4) resulted in the production of longer dsRNAs in the plant that were more effective in gene repression of CYP6AE14. This result shows that optimal efficiency of repression of targeted genes in pests might require stabilization of dsRNAs (Fig. 6.3). The group of Mao et al. (2007) has recently reported that they have engineered cotton to express the cotton bollworm CYP6AE14 dsRNA and that the plants show partial resistance to *Helicoverpa armigera*, as expected (Price and Gatehouse 2008).

6.10 The Insect Gut

The insect gut is divided into three regions: foregut, midgut and hindgut. Of these, the first two are continuations of the 'outside' of the insect and are chitin-lined, so that their surfaces do not present areas of exposed cells (although receptors and transporters are present to allow processes, such as taste recognition in the mouth cavity and water transfer in the hindgut to occur). The midgut region is the only part of the gut that contains surfaces of exposed cells, and it is the main site of exchange between the circulatory system (haemolymph) and the gut contents. The midgut itself is responsible for nutrient absorption, whereas excretion and water balance take place primarily in the Malpighian tubules attached to the hind end, which carry out a function similar to that of the kidney in higher animals. RNAi effects occurring in insects as a result of oral delivery of dsRNA are presumably mediated by the midgut surfaces through exposure of cells of the midgut epithelium and the Malpighian tubules to dsRNA in the gut contents. Conditions in the gut vary considerably between insect orders. Gut pH is an important factor in insect digestion and can vary from predominantly acidic (coleopteran larvae) to strongly alkaline (up to pH 10.5 in some species of Lepidoptera). In addition, within a single insect, the pH changes along the gut and with distance from the gut epithelium. The stability of ingested dsRNA in the insect gut could be affected both by chemical hydrolysis (which increases with increasing pH) and by enzymes present in the gut contents.

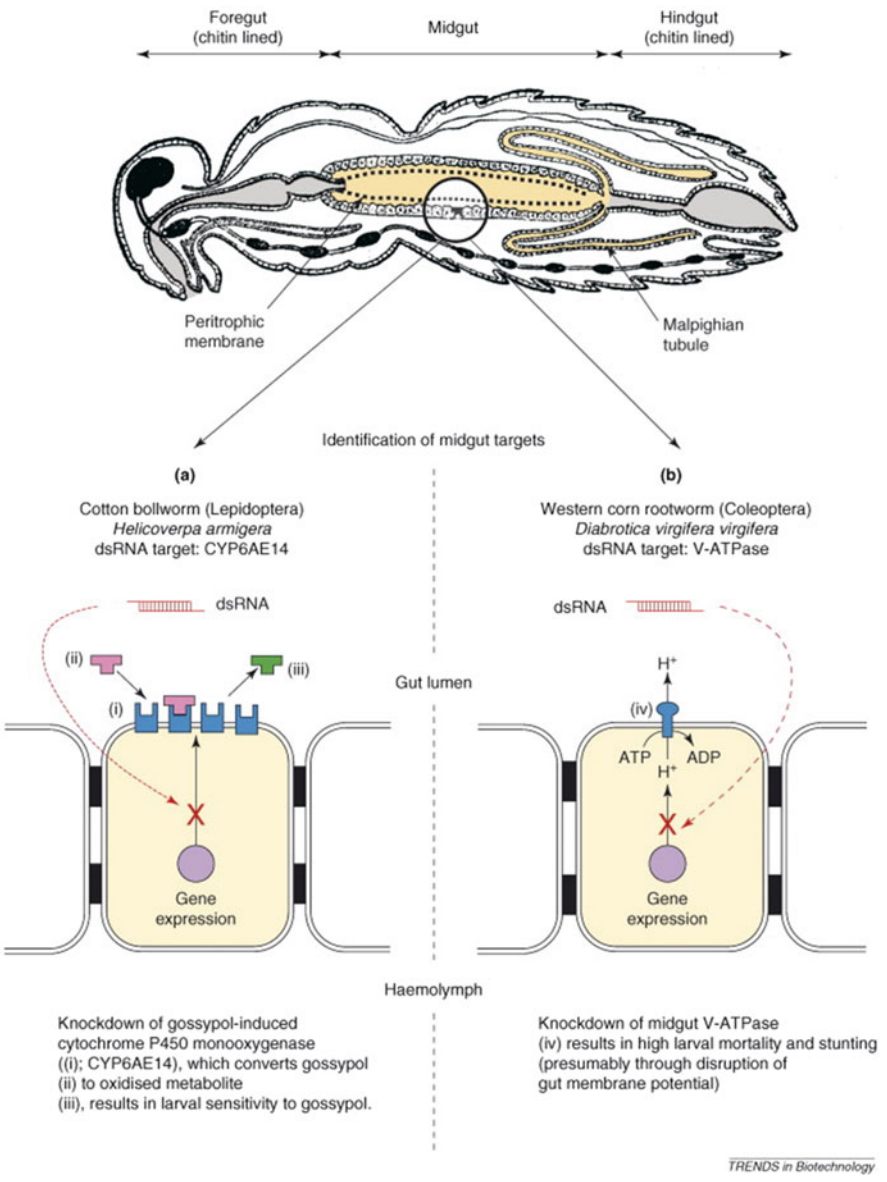

Fig. 6.3 Overview of RNAi approaches for insect-resistant transgenic plants. Double-stranded RNA (dsRNA) produced in planta can lead to targeted gene silencing in Lepidoptera and Coleoptera pest species (Baum et al. 2007; Mao et al. 2007). dsRNAs corresponding to specific insect targets are expressed in planta and are cleaved by endogenous plant dicer enzymes to produce short interfering RNAs (siRNAs) of around 21 nucleotides. Large dsRNA and siRNA cleavage products are expressed throughout plant tissues and are orally delivered to insect herbivores feeding on transgenic plant material. For gene silencing to initiate in targeted insect pests, large dsRNAs and siRNAs must persist in the insect gut, and sufficient quantities must be present for uptake into cells in contact with RNAs (the exact uptake mechanism in target insects remains unknown). Approach (a): a gut-specific cytochrome monooxygenase, CYP6AE14, has been identified (i) whose expression correlates with larval growth on diets containing gossypol (ii), a cotton secondary metabolite.

6.11 Applications of RNAi

6.11.1 Gene Knockdown

The RNA interference pathway is often exploited in experimental biology to study the function of genes in cell culture and in vivo in model organisms (Daneholt 2006). Double-stranded RNA is synthesized with a sequence complementary to a gene of interest and introduced into a cell or organism, where it is recognized as exogenous genetic material and activates the RNAi pathway. Using this mechanism, researchers can cause a drastic decrease in the expression of a targeted gene. Studying the effects of this decrease can show the physiological role of the gene product. Since RNAi may not totally abolish expression of the gene, this technique is sometimes referred to as a 'knockdown', to distinguish it from 'knockout' procedures in which expression of a gene is entirely eliminated (Voorhoeve and Agami 2003). In a recent study, validation of RNAi silencing efficiency using gene array data showed 18.5% failure rate across 429 independent experiments (Munkácsy et al. 2016).

6.11.2 Functional Genomics

Most functional genomics applications of RNAi in animals have used *C. elegans* (Kamath and Ahringer 2003) and *Drosophila* (Boutros et al. 2004), as these are the common model organisms in which RNAi is most effective. *C. elegans* is particularly useful for RNAi research for two reasons: first, the effects of gene silencing are generally heritable, and second, because delivery of the dsRNA is extremely simple. Through a mechanism whose details are poorly understood, bacteria such as *E. coli* that carry the desired dsRNA can be fed to the worms and will transfer their RNA payload to the worm via the intestinal tract. This 'delivery by feeding' is just as effective at inducing gene silencing as more costly and time-consuming delivery methods, such as soaking the worms in dsRNA solution and injecting dsRNA into the gonads (Fortunato and Fraser 2005). Although delivery is more difficult in most other organisms, efforts are also underway to undertake large-scale genomic screening applications in cell culture with mammalian cells (Cullen and Arndt 2005).

Fig. 6.3 (continued) CYP6AE14 is presumably involved in detoxification of gossypol (iii) because specific knockdown of this gene product by dsRNAs delivered in artificial diet and by transgenic plant material increases larval sensitivity to gossypol (May et al. 2005). Approach (b): a related study (Mao et al. 2007) used a screening approach to identify a lethal phenotype in *Diabrotica virgifera virgifera* when midgut V-type ATPase A (V-ATPase) (iv) was downregulated by dsRNAs delivered in artificial diet feeding trials and transgenic corn. Although no direct evidence was presented for the deleterious effects observed in larvae, it is tempting to speculate that knockdown of V-type ATPase A results in disruption of electrochemical gradient across the gut epithelia, which results in high larval mortality

6.11.3 Insecticide

RNAi is under development as an insecticide, employing multiple approaches, including genetic engineering and topical application (Kupferschmidt 2013). Cells in the midgut of some insects take up the dsRNA molecules in the process referred to as environmental RNAi. In some insects, the effect is systemic as the signal spreads throughout the insect's body (referred to as systemic RNAi). Animals exposed to RNAi at doses millions of times higher than anticipated human exposure levels show no adverse effects. RNAi has varying effects in different species of Lepidoptera (butterflies and moths). Possibly because their saliva and gut juice is better at breaking down RNA, the cotton bollworm, the beet armyworm and the Asiatic rice borer have so far not been proven susceptible to RNAi by feeding (Fig. 6.4).

Recent evidence suggests that resistance to RNAi could be broad-spectrum, meaning that resistance to one sequence could confer resistance to other dsRNA sequences. In one laboratory population of western corn rootworm, resistance occurred through lack of uptake of DvSnf7 dsRNA through the gut. When other dsRNA sequences were tested against DvSnf7, the other sequences were no longer effective which suggests that resistance management would be more difficult than simply switching out dsRNA sequences. Combining multiple strategies, such as engineering the protein Cry, derived from a bacterium called *Bacillus thuringiensis* (Bt), and RNAi in one plant delay the onset of resistance (Zhang 2017).

6.11.4 Transgenic Plants

Transgenic crops have been made to express dsRNA, carefully chosen to silence crucial genes in target pests. These dsRNAs are designed to affect only insects that express specific gene sequences. As a proof of principle, in 2009, a study showed RNAs that could kill any one of four fruit fly species while not harming the other three. In 2012, Syngenta bought Belgian RNAi firm Devgen for $522 million and Monsanto paid $29.2 million for the exclusive rights to intellectual property from Alnylam Pharmaceuticals. The International Potato Center in Lima, Peru, is looking for genes to target in the sweet potato weevil, a beetle whose larvae ravage sweet potatoes globally. Other researchers are trying to silence genes in ants, caterpillars and pollen beetles. Monsanto will likely be first to market, with a transgenic corn seed that expresses dsRNA based on gene Snf7 from the western corn rootworm, a beetle whose larvae annually cause 1 billion dollars in damage in the United States alone. A 2012 paper showed that silencing Snf7 stunts larval growth, killing them within days. In 2013, the same team showed that the RNA affects very few other species (Kupferschmidt 2013).

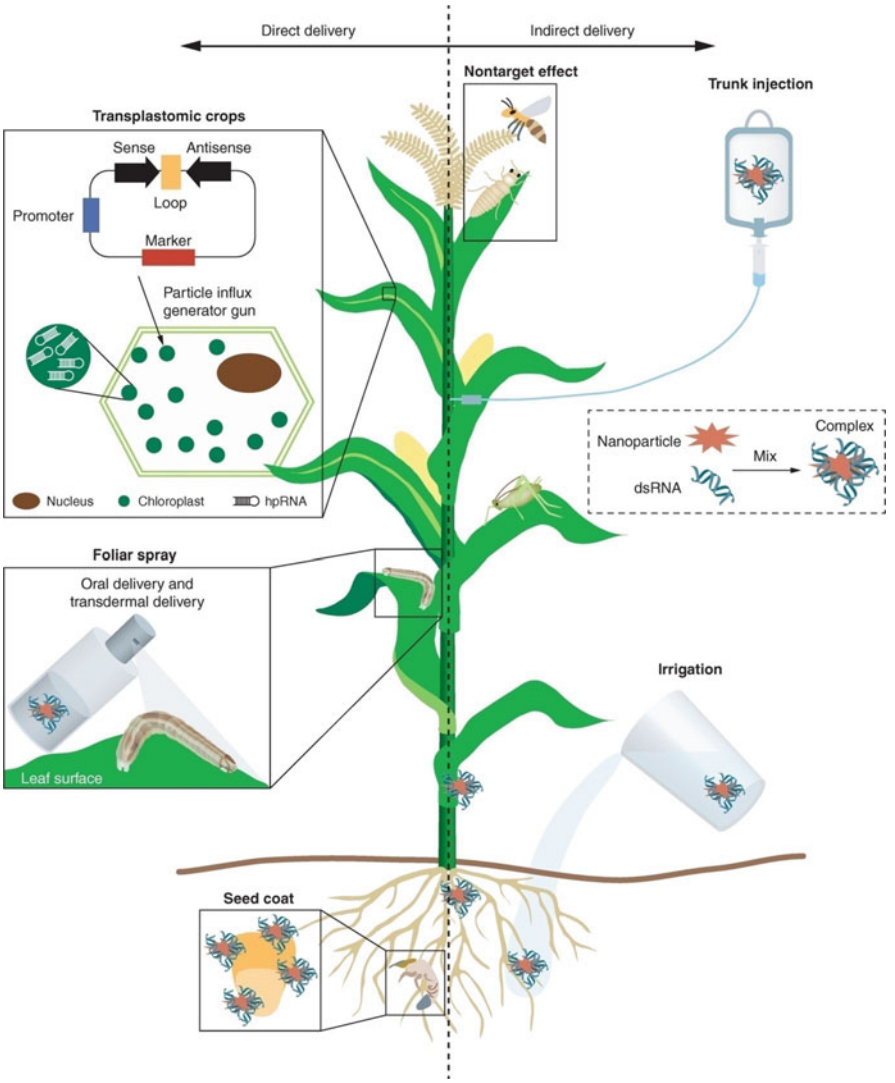

Fig. 6.4 Application of transplastomic technology and nanotechnology to improve RNAi efficiency for insect pest management. (When insecticidal dsRNAs are applied in practical production, they need to enter the target cells to work. Genetically modified (GM) crops and topical application, known as direct uptake, possess good prospects for wider application, and there have been some successful cases. However, dsRNA delivery efficiency is often low in topical application, and during conventional transgenesis, it is difficult to produce sufficient amounts of stable dsRNA owing to the plant RNAi machinery. However, dsRNAs can also enter the plant vascular system and undergo uptake by insect pests, known as indirect uptake. However, dsRNAs may be restricted to the xylem vessels, and dsRNA delivery inside the plant vascular system is limited. Therefore, some limitations exist when applying this indirect uptake method. Introduction of transplastomic technology and nanotechnology may overcome these current difficulties and could improve RNAi efficiency, promoting the development and practice of RNAi-based pest management strategies)

6.12 Future Prospects for RNAi-Based Control of Insect Pests

During the past two decades, RNAi has become an effective tool in functional genomics studies. Fast forward to today, the application of RNAi has helped scientists to find a possible solution to the global problems of agricultural losses attributed to insects and pathogens in a sustainable way. Recent studies reveal that this technology has raised enough attention and received ample funding support (Jalaluddin et al. 2019). For GM crops expressing dsRNA, transplastomic crops seem to be a preferable strategy to achieve the improved effects. However, they are still considered a GM product in most countries, which requires the crops to undergo a rigorous evaluation before approval, and the extensive regulatory process is constraining the extension of transplastomic technology. The commercialization of SMARTSTAX PRO maize seems to be a good beginning. Furthermore, scientists should develop new chloroplast transformation protocols for major crops to promote the expansion of chloroplast-transformed crop range. For nontransformative RNAi products, the supply of dsRNAs associated with nanoparticles through foliar spray, irrigation and trunk injection would be a great strategy to improve insecticidal activity, and other delivery methods, especially seed coats, still need to be evaluated. Bacteria-based expression of dsRNA is regarded as the most cost-effective method to produce large batch dsRNA, and some biotech companies are investing in this production method to produce affordable dsRNA for small and large farms (Joga et al. 2016). Scientists should also pay more attention to public concerns regarding the specificity of dsRNA, fate of nanoparticle/dsRNA formulation in the environment, effects of RNAi-based products on nontarget organisms and so on.

The feasibility of using RNAi in the protection of crops against insect herbivores has been demonstrated. This approach holds great promise for the future because it allows a wide range of potential targets for suppression of gene expression in the insect to be exploited. However, at the moment, the method compares unfavourably with existing transgenic technologies giving resistance to coleopteran and lepidopteran herbivores. From the limited data currently available for whole-plant bioassays in laboratory trials, protection of maize against corn rootworm, even in the best-performing RNAi-expressing plants, is not as effective as in transgenic maize engineered to produce a modified Cry3Bb *Bacillus thuringiensis* (Bt) toxin (Vaughn et al. 2005). Although it is unfair to compare the resistance of non-optimized research material with a commercial product, RNAi-expressing maize is unlikely to replace Bt maize in the short term, especially as the effectiveness of the new crop protection strategy at the field level remains to be determined. However, recent reports of resistance to Bt toxins being observed in field populations of insects exposed to transgenic plants will provide an additional impetus for the development of alternative crop protection strategies (Tabashnik et al. 2008; Gahan et al. 2001). Which insect genes should be targeted? The screening approach used by Baum et al. (2007) has already identified a series of potential targets in corn rootworm, of which a gene encoding the β-subunit of a COPI coatomer complex was the most effective in terms of LC_{50} for RNAi in artificial diet. The COPI complex is involved in translocation of proteins from endosomes to the cytoplasm, as well as other potential

roles in protein trafficking in the cell, but it is not obvious why interference with this function should be lethal. The screening approach can thus identify targets that would not necessarily be predicted from functional considerations but has the drawback of being very labour intensive if large numbers of insect bioassays are required. However, the demonstrated efficacy of targeting V-type ATPase A could easily be extended to other insect species. The approach of Mao et al. (2007), in which insect detoxification mechanisms towards plant secondary metabolites are targeted, has the advantage of being predictable and specific to pests that feed on a crop producing a defined defensive chemical (Gatehouse 2002; Wittstock et al. 2004). It can be readily extended to detoxification mechanisms in other plant–insect interactions. Further development of RNAi biotechnology could also seek to complement existing crop protection strategies; for example, it might be possible to use technologies in combination to counter broad-range, protein-degradation-based resistance to Bt toxins (observed in highly polyphagous insect pests such as *Heliocaverpa virescens*, which gains resistance through the upregulation of specific proteinase genes (Karumbaiah et al. 2007). Further increases in the effectiveness of RNAi strategies might be achieved by utilizing multiple targets. The feasibility of pyramiding multiple targets by RNAi has been demonstrated in *Drosophila* (Schmid et al. 2002) but has yet to be applied to crop protection strategies. The development of an understanding of the specificity of RNAi gene knockdown in insects should allow crops to be produced that express a cocktail of dsRNAs that are highly effective against target insect pest species. The sequence specificity of dsRNAs can be maximized by a careful bioinformatic approach, although multiple gene knockdown events might be achieved with a single dsRNA by targeting genes belonging to large families with high sequence similarity. However, care must be taken to avoid the possibility that loss of function is compensated for by another untargeted gene. Although RNAi is unlikely to have an immediate effect on crop protection against lepidopteran and coleopteran plant pests, for which Bt-based strategies offer a high degree of protection, the technology is likely to be taken up for applications where Bt-based approaches have proved difficult, for example, protection against flies (dipterans), or where no effective Bt toxins are known, for example, protection against sap-sucking homopteran pests such as aphids, leafhoppers and whitefly. Targeting these phloem-feeding insect pests would require in planta expression of dsRNAs and transport of dsRNAs in phloem sieve elements. The transport of RNA in plant phloem is well documented; viral RNA genomes, endogenous cellular mRNAs and small noncoding RNAs are known to be transported in plant phloem elements (Kehr and Buhtz 2008). However, there is no evidence for phloem transport of dsRNA; even though systemic RNAi-based gene silencing occurs in plants, recent evidence suggests that siRNAs are transported as single-stranded sense and antisense molecules (Yoo et al. 2004) and that all RNA in phloem is single stranded. It is possible that dsRNA expressed in phloem cells could be converted to single-stranded RNA (ssRNA) for transport in the phloem by the plant endogenously, but the stability and uptake of ssRNA into insect cells after feeding might then prove a problem. Further experimentation will be required to

determine whether dsRNAs can be introduced into plant phloem sap to make targeting specialist phloem feeders by RNAi feasible with current technology.

6.13 Conclusions

Downregulation of the expression of specific genes through RNA interference (RNAi) has been widely used for genetic research in insects. The method has relied on the injection of double-stranded RNA (dsRNA), which is not possible for practical applications in crop protection. By contrast, specific suppression of gene expression in nematodes is possible through feeding with dsRNA. This approach was thought to be unfeasible in insects, but recent results have shown that dsRNA fed as a diet component can be effective in downregulating targeted genes. More significantly, expression of dsRNA directed against suitable insect target genes in transgenic plants has been shown to give protection against pests, opening the way for a new generation of insect-resistant crops.

Points to Remember

- Promises and limitations of RNAi has become one of the most widely used tools to study loss-of-function phenotypes of genes of interest in *C. elegans*.
- Development of feeding RNAi method enabled researchers to perform genome-wide RNAi screening. With this, many researchers could find genes involved in lifespan (Dillin et al. 2002; Lee et al. 2003), synaptic function (Gottschalk et al. 2005), fat regulation (Ashrafi et al. 2003) and development (Zipperlen et al. 2001) in *C. elegans*.
- However, there are several important factors that must be considered when interpreting RNAi results. First, knockdown of gene activities often resulted in different phenotypes depending on RNAi methods (Kamath et al. 2001).
- The feeding of RNAi and injection of RNAi may downregulate gene expression in different manners, which remains unclear.
- The knockdown phenotypes by RNAi are often different from that of a genetic mutant phenotype, for example, genes in chromosome I of *C. elegans* that showed embryonic lethal phenotype in their mutants but resulted in normal or mild phenotypes when they were knocked down by RNAi.
- This result could represent the different sensitivities of each gene to RNAi. It is possible that genes that are highly expressed in some tissues can be difficult to silence or that genes encoding proteins with long half-lives may have little chance to show its knockdown phenotypes by RNAi since mRNA degradation does not reduce the quantity of the protein.
- Despite all these limitations, RNAi still is a favoured way of gene silencing for genes with no mutations identified, because gene targeting by homologous recombination is not available in *C. elegans* and it will take a long time before researchers will get genetic mutations in the gene of interest.
- Furthermore, RNAi is a way of choice for genome-wide screening, such as searching for interacting genes or for chemical target identification.

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Proteinase Inhibitors

7

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Omkar (ed.), *Molecular Approaches for Sustainable Insect Pest Management*,
https://doi.org/10.1007/978-981-16-3591-5_7

Abstract

Plant proteinase inhibitors (PIs) are defense proteins predominantly found in storage tissues. They play defensive role against insect pests by inhibiting the activity of digestive proteases in the larval midgut. The present chapter provides a general overview of PIs including their purification, classification and basic characteristic features of each class, mechanism of action on gut proteases, and signalling pathways involved during induction of PIs under biotic/abiotic stress conditions. The rapid development of resistance in insects towards pesticides was a major limitation for the generation of pest-resistant crop plants. The role of PIs as biopesticides in the management of lepidopteran pests *Helicoverpa armigera*, *Spodoptera litura*, *S. frugiperda*, *Achaea janata*, *Chilo partellus* and *C. suppressalis* was revealed in detail. Further, the transgenic expression of PI genes alone or by pyramiding with other defense genes paved way for the development of sustainable resistance in crop plants against insect pests.

Keywords

Achaea janata · Biopesticide · Bowman-Birk inhibitor · *Chilo partellus* · *C. suppressalis* · Elicitor · *Helicoverpa armigera* · Host-plant resistance · Induced expression · Insect resistance · Isoforms · Kunitz inhibitor · Lepidopteran pests · Oligomerization · Digestive proteases · Polyphagous pests · Purification · Serine protease inhibitors · *Spodoptera frugiperda* · *S. litura* · Transgenic plants · Zymogram

Learning Objectives

1. ‘Proteinase inhibitors’ (PIs) are the natural plant defense proteins, which show antagonistic effects against insect pests by irreversible inhibition of digestive protease activities that eventually lead to the developmental abnormalities and insect death.
2. They are classified into serine, cysteine, aspartic and metallo-PIs based on the amino acid residue (P1) present at the reactive site. However, Kunitz inhibitors (KIs) and Bowman-Birk inhibitors (BBIs) are well-characterized serine PIs and pronounced as the ‘biopesticides’.

3. They are constitutively expressed among various plant storage organs including seeds, tubers and induced in vegetative organs through the methyl jasmonate (MeJA) signalling pathway upon wounding or insect herbivory.
4. PIs can be screened from the various wild, non-host and host plant sources and purified using various chromatography techniques.
5. The structural and functional properties of PIs including insecticidal activity are evaluated by in vitro spectroscopic assays and in vivo feeding bioassays.
6. Transgenic pest-resistant plants were generated successfully using host or non-host sources of candidate PI genes either alone or in combination with other defense genes, such as *Bacillus thuringiensis* entomotoxins (Bt toxins) or chitinases or lectins, to target the polyphagous pests, such as *Helicoverpa* and *Spodoptera*.

7.1 Introduction

Insect pests are accountable for one-fifth of the global annual yield loss of several economically important crops. A substantial rise in food production is required to meet the demand for global food security. The use of synthetic pesticides as an instant pest controlling agent is potentially harmful to the environment and other nontarget organisms (Kumar and Kumar 2019). Hence, it is essential to enable sustainable agricultural practices to protect crop plants from devastating insect pests. In this context, the enhancement of host-plant resistance is of importance among the economic and environment-friendly approaches available for sustainable pest management (Stout 2014). A breakthrough in pest management is the launch of Bt toxins such as 'Cry' proteins into the crop plants through transgenic technology (Mishra and Kumari 2018). Nevertheless, the consumers have their potential concerns on biosafety issues while consuming the product developed from transgenic crops. In this scenario, overexpression of plants' own defense genes, such as PIs, arcelins, chitinases and defensins, in host plants by conventional breeding or introducing them by transgenic technology into other crop plants is advisable as a foremost possibility in developing sustainable agricultural practices (Arora and Sandhu 2017).

In plants, PIs are small defense proteins, which play a significant role in combating insect pests and pathogens. A majority of PIs exist in three prominent plant families; Fabaceae, Solanaceae and Poaceae of the plant kingdom (Bateman and James 2011). They are constitutively synthesized as seed storage proteins while induced in vegetative plant tissues upon herbivory or wounding. PIs have great demand in the field of medicine as well as in agriculture due to their selective inhibitory potential against specific enzymes, such as trypsin, chymotrypsin, elastase, thrombin, plasmin, kallikrein etc., along with insect digestive proteases (Choi et al. 2012; Scott and Taggart 2010). Plant PIs influence the insect growth by irreversible inhibition of digestive proteases that lead to starvation for free amino acids, which further results in stunted growth and pest mortality (Zhu-salzman and Zeng 2015). The defensive role of PIs in crop protection is evident

from pioneer studies using soybean flour in 1947, and subsequently several in vitro and in vivo studies from various plant sources confirmed their protective role in the management of insect pests (Fan and Wu 2005; Singh et al. 2020).

PIs are divided into several classes based on their sequence relationship and inhibitory domains, as serine, cysteine, aspartic and metallo-PIs (Ryan 1990). Nevertheless, serine PIs such as BBIs and KIs are characterized well and attained considerable attention as biopesticides. In general, BBIs possess ~8 kDa molecular mass with seven disulphide bridges, while Kunitz-type inhibitors are ~19 kDa proteins with two disulphide linkages (Macedo et al. 2015). Serine PIs exist in several isoforms that might result from multigene products and post-translational modifications (Jamal et al. 2013). The emergence of PI isoforms generally varies in its number, based on the selectivity of reacting protease and type of PI family existing within the plant, which together might emerge as counter-defense during host-pest coevolution (Lawrence and Koundal 2002). In general, BBIs have the propensity to self-associate and form homodimers, trimers or more complex oligomers, and this was demonstrated as one of its characteristic features (Mohanraj et al. 2019; Lokya et al. 2020). The oligomeric tendency of BBIs facilitates improved temperature tolerance, decreased entropy and enhanced resistance to pest digestive proteases (Kumar et al. 2004).

Lepidopteran insects are the most destructive pests of several economically important crops. In their larval form, they predominantly use serine proteases particularly trypsin-like (90%) and chymotrypsin-like (5%) enzymes as their major digestive proteases (Patankar et al. 2001; Srinivasan et al. 2006). However, as part of host-pest coevolution, the larvae show several adaptive dynamics by altering the gene expression pattern and protease spectrum in their midgut system to overcome the anti-nutritional effects of dietary PIs. In general, monophagous pests, such as *Achaea janata*, are known to show constitutive expression of proteases. In contrast, polyphagous pests such as *H. armigera* and *S. frugiperda* that feed on a broad host range, often undergo post-ingestive adaptations to release their gut enzymes so as to overcome plant defense mechanisms (Sarate et al. 2012). Hence, identifying and evaluating new PIs, preferably from a non-host or wild plant source, and formulating novel biopesticides, stacking selective insecticidal genes to target these widespread digestive proteases through transgenic technology are promising approaches to manage the resistance in polyphagous pests. In this context, the present chapter summarizes the main classes of PIs, their characteristic features, signalling pathways involved in their induction and possible application to use them as biopesticides in managing common lepidopteran pests.

7.2 Classification of Proteinase Inhibitors (PIs)

Plant PIs contain small and compact structure with one or more inhibitory domains specific to a diverse class of proteases (Macedo et al. 2015). Based on their catalytic mechanism, PIs are broadly grouped into four classes and recognized by the 'International Union of Biochemistry and Molecular Biology' (IUBMB). They are

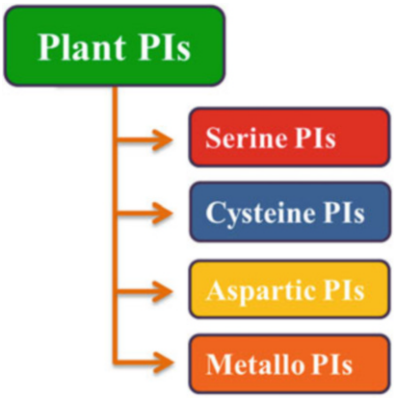

Fig. 7.1 General classification of plant PIs based on their inhibitory specificity towards protease classes

(1) serine PIs, (2) cysteine PIs, (3) aspartic PIs and (4) metallo-PIs (Ryan 1990; Fig. 7.1). Serine family includes Bowman-Birk, Kunitz, cereal, cysteine, mustard, sunflower, squash and potato-type PIs. The biochemical features of purified PIs belonging to different classes/families are described in Table 7.1. However, BBIs and KIs are well characterized among them and reported in both dicot and monocot plants. De Leo et al. (2002) categorized PIs into diverse protein families based on their amino acid sequence, structural and functional properties for easy access of information. The PLANT-PIs database (http://plantpis.ba.itb.cnr.it/) contains 495 inhibitors and their isoinhibitors from 195 plant species. Krowarsch et al. (2003) put forward a different type of classification for serine PIs based on their mechanism of action: (1) canonical inhibitors with convex inhibitory loop complementary to the active site of the proteases involving tight, non-covalent interactions and minimal conformational changes; (2) non-canonical inhibitors where the PIs interact with proteases involving their N-terminal segment in addition to extensive secondary interactions outside the active site; and (3) serpins form an irreversible acyl-enzyme complex, and their interaction with proteases triggers profound conformational changes in the inhibitor structure. Since PIs gained significant applications in agriculture and medicine due to their precise inhibitory potential, there was a need for a more organized PIs database to enable storage and retrieval of information. Therefore, Rawlings et al. (2004) developed the MEROPS database (https://www.ebi.ac.uk/merops/inhibitors/), a comprehensive classification system with a unified source of different proteases, their substrates and inhibitors. The MEROPS database includes 48 families of PIs classified based on similarity in their primary structure. Further, 31 families are clustered into 26 clans based on their three-dimensional structure to simplify the nomenclature system. Subsequently, the MEROPS database is relocated to EMBL-EBI (http://www.ebi.ac.uk/merops/) to facilitate hierarchical classification, such as protein species, families, clans and their identifier at each level.

Table 7.1 Biochemical characteristic features of purified *PIs* from different classes

Plant source	Origin	Class/ family of PIs	Molecular mass (kDa)	Stability of PI		References
				Temp (°C)	pH	
Vigna mungo	Seeds	BBI	8	20–80	2–12	Prasad et al. (2010a)
Dolichos biflorus	Seeds	BBI	8	4–100	2–12	Kuhar et al. (2013)
Glycine max	Seeds	BBI	8.8	20–60	2–12	Latif (2015)
Cajanus cajan	Seeds	BBI	8.5	20–80	2–12	Prasad et al. (2010b), Swathi et al. (2014)
Rhynchosia sublobata	Seeds	BBI	9.21	37–100	2–12	Mohanraj et al. (2018)
Arachis hypogaea	Seeds	BBI	6.73	20–90	2–12	Lokya et al. (2020)
Archidendron ellipticum	Seeds	Kunitz	20	4–60	1–10	Bhattacharyya et al. (2006)
Tamarindus indica	Seeds	Kunitz	21	20–80	1–10	Pandey and Jamal (2014), Medeiros et al. (2018)
Butea monosperma	Seeds	Kunitz	14	10–80	4–10	Jamal et al. (2015)
R. sublobata	Seeds	Kunitz	19.4	10–80	4–10	Mohanraj et al. (2019)
Oryza sativa	Seeds	Cystatin	12	–	–	Abe and Arai (1985)
Cicer arietinum	Seeds	Cystatin	25.3	30–70	3–10	Sheraz et al. (2017)
Prunus dulcis	Seeds	Cystatin	63.4	4–90	3–12	Siddiqui et al. (2016)
Helianthus annuus	Seeds	Aspartic	29, 9 (sub units)	–	–	Park et al. (2000)
Cynara cardunculus	Flowers	Aspartic	31, 15 (sub units)	–	–	Frazao et al. (1999)
Solanum tuberosum	Tuber	Metallo	>20	–	–	Rancour and Ryan (1968)
S. tuberosum	Leaves	Metallo	–	–	–	Graham and Ryan (1981)
S. tuberosum	Tuber	Metallo	40 and 20.5	–	–	Hass et al. (1975)

7.2.1 Serine PIs

The PIs from serine class interact with serine proteases of plant and animal origin, such as trypsin, chymotrypsin and elastase. Among serine PIs, the BBIs and KIs are studied in detail for structural-functional relationships, biological significance and

evolution. Besides for their biotechnological applications in crop protection, serine PIs are also known to possess several pharmacological properties as evident by their selective inhibitory activity towards serine proteases involved in human pathogenesis (Clemente and Arques 2014; Srikanth and Chen 2016).

7.2.2 Bowman-Birk Inhibitors

BBIs are the most widely studied plant PIs besides KIs. They are often found in both dicotyledonous and monocotyledonous plants and grouped in 'I12 family' of MEROPS database (Garcia et al. 2004; Rawlings et al. 2014). The BBIs from dicots possess two inhibitory domains with the molecular mass range of ~6–9 kDa. The primary structure of various BBIs shows two inhibitory domains with P1 and P1′ residues specific to proteases and a conserved cysteine residue framework (Fig. 7.2a). As shown in Fig. 7.2b, c, the two inhibitory reactive sites can interact simultaneously and independently with one or more protease molecules, through a standard mechanism of protease inhibition proposed by Laskowski and Kato (1980). Likewise, BBIs in monocots exist in two distinct forms, i.e. one of the forms contain ~16 kDa molecular mass with two reactive sites. The second form possesses ~8 kDa mass with one functional reactive site and a second non-functional reactive site due to the absence of four conserved cysteine residues (C_3–C_{13}, C_{10}–C_{11}). The conserved sequence homology of BBIs suggests that double-headed inhibitors evolved from ancestral single-headed BBIs through gene duplication and mutations in their reactive site loop residues (Prakash et al. 1996; Mello et al. 2003). Among several three-dimensional (3D) structures available so far, the soybean BBI is considered as the classical and well-studied model, represented in Fig. 7.2c (Werner and Wemmer 1992; Voss et al. 1996; Catalano et al. 2003; Ragg et al. 2006).

The structure of BBIs possesses highly conserved cysteine residues framework (e.g. C_1–C_{14}, C_2–C_6, C_3–C_{13}, C_4–C_5, C_7–C_9, C_8–C_{12}, C_{10}–C_{11}) with seven disulphide bonds (Chen et al. 1992; Mello et al. 2003; Qi et al. 2005) as shown in Fig. 7.2d. These conserved disulphide bonds form a compact-globular structure and stabilize the inhibitory reactive sites that possess nine amino acid residues and the overall structure of the BBIs. In solution, the compact structures of BBIs self-associate to form homodimers, trimers and more complex oligomers (Swathi et al. 2014; Mohanraj et al. 2019). Such characteristic oligomerization is a prominent feature of BBIs which is stabilized by polar network between amino acid side chains, hydrophobic interactions and disulphide bonds (Silva et al. 2005; Rao and Suresh 2007; Joshi et al. 2013). The self-association mechanism is characterized well in BBIs of horse gram (*Macrotyloma uniflorum*), where the interaction of Lys^{24} and $Asp^{75/76}$ played a key role (Kumar et al. 2004; Muricken and Gowda 2010). The self-association pattern further contributed to high structural and functional stability at elevated temperatures and wide pH range including the digestive system of insect pests and other animals (Tamura et al. 1994; Zavodszky et al. 2001; Chye et al. 2006).

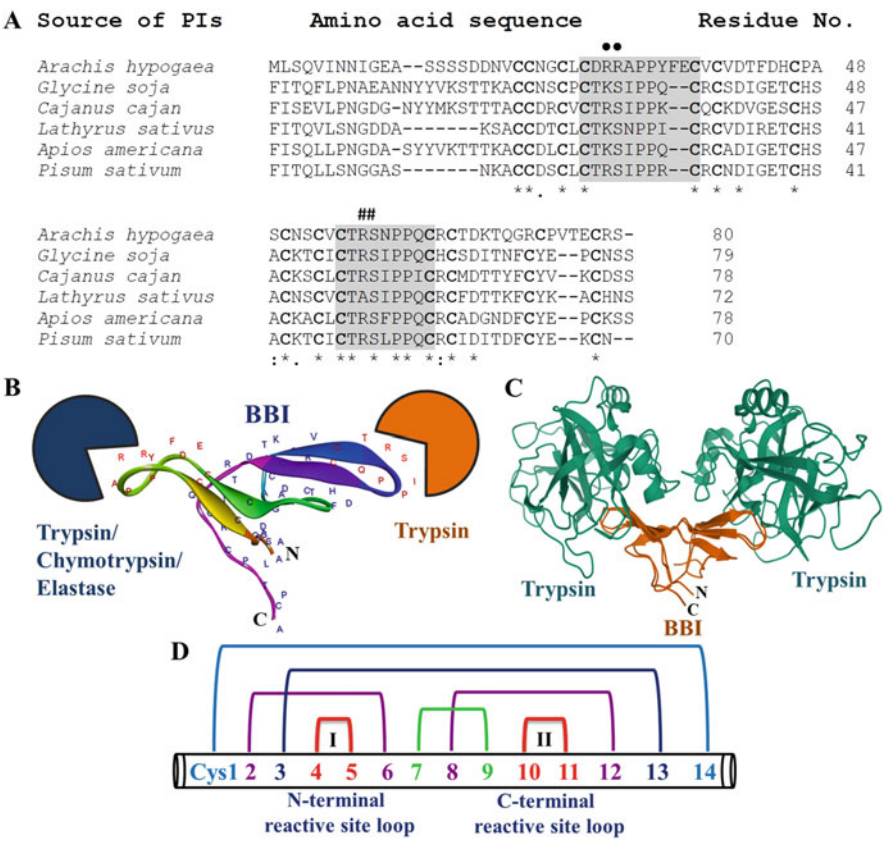

Fig. 7.2 (**a**) Primary structure of BBI from various plant sources. Conserved cysteine residues are highlighted in bold, while N- and C-terminal reactive centre loop residues are highlighted in light grey colour. The dots and hash symbols indicate P1, P1′ amino acid residues in the reactive site (identical residues are indicated by asterisks '*' and conserved residues are indicated by a colon '**:**'); (**b**) three-dimensional structure of peanut BBI (B-II) depicting its two reactive sites interacting with two molecules of proteases; (**c**) ternary complex of double-headed soybean BBI (PDB ID: 1D6R); and (**d**) intramolecular disulphide bonding pattern among the conserved 14 cysteine residues in BBI family. The numbers 1–14 represent the serial number of cysteine residues, and Roman numbers I and II represent N- and C-terminal RCL

PIs exist as several isoforms, which might vary within the plant in number, size and specificity towards the protease class (Voss et al. 1996; Kumar et al. 2004; Barbosa et al. 2007; Muricken and Gowda 2010; Mohanraj et al. 2019). Such variation observed in *Psophocarpus tetragonolobus* where Kunitz-type (five isoforms) and BBI-type (four isoforms) PIs existed with a mass range of 6–28 kDa. Similarly, isoforms from *Nicotiana tabacum* are specific for trypsin (four isoforms) and chymotrypsin (two isoforms). Thus, the evolution of PIs in

multiple isoforms is suggested as a defensive strategy to combat against phytophagous insect pests.

The dicot BBIs comprise broader specificity towards proteases with characteristic P_1–P_1' residues, such as Arg-Ser/Lys-Ser at the N-terminal and Arg-Ser at the C-terminal reactive sites (Qi et al. 2005). The BBIs attain the structure of canonical reactive site loop with cis-Pro at P3′ and an antiparallel β-strand, which exists among all known BBI structures (Bode and Huber 1992; Richardson 1977). They bind to their cognate proteases with exposed convex reactive site loop complementary to the protease' active site. In general, the legume BBIs' N-terminal reactive site is specific for trypsin, while the second reactive site located towards C-terminal binds to trypsin or chymotrypsin or elastase (Fig. 7.2b). Nevertheless, the structural characteristics of peanut BBIs are exceptional in several aspects with other legume BBIs, such as the presence of 11 residue N-terminal reactive site loops and their specificity towards protease (Suzuki et al. 1987; Qi et al. 2005).

Overall, BBIs from legumes share the following standard features: (1) low molecular mass with large cysteine content, (2) occurrence of several isoforms and self-association tendency, (3) double-headed inhibitor structure with two reactive sites, and (4) high stability towards a wide range of pH and thermal conditions. However, BBIs with 3 inhibitory domains and cyclic peptide of 14 amino acids were reported from rice and sunflower seeds (Luckett et al. 1999; Qu et al. 2003). In dicotyledonous plant *Maclura pomifera* a BBI with unique structural features such as presence of two non-identical reactive site loops and five disulphide bonds was identified (Indarte et al. 2017).

7.2.3 Kunitz Inhibitors (KIs)

KIs are reported widely in Fabaceae, Poaceae and Solanaceae families (Habib and Fazili 2007). In general, KIs display the following characteristic features: (1) molecular mass of 14–24 kDa; (2) single polypeptide chain with one reactive site; (3) β-trefoil structure stabilized by two disulphide bonds; and (4) capable of inhibiting serine, cysteine and aspartic proteases with reactive site amino acids Arg-Ser/Arg-Lys/Ala (Oliva et al. 2010; Bendre et al. 2018) as represented in Fig. 7.3a, b. However, KI with two polypeptide chains, which are held by either single disulphide bond (Bhattacharyya et al. 2006) or two disulphide bonds (Oliva et al. 1999; Hansen et al. 2007) or three disulphide bonds (Bronsoms et al. 2011), occurred in nature. KIs in general possess one active site. However, asparagus pea (*P. tetragonolobus*) was reported to bind two chymotrypsin molecules simultaneously. They exist in various isoforms, as observed in the soybean genome, consisting of 10 Kunitz PI isoforms. They are expressed in different parts of the plant during various developmental stages (Krishnan 2001). According to the MEROPS database, KIs reside in 'I3 family' and often exist in higher plants while being absent in green algae (Rawlings et al. 2014). Similar to BBIs, KIs also play an essential role as insecticidal agents by retarding lepidopteran larvae's growth and development (Pandey et al. 2015; Silva et al. 2014). KIs also possess anti-microbial

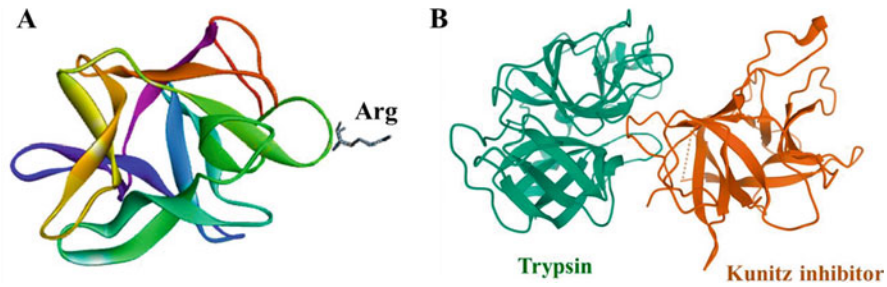

Fig. 7.3 (**a**) Three-dimensional structure of soybean KI showing reactive site amino acid arginine (Arg) and (**b**) complex of KI and trypsin (PDB ID: 1AVW)

(Macedo et al. 2016) and anti-fungal activities (Wang and Ng 2006; Müller et al. 2017; Oliveira et al. 2018). They are known to be induced upon exposure to biotic (Islam et al. 2015) or abiotic stress (Malefo et al. 2020).

7.2.4 Cysteine PIs

Cysteine PIs are small proteins with a molecular mass range of 12–16 kDa characterized in several plant species, including rice, maize, soybean, cowpea, apple and Chinese cabbage, and other mono- and dicotyledonous plants (Barrett 1987; Jacinto et al. 1998). Phytocystatins are grouped into 'I25 family' of MEROPS database (Martinez et al. 2016). Despite the single inhibitory unit of cystatin, several multicystains with 85–87 kDa harbour eight cystatin domains in dicots (Green et al. 2013). The physiological role of these PIs is described primarily in controlling the cysteine protease activity during seed germination and development. Besides, the defensive role of cysteine PIs against coleopteran insects, which depends on cysteine proteases for digestion was well documented (Jacinto et al. 1998). The cystatin superfamily contains stefin, cystatin, kininogen and phytocystatin families, which inhibit cysteine and thiol proteases that play multiple regulatory roles under normal and diseased conditions (Habib and Fazili 2007; Kennedy et al. 2012).

7.2.5 Aspartic PIs

Aspartyl PIs display inhibitory activity towards proteases' such as pepsin and retropepsin, which contain aspartate residue in their catalytic sites. They are found in barley, sunflower and potato tubers. Pepstatin found in potato tubers is described as a potent inhibitor of aspartyl midgut proteases of Colorado potato beetle (Park et al. 2000; Lawrence and Koundal 2002; Wolfson and Murdock 1987). The second type of aspartyl PI isolated from potato tubers is known to inhibit cathepsin D along with serine proteases trypsin and chymotrypsin but not pepsin, cathepsin E or rennin

(Lawrence and Koundal 2002). It has a molecular mass of 27 kDa and shares the considerable sequence homology with soybean trypsin inhibitor.

7.2.6 Metallo-PIs

Metallo-PIs are small in size consisting of ~38 amino acid residues with a molecular mass of 4.2 kDa (Hass et al. 1975; Hass and Hermodson 1981). They potentially inhibit a broad range of carboxypeptidases of both animal and plant origins but not from yeast (Havkioja and Neuvonen 1985). They are mostly described in tomato and potato plants and accumulate during potato tubers' development in response to wounding (Ryan 1990). Metalloproteinase inhibitors exhibit varying degree of inhibitory activity towards metalloproteinases in the different plant tissues. They play an essential role in the degradation of the extracellular matrix during tissue differentiation, wound healing and morphogenesis and possibly useful in treating cancer and arthritis (Browner et al. 1995; Winer et al. 2018).

7.3 Mechanism Underlying Insecticidal Activity of PIs

Plant PIs interact with target proteases by the standard Laskowski mechanism of protease inhibition (Rawlings et al. 2014). According to this mechanism, the inhibitor acts as a pseudosubstrate undergoing limited proteolysis upon complex formation. The reactive peptide bond of inhibitor interact with the target protease's active site, forming acyl intermediate with a high association constant (Laskowski and Kato 1980). The protease-inhibitor complex shows very low dissociation constant, while the cleaved and intact inhibitors can bind and inhibit the proteases. Thus, Plant PIs act as feeding deterrent for phytophagous insects and grazing animals. PIs accumulate in storage organs, such as seeds and tubers, contributing to 1–10% of the total protein content in many plants (Ryan 1990). They are often present in all parts of the plant and are also induced in response to wounding, insect/pathogen attack and during abiotic stress (Shewry 2003; Drame et al. 2013; Rehman et al. 2017). Ingestion of PIs impaired protein digestion in insect midgut by inhibiting the activity of digestive proteases, which affects larval growth and development (Dunse et al. 2010; War et al. 2018). This inhibitory action leads to a decrease in essential amino acid availability, which further causes retarded larval growth and development/deformities during metamorphosis/leading to larval death (De Leo and Gallerani 2002). Though the PIs are small and compact in their structure, they are functionally active at all physiological pH, wide range of temperature, and resistant to proteolytic digestion. These properties add further advantage to plant PIs, which could be a potential choice for controlling phytophagous insect pests. Despite insect counter-defense/adaptation mechanism, several transgenic plants expressing plant PIs have been generated to confer resistance against several

phytophagous insect pests (Macedo et al. 2015; Tanpure et al. 2017; Hamza et al. 2018; Clemente et al. 2019; Singh et al. 2020).

7.4 Expression of PIs

Expression of certain genes takes place continuously disregarding their state of cell cycle, tissue type, developmental stage or biotic/abiotic stimuli needed to maintain basic cellular functions essential for the survival of cells. These genes are called constitutive genes, and the mode of expression is known as 'constitutive expression' (Moein et al. 2017). On the other hand, certain genes are expressed specifically in response to external stimuli. However, they quickly return to their basal state once the stimulus is removed. These genes are turned off under normal conditions, but, any change in the surrounding environment triggers their expression, which is known as 'inducible expression' (Nadal and Posas 2010).

Apart from constitutive accumulations, PIs are also synthesized in vegetative tissues as defense proteins upon transcriptional activation of specific genes when exposed to various biotic and abiotic stress conditions (Leon et al. 2001). During defense mechanism, the activation of these specific genes occurs between few minutes to several hours followed by stress. It leads to the establishment of an immense resistance against the insect attack, systemically during subsequent invasions (Pieterse et al. 2001). Different families of PIs are induced when exposed to biotic stress, such as a pathogen or herbivorous insect attack, and abiotic stresses like heat, floods, drought, salinity and other unfavourable environmental conditions. Induction of defense response at damaged and undamaged sites that are far from the attack site indicated the presence of a systemic signalling system that plays a crucial role in inducing the defense gene expression in plants (Ryan 1992).

7.4.1 Induction of PIs Under Biotic and Abiotic Stress Conditions

The different classes of PIs induced in leaf tissues upon exposure to various biotic and abiotic stress conditions are indicated in Table 7.2. Among them, trypsin and chymotrypsin inhibitors were induced upon insect damage and mechanical wounding in tomato and potato leaves, indicating their defensive role against Colorado potato beetle insect pests (Green and Ryan 1972). The experiments conducted by Mishra et al. (2012) in *Capsicum annum* leaves under various biotic/abiotic stress conditions, such as mechanical wounding accompanied by treating with oral secretions of *H. armigera* and infestation with *Myzus persicae*, reported the expression of serine PIs. Wounding or treatment of MeJA to leaves systemically induced BBI type of PIs in pigeon pea (Lomate and Hivrale 2012). The induced BBIs possess the molecular mass of 16.5 kDa and exhibited potent in vitro inhibitory activity against gut extracts of *H. armigera*. Similarly, Brown et al. (1985) observed wound-induced PIs in alfalfa leaves with a molecular weight of ~6.5 kDa which are shown to be trypsin-specific but not active against chymotrypsin. Apart from biotic

stress tolerance, PIs are also reported to have a leading role in abiotic stress resistance. Induction of Kunitz PIs in white clover plants during water stress indicated their role to protect the plant from drought conditions (Islam et al. 2017).

Apart from serine PIs, the defensive role of cysteine PIs in abiotic stress tolerance was evidenced by overexpression of cystatin gene in *Jatropha curcas* during salinity stress (Li et al. 2015). Zhao et al. (1996) observed that mechanical wounding and MeJA treatment induced cysteine PIs in soybean. Further, seven small potato inhibitor II family PIs were induced upon wounding and treatment with systemin and MeJA in pepper (Moura and Ryan 2001).

7.4.2 Signalling Pathways Involved During Induction of PIs

Plants defend against various environmental stress conditions by switching on specific signalling molecules (Table 7.2). To cope with the external stress, plants tend to synthesize both membrane receptor molecules and chemical signalling molecules. These signalling molecules involved during the synthesis of stress-inducible PIs in tomato and potato include the plant growth hormones, such as peptide systemin, ABA and MeJA. However, the plant's deficit in ABA did not respond to wounding, which indicated its role as a vital signalling molecule in the synthesis of wound-inducible PIs (Cortés et al. 1995). Plants also produce reactive oxygen species (ROS) as a primary signalling molecule during tissue damage. The ROS, e.g. superoxide anions, is produced at the site of damage, while peroxide is identified at both damaged and undamaged parts of the plant. The inducible expression of PIs is known to occur via an octadecanoid pathway involving jasmonic acid (JA), which is formed by the breakdown of linolenic acid (Koiwa et al. 1997). Thus the foremost step before PI synthesis is the production of JA, which is regulated by the interaction of several signalling molecules with their cognate receptors in the plasma membrane. Apart from ROS, the signalling molecules known to participate are ABA, peptide systemin, chitosan, oligo-galacturonic acid and other electrical and hydraulic signals, while receptors include β-glucan-elicitor-binding protein and systemin-binding protein. During wounding, lipases are activated, releasing the linolenic acid from membranes and converting it into oxylipin, the central precursor molecule in JA biosynthesis, leading to the intracellular transduction pathway of PI synthesis (Sivasankar et al. 2000). Different signalling molecules mediate the local and systemic defense responses. Usually, local defense response uses systemin, an 18-mer peptide molecule, as a signal transmitter to the shorter distance through the apoplast and phloem and activates the JA pathway. In contrast, systemic response is reconciled by salicylic acid and their methyl esters (Hunt et al. 1996). The induction of plant PIs during biotic and abiotic stresses involving different signalling molecules is illustrated in Fig. 7.4.

Table 7.2 Different types of *PIs* and signalling molecules induced in leaves upon exposure to biotic/abiotic stresses

Name of the plant	Type of PI(s) induced	Nature of the stress/ chemical treatment	Signalling molecules/ pathway involved	References
Cajanus cajan (1R & 1S)	BBIs	Mechanical wounding and methyl jasmonate	Octadecanoid pathway	Lomate and Hivrale (2012)
Capsicum annum (pepper)	Serine PI	Aphid infestation, mechanical wounding, oral secretions of *H. armigera*	Salicylic acid	Mishra et al. (2012)
Trifolium repens (white clover)	Kunitz	Drought stress	ABA, ethylene	Islam et al. (2017)
Jatropha curcas	Cysteine PIs	Salinity	ROS	Li et al. (2015)
C. annuum (pepper)	Potato inhibitor II	Wounding, systemin and methyl jasmonate	Octadecanoid pathway	Moura and Ryan (2001)
Glycine max (soybean)	Cysteine PIs	Wounding and methyl jasmonate treatment	Methyl jasmonate	Zhao et al. (1996)
Medicago sativa (alfalfa)	BBI	Wounding	Wound-induced communication system	Brown et al. (1985)
Solanum lycopersicum, *S. tuberosum*	Trypsin and chymotrypsin inhibitors	Mechanical wounding and Colorado potato beetle insect damage	Proteinase inhibitor-inducing factor	Green and Ryan (1972)

7.4.3 Induced Expression of PIs Through Elicitors

Elicitors are foreign molecules associated with pests and pathogens that can attach to specific receptor proteins on plant cell membrane resulting in enhanced metabolites synthesis and improving plant resistance against biotic and abiotic stress. Any herbivorous compound that affects the plant at the cellular level is a potential elicitor. Active molecules from insects' oral secretions, such as frass, ovipositional fluids and endosymbionts of insects, act as elicitors. The major elicitor molecules reported so far from lepidopteran insects are lytic enzymes and fatty acid-amino acid conjugates (FACs). β-Glucosidase and glucose oxidase are lytic enzymes isolated from salivary secretions of *Pieris brassicae* infected upon cabbage and *Helicoverpa zea* infected upon tomato, respectively. The induced defense mechanism behind lytic enzyme elicitation was mediated by enhanced ROS levels, and induction of JA regulated defense genes called proteinase inhibitors (Pin 2) (Tian et al. 2012). Further, FACs also act as potent elicitors against insect pests. The first identified FAC elicitor volicitin was isolated from oral secretions of *Spodoptera exigua*. It induced the

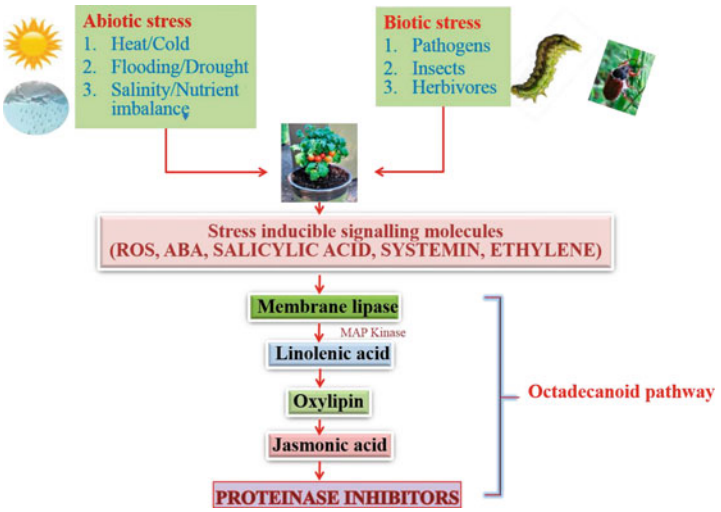

Fig. 7.4 Induction of plant PIs in response to various abiotic and biotic stress conditions. The PI genes are expressed through the octadecanoid pathway initiated by multiple signalling molecules

release of volatiles in maize to attract predators. FACs isolated from *Manduca sexta* induced JA and ethylene bursts and thereby enhanced the defense response by activating the MAPK signalling pathway through octadecanoid cascade (Halitschke et al. 2001). Acevedo et al. (2018) reported that phospholipase C, an elicitor molecule from *S. frugiperda*, induced defense response in maize through the synthesis of PIs.

Some fungal elicitors, such as chitin and ethylene-induced xylanase, trigger defense responses in a wide variety of plant species through the expression of JA, ethylene and salicylic acid (Sanabria et al. 2010). Endopolygalacturonase isolated from the fungus *Rhizopus stolonifer* is a potent elicitor of proteinase inhibitor I in tomato leaves (Simmons et al. 1984). Study of composition and secretions of herbivore elicitors may have a potential influence in understanding the plant-insect interactions, which may be useful in breeding programs to generate pest-resistant crops.

7.5 PIs in the Management of Lepidopteran Pests

7.5.1 *Helicoverpa armigera*

H. armigera Hübner (Lepidoptera: Noctuidae), cotton bollworm, is one among the most devastating pests, damaging the yields of around 181 economically important agricultural plant species belonging to 45 families including sorghum, maize, chickpea, soybean, sunflower, cotton, tobacco and several other pulses and vegetable crops worldwide. The polyphagous nature, high fecundity, facultative diapauses and

tendency to increase resistance to pesticides contribute firmly to their survival and adaptation to diverse cropping systems (Rajapakse and Walter 2007). Though various synthetic pesticides and biotechnological applications are in use, the dynamic compliance to unsteady territories makes the management of *H. armigera* a continued challenge (Fitt 1989; Tabashnik and Carriere 2017). Early-stage *H. armigera* feeds on vegetative parts of the plant, while later developing stage feeds on the nutrient-rich reproductive structures, causing a massive yield reduction. The estimated annual damage caused by *H. armigera* accounting for 2 billion US dollars globally demonstrates the importance to understand the adaptability dynamics and implementation of novel strategies to manage this devastating pest (Tay et al. 2013; EPPO 2019). In compliance with various feeding materials from several host plants, the flexibility in its gut protease expression is the main strength for its adaptability and polyphagous nature. Predominantly, a broad range of digestive proteases' selectivity is considered as the primary source for its adaptation. In this scenario, PIs may play a promising role in designing novel strategies to target these differentially expressed proteases as part of host-plant resistance.

7.5.2 Gut Proteases of *H. armigera*

The gut physiology and digestive proteases of *H. armigera* are one of the prominent areas well studied due to its high polyphagous nature and the extent of damage that it causes to crop yield. *H. armigera* hosts various specificities towards proteases, like trypsin, chymotrypsin, elastase, carboxypeptidases, aminopeptidases and cathepsin B, which are known to play a role in digestion. They are released as extracellular proteases into the gut lumen and exhibit optimal activity at alkaline pH (10–12). Among proteases, serine proteases including ~21 isoforms of trypsin-like and ~14 isoforms of chymotrypsin-like proteases account for 90% and 5% of total gut protease activity. Besides, elastases account for 1%, while the remaining proteases including carboxypeptidases, aminopeptidases and cathepsins are responsible for the rest of 4% gut digestive activity (Srinivasan et al. 2006; Patankar et al. 2001). A range of these protease isoforms express in dynamic flux and substitute for functional selectivity based on their nutritional stress and developmental stage of the larvae. Further, protease activity levels are known to increase from the third instar to late instar stages (Kipgen and Aggarwal 2014). Also, gut protease profiles may vary with an intake of host or non-host plant diets (Dawkar et al. 2011).

7.5.3 Insect Resistance

The rapid development of insect resistance is one of the significant limitations in developing pest-resistant crops in agriculture. *H. armigera* has developed resistance against several synthetic pesticides including cyclodienes, organophosphates, carbamates and pyrethroids. The gut system of *H. armigera* is well equipped to minimize the deleterious effects generated from defense compounds, such as PIs.

Several studies reported on multiple resistance strategies of *H. armigera* to bypass the anti-nutritional effect of PIs. Recent in vivo studies of Saikhedkar et al. (2018) using reactive centre loop (RCL) peptide of potato inhibitor II family (Pin-II family) PIs demonstrated that major trypsin- and chymotrypsin-like genes were downregulated but substituted by higher expression of HaTry1. The gut protease profiles may also switch over among various gene copies that support to survive on a broad range of host plants (Lopes et al. 2004; Kipgen and Aggarwal 2014). Nevertheless, triggering of the overall adaptive response among digestive proteases against ingested PIs is the main impediment in the strategic management of *H. armigera*, which is proven by the presence of a large number of digestive protease genes in polyphagous insect pests (Zhu-Salzman and Zeng 2015). In *H. armigera*, ~48 different gene copies belonging to serine protease family were identified (Mahajan et al. 2013). Therefore, the PI-incorporated transgenic lines might show diminished effectiveness towards the targeted pest, though the in vitro studies with PIs are competitive enough in inhibiting the digestive proteases of the same pest. Further, several other studies demonstrated the pest digestive system's intrinsic ability to synthesize detoxification enzymes to inactivate the antagonistic compounds from natural or chemical sources. Among them, esterase (EST), glutathione *S*-transferase (GST), Cyt p450 and phenoloxidase (PO) are the most common detoxification enzymes. Further, an enhancement in these enzyme activities was found to be the strategic mechanism for *H. armigera* pest resistance (Zibaee et al. 2009; Chen et al. 2019). The detoxification enzymes chemically modify the active site of pesticidal compounds to enhance their water solubility and promote further metabolism and excretion (Allocati et al. 2009). Thus several studies demonstrated an increase in the concentrations of glutathione-*S*-transferase (Bilal et al. 2018; Ottea and Plapp Jr 1984), carboxylesterases (Konus et al. 2013) and Cyt-P450 (Agosin et al. 1985; Mao et al. 2011; Rashid et al. 2013; Brun-Barale et al. 2010; Dawkar et al. 2016) as part of the process of pesticidal metabolic resistance.

7.5.4 PIs Against *Helicoverpa armigera* Trypsin-Like Gut Proteases (HGPs)

H. armigera gut system is intricate to flip-flop their proteases based on the influenced nutritive stress it possessed. A minor alteration at the active site chemistry of proteases may fetch into entirely new proteases that are insensitive to ingested PIs. Thus, despite the flexibility exhibited by HGPs, PIs with potential in vitro and in vivo inhibitory activities are well reported in several previous studies (Table 7.3). PIs from various plant sources, particularly from wild relatives and non-host sources, were shown to be more effective over host plant PIs since they are not exposed earlier to the pest digestive proteases (Chougule et al. 2003; Parde et al. 2012; Swathi et al. 2015).

Wild relatives are endowed with various agronomically desirable traits for crop improvement and protection, including biotic and abiotic resistance. Several studies reported that PIs from pigeon pea wild relatives, such as *C. platycarpus*,

Table 7.3 Insecticidal potential of *PIs* against lepidopteran pests

Genotype source	PI source	Class of PIs	Type of study	In vitro (%) inhibition	Targeted pest	References
[a]Wild relatives	*Cajanus platycarpus* (Leguminosae) (ICPW 60–ICPW 72)	–	In vitro and in vivo	44–80%	*H. armigera*	Swathi et al. (2015)
[a]Wild and cultivars	*C. cajan* (Leguminosae) (13 wild and 2 cultivars)	–	In vitro	40–83%	*H. armigera*	Parde et al. (2012)
[a]Wild and cultivars	*C. cajan* (Leguminosae) (17 wild and 36 cultivars)	–	In vitro	9–87%	*H. armigera*	Chougule et al. (2003)
[a]Wild and cultivars	*Cicer arietinum* (11 wild and 7 cultivars)	–	In vitro	2–36%	*H. armigera*	Patankar et al. (1999)
Wild relative	*R. sublobata* (Leguminosae)	Kunitz	In vitro	~81%	*H. armigera*	Mohanraj et al. (2019)
Wild relative	*Cajanus C. platycarpus* (Leguminosae)	Kunitz and miraculin-like	In vitro	100%	*H. armigera*	Swathi et al. (2016)
Non-host	*Arachis hypogaea* (Leguminosae)	BBI	In vitro and in vivo	86%	*H. armigera*	Lokya et al. (2020)
Non-host	*Psophocarpus tetragonolobus* (Leguminosae)	Kunitz	In vitro and in vivo	50%	*H. armigera*	Banerjee et al. (2017)
Non-host	*Datura metel* (Solanaceae)	Kunitz	In vitro	90%	*H. armigera*	Bonab et al. (2017)
Non-host	*Butea monosperma* (Fabaceae)	Kunitz	In vitro and in vivo	90%	*H. armigera*	Jamal et al. (2015)

Non-host	*Acacia nilotica* (Leguminosae)	–	In vitro and in vivo	86%	*H. armigera*	Babu et al. (2012)
Non-host	*Cocculus hirsutus* (Menispermaceae)	–	In vitro and in vivo	59%	*H. armigera*	Bhattacharjee et al. (2009)
Host plant	*Cassia fistula* (Leguminosae)	–	In vitro and in vivo	92% (CFTI-I) 78% (CFTI-II)	*H. armigera*	Pandey et al. (2015)
Host plant	*Sorghum bicolor* (Poaceae)	Amylase inhibitor	In vitro	~96%	*H. armigera*	Kotkar et al. (2009)
Host plant	*Momordica charantia* (Cucurbitaceae)	BBI	In vitro and in vivo	>80%	*H. armigera*	Telang et al. (2003)
Synthetic peptides	RCL peptide (reactive centre loop)	Pin-II family	In vitro and in vivo	60%	*H. armigera*	Saikhedkar et al. (2018)
[a]Host and wild relatives	*C. cajan* (8 wild and 14 cultivars)	–	In vitro	–	*S. litura*	Prasad et al. (2009)
Non-host	*Adenanthera pavonina*	–	In vitro and in vivo	–	*S. litura*	Velmani et al. (2019)
Non-host	*C. obtusifolia* (Leguminosae)	Kunitz	In vitro	60%	*S. litura*	Liu et al. (2015)
Non-host	*Archidendron ellipticum* (Fabaceae)	Kunitz	In vitro and in vivo	80%	*S. litura*	Bhattacharyya et al. (2007)
Non-host	*C. glauca* (Fabaceae)	Kunitz	In vitro and in vivo	70%	*S. litura*	Vasudev and Sohal (2019)

(continued)

Table 7.3 (continued)

Genotype source	PI source	Class of PIs	Type of study	In vitro (%) inhibition	Targeted pest	References
Host plant	*V. umbellata*	Kunitz	In vitro and in vivo	>80% (trypsin) ~69% (chymotrypsin)	*S. litura*	Sharma (2019), Telang et al. (2019)
Non-host	*C. glauca* (Fabaceae)	Kunitz	In vitro and in vivo	30%	*S. litura*	Mittal et al. (2014)
Host plant	*C. cajan* (Fabaceae) *Vigna mungo*	BBI	In vitro	14% (*C. cajan*) 28% (*V. mungo*)	*S. litura*	Prasad et al. (2010c)
Host plant	*V. umbellate* (Fabaceae)	BBI	In vitro	97% (trypsin) 81% (chymotrypsin)	*S. litura*	Katoch et al. (2015)
Non-host	*Momordica charantia* (Cucurbitaceae)	Kunitz	In vitro and in vivo	–	*S. litura*	Telang et al. (2003)
[a]Host plants	*Actinostemon concolor* (Euphorbiaceae), *Geonoma schottiana* (Arecaceae), *Palicourea rigida* (Rubiaceae), *Rudgea viburnoides* (Rubiaceae)	–	In vivo	32.7–81.1%	*S. frugiperda*	Alves et al. (2018)
Non-host	*Inga laurina* (Fabaceae)	Kunitz	In vitro/ in vivo	80%	*S. frugiperda*	Machado et al. (2017)
Host plant	*Platypodium elegans* (Fabaceae)	Kunitz	In vitro/ in vivo	95%	*S. frugiperda*	Ramalho et al. (2018)
Host plant	*Entada acaciifolia* (Fabaceae)	Kunitz	In vitro and in vivo	31.5%	*S. frugiperda*	Oliveira et al. (2013)
Host plant	*Poecilanthe parviflora* (Fabaceae)	Kunitz	In vitro	63%	*S. frugiperda*	Garcia et al. (2004)

Host plant	*Glycine max* (Leguminosae)	BBI and Kunitz	In vitro and in vivo	85% (trypsin-like) 45% (chymotrypsin-like)	*S. frugiperda*	Paulillo et al. (2000)
Host plant	*Ricinus communis* (Euphorbiaceae) (leaves)	Kunitz	In vitro and in vivo	66%	*S. frugiperda*	Carvalho et al. (2015)
Non-host PIs (purified)	*Ipomoea batatas* (Convolvulaceae) (tubers)	–	In vitro and in vivo	80%	*C. partellus*	Panchal and Kachole (2016)
Non-host PIs	*Capsicum annuum* (Solanaceae)	–	In vitro and in vivo	–	*C. partellus*	Jadhav et al. (2016)
[a]Non-host and wild relatives	*C. cajan* cultivars and wild relatives	–	In vitro	–	*A. janata*	Prasad et al. (2009)
Non-host	*C. cajan* (ICP 14770) and *V. mungo* (TAU-1)	BBI	In vitro and in vivo	70%	*A. janata*	Prasad et al. (2010c)
Non-host	*C. cajan* (ICP 7118)	BBI	In vitro and in vivo	~90%	*A. janata*	Swathi et al. (2014)
Wild relative	*R. sublobata*	BBI and Kunitz inhibitor	In vitro and in vivo	83% (BBI); 88% (rBBI); 73% (KI)	*A. janata*	Mohanraj et al. (2018, 2019)

[a]Screening studies

R. sublobata and C. acutifolius, are recognized as reservoirs of defense genetic source against several insect pests including *H. armigera* (Parde et al. 2012). Further, PIs from *C. platycarpus* and *R. sublobata* are well characterized, and their functional role against HGPs was evident by both in vitro and in vivo studies. The different accessions (13 genotypes) of *C. platycarpus* showed an abundance of PIs active against HGPs (HGPIs) among different plant organs in the following order: mature dry seeds > DAP-III > DAP-II > DAP-I > flowers > pods > leaves [DAP, days after pollination I (8–10 days), II (18–20 days) and III (28–32 days)]. The accumulation of PIs was in line with the 'optimal defense theory', which states that the distribution of constitutive and inducible chemical defenses among the several plant organs is based on their putative value, expectedness and the threat of herbivore attack to ably employ the energy resources (Karban 2011; Moreira et al. 2012). This hypothesis is sustained by the movements and feeding preference of early larval stages of *H. armigera* from the flowers towards developing seeds in the host plant.

Further, the in vivo feeding assays using crude protein extracts of *C. platycarpus* accessions demonstrated a significant reduction in larval and pupal body weights, delay in pupal formation and appearance of larval-pupal intermediates parallel to high mortality rates. Subsequently, the PIs purified from *C. platycarpus* (ICPW 63) showed a significant insecticidal (3180 HGPI units/mg protein) potential (Swathi et al. 2016). Similarly, KIs purified from *R. sublobata* exhibited significant inhibitory (15,000 HGPI units/mg protein) potential against HGPs (Mohanraj et al. 2019).

Apart from pigeon pea wild relatives, host plants, such as *Sorghum bicolour*, *Cicer arietinum*, *Gossypium herbarium* and *Momordica charantia*, and non-host plants, like *Acacia nilotica*, *Albizia lebbeck*, *Arachis hypogea*, *Butea monosperma*, *Cocculus hirsutus*, *Glycine max*, *P. tetragonolobus* and *Solanum tuberosum*, possessed PIs active against gut trypsin-like proteases of *H. armigera*. Recruitment of these PIs would be helpful to cultivate transgenic plants tolerant to *H. armigera* (Harsulkar et al. 1999; Patankar et al. 2001; Mulimani and Sudheendra 2002; Chougule et al. 2003; Babu et al. 2012; Padul et al. 2012; Stevens et al. 2013; Shaikh et al. 2014). Further, a trypsin-specific BBI purified from *Arachis hypogaea* (PnBBI) retarded *H. armigera* larvae's growth despite modulation in the expression of its midgut trypsin- and chymotrypsin-like proteases (Lokya et al. 2020).

7.5.5 *Spodoptera litura*

S. litura, commonly called as oriental leafworm moth, cotton leafworm or tobacco cutworm, is among the most important insect pests of agricultural crops in the Asian tropics. It is a polyphagous and herbivorous pest that can infest on 112 host species belonging to 40 plant families including tobacco, potato, cotton, soybean, beetroot, cabbage, chickpea, groundnut, jute, maize, rice etc. Though the habitable temperatures for *S. litura* are known to be between 10 and 37 °C, it can survive in tropical and temperate climate regions (Yu-Cui et al. 2014). *S. litura* larvae are peripheral feeders but sometimes bore into plant parts, such as buds, flowers and fruits (EPPO 2015). The early instar larvae feed on the inferior surface of the leaf,

which is softer and easily digestible leaving the upper epidermis intact leading to a condition called 'windowing'. The later instar larvae can digest and feed on mature leaves by leaving the midrib and leaf veins causing a condition called 'skeletonising'. The larval stage varies from five to seven instars of around 27 days (Gupta et al. 2015). The older larvae feed only during night time and borrow into the soil for pupation, and the adults emerge after 12 days at 25 °C.

S. litura is responsible for 71% yield loss of groundnut during pegging, podding and pod maturation stages of growth. Field experiments by Panchabhavi and Raj (1987) revealed that infestation of *S. litura* egg masses (12 per plot) led to haulm yield reduction of 43% and pod yield reduction of 27%, respectively. According to Patnaik (1998), *S. litura* caused 9–24% damage to tomato and 20–100% damage to potato (45 days old) crops during various developmental stages. It also causes severe damage to roots of sugar beet and makes it virtually unfit for marketing. It is responsible for 85% leaf consumption in cowpea (Ram et al. 1989), extensive defoliation in soybean (Bhattacharjee and Ghude 1985), brown flag syndrome in banana (Ranjith et al. 1997), fruit damage in grapes (Balikai 1999) and 20–35% yield loss in tobacco. Since it is the major insect pest of many economically important crops, several pesticides are in use. However, it has developed resistance against chemical insecticides, such as pyrethroids and carbamates (Imran et al. 2017; Ahmad 2007). Hence, several integrated pest management (IPM) technology protocols, such as pheromone traps for controlling egg-laying, application of fungicides such as neem kernel extracts (Wightman et al. 1990) and enhancing host-plant resistance are cast off to control *S. litura* on groundnut.

Several studies reported on the application of *Bt* toxins in developing *S. litura*-resistant transgenic lines by introducing *Cry1A* gene in crop plants including sweet potato (Zhong et al. 2019), cotton hybrids (Wan et al. 2008) or *Cry1Ab* gene in corn hybrids (Yinghua et al. 2017). However, larvae fed on Bt-transgenic lines showed marginal mortality rates (<10%), possibly due to increased resistance or decreased binding sites in gut epithelial tissue (Hallad et al. 2011). In this context, several studies reported on the exploration of PIs as an alternative approach to combat polyphagous insect pests, including *S. litura* (Table 7.3). BBIs purified from cultivars of host plants, *C. cajan* (red gram) and *V. mungo* (black gram), showed low to moderate inhibitory effects during in vitro (trypsin-like 14% and chymotrypsin-like 28%) and in vivo studies (Prasad et al. 2010c). However, PIs from other host plants exhibited moderate to significant antagonistic effects against *S. litura*. PIs purified from host plant *V. umbellate* with a molecular weight of 24 kDa exhibited in vitro inhibitory activity against both trypsin-like (80%) and chymotrypsin-like (69%) proteases of the *S. litura* larval gut extracts (Katoch et al. 2015; Sharma 2019). Further, in vivo bioassays using soybean Kunitz trypsin inhibitor (SKTI) showed retarded larval growth rate and increased mortality of *S. litura* (McManus and Burgess 1995). Nevertheless, bioassays with inhibitors showed a high mortality rate of *S. litura* after 72 h of feeding. The studies of Telang et al. (2003) also reported that PIs purified from the seeds of bitter gourd (*Momordica charantia*) showed retardation in the growth and development of *S. litura*.

Contrary to PIs from host plants, PIs from wild relatives of pigeon pea (*C. cajan*) are found to be more active against trypsin-like gut proteases of *S. litura* (SGPs) as compared to cultivars during the in vitro screening (8 wild and 14 cultivars) studies. This achievement could be due to less or non-exposure of wild species PIs to SGPs (Prasad et al. 2009). Nevertheless, apart from wild relatives, non-host PIs are also shown to be effective against *S. litura*. Recent in vivo studies of Velmani et al. (2019) using PIs from *Adenanthera pavonina* showed potent inhibitory activity against both trypsin-like and chymotrypsin-like proteases of *S. litura* upon feeding on PI-incorporated diet (0.25%, 0.50% and 1.0%), where high concentration (1.0%) of PIs showed a significant reduction in *S. litura* larval (41%), pupal (38%) and adult (44%) weights.

7.5.6 *Spodoptera frugiperda*

S. frugiperda commonly called as 'fall armyworm' belongs to the Lepidoptera order and Noctuidae family. It is an inhabitant of tropical regions from the United States to Argentina. It is a highly polyphagous pest of 76 plant families mainly Poaceae (106), Asteraceae (31) and Fabaceae. However, most tremendous damage was observed with sorghum and maize (Montezano et al. 2018). It has a climate-dependent life cycle with duration of 30 days during summer and 80–90 days during winter with six larval instars in the life cycle. Since it has developed high resistance against Bt toxins and chemical pesticides, it reemphasizes the necessity to exploit host-plant resistance. In this situation, several reports underscored the antagonistic efficacy of PIs in the management of *S. frugiperda* (Table 7.3).

In vivo screenings using methanol/water extracts of leaves from *Actinostemon concolor*, *Geonoma schottiana*, *Palicourea rigida* and *Rudgea viburnoides* incorporated into the larval diet showed a significant reduction in larval and pupal weights along with 33–81% inhibition in the gut trypsin-like activity. This study highlighted the critical role of trypsin inhibitors in hampering the growth of *S. frugiperda* (Alves et al. 2018). Further, PIs purified from seeds of non-host plant *Poecilanthe parviflora* showed significant (63%) in vitro inhibitory activity against trypsin-like proteases of *S. frugiperda* (Garcia et al. 2004). Similarly, in vitro and in vivo studies using trypsin inhibitors purified from *Ricinus communis* leaves exhibited 66% inhibitory activity against gut proteases followed by deleterious effects on growth and development of *S. frugiperda* (Carvalho et al. 2015). Bioassays using KI (19 kDa) purified from *Platypodium elegans* seeds showed 98% and 30% inhibition in trypsin-like and chymotrypsin-like gut proteases, respectively, along with a reduction in larval weight and extension of the life cycle of *S. frugiperda*. Also, the high stability of this TI over a wide range of temperature (37–80 °C) and pH (2–10) aided its adaptability to climate change and alkaline gut pH environment of insect pests (Ramalho et al. 2018). Nevertheless, the extraordinary polyphagous nature of *S. frugiperda* allowed it to feed and adapt even on crops, such as soybean, with high PI content. Thus, acquired PI-based resistance in *S. frugiperda* could be due to the existence of various isoforms of gut trypsin-like

and chymotrypsin-like proteases, while several of them might be insensitive to PIs (Paulillo et al. 2000). Further, it is clearly evident in studies of Oliveira et al. (2013) that feeding KI purified from seeds of *Entada acaciifolia* (EATI) to *S. frugiperda* displayed a switch over to expression of novel types of trypsin and chymotrypsin proteases that are insensitive to EATI inhibition. Likewise, similar results were observed from studies of Paulillo et al. (2000) where the *S. frugiperda* larvae fed on diet incorporated with soybean PI induced new types of PI-insensitive trypsin and had fourfold higher activity in chymotrypsin. Nevertheless, a trypsin inhibitor from *Inga laurina* (ILTI) significantly inhibited the activity of various trypsin proteases induced in larvae when fed upon the diet supplemented with SKTI and ILTI (Machado et al. 2017). High stability of ILTI against broad temperature, pH and DTT (dithiothreitol, a reducing agent) revealed the environmental resilience of ILTI and its further use in the management of *S. frugiperda*.

7.5.7 *Achaea janata*

A. janata commonly called as 'castor semilooper' is a major pest on castor. However, it can infest other hosts including tomato, sugarcane, citrus, mango, croton, banana, cabbage, *Ficus*, pomegranate, mustard, some legumes and other *Brassica* species. It infests leaves and can defoliate plants quickly, leaving stems and midribs by feeding disproportionately, and cause severe crop damage that younger plants may not tolerate. Adult insects are known to suck the juice from mango and citrus fruits (Bilapate 1982). The life cycle of these insects ranges between 48 and 50 days from egg to adults. Eggs hatch in 3–4 days resulting in yellowish-green tiny larvae. There are a total of six instar stages before pupation, and the whole larval period may last after 15–20 days. However, the duration mainly depends on the availability of food. Pupae are reddish-brown in colour covered by a silk cocoon, and pupation occurs in the soil or among fallen leaves. The pupal period lasts between 10 and 15 days and is mainly influenced by temperature conditions. As the adult moths are nocturnal, they lay eggs during the night time on an average of 1300 during its lifetime (Karmawati and Tobing 1988).

Several synthetic pesticides, such as pyrethrin, diazinon and neem seed kernel suspensions, are effective against castor semilooper besides their implications on human health and environment. However, strengthening the plant defense by incorporating insecticidal genes such as lectins, polyphenol oxidases, and PIs is alternative from natural sources. Several in vitro and in vivo studies revealed the potential of pigeon pea as a non-host resource for PIs active against gut proteases of *A. janata* (Table 7.3). An in vitro screening study using 14 cultivars and 8 wild relatives of pigeon pea showed 10–50 fold higher inhibitory activity against trypsin-like gut proteases of *A. janata* (AGPs) as compared to standard bovine trypsin (Prasad et al. 2009). Also, a comparative in vivo feeding experiment to establish the pesticidal characters of PIs isolated from red gram (RgPI) and black gram (BgPI) revealed the effectiveness of RgPI over BgPI in decreasing the larval body weights and survival rate in *A. janata* (Prasad et al. 2010c). Likewise, screening studies of

13 wild accessions of *C. platycarpus* and 5 cultivars reemphasized pigeon pea as a potential source of PIs active against AGPs. Among the screened lines, PIs from *C. cajan* (ICP 7118 or C11) were identified as a potential non-host source to enhance the resistance against *A. janata* (Swathi et al. 2012). Further, experiments with purified PIs from C11 (C11PI) variety against *A. janata* by performing leaf coating assays and in vivo feeding studies, which revealed a reduction in larval and pupal body masses together with developmental abnormalities, suggested C11PI as a potential candidate gene to exploit the ecofriendly biopesticides against *A. janata* (Swathi et al. 2014).

Several recent studies on *R. sublobata*, a wild relative of pigeon pea, identified that it is a prominent source of PIs effective against various lepidopteran insects (Prasad et al. 2009; Chougule et al. 2003; Parde et al. 2012). Further, the BBIs purified from *R. sublobata* seeds and recombinant BBI (rRsBBI) cloned from the immature seeds of *R. sublobata* (rRsBBI) and expressed in *E. coli* exhibited potent inhibitory activity against AGPs with an IC_{50} of 29 ng and 70 ng, respectively (Mohanraj et al. 2018, 2019). These results underscored that PIs from legume plants are a potential source of defense proteins to combat *A. janata* and their application in resistance breeding and transgenic technology.

7.5.8 *Chilo partellus* and *Chilo suppressalis*

Among lepidopteran stem borers, 27 species of *Chilo* are economically important as they cause substantial yield losses in Gramineae family crops. *C. partellus*, a 'spotted stem borer', is oligophagous and mainly infests maize and sorghum despite possessing a broad host range. Thus, it is known to infest rice, sugarcane and wild species, such as napier grass (*Pennisetum purpureum*), sudan grass (*Sorghum vulgare sudanense*) and vossia (*Vossia cuspidata*) (Harris 1990). Earlier, this insect was named as 'Swinhoe' as Charles Swinhoe first described it in 1885. It is one of the most economically important pests in Asia (India, Cambodia, Pakistan, Afghanistan, Nepal, Sri Lanka, Thailand, Vietnam) and Africa (Sudan, Ethiopia, Somania, South Africa, Tanzania, Uganda, Zambia). This species is believed to have originated from India and later spread to East and southern Africa and Madagascar (Harris 1990). *Chilo* takes 4–5 weeks to complete its life cycle based on the ambient temperature. The early larval stages feed on young leaves, while later stages damage the stems and cobs. It causes approximately 33% and >70% yield losses in maize and sorghum, respectively. However, the extent of loss is dependent on water stress and cropping patterns. Further, it affects biomass production and forage quality.

PIs extracted from the host and non-host plants, plant metabolites and elicitor molecules capable of inducing the expression of PIs showed an essential role in insect pest management. PIs isolated from non-host plants are reported to show inhibitory activity against gut proteases of *C. partellus* (Table 7.3). PIs purified from *Ipomoea batatas* are potent antagonists of *C. partellus* gut proteases (Panchal and Kachole 2016). A non-host PI from *Capsicum annuum* (CanPIs) also showed the remarkable potential to retard the growth and development of *C. partellus* larvae.

Among the two CanPIs reported, CanPI-7 which was shown to be more active in controlling the development of *C. partellus* can be used as a molecular tool in controlling this pest (Jadhav et al. 2016). Apart from PIs, *Cry1Ac* Bt toxin expressed in *Sorghum bicolor* exhibited larval mortality up to 40% in *C. partellus* (Girijashankar et al. 2005). As insect bioassays showed that a combination of Cry1Ab and Cry1Ac was more effective when compared with other Cry gene products, they may be used in a strategic approach of stacking along with PI genes (Sharma et al. 2010).

C. suppressalis (rice stem borer) is another stem borer and economically important pest of rice, highly responsible for reducing crop forage and yield. Other plant species affected by *C. suppressalis* are amaranthus, millets, grasses, sorghum, maize, radish etc. Symptoms of *C. suppressalis* infestation vary with the age of plants. Generally, it infests the growing ends of the young plants and surrounding leaves of young shoots (Dead hearts). The infested older leaf sheaths first show transparent patches followed by turning yellow-brown, and finally leaves fall off. The larvae feed inside the stem, around the nodes, which resulted in weak and brittle stems. Heavy infestation leads to a condition called white head involving the formation of empty panicles or with a few filled grains (Ishida et al. 2000). Life cycle ranges from 35 to 60 days, and female adults are larger and live longer than males.

The midgut of *C. suppressalis* contains various proteases including amylases, glycosidases, lipases and trypsin-like and chymotrypsin-like proteases. The midgut transcriptome study of *C. suppressalis* using qRT-PCR showed the expression dynamics of 12 digestion-related, 4 immune-responsive and 3 detoxification-related UniGenes. Several cultural methods, light traps, pheromone traps, biological control and chemical methods are used to decrease the damage caused by *C. suppressalis* (Alfaro et al. 2009). However, these methods can not control *C. suppressalis* due to penetration of the larvae into the plant stem immediately after hatching and getting protected from the chemical treatment. Lack of specificity is also one of the primary reasons for the failure of chemical treatments of this insect. Hence, several Bt toxin approaches have been developed to combat *C. suppressalis.* Transgenic rice lines expressing *Cry1A* (Cheng et al. 1998), *Cry1B* (Kiani et al. 2008) and five Bt protoxin (*Cry1Aa*, *Cry1Ab*, *Cry1Ac*, *Cry1Ba and Cry1Ca*) genes have been developed with great success against stem borers (Gao et al. 2010).

Further, Mochizuki et al. (1999) developed *C. suppressalis*-resistant rice plants by introducing synthetic gene coding for Kunitz-type trypsin inhibitor sourced from winged bean (WTI). The resultant transgenic plants showed a reduction in larval growth and development of *C. suppressalis* damage. Similarly, maize PI gene's introduction into two rice cultivars 'Senia' and 'Ariete' which are highly susceptible to *C. suppressalis* showed enhanced resistance (Vila et al. 2005). Recent advances in the knowledge related to plant-insect interactions suggested the gene pyramiding approach to develop transgenic rice lines expressing fusion genes consisting of maize PI and potato carboxypeptidase inhibitor (Quilis et al. 2014). The resultant lines conferred resistance to *C. suppressalis* and devastating rice blast fungal pathogen, *Magnaporthe oryzae* (rice blast disease). However, it warranted the

detailed study, strategic selection and application of various PIs or other defense genes to effectively manage this pest.

7.6 PIs in the Development of Pest-Resistant Transgenic Crops

The use of transgenic plants expressing PI genes solely or in combination with other defense genes related to biotic or abiotic stresses offers the opportunity to engineer the efficient long-term horizontal resistance in crop plants. The structural and functional characteristics of PIs, such as compactness, low molecular mass, existence in several isoforms, striking stability to elevated temperature and a wide pH range with highly efficient and selective inhibitory potential against digestive proteases unlocked the possibility to select PIs as ideal candidates to increase the host-plant resistance against a broad spectrum of pests and pathogens. In this context, several studies reported the successful integration of two or more pest-resistant genes using various gene stacking approaches employing conventional breeding between parental lines or the co-expression of distinct and distant transgenes as gene cassettes under the constitutive or wound-inducible promoter of the host (Boulter 1993; Sandhu and Kang 2017; Table 7.4).

Since serine and cysteine types are the major digestive proteases in most economically important pests belonging to the orders Lepidoptera, Coleoptera and Diptera, several studies are directed to develop transgenic plants by pyramiding the PI genes active against these mechanistic classes of proteases. Transgenic overexpression of single serine PI gene from *Beta vulgaris* under constitutive expression of CaMV using 35S promoter in *Nicotiana benthamiana* showed decreased larval and pupal masses and developmental abnormalities in lepidopteran pests including *S. frugiperda*, *S. exigua* and *M. sexta* (Smigocki et al. 2013). However, targeting multiple digestive proteases often produce sustainable defensive responses in plants. Further, *Nicotiana benthamiana* plants transformed with serine- and cysteine-specific Kunitz TIs (AtKT14 and AtKT15) from *Arabidopsis thaliana* showed improved resistance against spider mite (*Tetranychus urticae*), which is a highly polyphagous pest in agriculture (Arnaiz et al. 2018).

Apart from PIs, other defense genes including Cry toxins, chitinases, thiopins and sporamines are introduced into crop plants to strengthen the plant defense against a wide range of pests parallel to other biotic and abiotic stresses (Chen et al. 2014; Senthilkumar et al. 2010; Outchkourov et al. 2004). Such combination of defense genes expression was reported by stacking the genes of sporamine (potato), cysteine PI (taro) and chitinase (*Paecilomyces javanicus*) in *Nicotiana benthamiana*, which showed enhanced resistance against biotic stresses caused by pests (*S. litura* and *S. exigua*) and pathogens, such as *Alternaria alternata* that cause leaf spot disease and *Pectobacterium carotovorum* that cause soft rot disease, as well as abiotic (salt/osmotic) stresses (Chen et al. 2014). Notably, the gene pyramiding approach is more advantageous in controlling polyphagous pests, such as *H. armigera* and *S. frugiperda* (Luo et al. 2009; Pujol et al. 2005). On the other hand, transgenic tobacco overexpressing the stacked genes of sporamine (trypsin inhibitor) from

Table 7.4 PIs used in development of transgenic crops

PI plant source	Trans genes selected	Targeted pest	Transformed plant	Evidence	References
Beta vulgaris	Serine PI	*S. frugiperda* *S. exigua* *M. sexta*	*Nicotiana benthamiana*	In vitro	Smigocki et al. (2013)
Nicotiana alata *Solanum tuberosum*	Potato type I and type II	*H. armigera* *H. punctigera*	*Gossypium herbaceum*	In vivo and in field	Dunse et al. (2010)
Potato, taro, *Paecilomyces javanicus*, respectively	Sporamine, cystatin, chitinase	*S. litura* *S. exigua*	*N. benthamiana*	In vitro	Chen et al. (2014)
Sweet potato and taro, respectively	Sporamine + cysteine PI	*H. armigera*	*N. tabacum*	In vitro	Senthilkumar et al. (2010)
Zea mays	*mpiC1* (maize PI) + *Cry1Ac*	*C. partellus*	*Sorghum*	Greenhouse	Girijashankar et al. (2005)
Oryza sativa and *S. tuberosum*	Oryzacystatin + potato carboxypeptidase inhibitor	*Tetranychus urticae* (spider mite)	–	In vitro	Benchabane et al. (2008)
N. alata	Serine PI	*Epiphyas postvittana* (apple moth)	*Royal gala* (apple)	In vitro	Maheswaran et al. (2007)
Glycine max (soybean)	Kunitz + BBI	*Diatraea saccharalis* (sugarcane borer)	*Saccharum officinarum* (sugarcane)	Greenhouse	Falco and Silva-Filho (2003)
Arabidopsis thaliana	Kunitz PI (*AtKT14* and *AtKT15*) (serine + cysteine PIs)	*Tetranychus urticae* (spider mite)	*N. benthamiana*	In vitro	Arnaiz et al. (2018)
Triticum aestivum (wheat)	*TaMDC1* (multi-domain cysteine PI)	*Leptinotarsa decemlineata* (Colorado potato beetle)	Tomato	In vitro	Christova et al. (2018)
S. tuberosum	Cystatin + thyropin	*Frankliniella occidentalis*	Potato	In vitro	Outchkourov et al. (2004)

sweet potato and phytocystatin from taro under control of wound- and pathogen-responsive promoter of sporamine 'pMSPOA' showed enhanced resistance against polyphagous pest, *H. armigera* (Senthilkumar et al. 2010). It also showed potent tolerance to bacterial soft rot disease caused by *Erwinia carotovora* and damping-off disease caused by *Pythium aphanidermatum*. Similarly, transient expression of cowpea TI in tobacco showed retarded growth and development in *S. frugiperda* (Pujol et al. 2005).

7.7 General Methods for PI Analysis, Purification and Characterization

7.7.1 Preparation of Crude PI Extract

The crude PI extract is prepared from the plant source (e.g. seeds/leaf etc.) by grinding into a fine powder, which is depigmented and defatted by several washes with acetone and hexane. The filtered dry powder is extracted into a suitable buffer containing 1% polyvinylpyrrolidone (PVP) and stirred overnight and subjected to centrifugation to obtain a clear solution of crude PI extract.

7.7.2 Purification of PIs

The crude PI extract is subjected to ammonium sulphate fractionation by using the principle of salting out, and the presence of PI is tracked by monitoring its inhibitory activity against specific proteases, such as trypsin/chymotrypsin, as described in the following section. The fractions containing maximum PI activity are dialysed and subsequently passed through an ion-exchange, affinity and size exclusion chromatography columns to obtain purified PI as described by Prasad et al. (2010a). The purification procedure is briefly described in the flow chart (Fig. 7.5).

7.7.3 Assay of PIs

The PI activity is indirectly determined by monitoring the decrease in the formation of *p-nitroanilide* (at 410 nm) by the action of proteases on specific substrates in its presence. Thus, PI activity is determined by incubating the crude PI extract or purified PI with specific proteases, such as trypsin/chymotrypsin/insect midgut proteases for 15 min to allow the inhibitor to interact with the active site of the protease. The quantification of residual protease activity is followed by addition of suitable (1 mM) chromogenic substrate N-α-benzoyl-DL-arginine-*p*-nitroanilide (BA*p*NA) for trypsin and *N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GLUPHEPA) for chymotrypsin at 37 °C. The colour (yellow) intensity of the reaction mixture is quantified by using spectrophotometer at 410 nm after terminating the reaction with acetic acid. The relative inhibition in protease activity is determined by comparing

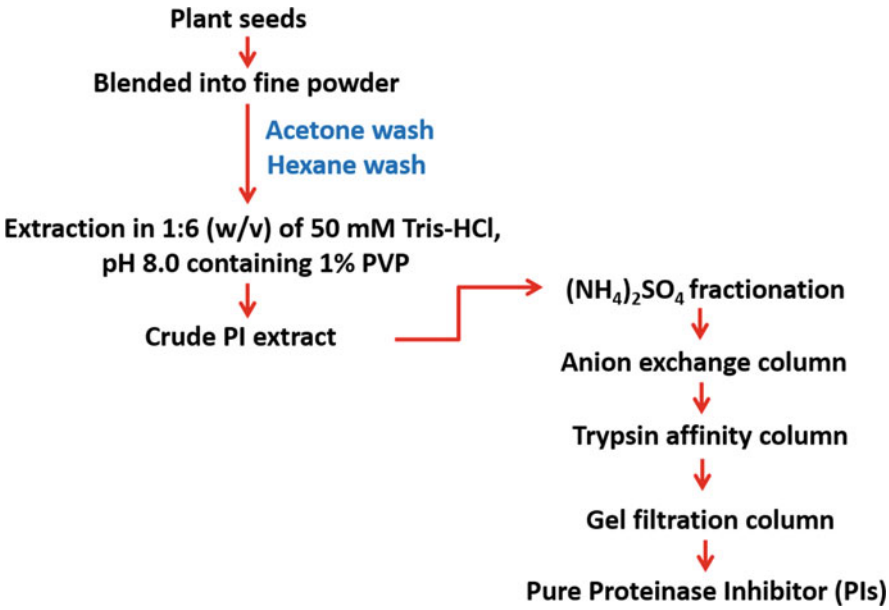

Fig. 7.5 Schematic representation for purification of PIs. A fine seed powder is subjected to overnight extraction in Tris buffer with PVP and the clear supernatant containing crude PI is passed through various chromatography columns to obtain homogeneous pure PIs

the absorbance of the assay reaction mixture (with PI) to the control reaction mixture (without PI) and expressed as PI units. One PI unit is defined as the amount of PI required to inhibit 40–50% of the corresponding protease activity (Swathi et al. 2014; Mohanraj et al. 2019).

7.7.4 Electrophoretic Separation and Identification of PIs

The homogeneity of purified PI would be determined by Tricine SDS-PAGE followed by silver nitrate staining (Schägger 2006; Lokya et al. 2020). The various PI isoforms present in purified protein are further resolved by two-dimensional (2D) gel electrophoresis. Their specific in-gel inhibitory activity was determined using copolymerized gelatin SDS-PAGE, i.e. a 'reverse zymography' technique (Felicioli et al. 1997; Prasad et al. 2010a). After separation, the PIs are identified by subjecting to matrix-assisted laser desorption ionization-mass spectroscopy (MALDI-MS) analysis (Prasad et al. 2010a; Swathi et al. 2014).

7.7.5 Determination of Biochemical and Biophysical Properties of PIs

The biochemical properties of PIs, i.e. their stability against pH, temperature and to a reducing agent (DTT), would be determined by incubating them at a wide range of pH (2–12), temperature (4–90 °C) or DTT, and their residual inhibitory activities would be determined by performing the standard proteinase inhibitor assay as described above. The structural stability is determined by monitoring the secondary structures of inhibitor protein using circular dichroism (CD) spectropolarimeter. The Far-UV (190–260 nm) CD spectra of PI is recorded under varying pH, temperature or DTT as described above.

7.7.6 Evaluation of the Insecticidal Potential of PIs

Insect midgut protease extract is prepared by dissecting the insect midgut in a suitable buffer followed by homogenization and centrifugation at ice-cold conditions. The in vitro inhibitory potential of PI towards the larval midgut digestive proteases is determined by the standard protease inhibition assay described above. Later, the in vivo insecticidal potentia is assessed by feeding PI-supplemented chickpea-based artificial diet or detached leaf coating assay with different concentrations of PI protein. The resultant deleterious effects would be monitored by recording the periodic changes in larva and pupal body masses along with developmental abnormalities and changes in digestive metabolism as evident by the changes in expression of trypsin-like and chymotrypsin-like proteases (Prasad et al. 2010b; Lokya et al. 2020).

7.8 Conclusions

The present chapter summarizes the significant role of ‘proteinase inhibitors’ in enhancing plant resistance to lepidopteran insect pests. The characteristic features of the four major classes of PIs serine, cysteine, aspartic and metalloproteases—are deliberated to understand the mechanism of action against insect digestive proteases. Further, the protocols related to PI extraction, purification and their analysis against insect gut proteases provide a better understanding of protease inhibitors and proteases’ structure-function relationships. The role of signalling molecules involved in the induction of PIs further strengthens the proteases’ dynamic interactions with proteases inhibitors. Despite the dynamic flexibility in the expression of various protease classes, PIs from Leguminosae, Solanaceae and Graminaceae are proven to be very efficient in inhibiting several lepidopteran insect gut proteases. Furthermore, the transgenic expression of PI genes alone or by pyramiding with different classes of PIs / other defense genes such as Cry toxins, chitinases and thiopins, in crop plants opened up the possibility to target expression dynamics of digestive proteases and thereby combat highly polyphagous insects,

such as *Helicoverpa* and *Spodoptera*. Also, as the host-pest coevolution is a continuous process during the course of evolution, it warrants searching for new PIs from all types of plants including wild, non-host and host resources to combat the highly polyphagous lepidopteran pests.

Points to Remember

- PIs are natural and biodegradable defense molecules that can be used as biochemical markers to identify various crop plants with differential resistance against insect pests.
- BBIs and KIs belonging to serine group of PIs are promising molecules in controlling lepidopteran insect pests.
- The exploitation of PIs in resistance breeding program might lead to the development of novel defense strategies in domestic varieties.
- PIs also offer a way to engineer the plant resistance against several devastating insect pests through rDNA technology.
- Gene stacking approach using different mechanistic classes of PIs is useful to target the dynamic expression of digestive proteases of highly polyphagous pests, such as *Helicoverpa* and *Spodoptera.*
- The use of PIs in transgenic technology is also envisaged in molecular farming and mass production as biopesticides.
- Identification of new elicitor molecules from different insect pests might pave a new path to improve upon natural defense role of PIs in crop plants.

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Hormone Analogues and Chitin Synthesis Inhibitors

8

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Contents

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Omkar (ed.), *Molecular Approaches for Sustainable Insect Pest Management*,
https://doi.org/10.1007/978-981-16-3591-5_8

Abstract

The complexity of insect endocrine system can be well understood by studying different types of hormones, which include juvenile hormones, ecdysteroids and neuropeptide hormones. Juvenile hormone is basically a controlling hormone (control moults induced by ecdysone) for metamorphosis in insects. It also plays an important role in reproduction, diapauses of insects and caste determination. Ecdysteroids play a vital role in moulting, growth and development of insects. Depending upon the developmental stage of insect, they can act either as sole hormone or precursor for other ecdysteroid hormones. Neuropeptides commonly known as brain hormones are produced by neurosecretory cells of the central nervous system. The management of insect pests has become a greater challenge due to their ability to develop resistance to many insecticides. To conserve efficacy of insecticides for the control of insect pests, it is necessary to add diversity to the insecticidal pool by introduction of novel insecticides that are specific for biochemical sites or physiological processes in the target pest. Use of insect growth regulators (IGRs) is one of the approaches towards this kind of strategy. IGRs are biorational insecticides, which have novel modes of action causing disruption in the physiology and development of the target pest. IGRs are advantageous over conventional insecticides, as they are specific in action and have low toxicity towards nontarget organisms and mammals and lower rate of persistence in the environment. IGRs have been shown to cause numerous sublethal effects, viz. larval-pupal intermediate, adultoids, increase/decrease in fecundity, transovarial effects and developmental rate as well as changes in sex ratio, diapauses and morphology. Insect growth regulators are categorized into three types based on their mode of action, i.e. juvenile hormone analogues, ecdysone antagonists and chitin synthesis inhibitors. Presently, a number of commercial IGRs are available, but there is need for exploring more IGRs to expand our knowledge regarding their chemistry and effects on insect pests so that the use of these compounds could be expanded in integrated pest management programmes.

Keywords

Juvenile hormone · Ecdysteroids · Hormone antagonists · Chitin synthesis inhibitors · Integrated pest management

Learning Objectives

1. Categorization and functions of insect hormones, i.e. juvenile hormone, ecdysteroids and neurohormones.
2. Need of introduction of insect growth regulators (IGRs).
3. Different types of IGRs, i.e. juvenile hormone analogues, anti-juvenile hormones, ecdysone antagonists and chitin synthesis inhibitors.
4. Role of IGRs in integrated pest management.
5. Scope of anti-juvenoids in integrated pest management.

8.1 Introduction

Insect endocrine system is simpler, comprising of limited number of glands and tissues (Highnam 1967; Doucet et al. 2009). The secretions of the endocrine system, i.e. hormones, are chemical messengers or signals that play important role in coordination of various life processes, viz. development, physiological and behavioural processes, in insects (Highnam 1967; Doucet et al. 2009; Hoffmann and Lorenz 1998). Insect central nervous system (CNS) plays a crucial part in controlling hormonal secretions either directly or indirectly (Nijhout 1994; Reynolds 2013).

Integrated pest management was introduced in the twentieth century as a result of the negative impacts of broad-spectrum pesticides, such as organochlorines, organophosphates and carbamates (Kogan 1998; Doucet et al. 2009). These insecticides induced many ill effects on the environment, nontarget organisms and human health, via bioaccumulation, biomagnifications, persistence in the environment and toxicity. Along with these factors, the major issues were insecticide resistance and resurgence of new pest species. The main focus of IPM strategies was to lower the use of synthetic insecticides and application of safe alternatives. All this led to the introduction of chemicals to insecticidal pool, which were more specific in their mode of action (targeting particular physiological processes) and environment friendly (Doucet et al. 2009). The discovery of molecules that target insect endocrine system was part of this approach. The hormone analogues or antagonists are hormone mimics, which interfere in normal functioning of hormones and affect various physiological events in insect pests (Bowers 1971; Singh and Kumar 2011; Perner and Dhadialla 2012). This chapter will emphasize the role of these chemicals in integrated pest management.

8.2 Insect Hormones: Chemical Nature and Mode of Action

The principal hormones secreted by the endocrine system of insects are:

1. Juvenile hormones
2. Ecdysteroids
3. Neurohormones

8.2.1 Juvenile Hormone

This hormone is secreted by the corpus allatum and was first extracted by Williams in 1956 from the abdomens of adult male cecropia silk moth, *Hyalophora cecropia* (Highnam 1967; Roller et al. 1967; Minakuchi and Riddiford 2006). This acyclic sesquiterpene (Reynolds 2013; Goodman and Cusson 2012) is synthesized via the mevalonate pathway from farnesyl diphosphate or from one of its ethyl-branched

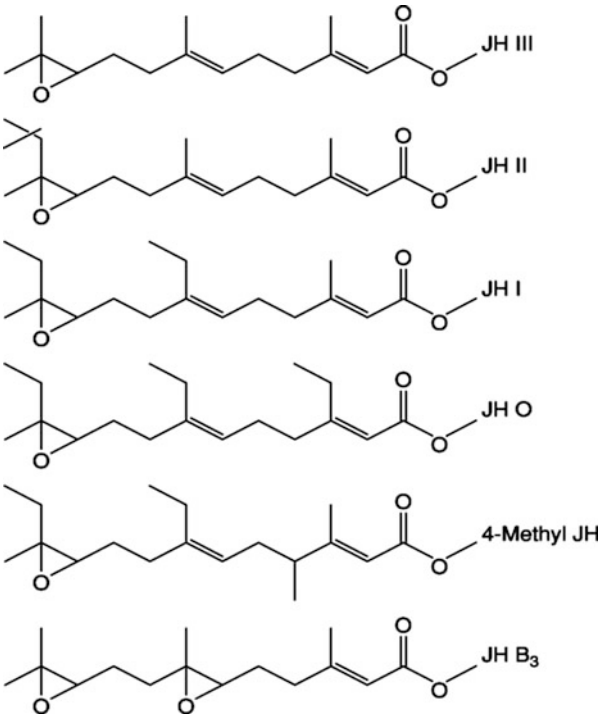


Fig. 8.1 Structure of juvenile hormones (Dhadialla et al. 2005)

homologues (Belles et al. 2005; Minakuchi and Riddiford 2006; Doucet et al. 2009; Singh and Kumar 2011; Goodman and Cusson 2012). Upon secretion juvenile hormone binds to juvenile binding proteins in the haemolymph of insect; this complex acts as a transportation source of juvenile hormones to target sites in insect's body (Mirth et al. 2005; Caldwell et al. 2005; Minakuchi and Riddiford 2006). There is no clarity about the molecular mechanism involved in mode of action of juvenile hormone (Minakuchi and Riddiford 2006; Reynolds 2013), as exact juvenile receptors are not identified.

There are different types of juvenile hormone identified in insects, i.e. JH 0, JH I, JH II and JH III. Most insects produce JH III, but Lepidoptera order is an exception, as it can synthesize JH 0, JH I, JH II and 4-methyl JH I (Fig. 8.1) (Schooley et al. 1984; Baker 1990). Bis-epoxy form of JH III is found in Diptera (Richard et al. 1989; Cusson et al. 1991; Minakuchi and Riddiford 2006; Goodman and Cusson 2012; Reynolds 2013). Juvenile hormones play an important role in regulation of development (growth and prevention of metamorphosis in larva), reproduction, stress response, behaviour, polyphenism and diapause (Goodman and Granger 2005a, b; Goodman and Cusson 2012; Noriega 2014).

8.2.2 Ecdysteroids

Ecdysteroids commonly termed as moulting hormones are polyhydroxylated derivatives of 7-dehydrocholestrol (Slama 2015; Gilbert et al. 1980; Milner et al. 1986) which are produced by the prothoracic glands in immature insects. In most adult insects, gonadal and other tissues may produce ecdysteroids upon degeneration of the prothoracic glands. The major ecdysteroid is 20E, but some insect species contain its homologues, i.e. makisterone A and makisterone C, respectively (Hoffmann and Lorenz 1998; Lafont et al. 2012). Ecdysone is generally considered to be a prohormone, being converted into the fat body or epidermis in most insects to the active hormone 20-hydroxyecdysone, by cytochrome P450 enzyme CYP314A. The steroids required for the synthesis of ecdysteroids are part of insect diet, as insects cannot produce steroids (Hoffmann and Lorenz 1998). Cholesterol is converted into ecdysteroid by a series of steps catalysed by P450 and several other enzymes. Phytophagous insects produce their own phytosterols as their diet lack cholesterol; as a result in some insects ecdysteroidogenesis begins with a different precursor, and the prothoracic glands secrete ecdysteroids other than ecdysone (Gilbert 1964; Highnam 1967; Hoffmann and Lorenz 1998; Reynolds 2013). The ecdysteroids form ecdysteroid receptor complex by binding with receptor molecule, which are site-specific DNA binding proteins (−100 kDa) in nucleus of the target cell. This complex further interacts with DNA to induce formation of new transcripts of RNA (Gade et al. 1997; Reynolds 2013; Uryu et al. 2015). Ecdysteroids act as moulting hormones, playing vital role in moulting of larvae and metamorphosis (Niwa and Niwa 2014; Uryu et al. 2015).

8.2.3 Neurohormones

Neurohormones also known as brain hormones of insects are peptides released by the neurosecretory cells of the central nervous system of insects (Highnam 1967; Hoffmann and Lorenz 1998). The diversity of these hormones is very large in insects (Reynolds 2013). There is a great variation in size of insect peptides according to the number of amino acid residues present in them, varying from lesser number of 5 residues (proctolin) to larger number of 62 residues found in eclosion hormone. The neuropeptide hormones can be either in the form of simple amino acid chains or modified post-translationally (Reynolds 2013). The neurohormones may act directly (adipokinetic hormone) on effector organs, or they may stimulate (prothoracicotropic hormone) other endocrine organs for the synthesis of hormones (Highnam 1967; Reynolds 2013). These hormones are also termed '**master regulators**' (Hoffmann and Lorenz 1998; Perić-Mataruga et al. 2006) as they regulate most of the physiological processes in insects, such as reproduction, development, behaviour, metabolism and homeostasis (Hoffmann and Lorenz 1998; Perić-Mataruga et al. 2006). Biogenic amines and adipokinetic hormones, neurohormones, control metabolism of carbohydrates and lipids. Ecdysiotropins or prothoracicotropic neurohormones (PTTH) stimulate the biosynthesis of ecdysteroid

in the prothoracic glands (Borovsky 2003; Gade and Goldsworthy 2003; Perić-Mataruga et al. 2006).

8.3 Concept of Insect Growth Regulators (IGRs) and Insect Growth Disruptors (IGDs)

Insect growth regulators (IGRs) were the result of quest to find insecticides with specific mode of action and which are safer for the environment and nontarget organisms with more selective modes of action (Staal 1975; Williams 1967; Altstein et al. 1993; Hoffmann and Lorenz 1998). Carroll Williams proposed the term '**third-generation pesticide**' in 1967 keeping in view the role of insect juvenile hormone (JH) as an insecticide (Dhadialla et al. 2005).

In the 1970s the term 'IGRs' was cited first time; Schneiderman (1972) used this term for hormone analogues or antagonists (juvenile hormones and ecdysones) that interrupt the regulation of growth and development in insects. Dhadialla et al. (2005, 2010) used the term insect growth disrupters instead of IGRs, as according to them these chemicals do not regulate endocrine processes but rather disrupt normal endocrine activities and, moreover, some chemicals such as chitin synthesis inhibitors (CSIs) are not involved directly in endocrine processes (Ioriatti et al. 2006; Slowik et al. 2001; Perner and Dhadialla 2012). Hence, these chemicals are a type of insecticides that disrupt the normal activity of the endocrine system, resulting in influences on growth, development, metamorphosis and reproduction of the target insect pests, and have slower mode of action as compared to the synthetic chemical insecticides (Staal 1982; Hoffmann and Lorenz 1998; Dhadialla et al. 2005). There are basically three types of IGRs that are commercially available:

1. Juvenile hormone analogues
2. Ecdysone agonists
3. Chitin synthesis inhibitors

8.3.1 Juvenile Hormone Analogues (JHA)

In the 1960s Schmialek (1961) discovered the first JHAs, farnesol and farnesal. Slama et al. (1974) found that both acyclic and cyclic compounds may act as JHAs. In 1972, methoprene became the first commercially available JHA. Most of the early JHAs were either synthesized (terpenoids) or procured naturally (juvabione) (Slama et al. 1974; Staal 1975; Henrick 2007; Ramaseshadri et al. 2012). The latter JHAs, i.e. fenoxycarb and pyriproxyfen, were more photostable and had broad-spectrum activity (Dorn et al. 1981; Masner et al. 1981; Grenier and Grenier 1993; Hatakoshi et al. 1986; Dhadialla et al. 1998; Perner and Dhadialla 2012).

The general assumption about JHAs is that they mimic the action of naturally occurring JH and affect all functions. However, only few of such functions are

explored for the management of insect pests (Retnakaran et al. 1985). The hormonal effects that are exploited for the control of insect pests are:

1. Interference of normal metamorphosis of last instar larva, resulting in larva-pupal intermediates (Retnakaran 1973a, b; Retnakaran et al. 1985; Dhadialla et al. 2005).
2. JHAs block embryonic development at blastokinesis stage and induce ovicidal effects (Riddiford and Williams 1967; Masner et al. 1968; Retnakaran 1970; Riddiford 1971; Dhadialla et al. 2005).
3. Induction of sterility in adults (Langley et al. 1990; Dhadialla et al. 2005).
4. Termination of reproductive diapauses (De Wilde et al. 1971; Retnakaran 1974; Dhadialla et al. 2005).

8.3.1.1 Commercially Available Juvenile Analogues and Their Role

Methoprene

Methoprene is terpenoid, which lacks the epoxide function present in JH (Ashok et al. 1998; Wilson and Ashok 1998; Hoffmann and Lorenz 1998; Dhadialla et al. 2005). Methoprene is most studied and relatively nontoxic to most nontarget organisms. Methoprene half-life is 10 days in soil and is rapidly broken down and excreted. This JHA also shows larvicidal property for controlling many insects of the order Coleoptera, Diptera, Homoptera and Siphonaptera (Harding 1979; Hoffmann and Lorenz 1998; Dhadialla et al. 2005) (Fig. 8.2).

Kinoprene

This JHA has very low or no toxicity. It is non-persistent, easily decomposes on sun exposure and is nontoxic to nontargets and beneficial insects. It induces ovicidal, morphological and sterilant effects in insect pests and is effective in the control of whiteflies, scales, aphids, mealybugs and fungal gnats (Harding 1979; Dhadialla et al. 2005).

Fenoxycarb

Fenoxycarb is phenoxy JHA having carbamate moiety, which is very effective in the control of cockroaches, sucking insects, fleas, fire ants, mosquitoes and scale insects (Grenier and Grenier 1993). Unlike other JHAs, it is slightly toxic to nontargets (aquatic crustaceans and beneficial insects (neuropterans)) (Liu and Chen 2001; Dhadialla et al. 2005) (Fig. 8.2).

Pyriproxyfen

This JHA, also a phenoxy analogue, is one of the most potent JHAs. It causes morphogenetic and sterility in target insects. It has been used for controlling aphids, scales, fire ants, whiteflies and pear psylla. It is, however, mildly toxic to some aquatic organisms but nontoxic to beneficial insects, like bees (Langley et al. 1990; Dhadialla et al. 2005) (Fig. 8.2).

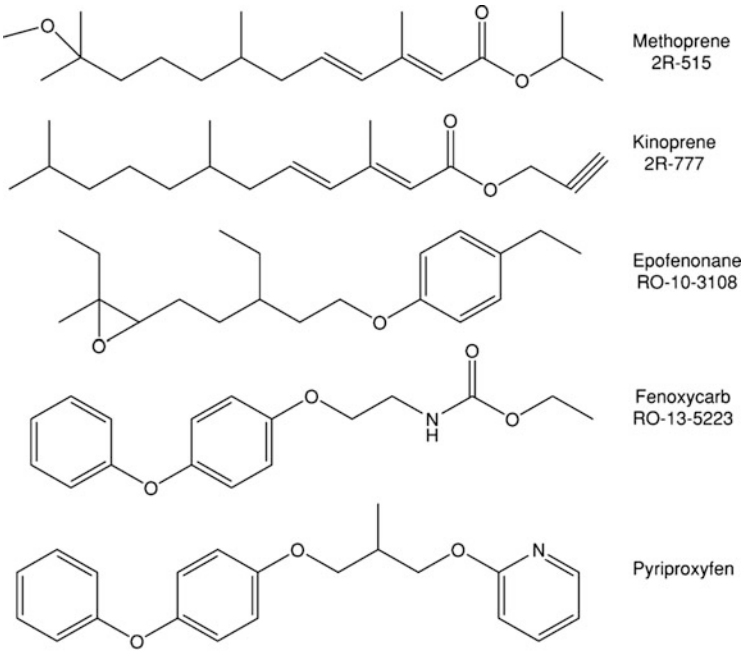


Fig. 8.2 Structures of juvenile hormone analogues (Dhadialla et al. 2005)

8.3.2 Ecdysone Antagonists

Hsu (1991) discovered the first bisacylhydrazine ecdysone agonist, which was further altered to more potent and unsubstituted analogue RH-5849. This analogue possessed broad-spectrum activity and was effective against insect pests of Lepidoptera, Coleoptera and Diptera orders (Aller and Ramsay 1988; Wing et al. 1988; Wing and Aller 1990; Dhadialla et al. 2005). Further research resulted in discovery of cost-effective, highly selective and more potent bisacylhydrazines, such as tebufenozide, methoxyfenozide and halofenozide (Dhadialla et al. 1998, 2005). Tebufenozide and methoxyfenozide are selectively toxic to larvae of lepidopteran insect pests (Hsu 1991). However, methoxyfenozide is more efficacious as compared to tebufenozide and is toxic to a wider range of lepidopteran and other insect pests (Ishaaya et al. 1995; Le et al. 1996; Trisyono and Chippendale 1997; Dhadialla et al. 2005). Halofenozide has a broad spectrum and is effective for the control of cutworms, scarab beetle larvae and webworms (RohMid LLC 1996). Chromafenozide is another bisacylhydrazine used for the control of lepidopteran larvae (Yanagi et al. 2000; Ichinose et al. 2000; Toya et al. 2002; Dhadialla et al. 2005).

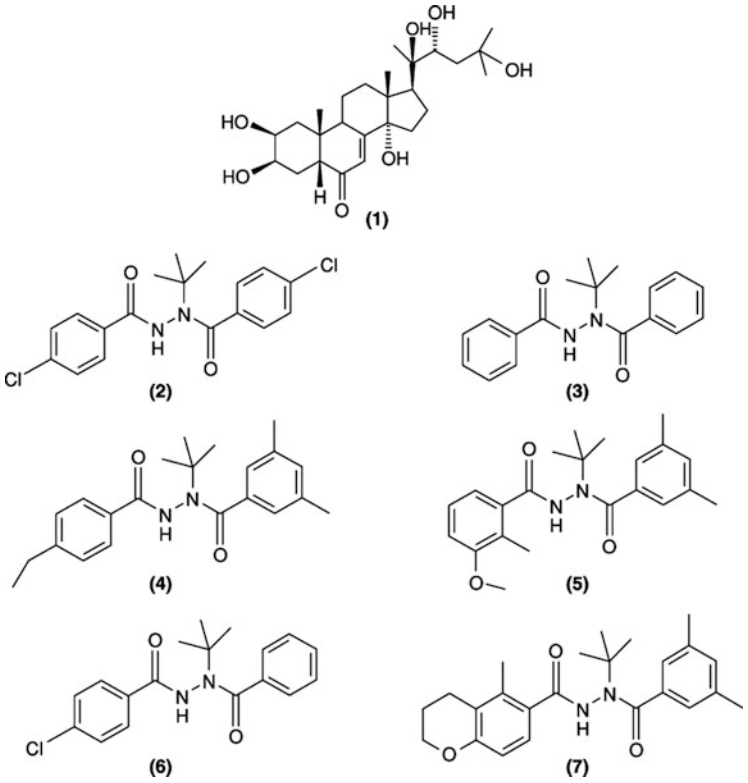


Fig. 8.3 Chemical structures of 20-hydroxyecdysone (1), symmetrically substituted dichlorodibenzoylhydrazine (2), RH-5849 (3), tebufenozide (4), methoxyfenozide (5), halofenozide (6), chromafenozide (7) (Dhadialla et al. 2005)

8.3.2.1 Commercially Available Ecdysone Antagonists and Their Role

Chromafenozide

It is a nonsteroidal ecdysone agonist developed jointly by Nippon Kayaku Co., Ltd. (Saitama, Japan), and Sankyo Co., Ltd. (Ibaraki, Japan). It is registered for the management of lepidopteran pests on vegetables, fruits, vines, tea, rice, arboriculture, ornamentals and other crops in Japan (Yanagi et al. 2000; Ichinose et al. 2000; Toya et al. 2002). Chromafenozide is safe for mammals, birds, aquatic animals and other nontarget and beneficial insects (Dhadialla et al. 2005) (Fig. 8.3).

Halofenozide

It is a systemic compound having broad-spectrum activity. It is effective for the control of beetle grubs (Japanese beetle, oriental beetle, June beetle, northern and southern masked chafer, green June beetle, black turfgrass ataenius beetle, annual bluegrass weevil larvae, *Aphodius* beetles, European chafer and bill bugs) and

lepidopteran larval pests (cutworms, sod webworms, armyworms and fall armyworms) (Cowles and Villani 1996; Cowles et al. 1999; Dhadialla et al. 2005) (Fig. 8.3).

Tebufenozide and Methoxyfenozide

Tebufenozide is used for the control of lepidopteran larvae and insect pests from families Noctuidae, Pyralidae, Tortricidae and Pieridae (Le et al. 1996; Dhadialla et al. 1998; Carlson et al. 2001). Both tebufenozide and methoxyfenozide act primarily by ingestion mode but also possess contact and ovicidal activity (Trisyono and Chippendale 1997; Sun and Barrett 1999; Sun et al. 2000; Dhadialla et al. 2005) (Fig. 8.3).

8.3.3 Chitin Synthesis Inhibitors

Chitin is a β-1,4-linked amino polysaccharide homopolymer of *N*-acetylglucosamine (GlcNAc) and cross-linked to proteins via biphenyl linkages to form chitin microfibers–protein complex which acts as a protective matrix (Lotmar and Picken 1950; Rudall and Kenchington 1973; Dhadialla et al. 2005; Doucet and Retnakaran 2012). Chitin is a major component of the outermost layer of insect integument called cuticle. Insect's peritrophic matrix is also constituted of chitin, which acts as a permeability barrier between the food bolus and epithelium of the midgut and protects the gut from injury, toxins and pathogens. The chitin synthesis and degradation in insect body is consistent in a highly controlled manner to allow both regeneration and ecdysis of the peritrophic matrix (Locke 1991; Moussian 2010; Vincent and Wegst 2004; Doucet and Retnakaran 2012).

Chitin biosynthesis is initiated with the disaccharide trehalose, finally resulting in the *N*-acetylglucosamine subunit polymerization by enzyme chitin synthase leading to the production of chitin microfibrils. Enzymes, such chitinases, deacetylases and hexosaminidases, help in the degradation and recycling of old chitin exoskeleton. Chitin synthesis is a key target process used for the development of biorational insecticides, such as benzoylphenyl ureas, which act as chitin synthesis inhibitors (Doucet and Retnakaran 2012).

In the 1970s the first chitin synthesis inhibitor, diflubenzuron, belonging to the benzoylphenyl urea class of chemistry, was discovered by Philips-Duphar Company (Miyamoto et al. 1993; Tunaz and Uygun 2004; Subramanian and Shankarganesh 2016). The discovery of diflubenzuron resulted in the development of a number of other derivatives of BPU, such as triflumuron, chlorfluazuron, teflubenzuron, hexaflumuron, flufenoxuron, novaluron and lufenuron (Hamman and Sirrenberg 1980; Haga et al. 1982; Becher et al. 1983; Sbragia et al. 1983; Anderson et al. 1986; Ishaaya et al. 1996; Subramanian and Shankarganesh 2016). The non-BPU compounds, which are developed recently, include etoxazole, buprofezin, cyromazine and dicyclanil (Ishida et al. 1994; Dhadialla et al. 2005; Subramanian and Shankarganesh 2016).

Chitin synthesis inhibitor compounds act on insects through inhibition of chitin formation, abnormal endocuticular deposition and abortive moulting (Ishaaya and Casida 1980; Dhadialla et al. 2005; Merzendorfer 2013).

These are divided into two categories on the basis of their chemistry, i.e.:

1. Benzoylphenyl ureas (BPUs)
2. Non-benzoylphenyl ureas (non-BPUs)

8.3.3.1 Benzoylphenyl Ureas

Benzoylphenyl urea compounds have a central urea moiety; the phenyl end generally is the site of most complex substitutions, while the benzoyl part remains relatively simple. It is assumed that the benzoyl part of BPUs gets attached to the unidentified receptor, which results in chitin synthesis inhibition (Nakagawa et al. 1991; Dhadialla et al. 2005; Doucet and Retnakaran 2012; Subramanian and Shankarganesh 2016). Benzoylphenyl urea compounds generally have a common mode of action and block a postcatalytic step in chitin biosynthesis process (Nauen and Smagghe 2006; Van Leeuwen et al. 2012), e.g. diflubenzuron, bistrifluron, chlorbenzuron, novaluron, lufenuron, hexaflumuron etc. (Doucet and Retnakaran 2012) (Fig. 8.4).

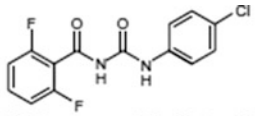
Commercially Available Benzoylphenyl Ureas and Their Role in Pest Management

Chlorfluazuron

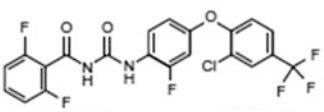
Chlorfluazuron is a broad-spectrum BPU compound, being actively used against most lepidopteran, coleopteran, hymenopteran and dipteran insect pests along with thrips and whiteflies. It is an environmentally safe compound and has ingestion as route of action. It also has a very low toxic effect on adult egg of parasitoids and is safe for beneficial insects as compared to other synthetic insecticides (Wang et al. 2012; Rabea et al. 2010). Chlorfluazuron is also helpful in controlling the Formosan subterranean termite, *Coptotermes formosanus*, and the eastern subterranean termite, *Reticulitermes flavipes* (Dhadialla et al. 2005; Osbrink et al. 2011; Doucet and Retnakaran 2012).

Diflubenzuron

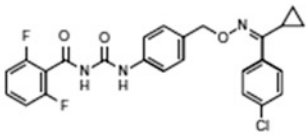
Diflubenzuron is nonsystemic and is the most studied and extensively used BPU worldwide (Doucet and Retnakaran 2012). This highly water-insoluble compound has stomach and contact toxicity. It has to be ingested to be effective. It does not affect sap-sucking insects, as it is nonsystemic to plants. It is not effective for all lepidopteran larvae due to variation in detoxification processes among different species. The developmental stage of larvae also influences the effectiveness of the compound, as in the case of spruce budworm, *Choristoneura fumiferana*, in which the larvae of the fifth and sixth instars were more susceptible to diflubenzuron as compared to the earlier stages (Granett and Retnakaran 1977). The fruit tortrix moths *Adoxophyes orana* and *Pandemis heparana* are relatively insensitive to



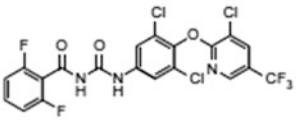
Diflubenzuron (Dimilin)— Philips-Duphar BV 1972



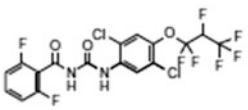
Flufenoxuron — Shell International Co.Ltd. 1987



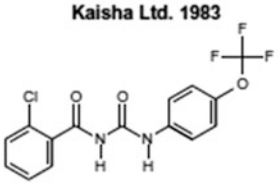
Fluxyloxuron (PH 60-23)— Philips-Duphar BV 1988



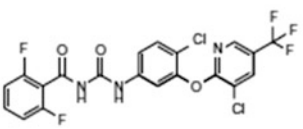
Chlorfluazuron— Ishihara Sangyo Kaisha Ltd. 1983



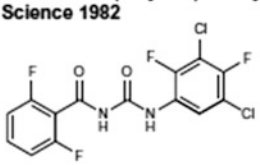
Lufenuron — Novartis A.G. 1977



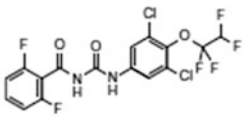
Triflumuron (Alsystin) — Bayer Crop Science 1982



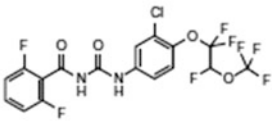
Fluazuron — Novartis A.G. 1990



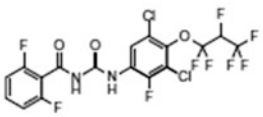
Teflubenzuron — Celamerck GmBH. 1982



Hexaflumuron — Dow Elanco Ltd. 1984



Novaluron — Makhteshim Agan Industries 1990



Noviflumuron — Dow Agro Sciences LLC. 2001

Fig. 8.4 Chemical structures of commercialized benzoylphenyl ureas (Doucet and Retnakaran 2012)

diflubenzuron, while the forest tent caterpillar, *Malacosoma disstria*, and the gypsy moth, *Lymantria dispar*, are sensitive (Eck 1981; Retnakaran et al. 1985). It has been used to control cockroaches, locusts, grasshoppers, larvae of sciarid flies, phorid flies, mosquitoes and insect pests of cotton, horticultural crops and soybean (Weiland et al. 2002). Diflubenzuron is less effective for the control of Colorado

potato beetle, *Leptinotarsa decemlineata*, than other BPU, such as lufenuron (Karimzadeh et al. 2007). Diflubenzuron is nontoxic to beneficial insects (bees), mammals and birds; however, crustaceans are sensitive to it (Dhadialla et al. 2005; Gartenstein et al. 2006; Doucet and Retnakaran 2012).

Flucycloxuron

This BPU compound has topical contact activity and is mainly used as an acaricide (Doucet and Retnakaran 2012). Flucycloxuron is used for the control of both tetranychid and eriophyid mites. It penetrates the leaf cuticle and is shown to have ovicidal, transovarial-ovicidal and ovo-larvicidal effects in target organisms. According to Grosscurt (1993), it was effective on the two-spotted spider mite, *Tetranychus urticae*, and the European red mite, *Panonychus ulmi*, on apple leaves. It is similar to diflubenzuron in terms of toxicity but might be more toxic to aquatic organisms, such as rainbow trout, *Oncorhynchus mykiss*, and water flea, *Daphnia* (Darvas and Polgar 1998; Dhadialla et al. 2005; Doucet and Retnakaran 2012).

Fluazuron

Fluazuron has been shown to be effective against ticks (*Rhipicephalus sanguineus*) and mites (*Sarcoptes scabiei*) (De Oliveira et al. 2012; Pasay et al. 2012). The population of flea was successfully lowered in squirrels and mice by application of fluazuron (Dhadialla et al. 2005; Davis et al. 2008; Doucet and Retnakaran 2012).

Flufenoxuron

Flufenoxuron is used against the larvae of lepidopteran insects on vegetables, fruits, cotton and grain crops (Doucet and Retnakaran 2012). It is second best control measure for *Spodoptera littoralis* after lufenuron (El-Sheikh and Aamir 2011). It is also very effective as a control of mushroom sciarid fly, *Lycoriella ingenua*, as compared to novaluron, diflubenzuron and teflubenzuron (Dhadialla et al. 2005; Doucet and Retnakaran 2012; Erler et al. 2011).

Hexaflumuron

Hexaflumuron has been used against the larvae of Lepidoptera, Coleoptera and Diptera (Doucet and Retnakaran 2012). It is also effective against termite, *Reticulitermes flavipes* and *Coptotermes formosanus*, following incorporating it in bait (Dhadialla et al. 2005; Messenger et al. 2005; Ripa et al. 2007; Doucet and Retnakaran 2012).

Lufenuron

This BPU is extensively used in controlling fly pests (*Lycoriella ingénue*) of common mushroom, *Agaricus bisporus* (Erler et al. 2011; Doucet and Retnakaran 2012). Lufenuron has been also effective against termites, *Reticulitermes hesperus* (Haverty et al. 2010). Lufenuron causes transovarial-ovicidal and larvicidal effects; due to this property, it has been used against many lepidopteran pests. It has low toxicity against many parasitoids and has adequate persistence making it effective on many pests. Tortricid, the light brown apple moth, *Epiphyas postvittana*, can also be

controlled by lufenuron (Whiting et al. 2000; Dhadialla et al. 2005; Doucet and Retnakaran 2012).

Triflumuron

Triflumuron is a broad-spectrum BPU, which is effective against cabbage moth, apple leaf miner, boll worm, codling moth, psyllids, cotton leafworm, tortrix moth, summer fruit moth and many other insect pests (Doucet and Retnakaran 2012). Triflumuron is the most effective among BPU compounds for the management of mushroom sciarid, *Lycoriella ingenua* (Erler et al. 2011). It is used successfully for the control of mealworm, *Alphitobius diaperinus*, when used in combination with pyrethroid insecticides (Salin et al. 2003). It induces ovicidal and larvicidal activities making it an ideal candidate for the control of flies also (Smith and Wall 1998; Broadbent and Pree 1984; Hejazi and Granett 1986; Asher and Nemny 1984; Dhadialla et al. 2005; Vazirianzadeh et al. 2007; Doucet and Retnakaran 2012).

Teflubenzuron

Teflubenzuron hindered the egg hatching in females of migratory locust, *Locusta migratoria* (Acheuk et al. 2012; Doucet and Retnakaran 2012). It also reduces sea lice (ectoparasite), *Lepeophtheirus salmonis*, population in Atlantic salmon fish farms (Dhadialla et al. 2005; Campbell et al. 2006; Doucet and Retnakaran 2012).

Noviflumuron

This BPU is effective against cockroaches and termites (*C. formosanus*) (Ameen et al. 2005; Dhadialla et al. 2005; Husseneder et al. 2007; Doucet and Retnakaran 2012).

Novaluron

It is an effective agent in the control of several lepidopteran, dipteran, coleopteran and homopteran pests (Doucet and Retnakaran 2012). It has low acute toxicity against mammals and poses low risk to nontarget organisms and the environment. It is an ideal candidate for IPM and integrated resistance management (IRM) programmes (Cutler and Scott-Dupree 2007). Novaluron is used for the management of many important pests, such as leaf miners, whiteflies and beet armyworm (Ishaaya and Horowitz 1998; Ishaaya et al. 1996). In Brazil, it is successfully used to reduce the population of mosquito, *Aedes aegypti* (Dhadialla et al. 2005; Doucet and Retnakaran 2012; Farnesi et al. 2012).

8.3.3.2 Non-benzoylphenyl Ureas

The non-benzoylphenyl urea class of compounds, viz. buprofezin, etoxazole, cyromazine and dicyclanil, has been used widely for the control of insect pets in agricultural and public health systems (Subramanian and Shankarganesh 2016). Buprofezin belonging to the group of thiadiazines acts on insects by inhibition of cuticle deposition, chitin biosynthesis, lamellate cuticle formation and inhibition of cholinesterase activity (Cottage and Gunning 2006; Subramanian and Shankarganesh 2016). Cyromazine and dicyclanil interfere with cuticle formation

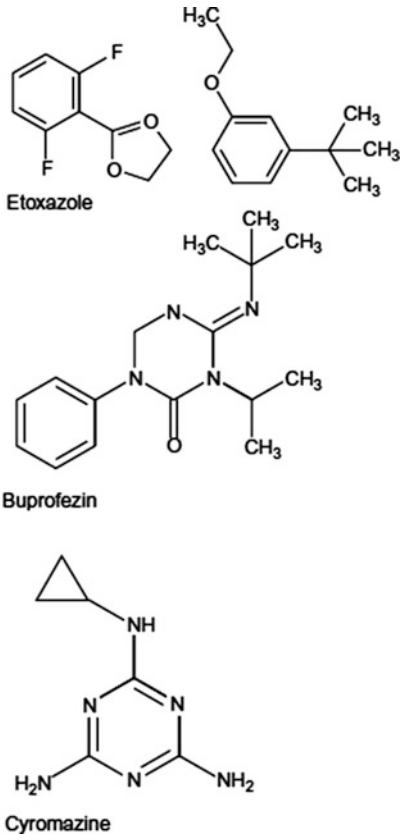


Fig. 8.5 Chemical structures of commercialized non-benzoylphenyl ureas (Doucet and Retnakaran 2012)

and do not inhibit chitin synthesis, and so are considered as moult inhibitors. Cyromazine, an aminotriazine and a cyclopropyl derivative of melamine, is commercially available under the trademarks Neoprex, Trigard and Vetrazin and provides a good control measure for stable flies in winter hay (Taylor et al. 2012). Dicyclanil (CliK) is efficacious against sheep and lamb blowflies (Dhadialla et al. 2005; Cohen 2010; Doucet and Retnakaran 2012; Subramanian and Shankarganesh 2016) (Fig. 8.5).

Commercially Available Non-benzoylphenyl Ureas and Their Role in Pest Management

Buprofezin

Buprofezin, 2-tert-butylimino-5-phenyl-3-propan-2-yl-1,3,5-thiadiazinan-4-one, developed by Hoechst acts specifically on immature developmental stages of some homopteran (scale insects, mealybugs and whiteflies) pests by inhibiting *N*-acetyl-[D-H3] glucosamine incorporation into chitin and thus disrupting the cuticle formation, which leads in nymphal mortality during ecdysis (Ishaaya and Horowitz 1998;

Kanno et al. 1981; Nasr et al. 2010; Doucet and Retnakaran 2012). This compound also acts on cholinesterase, suppresses oviposition in adults and reduces viability of eggs. It has been used extensively against the whitefly *Bemisia tabaci* (Cottage and Gunning 2006). It is mildly toxic to mammals but generally nontoxic to birds (Palli and Retnakaran 1998; Dhadialla et al. 2005; Doucet and Retnakaran 2012).

Etoxazole

Yashima Chemical Industry Co., Japan, developed this non-BPU compound in 1994. It acts as acaricide for the control of tetranychid spider mites (*Panonychus* and *Tetranychus* species) (Yagi et al. 2000; Suzuki et al. 2001, 2002; Tisdell et al. 2004; Hirose et al. 2010; Doucet and Retnakaran 2012; Li et al. 2014). It inhibits moulting during the development of insects and mites (Lee et al. 2004; Asahara et al. 2008; Sun et al. 2008). It is also effective against leafhoppers, aphids, fall armyworm and diamond back moth (Nauen and Smagghe 2006). In case of spider mites, it affects only the eggs, larvae and nymphs but not adults. Etoxazole degradation in the soil is slow and also undergoes partial photolysis (Dhadialla et al. 2005; Doucet and Retnakaran 2012).

Cyromazine

Cyromazine (CGA 72662, *N*-cyclopropyl-1,3,5-triazine-2,4,6-triamine) discovered by Ciba-Geigy, Ltd., in the 1970s is an aminotriazine and a cyclopropyl derivative of melamine (Shen and Plapp 1990; Vazirianzadeh et al. 2007; Doucet and Retnakaran 2012). It has both insecticidal and acaricidal activity and has contact activity that inhibits moulting and pupation in target pests (Patakioutas et al. 2007). It has been successfully used for the control of insect pests of vegetables, mushrooms and ornamentals. It is also helpful in the management of stable fly maggots in winter hay (Dhadialla et al. 2005; Doucet and Retnakaran 2012; Taylor et al. 2012).

8.4 Anti-juvenile Hormones

The anti-JH agents are compounds that have property of inhibiting the biosynthesis of JH in insects, eventually leading to halting of biological processes under the control of JH (Staal 1986; Darvas et al. 1990; Goodman and Granger 2005a, b; Ghoneim and Bakr 2018). The sublethal affects include inhibition of growth and development, deranged morphogenesis, precocious metamorphosis, lower rates of adult emergence and reduced survival of adults (Ghoneim and Bakr 2018). These compounds also possess anti-gonadotropic activity, affecting oocyte maturation, oviposition and reproductive capacity in insects (Ghoneim and Bakr 2018). Bowers et al. (1976) were first to discover the insect anti-JHs, i.e. precocenes I and II (Minakuchi and Riddiford 2006; Ghoneim and Bakr 2018). Further research leads to the synthesis of synthetic precocenoids and other anti-JH compounds including fluoromevalonate, ethyl-4-[2-(tert-butylcarbonyloxy)butoxy]benzoate (ETB), compactin, EMD, dichloroallyl hexanoate, KK-42, KK-110, brevioxime, terpenoid and 1,5-disubstituted imidazoles (Quistad et al. 1981; Staal et al. 1981; Farag and

Varjas 1983; Hiruma et al. 1983; Staal 1986; Kuwano et al. 1988; Darvas et al. 1990; Castillo et al. 1998). Most of these compounds induce precocious metamorphosis, but black pigmentation (piperonyl butoxide and thiolcarbamates) was also reported in few cases (Kramer et al. 1983; Ghoneim and Bakr 2018).

8.4.1 Precocenes

Precocenes, plant-derived chromenes (Ghoneim and Bakr 2018), were isolated by Bowers et al. (1976) from *Ageratum houstonianum* and termed them as precocenes I (7-methoxy-2,2-dimethylchromene) and precocenes II (6,7-dimethoxy-2,2-dimethylchromene) (Bowers 1976, 1992; Proksch et al. 1983; Isman et al. 1986; Minakuchi and Riddiford 2006; Ghoneim and Bakr 2018). These compounds were known to induce cytotoxicity in corpora allata in insects, resulting in the prohibition of juvenile hormone biosynthesis (Pratt et al. 1980; Schrankel et al. 1982; Minakuchi and Riddiford 2006; Ghoneim and Bakr 2018). Holometabolous insect larvae are less susceptible to precocenes action, which could be due to sequestration and detoxification (Burt et al. 1979; Haunerland and Bowers 1985; Minakuchi and Riddiford 2006). However, some holometabolous insects, i.e. lawn armyworm, *Spodoptera mauritia*, and the Egyptian cotton leafworm, *Spodoptera littoralis*, are exceptions, as they are found to be susceptible (Mathai and Nair 1984; Khafagi and Hegazi 2001; Ghoneim and Bakr 2018). These compounds also affect non-social insects by inducing precocious metamorphosis during the pre-adult stages (Khan and Kumar 2000, 2005; Gaur and Kumar 2009; Ghoneim and Bakr 2018). They also halt vitellogenic development of the oocytes, leading to sterility, thus affecting the reproduction in many insect orders (Staal 1986; Kumar and Khan 2004; Amiri et al. 2010; Ghoneim and Bakr 2018). Precocenes induces early diapauses in insects and also influences insect behaviour, i.e. mating, flight, maternal defense and sexual behaviour (Bowers 1983; Walker 1978; Rankin 1980; Kight 1998; Pathak and Bhandari 2002; Ringo et al. 2005; Ghoneim and Bakr 2018). They also have property of inhibiting sex pheromone production and possess antifeedant and repellent activities (Bowers 1983; Khafagi 2004; Lu et al. 2014; Ghoneim and Bakr 2018). Precocenes are mainly used for experimental purposes only for studying activity of juvenile hormone on development and reproduction in insects (Minakuchi and Riddiford 2006).

8.4.2 Fluoromevalonate (FMeV)

FMev (tetrahydro-4-fluoromethyl-4-hydroxy-2*H*-pyran-2-one) is an anti-JH compound, highly effective and selective against various lepidopteran species, i.e. *Spodoptera exigua*, *Manduca sexta*, *Galleria mellonella*, *Samia cynthia*, *Hyphantria cunea*, *Phryganidia californica* and *Heliothis virescens* (Quistad et al. 1981; Edwards et al. 1983; Ghoneim and Bakr 2018). Non-lepidopteran species are not susceptible to FMeV (Menn 1985). The definite mode of action of this

compound in insects is not yet clear. It is assumed that FMev disrupts metabolism of mevalonate by inhibiting the initial steps in juvenile hormone biosynthetic pathway (Quistad et al. 1981; Baker et al. 1986). Precocious pupation is characteristic response of FMev treatment (Kramer and Staal 1981; Farag and Varjas 1983; Ghoneim and Bakr 2018).

8.4.3 Terpenoid Imidazoles

The major active anti-juvenile hormone compounds of this group were KK-22 and KK-42 (Kuwano and Eto 1983; Akai et al. 1984; Ghoneim and Bakr 2018). KK-22 induces precocious metamorphosis (Asano et al. 1984). KK-42 inhibits juvenile hormone and ecdysone synthesis and affects the growth and development of insect species (Kuwano et al. 1992; Kadano-Okuda et al. 1994; Kadono-Okuda et al. 1987; Minakuchi and Riddiford 2006; Ghoneim and Bakr 2018).

8.4.4 Derivative of Fungi and Bacteria Anti-juvenile Hormone Compounds

These includes brevioxime, compactin, fluvastatin (fungi-derived) and cycloheximide (bacteria-derived). Brevioxime is derivative of entomopathogenic fungus, *Penicillium brevicompactum*, and possesses strong anti-JH activity against *Oncopeltus fasciatus* (Castillo et al. 1999; Ghoneim and Bakr 2018). Compactin strongly inhibits JH biosynthesis in *Manduca sexta*, *Mamestra brassicae* and *Periplaneta americana* (Monger et al. 1982; Hiruma et al. 1983; Edwards and Price 1983; Ghoneim and Bakr 2018). Fluvastatin treatment results in the inhibition of JH-regulated metamorphosis in locust, *Locusta migratoria* (Debernard et al. 1994), and halts JH acid biosynthesis in the black cutworm, *Agrotis ipsilon* (Duportets et al. 1996; Ghoneim and Bakr 2018). Cycloheximide isolated from the bacterium *Streptomyces griseus* is a RNA (*L. migratoria*) and protein synthesis inhibitor (*Spodoptera frugiperda*) (Siegel and Sisler 1963; Baliga et al. 1969; Kelly and Lescott 1976; Phillips and Loughton 1979).

8.4.5 Benzoate and Methyl Dodecanoate Compounds

The benzoate compound ETB (ethyl-4-[2-(tert-butylcarbonyloxy)butoxy]benzoate) developed in 1975 (Minakuchi and Riddiford 2006; Ghoneim and Bakr 2018) reduces the level of juvenile hormone (anti-juvenile activity) in *M. sexta* and *B. mori* resulting in precocious metamorphosis (Kiguchi et al. 1984; Minakuchi and Riddiford 2006; Ghoneim and Bakr 2018). EMD (ethyl-[*E*]-3-methyl-2-dodecanoate) exhibits anti-JH effects on the tobacco budworm *Heliothis virescens* and *M. sexta* (Staal 1982). In a study conducted on *B. mori* larvae, no precocious metamorphosis was induced by EMD in the third and fourth instars (Kuwano et al.

1988). Balamani and Nair (1989) found the formation of larval-pupal intermediates in *Spodoptera mauritia* upon treatment with EMD (Ghoneim and Bakr 2018).

8.4.6 Bisthiolcarbamate and Sulphoxides

Bisthiolcarbamate treatment of the third instar larvae of *M. sexta* resulted in suppression of JH titre. Precocious pupation was not observed, but black pigmentation was reported with this compound. Rapid degradation was the main reason for the weak activity of bisthiolcarbamate (Kramer et al. 1983; Ghoneim and Bakr 2018). The anti-JH activity of the compound polyacetylene sulphoxide was first revealed by Bowers and Aregullin (1987). This compound induced sterility in adults of *O. fasciatus*. In the 1980s a number of fluorinated vinyl sulphoxides were developed, which were effective against Lepidoptera order (Carney and Brown 1989; Ghoneim and Bakr 2018).

Although anti-JH compounds possess advantage of being selectively toxic, halting major physiological processes in target insects, still the commercialization of these compounds has not been yet achieved as the majority of the studies on these compounds have been conducted in laboratory conditions, while the field investigations remained untouched (Minakuchi and Riddiford 2006; Ghoneim and Bakr 2018).

8.5 Neuropeptide Hormones as Potential Candidates for Pest Management: A Future

Neuropeptide hormones act as key regulators of vital physiological processes in insects, such as reproduction, growth, development, metabolism and homeostasis. The quality of these hormones could be explored for the development of their analogues or agonists, making them potential tool for insect pest control (Fonagy 2006; Altstein 2001). Analogues could possibly interfere with synthesis and secretion of neuropeptides and affect receptors (Gade and Goldsworthy 2003). Although use of neuropeptide antagonists could be very effective in the management strategy, it is not implemented till date due to few but major limitations:

1. Linear structure of peptides makes them nonselective, hinders penetration through tissues of target pests and increases susceptibility to proteolytic degradation (Altstein 2001).
2. Lack of knowledge about the three-dimensional structure of receptor-agonist complex and mechanism of activation of this receptor (Altstein 2001).

According to Altstein (2001), the backbone cyclic neuropeptide-based antagonist (BBC-NBA) approach could be effectively used to overcome limitations for the generation of neuropeptide antagonists. This technique is applied to the insect, pyrokinin (PK)/pheromone biosynthesis activating neuropeptide (PBAN), leading

to production of linear lead antagonist and metabolically stable backbone cyclic antagonists, which lack agonistic activity and inhibit activities in insects mediated by PBAN. This approach is adeptly used in inhibition of sex pheromone biosynthesis in adult female of *Helicoverpa peltigera* and cuticular melanin formation in larvae of *Spodoptera littoralis* (Altstein et al. 1996, 1999; Altstein 2001).

8.6 Conclusions

JHAs and CSIs among IGRs can become a viable component of IPM programme if used judiciously, and many commercial formulations of these are available. These are less toxic to natural enemies of insects. Low mammalian toxicity, biodegradability and specific nature of these compounds make them ecofriendly. The novel mode of action of IGRs reduces the risk of cross-resistance. There is an urgent need to have better field stable formulations of IGRs mainly photostable formulations, which should also be cost-effective for large-scale use.

Points to Remember

- The complexity of insect endocrine system can be well understood by studying different types of hormones, which include juvenile hormones, ecdysteroids and neuropeptide hormones.
- Juvenile hormone is basically a controlling hormone (control moults induced by ecdysone) for metamorphosis in insects. It also plays an important role in reproduction, diapauses of insects and caste determination.
- Ecdysteroids play vital role in moulting, growth and development of insects. Depending upon stage of insect, they act as either sole hormone or precursor for other ecdysteroid hormones.
- Neuropeptides, commonly known as brain hormones, are produced by neurosecretory cells of the central nervous system. The management of insect pests has become a greater challenge due to their ability to develop resistance to many insecticides.
- To conserve efficacy of insecticides for the control of insect pests, it is necessary to add diversity to the insecticidal pool by introduction of novel insecticides that are specific for biochemical sites or physiological processes in the target pest.
- IGRs are biorational insecticides, which have novel modes of action, causing disruption in the physiology and development of the target pests.
- IGRs are advantageous over conventional insecticides, as they are specific in action and have low toxicity to nontarget organisms and mammals and lower rate of persistence in the environment.
- IGRs have been shown to cause numerous sublethal effects, viz. larval-pupal intermediates, adultoids, increase/decrease in fecundity, transovarial effects and developmental rate as well as changes in sex ratio, diapauses and morphology.
- Insect growth regulators are categorized into three types on the basis of their mode of action, i.e. juvenile hormone analogues, ecdysone antagonists and chitin synthesis inhibitors.

- Analogues of hormones, i.e. juvenile and ecdysteroids, are being used at commercial level in integrated pest management programmes.
- Although the use of insect hormone analogues is limited, the qualities like species specificity, nonpersistence in the environment and safety to nontarget organisms make them ideal candidates for pest management programmes.
- Presently, a number of commercial IGRs are available, but there is need for exploring more IGRs to expand our knowledge regarding their chemistry and effects on insect pests, so that the use of these compounds could be expanded in integrated pest management programmes.
- Neuropeptide analogues and anti-juvenile hormone could be a bright future for insect growth regulators, if successfully commercialized.

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Phyto-Antifeedants

9

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Contents

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Omkar (ed.), *Molecular Approaches for Sustainable Insect Pest Management*,
https://doi.org/10.1007/978-981-16-3591-5_9

Abstract

Plants possess primary and secondary metabolites. Primary metabolites are required to maintain their basic physiological processes, which also serve as essential sources of nutrients for herbivores, whereas secondary metabolites help to protect plants from herbivore damage. Phyto-antifeedants, a type of secondary metabolite, are recorded from 43 families of plants, but stress has been given in 4 families—Meliaceae, Asteraceae, Labiatae and Leguminosae. Terpenes are classified depending on isoprene units. Terpenes are divided into monoterpenes, sesquiterpenes, diterpenes and triterpenes, and many compounds among these groups act as antifeedants. Flavonoids, alkaloids, steroids and coumarins from plant sources could also act as antifeedants. The lepidopteran larvae possess chemosensilla on the maxillary palp, and the test cells in the sensillum act as deterrent. Some insects possess P450 detoxification enzymes in the midgut to detoxify the antifeedants. One of the most commonly used antifeedant is azadirachtin A from *Azadirachta indica*, which is applied against ca. 400 insect species belonging to Blattodea, Coleoptera, Diptera, Dermaptera, Ensifera, Homoptera, Heteroptera, Hymenoptera, Lepidoptera, Isoptera, Phasmida, Thysanoptera and Siphonaptera. One of the best strategies to apply an antifeedant is in water- or oil-based formulations. Latex may also be used to apply antifeedants. At present 1000 antifeedants have been isolated from plants in laboratory conditions, but the efficacies of antifeedants in the field are low due to either habituation of insects towards antifeedants or variations in responses among different insects. The major hindrance in developing phyto-antifeedants is that they are not broad spectrum or they may not be effective in field conditions. Therefore, basic research in combination with field trials of the isolated phyto-antifeedants at different doses are necessary to get ecofriendly safe products for insect pest management.

Keywords

Phytochemicals · Antifeedants · Pest control · Mode of action · Commercialization

Learning Objectives

1. Application of synthetic insecticides to control insect pests poses threat to human health, nontarget organisms and the environment. Recently the European Union prohibited the use of certain pesticides. Now the question is asked whether phytochemicals as antifeedants can replace the synthetic pesticides.
2. Plants produce a diversity of compounds called secondary metabolites to cope with the feeding damage caused by herbivorous insects. Since the early days, humans are using plant extracts comprised of specific secondary metabolites to modulate insect behaviour.
3. A number of secondary metabolites acting as antifeedants could be used for pest management strategies, but commercial success of botanical pesticides using

secondary metabolites is meagre except for plant extracted oils, pyrethrum and neem.
4. An improved understanding of secondary metabolites acting as antifeedants to insects is one of the major focuses in integrated pest management strategies in the present scenario.

9.1 Introduction

The present century focuses on protecting crop plants from insect herbivores to safeguard plants from herbivore feeding damage. Plants have evolved during Devonian Period ca. 400 million years back, and since the beginning of plant evolution, plants have evolved different compounds, which may deter from insect feeding. Green plants produce carbohydrates by photosynthesis which are stored as sugars and considered as primary energy source. A part of this energy is used to transform nitrogen to amino acids. Sugars are also employed to build in cell walls. Primary metabolites represent a greater part of plant biomass. The primary metabolites mainly consist of carbohydrates, proteins and lipids, which are responsible for basic physiological process of plants and serve as essential sources of nutrients for herbivores. Depending on the primary metabolism, plants have an array of metabolic pathways to generate diverse secondary plant substances. These secondary plant substances do not possess a role in primary metabolism. As plants cannot move during insect attack as well as do not possess adaptive immune system like vertebrates during various infections, plants produce an array of diverse secondary metabolites to protect them from herbivore damage. The secondary metabolites are evolved during natural selection in plants in such a way that these compounds may intervene the metabolism, neural transmission, development and reproduction of insect herbivores. Besides production of secondary metabolites, plants have developed various morphological defensive mechanisms, such as impervious cuticles, thorns, spikes, trichomes, etc. against insect herbivores.

Green plants produce a wide structural diversity of secondary metabolites, such as terpenoids, phenolics, alkaloids, cyanogenic glycosides, glucosinolates, quinones, amines, peptides, non-protein amino acids, organic acids, polyacetylenes and peptides. A cursory review of literature documents that more than 100,000 compounds are on records (Wink 1988, 2003). These plants produced secondary metabolites can act on different molecular targets at a particular time and frequently in a synergistic manner (Wink 2008, 2015; Mason and Singer 2015). Therefore, the mixtures of secondary metabolites vary between different organs and developmental stages of a plant as well as within populations of a species.

Insects are one of the most important agents causing damage in agroecosystems. The USA, EU, China and Brazil are the largest agricultural producers in the world, and these four countries used 827 million, 831 million, 1.2 billion and 3.9 billion pounds of pesticides in 2016, respectively. Despite application of insecticides, it is estimated that 18–20% crop losses due to arthropod attack occur across the globe and result in an estimated loss of more than a value of US$ 400 billion. In India, crop

losses due to insect attack are estimated to be 15.7% at the present condition, and the agriculture sector of India loses an estimated value of about US$ 36 billion. Food plants throughout the world are affected by 10,000 insect species, 30,000 weed species, 1000 nematode species and 100,000 diseases, which are due to the attack by fungi, viruses, bacteria and other microorganisms. About 10% of the insect pests are generally predicted to be major pests, and herbivorous insects are reported to cause one-fifth of the world's crop loss per annum. Four major and 26 minor crops are responsible for ca. 95% of human sustenance, indicating that many of these crop plants are cultivated for a long time, and thus, these crop plants provide food for a vast array of insect species with a high degree of adaptation to the crop plants. It is found that most of the insect species are specialist feeders—75% of temperate and 80% of tropical lepidopteran insect pests are monophagous or oligophagous.

Entomologists have been searching for safe and ecofriendly insect control measures by underpinning the idea that in real world, many plants protect themselves from insect attack by secreting unpalatable substances, and it is feasible to apply such compounds as feeding or oviposition inhabitants to protect the crop plants. The progress on this concept has been slow. The idea is that 'suppressants' inhibit insects against biting activity, while 'deterrents' avert insects from further feeding. Generally most of the times, we are unable to understand the phase of feeding when it is interrupted, and subsequently, many authors concomitantly employ 'antifeedants' as well as 'feeding deterrents' for compounds present in plant tissues that inhibit or avert insect feeding activity. In this context, the expression 'rejectant' could not be used as it does not make a distinction between suppressants and deterrents. The word 'repellent' implicates an oriented movement from the source of stimulus (Dethier et al. 1960). An ideal antifeedant would be nontoxic secondary metabolites, not phytotoxic and nontoxic to human, animals, beneficial insects and organisms, as well as suppresses the feeding activity of as many as insect pests, practically applicable to a crop, and ultimately, low cost for commercial production as well as high availability.

After reviewing crop yield losses by the herbivorous insects, it is interesting to discuss about the origin of antifeedants in the perspective of plant origin, mode of action, formulations and applications of phyto-antifeedants, including the drawbacks and prospects on the use of phyto-antifeedants for insect pest control, which is an essential step towards developing safe and economical as well as sustainable methods of pest management programme for the food security and also for the future. This chapter discusses about phyto-antifeedants, not about the derivative antifeedants, which are prepared from antifeedants of plant origin.

9.2 Phyto-Antifeedants: Biochemical Diversity and Target Insects

Antifeedants in plants differ to a great extent in their chemistry and are comprised of inorganic compounds as well as secondary metabolites. The prospective of plant taxa to show antifeedant activity of insects has been demonstrated to be definite to

certain insect species as well as the effectiveness may be determined by their genotype and ecological environment.

To date, the insect antifeedant activity has been recorded from 43 families of plants, but more research has been performed in families Meliaceae (Fagoonee and Lange 1981), Asteraceae (Zalkow et al. 1979; Rose et al. 1981), Labiatae (Miyase et al. 1981) and Leguminosae (Bentley et al. 1984). Future researches are required to search all potential local plants depending on visual as well as chemotaxonomic basis, while simultaneously the industrial waste products of plants should be tested since they may possess substantial amounts of inhibitory compounds or new antifeedants arising due to processing (Jermy et al. 1981).

9.2.1 Terpenes

Terpenes, the largest class of compounds, consist of more than 30,000 compounds and show a wide variety of structures comprising isoprene molecules. Each isoprene molecule (isoprene unit) possesses five carbon atoms with double bonds. The carbon skeleton of terpene is formed by an enzyme class, the terpene synthases, which converts the acyclic prenyl diphosphates including squalene into an array of cyclic and acyclic forms. The diversity of terpenes is due to the large number of various terpene synthases, and at the same time, some terpene synthases create multiple products. Terpenes are subdivided into acyclic or cyclic according to the structure. Acyclic terpenes are linear, such as β-myrcene (monoterpene), while cyclic terpenes are ring-like, such as *p*-cymene (monoterpene). Based on isoprene units, terpenes are divided into monoterpene, sesquiterpene, diterpene and triterpene.

9.2.1.1 Monoterpenes

The simplest terpenes are known as monoterpenes, which are comprised of two isoprene molecules. Monoterpenes (C-10 compounds) are highly volatile, which are abundant in plants, and act as strong feeding deterrence as well as deterrent to predators (Table 9.1 and Fig. 9.1).

9.2.1.2 Sesquiterpenes

Sesquiterpenes develop from farnesyl pyrophosphate (C_{15}) containing three isoprene units (C_5) and present in plant essential oils. Sesquiterpenes consist of a large diversity of cyclic compounds and non-cyclic farnesyl derivatives. The cyclic sesquiterpenes consist of monocyclic, bicyclic and tricyclic compounds including the sesquiterpene lactones. A list of sesquiterpenes (Table 9.2 and Fig. 9.2) and sesquiterpene lactones (Table 9.3 and Fig. 9.3) acting as phyto-antifeedants were presented below.

9.2.1.3 Diterpenes

These compounds are derived from C_{20} isoprenoid geranylgeranyl pyrophosphate, which are heavy molecules with high boiling points. The diversity (structural and functional) of diterpenes is attributed to the different functions of diterpene cyclases

Table 9.1 A list of monoterpenes acting as phyto-antifeedants

Sl No.	Monoterpenes	Test insect	Origin	References
1	Ipolamiide	*Locusta migratoria*	*Stachytarpheta mutabilis*	Bernays and De Luca (1981)
		Schistocerca gregaria		
		Spodoptera littoralis		
2	Catalpol + catalposide	*Poanes hobomok*	*Catalpa speciosa*	Chang and Nakanishi (1983)
3	Specionin	*Choristoneura fumiferana*		
4	Xylomollin	*Spodoptera exempta*	*Xylocarpus moluccensis*	Kubo and Nakanishi (1977), Mabry et al. (1977)
5	Verbenone	*Hylobius abietis*		Klepzig and Schlyter (1999), Lindgren et al. (1996)
		Dendroctonus ponderosae		Gillette et al. (2014)
		Leptinotarsa decemlineata		Ortiz de Elguea-Culebras et al. (2017)
6	Carvone	*Hylobius abietis*	Essential oils of many plants and conifer plants	Klepzig and Schlyter (1999), Lindgren et al. (1996), Schlyter et al. (2004)
		Hylobius pales	*Carum carvi*, *Mentha spicata*	Schlyter et al. (2004)
7	Thymol	*Spodoptera litura*	*Thymus vulgaris, Origanum vulgare*	Hummelbrunner and Isman (2001), Erler and Tunc (2005), Kim et al. (2010), Ortiz de Elguea-Culebras et al. (2017)
		Ephestia kuehniella		
		Tribolium castaneum		
		Leptinotarsa decemlineata		
		Myzus persicae	*Senecio palmensis*	González-Coloma et al. (2002)
		Diuraphis noxia		
		Rhopalosiphum padi		
		Metopolophium dirhodum		
		Sitobion avenae		
8	*trans*-Anethole	*Spodoptera litura*	*Pimpinella anisum*	Hummelbrunner and Isman (2001)
9	Limonene	*Spodoptera litura*	*Chloroxylon swietenia*	Kiran et al. (2006)

(continued)

Table 9.1 (continued)

Sl No.	Monoterpenes	Test insect	Origin	References
		Leptinotarsa decemlineata		Khorram et al. (2011)
10	Carvacrol	*Ephestia kuehniella*	*Ocimum basilicum, Eugenia caryophyllus*	Erler and Tunc (2005), Kim et al. (2010), Saroukolai et al. (2014), Ortiz de Elguea-Culebras et al. (2017)
		Tribolium castaneum		
		Leptinotarsa decemlineata		
11	γ-Terpinene	*Ephestia kuehniella*		Erler and Tunc (2005)
12	Terpinen-4-ol	*Ephestia kuehniella*		Erler and Tunc (2005)
		Sitophilus zeamais		Yildirim et al. (2013)
		Leptinotarsa decemlineata		Ortiz de Elguea-Culebras et al. (2017)
13	α-Pinene	*Leptinotarsa decemlineata*		Rodilla et al. (2008), Khorram et al. (2011)
		Tribolium castaneum		Kim et al. (2010)
14	β-Pinene	*Leptinotarsa decemlineata*		Rodilla et al. (2008)
15	Eucalyptol	*Leptinotarsa decemlineata*		Rodilla et al. (2008)
16	Myrcene	*Tribolium castaneum*		Kim et al. (2010)
		Leptinotarsa decemlineata		Khorram et al. (2011)
17	Terpinolene	*Myzus persicae*	*Piper hispidinervum*	Andrés et al. (2017)
		Choristoneura fumiferana		Kumbasli and Bauce (2013)
		Tribolium castaneum		Wang et al. (2009)
		Sitophilus zeamais		Wang et al. (2009)
18	Pyrethrins	*Bemisia tabaci*, *Myzus persicae*	Pyrethrum	Prota et al. (2014)
19	Camphor	*Leptinotarsa decemlineata*		Ortiz de Elguea-Culebras et al. (2017)
20	Linalool	*Tribolium castaneum*, *Rhyzopertha dominica*, *Sitophilus oryzae*	Lamiaceae, Lauraceae	Kanda et al. (2017)

(continued)

Table 9.1 (continued)

Sl No.	Monoterpenes	Test insect	Origin	References
21	Menthone	*Sitophilus oryzae*	*Mentha piperita*	Rajkumar et al. (2019)
		Tribolium castaneum		
22	Menthol	*Sitophilus oryzae*		
		Tribolium castaneum		
23	1,8-Cineole	*Leptinotarsa decemlineata*		
24	Fenchone			
25	γ-Terpinene			

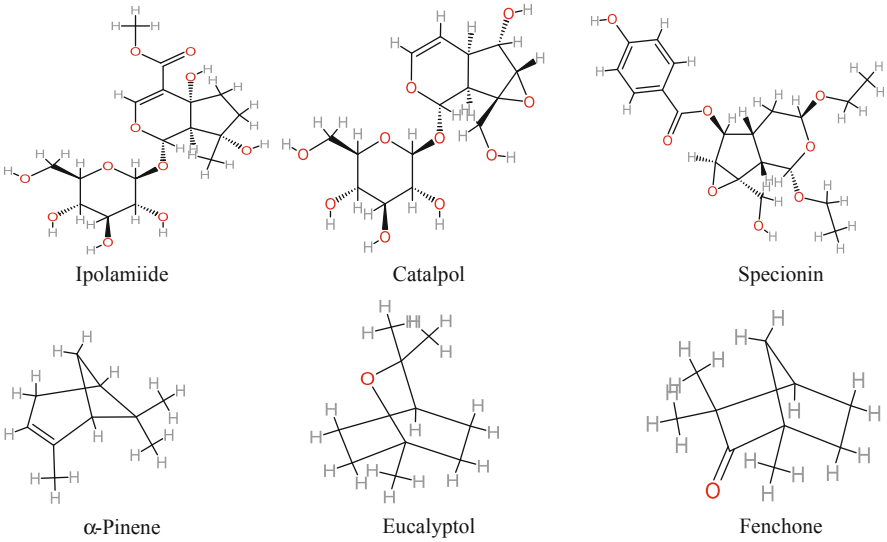


Fig. 9.1 Structure of some monoterpenes

as well as chemical modification of enzymes. Table 9.4 presents a list of diterpenes and the structure of some common diterpenes (Fig. 9.4) that act as phyto-antifeedants.

9.2.1.4 Triterpenes

Triterpenoids represent the largest groups in nature possessing 30 carbon atoms composed of 6 isoprene units. The extensive occurrence in plants is one of the main reasons for considerable interest with more than 14,000 compounds identified (Hamberger and Bak 2013). Triterpenoids are formed by cyclization of oxidized squalene predecessors by oxidosqualene cyclases, forming over 100 various cyclical

Table 9.2 A list of sesquiterpene acting as phyto-antifeedants

Sl No.	Sesquiterpenes	Test insect	Origin	References
1	Shiromodioldiacetate	*Spodoptera litura*	*Parabenzoin trilobum*	Wada et al. (1968)
2	Shiromodiolmonoacetate			
3	Plagiochiline A	*Spodoptera exempta*	*Plagiochila fruticosa*, *P. hattoriana*, *P. ovalifolia and P. yokogurensis*	Asakawa et al. (1980)
4	Drimanes	*Myzus persicae*		Caprioli et al. (1987), Gutiérrez et al. (1997)
5	Bisabolanes	*Myzus persicae*		
6	Bisabolangelone	*Peridroma saucia*	*Angelica sylvestris*	Nawrot et al. (1991)
		Mamestra configurata		
7	Bakkenolide-A	*Peridroma saucia*	*Homogyne alpina*	Isman et al. (1989)
		Coptotermes fornosanus		Kreckova et al. (1988)
8	Celangulin	*Spodoptera exempta*	*Celastrus angulatus*	Wakabayashi et al. (1988)
9	11β-Acetoxy-5α-angeloyloxysilphinen-3-one	*Leptinotarsa decemlineata*		González-Coloma et al. (1995, 1997)
10	11β,5α-Dihydroxysilphinen-3-one	*Leptinotarsa decemlineata*		
11	11β-Acetoxy-5α-isobutyryloxysilphinen-3-one	*Myzus persicae*	*Senecio palmensis*	González-Coloma et al. (2002)
		Diuraphis noxia		
		Rhopalosiphum padi		
		Metopolophium dirhodum		
		Sitobion avenae		
12	Germacranolides	*Spodoptera litura*	*Neurolaena lobata*	Passreiter and Isman (1997)
13	Neurolenin A, B, C, D	*Spodoptera litura*		
14	Lobatin A			
15	Lobatin B			
16	Polygodial	*Bemisia tabaci*	*Drimys winteri*	Prota et al. (2014)
		Myzus persicae		
		Leptinotarsa decemlineata		
		Spodoptera littoralis		Kubo and Ganjian (1981), Caprioli et al. (1987),

(continued)

Table 9.2 (continued)

Sl No.	Sesquiterpenes	Test insect	Origin	References
				Zapata et al. (2009)
		Spodoptera exempta		Kubo and Ganjian (1981), Caprioli et al. (1987)
17	Drimane sesquiterpenoids	*Spodoptera littoralis*		Zapata et al. (2009)
18	Drimendiol			
19	Isodrimeninol			
20	Isotadeonal			
21	Mansonone E	*Spodoptera litura*	*Mansonia gagei*	Mongkol and Chavasiri (2016)
22	Dehydrofukinone	*Myzus persicae*	*Senecio adenotrichius*	Ruiz-Vásquez et al. (2017)
		Spodoptera littoralis		
23	11-Hydroxyeremophila-6,9-dien-8-one	*Myzus persicae*		
24	Ligudicin A	*Myzus persicae*		
		Spodoptera littoralis		

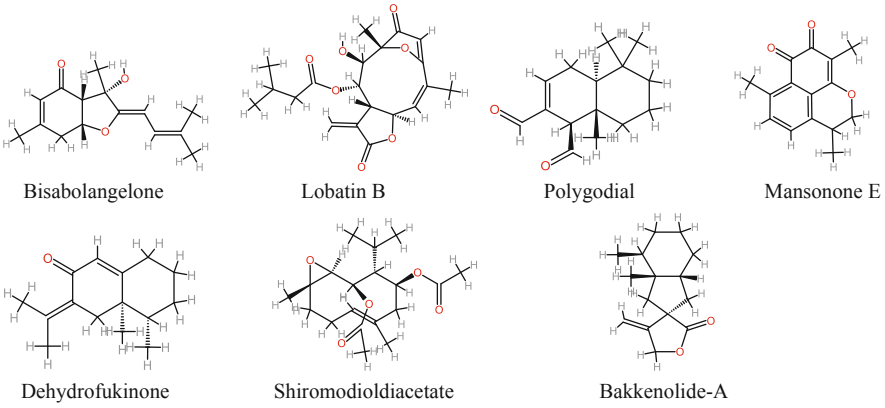


Fig. 9.2 Structure of some sesquiterpenes

triterpene scaffolds. These scaffolds are the initiators to create the wide diversity of triterpenoids followed by wide-ranging diversification, particularly by oxygenation and glycosylation (Cárdenas et al. 2019). On the other hand, the oxygenated terpenes are called limonoids, which are characterized by a 4,4,8-trimethyl-17-furanylsteroid skeleton. The first tetranotriterpenoid is limonin isolated from citrus, and the term limonoid is originated from limonin. Limonoids are created by the deletion of four

Table 9.3 A list of sesquiterpene lactones acting as phyto-antifeedants

Sl No.	Sesquiterpene lactones	Test insect	Origin	References
1	Schkuhrin I	*Spodoptera exempta*	*Schkuhria pinnata*	Pettei et al. (1978)
		Epilachna varivestis		
2	Schkuhrin II	*Spodoptera exempta*		
		Epilachna varivestis		
3	Vernodalin	*Spodoptera exempta*	*Vernonia amygdalina*	Ganjian et al. (1983)
4	Vernodalol			
5	11,13-Dihydrovernodalin	*Spodoptera exempta*		
6	Alantolactone	*Sitophilus granarius*	*Inula helenium*	Nawrot et al. (1986)
		Tribolium confusum		
		Trogoderma granarium		
7	Britanine	*Sitophilus granarius*	*Inula caspica*	
		Tenebrio molitor	*Inula caspica*	Adekenov et al. (2015)
8	Glaucolide-A	*Spodoptera eridania*	*Vernonia gigantea*, *V. glauca*	Mabry et al. (1977)
		Spodoptera frugiperda		
9	Parthenolide	*Spodoptera litura*	*Neurolaena lobata*	Passreiter and Isman (1997)
10	Buddlein A			
11	Neurolenin B			
12	(1*S*,6*R*)-2,7(14),10-Bisabolatrien-1-ol-4-one and (+)-7(14),10-bisaboladien-1-ol-4-one	*Locusta migratoria*	*Cryptomeria japonica*	Kashiwagi et al. (2007)
13	Cubebol and ferruginol		*Cryptomeria japonica*	Wu et al. (2008)
14	Inuchinenolide C	*Tenebrio molitor*	*Inula caspica*	Adekenov et al. (2015)
15	Arglabin		*Artemisia glabella*	Adekenov et al. (2015)
16	Bilobalide	*Hyphantria cunea*	*Ginkgo biloba*	Pan et al. (2016)
17	Eupatolide 13-*O*-β-d-glucopyranoside (eupatolide-II)	*Phyllotreta striolata*	*Inula salsoloides*	Bai et al. (2018)

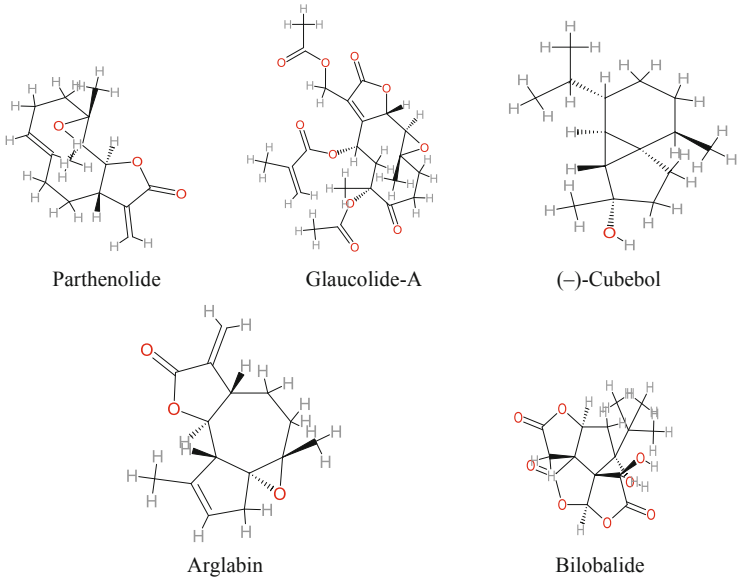


Fig. 9.3 Structure of some sesquiterpene lactones

carbon atoms from the terminal chain of apotirucallane or apoeuphane skeleton and changed to furan ring (Fang et al. 2011). The presence of limonoids is reported from plant families (Meliaceae and Rutaceae and sometimes in Cneoraceae and Simaroubaceae) of order Rutales (Roy and Saraf 2006). One-third of 300 limonoids isolated from plants is from *Azadirachta indica* (neem) and *Melia azedarach* (Chinaberry). Scientifically, the inhibitory feeding activity of neem tree was described first. In 1952, Heinrich Schmutterer exhibited that the desert locust [*Schistocerca gregaria* (Forskal)] refused to consume neem. David Morgan (Butterworth and Morgan 1968) isolated the active ingredient azadirachtin from the seeds of *A. indica*. Tables 9.5 and 9.6 present the lists of triterpenes and triterpene limonoids, respectively, which act as phyto-antifeedants, and some common structures of triterpenes are presented in Fig. 9.5.

9.2.2 Flavonoids

Flavonoids are compounds (1) consisting of derivatives of a phenyl-substituted propylbenzene containing a C15 skeleton; (2) having a C16 skeleton, which contain phenyl-substituted propylbenzene derivatives; and (3) flavonolignans containing derivatives of phenyl-substituted propylbenzene compressed with C6-C3 lignan precursors (Yonekura-Sakakibara et al. 2019). More than 9000 flavonoid compounds are identified having C6-C3-C6 carbon framework containing the structure of chromane or chromene, such as flavans, flavones, flavonols and

Table 9.4 A list of diterpenes acting as phyto-antifeedants

Sl No.	Diterpene clerodanes	Test insect	Origin	References
1	Tafricanin A, B	*Locusta migratoria*	*Teucrium africanum*	Hanson et al. (1982)
2	Clerodin (I)	*Spodoptera litura*	*Caryopteris divaricata*, *Scutellaria altissima*	Hosozawa et al. (1973, 1974)
		Leptinotarsa decemlineata	*Caryopteris divaricata*, *Scutellaria altissima*	Bozov and Georgieva (2017)
3	Caryoptin (II)	*Spodoptera litura*	*Caryopteris divaricata*	Hosozawa et al. (1973, 1974)
4	Dihydroclerodin-I (V)			
5	Dihydrocaryoptin (VI)			
6	Clerodin hemiacetal (VII)			
7	Caryoptin hemiacetal (VIII)			
8	Caryoptinol (IX)			
9	Dihydrocaryoptinol (X)			
10	Ajugacumbins A, B, C, D	*Pareba vesta*	*Ajuga decumbens*	Min et al. (1989)
11	Jodrellin A, B	*Spodoptera littoralis*	*Scutellaria woronowii*	Anderson et al. (1989)
12	Ajugarin I		*Ajuga remota*	Simmonds et al. (1989)
		Helicoverpa armigera	*Ajuga remota*	
13	6,19-Diacetylteumassilin	*Helicoverpa armigera*	*Teucrium*	
14	Deacetyl ajugarin II	*Spodoptera littoralis*		
15	Teucjaponin B			
16	12-Epl-teucvm			
17	Rhodojaponin III	*Leptinotarsa decemlineata*	*Rhododendron molle*	Klocke et al. (1991)
		Spodoptera frugiperda		
18	3,13*E*-clerodien-15-oic acid	*Reticulitermes speratus*	*Detarium microcarpum*	Lajide et al. (1995)
19	4(18), 13*E*-clerodien-15-oic acid			
20	18-Oxo-3,13*E*-clerodien-15-oic acid			
21	2-Oxo-3,13*E*-clerodien-15-oic acid			
22	Ryanodol	*Spodoptera litura*	*Persea indica*	González-Coloma et al. (1996)
23	Ryanodol 14-monoacetate	*Spodoptera litura*	*Persea indica*	
24	Cinnzeylanol	*Spodoptera litura*	*Persea indica*	
25	Cinnzeylanone	*Spodoptera litura*	*Persea indica*	

(continued)

Table 9.4 (continued)

Sl No.	Diterpene clerodanes	Test insect	Origin	References
26	Epicinnzeylanol	*Spodoptera litura*	*Persea indica*	
27	Tanabalin (=12*S*-acetoxyhautriwaic acid)	*Pectinophora gossypiella*	*Tanacetum balsamita*	Kubo et al. (1996)
28	Ajugapitin	*Spodoptera littoralis*	*Ajuga chamaepitys*, *Salvia lineata*	Belles et al. (1985)
29	Indicol	*Spodoptera litura*	*Persea indica*	Fraga et al. (1997)
30	Vignaticol			
31	Perseanol			
32	14,15-Dehydroajugareptansin	*Spodoptera littoralis*	*Ajuga reptans*	Bremner et al. (1998)
33	Scutecyprol B	*Spodoptera littoralis*	*Scutellaria rubicunda*	Bruno et al. (1999)
		Spodoptera frugiperda		
		Mamestra brassicae		
		Pieris brassicae		
		Helicoverpa armigera		
34	Isofruticolone	*Spodoptera littoralis*	*Teucrium fruticans*	
35	Clerodin	*Spodoptera littoralis*	*Caryopteris divaricata*	Hosozawa et al. (1974)
36	Caryoptin	*Spodoptera littoralis*		
		Henosepilachna vigintioctopunctata		Govindachari et al. (1999)
37	Dihydroclerodin-I	*Spodoptera littoralis*		Hosozawa et al. (1974)
38	Dihydrocaryoptin			
39	Clerodin hemiacetal			
40	Caryoptin hemiacetal			
41	Sideroxol	*Spodoptera frugiperda*	*Sideritis akmanii*, *S. rubriflora*	Bondì et al. (2000)
42	14,15-Dihydroajugapitin		*Ajuga iva*	
43	Ivain IV	*Spodoptera littoralis*	*Ajuga iva*	
		Spodoptera frugiperda		
44	Montanin D	*Spodoptera littoralis*	*Teucrium arduini*	Bruno et al. (2002)
45	6β-Hydroxyteuscordin			
46	*Cis*-cleroda-15,16-dihydroxy-3,13(*Z*)-dien-18-*O*-[β-D-galactopyranosil]-peracetylester	*Tenebrio molitor*	*Baccharis sagittalis*	Cifuente et al. (2002)

(continued)

Table 9.4 (continued)

Sl No.	Diterpene clerodanes	Test insect	Origin	References
47	*Cis*-cleroda-3,13(14)-dien-15,16-olide-18-*O*-[β-D-galactopyranosyl]-peracetylester			
48	Hastifolins A, B, C	*Spodoptera littoralis*	*Scutellaria hastifolia*	Raccuglia et al. (2010)
49	Clerodin	*Helicoverpa armigera*	*Clerodendrum infortunatum*	Abbaszadeh et al. (2014)
50	15-Methoxy-14,15-dihydroclerodin			
51	15-Hydroxy-14,15-dihyroclerodin			
52	Ginkgolide	*Hyphantria cunea*	*Ginkgo biloba*	Pan et al. (2016)
53	Scutecyprin	*Leptinotarsa decemlineata*	*Scutellaria altissima*	Bozov and Georgieva (2017)
54	11-Epi-scutecolumnin C			

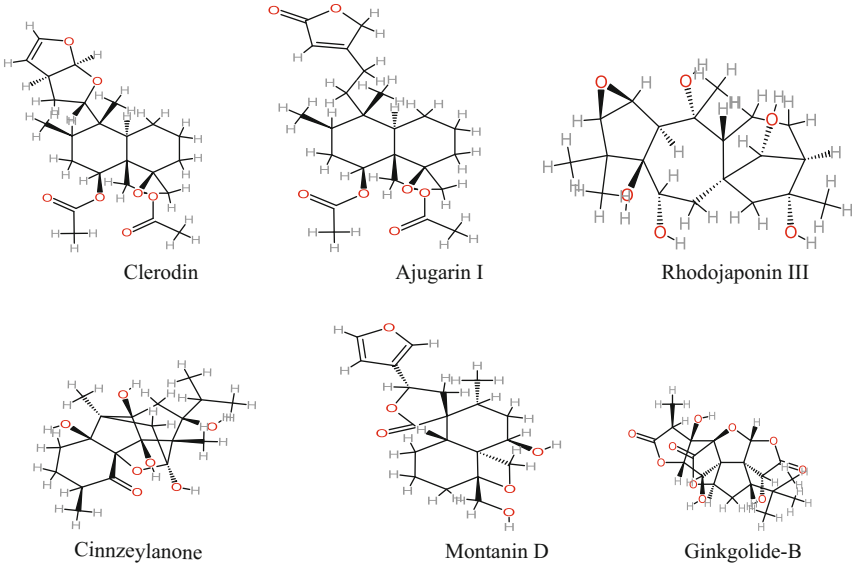


Fig. 9.4 Structure of some diterpenes

anthocyanidins (Anderson and Markham 2006). However, aurones, chalcones and dihydrochalcones are also under flavonoids in a wide sense, but truly not in a limited sense (Yonekura-Sakakibara et al. 2019). Table 9.7 presents a list of flavonoids, which act as phyto-antifeedants (Fig. 9.6).

Table 9.5 A list of triterpenes acting as phyto-antifeedants

Sl No.	Triterpene	Test insect	Origin	References
1	Betulin	*Myzus persicae*	*Betula* species	Schoonhoven and Derksen-Koppers (1976)
2	Harrisonin	*Eldana saccharina*	*Harrisonia abyssinica*	Hassanali et al. (1986)
		Maruca testulalis		
3	Obacunone	*Eldana saccharina*		
		Maruca testulalis		
4	Salannin	*Epilachna varivestis*	*Pieris brassicae*	Schwinger et al. (1984), Kraus et al. (1987)
5	Momordicine II	*Aulacophora foveicollis*	*Momordica charantia*	Chandravadana (1987)
		A. nigripennis		Abe and Matsuda (2000)
		Epilachna admirabilis		
		E. boisduvali		
		A. femoralis		
6	3,7,23-Trihydroxycucurbita-5,24-dien-19-al	*Aulacophora foveicollis*		Chandravadana (1987)
7	Betulinic acid	*Spodoptera litura*	*Zizyphus xylopyrus*	Jagadeesh et al. (1998)
8	Oleanolic acid	*Sitophilus oryzae*	*Junellia aspera*	Pungitore et al. (2005)
		Heliothis zea		Argandoña and Faini (1993)
9	Asiatic acid	*Oxya fuscovittata*	*Shorea robusta*	Sanjayan and Partho (1993)
10	Salannin	*Spodoptera litura*	Neem oil	Govindachari et al. (1996)
		Pericallia ricini		
		Oxya fuscovittata		
11	Nimbin	*Spodoptera litura*		
		Pericallia ricini		
		Oxya fuscovittata		
12	Deacetylnimbin	*Spodoptera litura*		

(continued)

Table 9.5 (continued)

Sl No.	Triterpene	Test insect	Origin	References
		Pericallia ricini		
		Oxya fuscovittata		
13	Momordicine I	*Aulacophora nigripennis*	*Momordica charantia*	Abe and Matsuda (2000)
		Epilachna admirabilis		
		Epilachna boisduvali		
14	Methyl 6,11β-dihydroxy-12α-(2-methylpropanoyloxy)-3,7-dioxo-14β,15β-epoxy-1,5-meliacadien-29-oate	*Spodoptera littoralis*	*Trichilia pallida*	Simmonds et al. (2001)
		Spodoptera exigua		
		Heliothis virescens		
		Helicoverpa armigera		
15	Betulinic acid	*Achaea janata*	*Vitex negundo*	Chandramu et al. (2003)
16	Ursolic acid			
17	Maslinic acid	*Sitophilus oryzae*	*Junelia aspera*	Pungitore et al. (2005)
18	Xylogranatins F, G, R	*Mythimna separata*	*Xylocarpus granatum*	Wu et al. (2008)
19	Catunarosides A, B, C, D	*Plutella xylostella*	*Catunaregam spinosa*	Gao et al. (2011)
20	Swartziatrioside			
21	Araliasaponin V			
22	Araliasaponin IV			
23	Ginsenoside	*Pieris rapae*	*Panax ginseng*	Zhang et al. (2017)
24	Ginsenosides (Rg1, Re, Rf, Rb1, Rg2, Rc, Rb2, Rb3 and Rd)	*Plutella xylostella*		Yang et al. (2018)
25	Ginsenosides Rb1, Rb2, Rc, Rd, Re and Rg1 [Rb1, Rb2, Rc, Rd, Rh2 and Rg3]	*Ostrinia furnacalis*		Liu et al. (2020)
26	Ginsenosides Re, Rg1 and Rg2			
27	Saponin CP4	*Plutella xylostella*	*Clematis aethusifolia*	Tian et al. (2021)
28	Clematoside S			
29	3-*O*-β-D-ribopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-β-D-xylopyranosyl hederagenin			
30	Lupeol	*Corcyra cephalonica*	*Hemidesmus indicus*	Pillai et al. (2020)

Table 9.6 A list of triterpene limonoids acting as phyto-antifeedants

Sl No.	Limonoids	Test insect	Origin	References
1	Toonacilin, toonacilid	*Epilachna varivestis*	*Toona ciliata*	Kraus et al. (1978)
2	Meliantriol	*Schistocerca gregaria*	*Melia azedarach*	Kraus et al. (1981)
3	Limonin	*Spodoptera frugiperda*	Citrus, grapefruit seeds	Klocke and Kubo (1982)
		Heliothis zea		
4	Sendanin	*Heliothis zea*	*Trichilia roku*	
5	7-Acetyltrichilin A	*Spodoptera eridania*		Nakatani et al. (1985a, b)
		Epilachna varivestis		
		Spodoptera littoralis		
6	Limonin	*Eldana saccharina*	Citrus, grapefruit seeds	Hassanali et al. (1986)
		Maruca testulalis		
		Chortstoneura fumtferana		Alford and Bentley (1986)
		Leptinotarsa decemlineata		Alford et al. (1987)
		Leptinotarsa decemlineata		Mendel et al. (1991)
7	Azadirachtin	*Schistocerca gregaria*	*Azadirachta indica*	Butterworth and Morgan (1968), Mordue (Luntz) and Nisbet (2000)
8	Obacunone	*Leptinotarsa decemlineata*	Grape fruit seeds	Mendel et al. (1991)
9	Nomilin			Mendel et al. (1991)
10	Sandoricin	*Spodoptera frugiperda*	*Sandwicum koetjape*	Powell et al. (1991)
11	Cedrelone	*Peridroma saucia*, *Mamestra configurata*	*Toona ciliata*	Koul and Isman (1992)
12	1-Deoxy-3-trigloyl-11-methoxymeliacarpinin	*Spodoptera exigua*	*Melia azedarach*	Nakatani et al. (1993)
13	Humilinolides A–D	*Tenebrio molitor*	*Swietenia humilis*	Segura-Correa et al. (1993)
14	Toosendanin	*Peridroma saucia*	*Melia toosendan*, *M. azedarach*	Chen et al. (1995)
15	Nimbolidins B, C, D, E	*Spodoptera eridania*	*Melia toosendan*	Nakatani et al. (1996)

(continued)

Table 9.6 (continued)

Sl No.	Limonoids	Test insect	Origin	References
16	Salannin			
17	Trichilins H, I, J, K and L			Zhou et al. (1996)
18	Azedarachin A and 12-*O*-acetyl-azedarachin B			
19	Ichangensin	*Leptinotarsa decemlineata*	Citrus molasses	Murray et al. (1999)
20	Melianoninol, melianone	*Pieris rapae*	*Melia azedarach*	Wang et al. (1994)
21	Melianol, meliandiol			
22	Meliantriol, toosendanin			
23	Trichilins B, D, H	*Spodoptera exigua*		Nakatani et al. (1994)
24	Lignanes	*Rhodnius prolixus*		Cabral et al. (1995)
25	Piscidinol B-F	*Spodoptera exigua*	*Walsura piscidia*	Govindachari et al. (1996)
26	Azedarachin C	*Spodoptera exigua*	*Melia azedarach*	Huang et al. (1995)
27	Azadirachtin	*Spodoptera litura*	*Azadirachta indica*	Li et al. (1995)
28	Toosendanin	*Peridroma saucia*	*Melia toosendan*	Xie et al. (1995)
29	Salannin, nimbin	*Spodoptera litura*	*Melia azedarach*	Govindachari et al. (1996)
30	Ruageanins A, B	*Spodoptera frugiperda*	*Ruafea fglabra*	Mootoo et al. (1996)
31	Azedarachin A, salannin	*Spodoptera eridania*	*Melia toosendan*	Nakatani et al. (1996)
32	Nimbolidins C–E	*Spodoptera eridania*	*Melia toosendan*	
33	Trichilins K, L, I, J, H	*Spodoptera eredania*	*Melia toosendan*	Zhou et al. (1996)
34	Azadirachtin	*Spodoptera littoralis*	*Azadirachta indica*	Mordue (Luntz) and Nisbet (2000)
		Spodoptera frugiperda		
		Heliothis virescens		
		Helicoverpa armigera		
		Pieris brassicae		

(continued)

Table 9.6 (continued)

Sl No.	Limonoids	Test insect	Origin	References
		Epilachna varivestis		
		Locusta migratoria		
		Melanoplus sanguinipes		
35	Meliartenin	*Spodoptera eridania*	*Melia azedarach*	Carpinella et al. (2002)
		Epilachna panuelata		
		Epilachna paenulata	*Melia azedarach*	Carpinella et al. (2003)
36	Dumsin	*Pectinophora gossypiella*	*Croton jatrophoides*	Nihei et al. (2002)
		Spodoptera frugiperda		
37	Zumsin	*Pectinophora gossypiella*		
		Spodoptera frugiperda		
38	Meliartenin	*Epilachna paenulata*	*Melia azedarach*	Carpinella et al. (2003)
39	Musidunin	*Pectinophora gossypiella*	*Croton jatrophoides*	Nihei et al. (2004, 2005, 2006)
		Spodoptera frugiperda		
40	Musiduol	*Pectinophora gossypiella*		
		Spodoptera frugiperda		
41	Zumketol	*Pectinophora gossypiella*		
		Spodoptera frugiperda		
42	Zumsenin	*Pectinophora gossypiella*		
		Spodoptera frugiperda		
43	Zumsenol	*Pectinophora gossypiella*		
		Spodoptera frugiperda		
44	Dumnin	*Pectinophora gossypiella*		

(continued)

Table 9.6 (continued)

Sl No.	Limonoids	Test insect	Origin	References
		Spodoptera frugiperda		
45	Dumsenin	*Pectinophora gossypiella*		
		Spodoptera frugiperda		
46	Xylogranatins F, G and R	*Mythimna separate*	*Xylocarpus granatum*	Wu et al. (2008)
47	2-Acetyl soymidin B	*Spodoptera litura*	*Soymida febrifuga*	Yadav et al. (2014)
		Achaea janata		
48	Soymidin D	*Spodoptera litura*		
		Achaea janata		
49	Soymidin E	*Spodoptera litura*		
		Achaea janata		
50	Trichanolide F	*Spodoptera litura*	*Trichilia connaroides*	Solipeta et al. (2020)

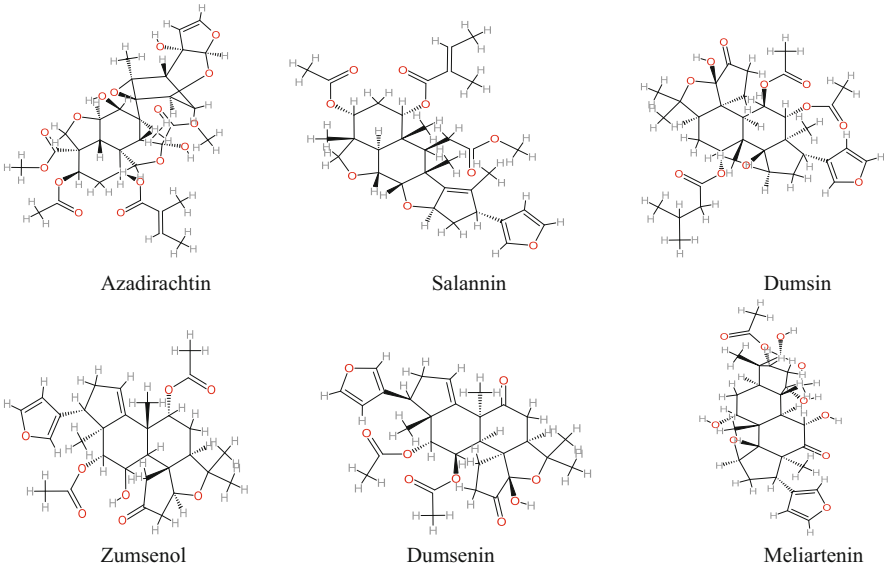


Fig. 9.5 Structure of some triterpenes

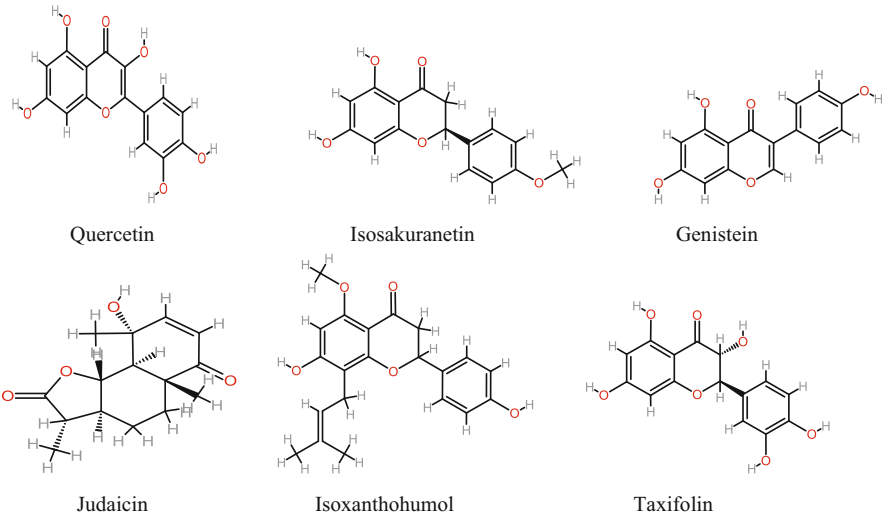


Fig. 9.6 Structure of some flavonoids

9.2.3 Alkaloids

Alkaloid compounds (nitrogen incorporated into a heterocyclic ring) are naturally occurring low-molecular-weight organic compounds. It was reported that ca. 20–30% of all alkaloids arise in higher plants, mostly in dicotyledonous angiosperms at concentrations of ca. 0.01% of the dry weight or more (Seigler 1998). These compounds could be stored in any part of the plant at different concentrations; they are most often intense in the most nutritious tissues, such as seed tissues (Bernays and Chapman 1994). It is reported that ca. 10% of plant species produce alkaloids as secondary metabolites, and these compounds primarily help to protect against herbivores as well as pathogens. Till date more than 16,000 alkaloids have been identified (Murphy 2017). However, some of them act as phyto-antifeedants (Table 9.8 and Fig. 9.7).

9.2.4 Steroids

Steroids possess the tetracyclic 1,2-cyclopentanoperhydrophenanthrene (5α- or 5-β-gonane) carbon skeleton, normally having methyl substituents at C-10 and C-13 and an alkyl substituent (side chain) at C-17. An array of diverse steroid compounds arises due to different oxidation states of carbons of its tetracyclic core and CH_3 groups and the framework of the side chain. All steroids are derived from *S*-squalene-2,3-epoxide (Gunaherath and Gunatilaka 2014). The major plant steroids are phytosteroids, withanolides, brassinosteroids, phytoecdysteroids, and steroidal alkaloids. Table 9.9 shows a list of steroids, which act as phyto-antifeedants (Fig. 9.8).

Table 9.7 A list of flavonoids acting as phyto-antifeedants

Sl No.	Flavonoids	Test insect	Origin	References
1	5-Hydroxy-3,6,7,8,4′-pentamethoxyflavone	*Spodoptera litura*	*Gnaphalium affine*	Morimoto et al. (2000, 2003)
2	5-Hydroxy-3,6,7,8-tetramethoxyflavone			
3	5,6-Dihydroxy-3,7-dimethoxyflavone			
4	4,4′,6′-Trihydroxy-2′-methoxychalcone			
5	5-Hydroxy-3,6,7,8,4′-heptamethoxyflavone			
6	5-Hydroxy-3,6,7,8-tetramethoxyflavone			
7	5,6-Dihydroxy-3,7-dimethoxyflavone			
8	Quercetin	*Coptotermes formosanus*	*Bobgunnia madagascariensis*	Ohmura et al. (2000)
		Tribolium castaneum		Adeyemi et al. (2010)
9	Taxifolin	*Coptotermes formosanus*		Ohmura et al. (2000)
10	Naringenin			
11	Isosakuranetin			
12	Aromadendrin			
13	Phloretin			
14	Myricetin			
15	Sakuranetin			
16	Eriodictyol			
17	Genistein	*Coptotermes formosanus*	*Trifolium pratense*	Ohmura et al. (2000)
		Acyrthosiphon pisum		Goławska and Łukasik (2012)
		Hylastinus obscurus		Quiroz et al. (2017)
18	Formononetin	*Hylastinus obscurus*		
19	Fisetin	*Coptotermes formosanus*		Ohmura et al. (2000)
20	Kaempferol			
		Sitophilus oryzae	*Calotropis procera*	Nenaah (2013)
		Rhyzopertha dominica	*Calotropis procera*	
21	Catechin	*Coptotermes formosanus*		Ohmura et al. (2000)
22	Catechinic acid			
23	Judaicin	*Helicoverpa armigera*	*Cicer judaicum*	Simmonds and

(continued)

Table 9.7 (continued)

Sl No.	Flavonoids	Test insect	Origin	References
		Spodoptera litura		Stevenson (2001)
		Spodoptera frugiperda		
24	Maackiain	*Helicoverpa armigera*		
		Spodoptera litura		
		Spodoptera frugiperda		
25	Luteolin	*Acyrthosiphon pisum*		Goławska and Łukasik (2012)
26	3-O-Rutinosides of quercetin	*Sitophilus oryzae*	*Calotropis procera*	Nenaah (2013)
		Rhyzopertha dominica		
27	3-O-Rutinosides of isorhamnetin	*Sitophilus oryzae*		
		Rhyzopertha dominica		
28	5-Hydroxy-3,7-dimethoxyflavone-4′-O-β-glucopyranoside	*Sitophilus oryzae*		
		Rhyzopertha dominica	*Calotropis procera*	Nenaah (2013)
29	Tephroapollin-F	*Sitophilus oryzae*	*Tephrosia apollinea*	Nenaah (2014)
		Rhyzopertha dominica		
		Tribolium castaneum		
30	Isoxanthohumol	*Myzus persicae*		Stompor et al. (2015)
31	Formononetin	*Hylastinus obscurus*		Quiroz et al. (2017)

Table 9.8 A list of alkaloids acting as phyto-antifeedants

Sl No.	Alkaloids	Test insect	Origin	References
1	Isoboldine (I)	*Spodoptera litura*	*Cocculus trilobus*	Munakata (1975)
		Abraxas miranda		
2	Wilforine	*Pieris rapae*	*Maytenus rigida*	Monache et al. (1984)
		Locusta migratoria		
3	Pterocarpan	*Maruca testulalis*	*Tephrosia hildebrandtii*	Lwande et al. (1985)
4	Hildecarpin			
5	Vasicine	*Aulacophora foveicollis*	*Adhatoda vasica*	Saxena et al. (1986)
		Epilachna vigintioctopunctata		
6	Vasicinol	*Aulacophora foveicollis*		
		Epilachna vigintioctopunctata		
7	Vasicinone	*Aulacophora foveicollis*		
		Epilachna vigintioctopunctata		
8	Tylophorine	*Spilosoma obliqua*	*Tylophora asthmatica*	Tripathi et al. (1990)
9	Dithyreanitrile	*Spodoptera frugiperda*	*Dithyrea wislizenii*	Powell et al. (1991)
		Ostrinia nubilalis		
10	3′-Acetyltrachelanthamine	*Leptinotarsa decemlineata*	*Heliotropium floridum*	Reina et al. (1997)
11	Europine	*Spodoptera littoralis*		Reina et al. (1995)
12	Cardiopetamine	*Spodoptera littoralis*	*Delphinium cardiopetalum*	González-Coloma et al. (1998)
13	15-Acetylcardiopetamine	*Leptinotarsa decemlineata*	*Delphinium cardiopetalum*	
14	Lycopsamine	*Leptinotarsa decemlineata*	*Heliotropium megalanthum*	Reina et al. (1998)
		Spodoptera littoralis		
15	Berberine	*Hyphantria cunea*	*Coptis japonica*	Park et al. (2000)
		Agelastica coerulea		
16	Palmatine	*Hyphantria cunea*		
		Agelastica coerulea		
17	Coptisine	*Hyphantria cunea*		
		Agelastica coerulea		

(continued)

Table 9.8 (continued)

Sl No.	Alkaloids	Test insect	Origin	References
18	Leptine	*Leptinotarsa decemlineata*	*Solanum chacoense*	Rangarajan et al. (2000)
19	Strychnine	*Spodoptera litura*	*Neurolaena lobata*	Passreiter and Isman (1997)
		Diabrotica virgifera virgifera		Simmonds (2003)
20	Matrine	*Coptotermes formosanus*	*Sophora flavescens*	Mao and Henderson (2007)
21	Oxymatrine	*Coptotermes formosanus*		
22	Atropine	*Spodoptera litura*	*Datura stramonium*, *Datura ferox*, *Datura innoxia*	González-Coloma et al. (2004)
		Leptinotarsa decemlineata	*Datura stramonium*, *Datura ferox*, *Datura innoxia*	
23	Atropine + Nicotine	*Lymantria dispar*	*Datura stramonium*, *Datura ferox*, *Datura innoxia*	Shields et al. (2008)
24	3-*O*-Acetyl-narcissidine	*Spodoptera littoralis*	*Hippeastrum puniceum*	Santana et al. (2008)
25	(+)-11β-Methoxy-10-oxoerysotramidine		*Erythrina latissima*	Cornelius et al. (2009)
26	(+)-10,11-Dioxoerysotramidine			
27	(+)-Erysotrine			
28	(+)-Erysotramidine			
29	(+)-Erythraline			
30	(+)-11β-Hydroxyerysotramidine			
31	Taxol	*Lymantria dispar*	Yew plant	Hu et al. (2011)
32	α-Chaconine	*Trogoderma granarium*	*Solanum tuberosum*	Nenaah (2011)
33	α-Solanine	*Trogoderma granarium*	*Solanum tuberosum*	Nenaah (2011)
34	(3β,7α)-Stigmast-5-ene-3,7-diol	*Leptinotarsa decemlineata*	*Echium wildpretii*	Santana et al. (2012)
35	(3β,7α)-7-Methoxystigmast-5-en-3-ol			

(continued)

Table 9.8 (continued)

Sl No.	Alkaloids	Test insect	Origin	References
36	7-Demethoxytylophorine	*Plutella xylostella*	*Cynanchum komarovii*	Guo et al. (2014)
37	6-Hydroxyl-2,3-dimethoxy phenanthroindolizidine			
38	Vasicine acetate		*Adhatoda vasica*	Paulraj et al. (2014)
39	2-Acetyl-benzylamine			
40	Pubescensine	*Pieris rapae*	*Aconitum soongaricum* var. *pubescens*	Chen et al. (2015)
41	3-Deoxyaconitine			
42	Aconitine			
43	15-α-Hydroxyneoline			
44	Taurenine			
45	Bullatine B			
46	Chasmanthinine	*Spodoptera exigua*	*Aconitum franchetii* var. *villosulum*	Zhang et al. (2017)
47	Apetaldine A	*Spodoptera litura*	*Aconitum apetalum*, *Aconitum franchetii var. villosulum*	
48	Apetaldine E		*Aconitum apetalum*, *Aconitum franchetii var. villosulum*	
49	Chasmaconitine		*Aconitum apetalum*, *Aconitum franchetii var. villosulum*	
50	Indaconitine		*Aconitum apetalum*, *Aconitum franchetii var. villosulum*	

9.2.5 Coumarins

Coumarin compounds are in the family of benzopyrones (1,2-benzopyrones or 2*H*-1-benzopyran-2-ones), which is a class of lactones containing a benzene ring fused to α-pyrone ring (Matos et al. 2015). The name 'coumarin' is derived from the French term of Tonka bean (*coumarou*), seeds of *Dipteryx odorata* (*Coumarouna odorata*) (Fabaceae/Leguminosae), which was first isolated in 1820. A list of coumarins is presented in Table 9.10. Figure 9.9 provides some structure of coumarins.

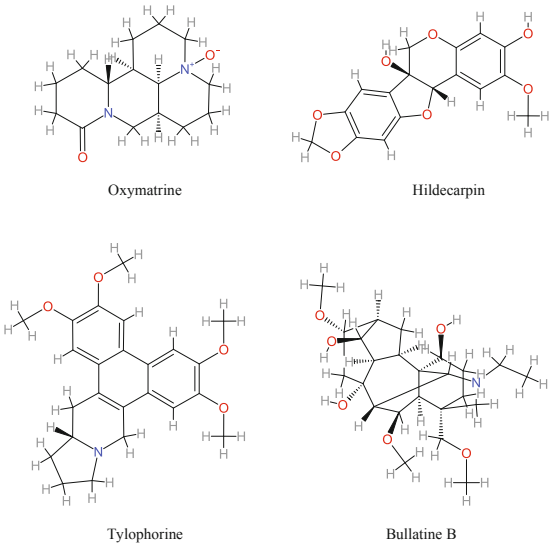


Fig. 9.7 Structure of some alkaloids

9.2.6 Other Compounds

Aglaroxin A isolated from the twigs with bark of *Aglaia elaeagnoidea* (syn. *A. roxburghiana*) had potent antifeedant activity against the gram pod borer, *Helicoverpa armigera* (Hübner) and Asian armyworm, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) (Koul et al. 2005).

Ononitol monohydrate, a class of glycoside, isolated from *Cassia tora* (Fabaceae) leaves showed antifeedant activity against the third instar larvae of *H. armigera* and *S. litura* (Baskar and Ignacimuthu 2012).

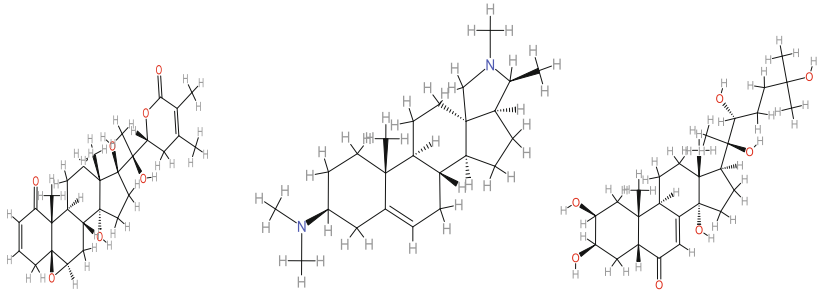
9.3 Phyto-Antifeedants: Mode of Action

The antifeedant effects of compounds on insects are generally measured by determining nutritional indices, such as consumption, digestion and growth rate of insects after consuming the foods provided. However to measure accurate estimate of nutritional indices, a series of control experiments with weighed quantity of food would have to be provided to determine whether the compound of interest has resulted in a reduction in food consumption.

In feeding inhibitory test of a compound, different methods have been employed, such as spraying of the compound on natural food (leaf disks), incorporating it with dried food (wheat flour for locusts) and adding it in artificial diets, which is palatable (mostly with sucrose). For chewing insects, sucrose is mixed with agar or agar cellulose substrates; filter paper or glass fibre disks have been employed, while an artificial medium in parafilm sachets is used for sucking insects. For heteropteran and lepidopteran larvae and coleopteran insects, antifeedants are provided in drinking water sources.

Table 9.9 A list of steroids acting as phyto-antifeedants

Sl No.	Steroids	Test insect	Origin	References
1	Withanolide E	*Spodoptera littoralis*	*Physalis peruviana*, *Withania somnifera*	Ascher et al. (1980)
		Epilachna varivestis	*Physalis peruviana*, *Withania somnifera*	
2	Nicalbin A, B	*Epilachna varivestis*	*Nicandra physalodes*	
3	4β-Hydroxywithanolide E	*Epilachna varivestis*	*Physalis peruviana*	
4	Nic-1 (nicandrenone)	*Epilachna varivestis*	*Nicandra physalodes*	
5	Azedarachol	*Agrotis segetum*	*Melia azedarach*	Nakatani et al. (1985b)
6	Conessine	*Spodoptera litura*	*Holarrhena antidysenterica*	Thappa et al. (1989)
		Pieris brassicae	*Holarrhena antidysenterica*	
7	Salpichrolide A, C, G	*Musca domestica*	*Salpichroa origanifolia*	Mareggiani et al. (2000)
8	Leptine I	*Leptinotarsa decemlineata*		Hollister et al. (2001)
9	Leptinines			
10	Luciamin	*Schizaphis graminum*		Dayan et al. (2009)
11	20-Hydroxyecdysone	*Phyllotreta striolata*	*Ajuga nipponensis*	Xu et al. (2009)
12	(3β,7α)-Stigmast-5-ene-3,7-diol	*Leptinotarsa decemlineata*	*Echium wildpretii*	Santana et al. (2012)
13	(3β,7α)-7-Methoxystigmast-5-en-3-ol	*Leptinotarsa decemlineata*	*Echium wildpretii*	



Withanolide E Conessine 20-hydroxy ecdysone

Fig. 9.8 Structure of some steroids

Table 9.10 A list of coumarins acting as phyto-antifeedants

Sl No.	Coumarins	Test insect	Origin	References
1	Xanthotoxin	*Spodoptera litura*	Umbelliferae	Yajima and Munakata (1979)
		Spodoptera exigua		Berdegue et al. (1997)
		Trichoplusia ni		Akhtar and Isman (2004)
2	8-Methoxypsoralen	*Spodoptera littoralis*	*Tetradium daniellii*	Stevenson et al. (2003)
		Heliothis virescens		
3	5-Methoxypsoralen	*Spodoptera littoralis*		
		Heliothis virescens		
		Cryptotermes brevis		Sbeghen-Loss et al. (2011)
4	5,8-Dimethoxypsoralen	*Spodoptera littoralis*		Stevenson et al. (2003)
		Heliothis virescens		
5	5-Geranyloxypsoralen	*Spodoptera littoralis*		
		Heliothis virescens		
6	Xanthotoxin	*Trichoplusia ni*	Umbelliferae plants	Akhtar and Isman (2004)
7	3(2″,2″Dimethyl butenyl) 3′-hydroxydihydrofuropsoralen	*Spodoptera littoralis*	*Ruta chalepensis*	Emam et al. (2009)
8	Rutamine	*Spodoptera littoralis*	*Ruta chalepensis*	
9	5,7-Dimethoxycoumarin	*Cryptotermes brevis*	Total citrus wax	Sbeghen-Loss et al. (2011)

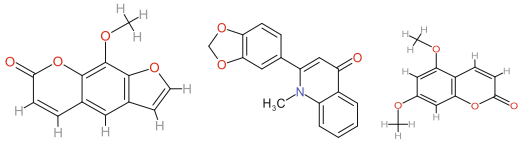


Fig. 9.9 Structure of some coumarins

In choice tests, the screening method is much sensitive. The peach aphid *Myzus persicae* feeds on artificial foods containing different allelochemicals, whereas in a choice experiment the aphids could not distinguish between the control without the test allelochemicals and substance with allelochemicals. This study indicated that experimental conditions would have to be chosen after careful considerations. According to Ma (1977), the threshold value of *Spodoptera exempta* towards warburganal was 1000 times higher when applied in sucrose-agar diet than warburganal present in natural leaf surface (Kubo et al. 1976). These results suggested that the compound mixed in agar caused the receptors to contact at lower concentrations than that present in the leaf surface. Further, the increased food intake may be due to poor nutritional value of agar (Dethier 1982).

Different methods have been applied by various researchers to describe antifeedant effects, such as the effect of antifeedants in concentrations (ppm—implicating a reduction in food intake by 50%) which reduce food intake by 50%, while a group of researchers reported that the effect of antifeedants would be taken into account when the compound of interest inhibited feeding of the insect pest between 80% and 100%; antifeedants in the context of leaf surface area are not fed by an insect (protective concentrations, PC) and the intensity of insect starvation (starvation concentration, SC), i.e., the effective antifeedant concentration was not taken into account when these values are below 95% level. Jermy et al. (1981) used a log 2 concentration series to state antifeedant activity in effective threshold concentrations. However, a number of reviews suggested that bioassays to observe the antifeedant effect of an insect towards a compound will not be more than 6 h as lower feeding for long-term test could cause post-ingestive toxicity rather than behavioural basis.

9.3.1 Cognition of Antifeedants

Different mechanisms are used by various insects at the sensory level for the cognition of antifeedants. Phytophagous insects possess taste cells to detect inedible and/or toxic secondary metabolites of plant origin, and specialized receptors are stimulated by the substances, or the activities of receptors are modified by tuning the other compounds, and in this way insects adjust the sensory code (van Loon and Schoonhoven 1999).

In lepidopteran larvae, the bitter-receptor (deterrent) taste cells possess four types of chemosensilla—the lateral and medial styloconic sensilla, epipharyngeal sensilla and gustatory sensilla, which are located on the maxillary palp. Each sensillum possesses three to four taste cells. One of the taste cells in each sensillum acts as deterrent. Overlapping molecular receptive ranges (MRRs) are present in some bitter-receptor taste cells (van Loon and Schoonhoven 1999). A bitter-receptor taste cell can respond to various secondary plant metabolites by the co-localization of a set of signalling pathways, each with distinct MRRs, such as the bitter-receptor taste cell located in the lateral styloconic sensillum of *M. sexta* and had at least two signalling pathways: one pathway reacts to phenolic glycosides (salicin and helicin)

and methylxanthines (caffeine, theophylline and theobromine), while the other pathway reacts to aromatic nitro derivatives (aristolochic acids) (Glendinning and Hills 1997). For example, caffeine—a deterrent to the monophagous larva of *Danaus plexippus*—responds to all eight receptors located in the maxillary sensilla styloconica. A number of literatures reveal that direct gated ion channels and G protein-coupled receptors are involved in sugar signalling pathways for dipteran taste cell (Murakami and Kijima 2000; Ishimoto et al. 2000; Dahanukar et al. 2001).

Phytophagous insects may employ post-ingestive response to detect toxic compounds in food, e.g. the larvae of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) initially start feeding on foods containing indole-3-carbinol (a toxic compound), which is present in cruciferous plants, but the larvae did not consume after 2–3 min and become motionless (Glendinning and Slansky 1995). This observation suggests that indole-3-carbinol does not deter the larvae initially through pre-ingestive (i.e. gustatory or olfactory) mechanism, and this compound deter the larvae to feed through post-ingestive response. Similar results were recorded in the case of *M. sexta* larvae. Larvae of *M. sexta* when provided with artificial diet mixed with nicotine then they initially consumed rapidly, but they did not feed after 24–30 s, and subsequently, the larvae started to tremble aggressively. The above fact is not an incident of pre-ingestive response but post-ingestive response of the *M. sexta* larvae, which is proved by these four facts: (1) taste-mediated inhibitory responses in the larvae generally onset more rapidly (in <6 s); (2) destroying the gustatory and olfactory chemosensilla of larvae had no effect on the time course or the nature of inhibitory response to the diet containing nicotine; (3) nicotine did not stimulate the deterrent taste cells in the larvae (Glendinning 1996); and (4) the larvae aggressively tremble when nicotine trespasses the central nervous system (Morris 1984).

9.3.2 Validating the Action of Inhibitory Response

Phytophagous insects tackle the inhibitory response of secondary metabolites by at least three different mechanisms—two are performed by the taste system, while the third is mediated by detoxication enzymes present in the midgut. It seems that these three mechanisms are helpful to combat against a wide array of secondary plant metabolites.

9.3.2.1 Carbohydrates Hide the Distasteful Taste of Secondary Plant Metabolites

When inedible secondary plant metabolites are provided with carbohydrates (sugars or sugar alcohols), then this mechanism is functional. The carbohydrates in the food can override the inedible taste of some plant secondary metabolites, which causes the inedible food to become edible or palatable food (Glendinning et al. 2000). The peripheral taste system helps to detect the mechanism as several reports are available, which proved that carbohydrates inhibit the response mechanism of deterrent taste cells (Blaney and Simmonds 1990; Shields and Mitchell, 1995a, b). Among the

two possibilities, one is that carbohydrate-sensitive taste cell inhibits the activity of deterrent taste cell present in the same chemosensillum, while in another possibility, carbohydrates attach to the receptor molecules, resulting in the inhibition of the response of the taste cells.

9.3.2.2 Longer Dietary Exposure Helps the Gustatory System to Consume Nontoxic Unpalatable Substances

If phytophagous insects are provided a diet with nontoxic unpalatable substances, then insects will repetitively check the diet, and after 12–48 h of tasting the diet, insects will ultimately adapt their inhibitory response towards these substances. In *M. sexta*, a diet containing caffeine has been provided for 24 h; then, the insect put an end to inhibitory response towards caffeine. This mode of mechanism is mediated peripherally as the prolonged exposure to the diet helps to desensitize all caffeine-response taste cells towards caffeine. Similar results were obtained if salicin is provided for 24 h, but this mechanism is performed centrally because of the absence of desensitization of salicin-response taste cells. Both these results suggest that the larvae of *M. sexta* employ peripheral and central gustatory mechanisms to adapt nontoxic unpalatable substances.

9.3.2.3 Longer Dietary Exposure Towards Toxic and Unpalatable Substances Causes Release of Detoxification Enzymes

It is common that phytophagous insects can overcome the inhibitory responses of toxic plant secondary metabolites by inducing the detoxification enzymes present in the midgut (Zangerl and Berenbaum 1993; Glendinning and Slansky 1995).

The larvae of *M. sexta* can overcome the neurotoxic effects caused by nicotine in the diet. Initially for a period of 30 h, the larvae deter from feeding towards ecologically relevant concentration of nicotine, but after that the midgut wall produces a huge amount of P450 detoxification enzymes, which catabolize the nicotine to excretal substance with less toxicity (Negherbon 1959; Morris 1983, 1984; Snyder et al. 1993, 1994). The above statement is supported by two reasons: (1) feeding of low amount of nicotine in diet does not induce release of P450 detoxification enzymes (Snyder and Glendinning 1996), and (2) when nicotine-fed larvae were provided piperonyl butoxide (PB) (an inhibitor of P450 detoxification enzymes), it results in consumption of nicotine at a lower rate that is similar to that of uninduced larvae.

9.4 Phyto-Antifeedant: Formulation

The use of natural antifeedants is growing in the world, and the choice of the ideal formulation is dependent on a series of factors: type of antifeedants (natural or synthetic), pharmaceutical forms (dust and spray), duration of action time (short or long) and environment of exposure. The most used antifeedant is azadirachtin A from *A. indica*. Other azadirachtin isomers are also reported to act as antifeedants, but activity of azadirachtin A is higher than other isomers. This compound is

effective against ca. 400 insect species belonging to Blattodea, Coleoptera, Diptera, Dermaptera, Ensifera, Homoptera, Heteroptera, Hymenoptera, Lepidoptera, Isoptera, Phasmida, Thysanoptera and Siphonaptera (Koul and Wahab 2004).

Liquid formulations of commercial neem-based insecticides—(1) Agroneem (Ajay Bio-Tech, Pune, India), (2) Ecozin (AmVaC, Los Angeles, CA) and (3) Neemix 4.5 (Certis, Columbia, MD)—and a neem seed extract formulation containing 1036, 16,506, 471 and 223μg/ml azadirachtin, respectively, caused lower feeding punctures by the gravid female boll weevils *Anthonomus grandis grandis* Boheman on the treated cotton square compared to control treatments (Showler et al. 2004). If the formulations are applied in outdoor environment 24 h before weevils were in touch, a decrease of 46–60% feeding compared with controls was recorded (Showler et al. 2004), indicating that repeated applications are needed to get the best result. A significant reduction in the feeding activity of the diamond-back moth, *Plutella xylostella*, larvae was recorded by feeding on Agroneem, Ecozin and Neemix (Liang et al. 2003).

AgriDyne Technologies Inc. (ATI) has developed a formulation, Align™ (an emulsifiable concentrate containing 3% azadirachtin), which is diluted with water before spraying to control insect pests of fruits and vegetables. The application of Align™ resulted in a significant reduction in feeding activity of cabbage looper, beet armyworm, diamondback moth, Colorado potato beetle, sweet potato whitefly, grape leafhopper, green peach aphid and onion thrips. Further, AgriDyne has formulated two neem-based insecticides, Azatin® EC and Turplex™, to control insect pests of greenhouse and ornamental plants, respectively.

In India, several neem-based products are available, such as Azadit; Biosol; Godrej; Achook [containing 2800 ppm of the compounds azadirachtin (aza) (0.03%; 300 ppm), azadiradione, nimbocinol and epinimbocinol]; Field Marshal (azadirachtin-enriched neem extract—water-miscible); neem-based emulsifiable concentrate, dust, water dispersible powder and granule (25% WDP are effective against *H. armigera*, *S. obliqua* and *E. cnejus*, while 5% dust are effective against *S. obliqua,* and 3.5% and 10% granules on China clay against sorghum stem borer, *Chilo partellus*); Neemhit prepared by Ayurvedic formula (effective against cotton, sugarcane, peanut, soybean, sunflower, corn, pulses, rice, vegetables, fruit trees, flowers and plantation crops according to manufacturer); Neem Oil Emulsion; Neem Plus; Neem Top; Neemark (water-miscible concentrate containing 80% neem biomass—give an emulsion on dilution with water); Neemasol; Neemgold; Neemguard; etc. Further, four neem-based insecticides—Neemix® (0.25% EC at 20 mg azadirachtin/litre), Ecozin® (3% EC at 20 mg azadirachtin/litre), Agroneem® (0.15% EC at 4.8 mg azadirachtin/litre) and neem oil (0.25% EC azadirachtin at 20 mg azadirachtin/litre)—are effective antifeedants against the larvae of *Pieris brassicae* (Hasan and Ansari 2011).

Zuleta-Castro et al. (2017) formulated the emulsion containing 0.76% p/p ethanolic extract using *A. indica* cell culture extract, 0.72% 8-hydroxyquinoline, 1% anthraquinone and epichlorohydrin, 0.20% Tween 8 and 50/50 aqueous phase/oil phase to control *S. frugiperda* insects, and the metabolite did not degrade in the light, which causes death of the insect pests in the field.

Neem seed extracts inhibited the feeding of rose aphid, *Macrosiphum rosae* (L.), and chrysanthemum aphid, *Macrosiphoniella sanborni* (Gillette), and subsequently resulted in a reduction in the aphid populations on host plants, while EC50 values were 0.88% and 0.96% for *M. rosae* and *M. sanborni*, respectively (Koul 1999).

It is essential that antifeedants must have properties like insecticides, i.e., effective only against the target insect pest (compounds that are nontoxic against mammals and nontarget mechanisms, such as beneficial insects), and they must possess residual property, so that crops can be protected against insect pests through its window of exposure. It is common problem of antifeedants that these compounds had been suffering from higher interspecific variations in bioactivity; for example, azadirachtin is an effective antifeedant against the desert locust (inhibiting feeding by 50% at a 0.05 ppm concentration), but the migratory grasshopper (a pest of cereal crops and rangeland grasses in North America) does not deter feeding at a concentration of 1000 ppm (Champagne et al. 1989). Further, the EC50 values of azadirachtin varied more than 30-fold between species; for example, the tobacco cutworm (*Spodoptera litura*) is the most sensitive, and the black army cutworm (*Actebia fennica*) is the least (Isman 1993).

González-Coloma et al. (2002) demonstrated that the antifeedant activities of silphinene sesquiterpenes are species dependent, such as the cotton leaf worm (*S. littoralis*), Colorado potato beetle (*L. decemlineata*) and five aphid species (*M. persicae*, *Diuraphis noxia*, *Rhopalosiphum padi*, *Metopolophium dirhodum* and *Sitobion avenae*). Several reports revealed that insects show habituation on antifeedants though these compounds initially act as antifeedants on the insects; for example, the larvae of tobacco cutworm initially did not feed on azadirachtin, but the antifeedant activity of this compound becomes half after prolonged exposure of the insect for 5 h (Bomford and Isman 1996). The antifeedant activity of toosendanin is destroyed after 4.5 h. These observations suggest that the application of antifeedants on plants might only protect the plant from insect pests during initial attack, but after that the antifeedants become ineffective.

According to Isman (2002), the habituation was observed in the armyworm larvae (*P. unipuncta*) when they were provided xanthotoxin or thymol alone, but larvae did not show habituation when they were exposed to a blend of these two compounds. It was also shown that the larvae of *S. litura* showed habituation on azadirachtin, but the larvae did not become habituated when they were exposed to neem extract containing the same amount of azadirachtin (Bomford and Isman 1996). In the same way, the larvae showed habituation to toosendanin (95%), but they did not show habituation to a blend of limonoids containing 60% toosendanin.

9.5 Phyto-Antifeedants: Potential Uses

The best method to apply an antifeedant is in water- or oil-based formulations like the application of an insect pesticide. It is noted that the beneficial effects of antifeedants are dependent on applying these compounds in more strategic ways. Latex, a natural hydrocarbon polymer, is a nontoxic material, which is used in paints,

surface coatings, furniture, packaging, textiles, construction and pharmacy. Further, pharmaceutical industries apply them to put together in controlled release drug delivery systems to protect dosage forms from UV exposure and moisture (Shtykova et al. 2008). Shtykova et al. (2008) used the latex dispersion Eudragit copolymer (EC) to prepare the coatings on the antifeedants 2,6-di-tert-butyl-4-methylphenol (BHT) and cisdihydropinidine (Alk), which were efficient to deter the feeding activity on conifer bark by *Hylobius abietis* (pine insect) both in laboratory and in fields. The applications of essential oils as antifeedants are not so fruitful because of the degradation and volatilization of the active ingredients in essential oils. El Asbahani et al. (2015) formulated essential oils as microspheres or microcapsules to protect them from degradation. The ethanolic crude extract of *Annona mucosa* Jacq. (ESAM) seeds contains a mixture of alkaloids, triglycerides and acetogenins, which is a prospective source of insecticidal compounds against agricultural pests (Ansante et al. 2015; Souza et al. 2017). Souza et al. (2019) demonstrated that the combination of ESAM and acetogenin-based commercial bioinsecticide Anosom® 1 EC had marked antifeedant and growth inhibitory activities on the larvae of *H. armigera*. Skuhrovec et al. (2020) prepared encapsulated formations of essential oils using anise (*Pimpinella anisum* L. [Apiales: Apiaceae]) against one of the major insect pests of potato, the Colorado potato beetle.

The strategy 'stimulo-deterrent diversion' (also called 'push-pull strategy') employs 'push' intercrop and 'pull' edge crop to protect crops from insect pests by promoting biocontrol agents. This strategy is applied to manage pea leaf weevils by applying neem antifeedant (push) to keep away the insect pest and edge planting of winter peas as trap crops (pull) to attract the insect pest (Smart et al. 1994). Aggregation pheromone can be applied on the edge trap crop to increase the attraction of insect pests. Clover can also be grown as trap crop instead of winter pea (Cook et al. 2007). Neem-based antifeedants (push) can be applied in stimulo-deterrent diversion strategy to control *L. decemlineata* by early boundary planting of trap crop (potato as pull) to attract the insect pests and natural enemies of the insect pests (Martel et al. 2005). The western flower thrips, *Frankliniella occidentalis*, are one of the major insect pests of greenhouse-grown chrysanthemums. The thrips were deterred from chrysanthemums by spraying the antifeedant procured from the plant, Dorrigo pepper on the main crop, and concentrating them onto trap plants (cv. 'springtime' of chrysanthemum is the most attractive) (Bennison et al. 2002).

Another approach is the joint action of antifeedant and insect growth regulators (IGRs) to control the insect pests (Griffiths et al. 1991). A blend of *Ajuga* spp. leaf extract (antifeedant) and teflubenzuron (IGR) was effective against *Phaedon cochleariae* (mustard beetle) and the larvae of *Plutella xylostella* feeding on mustard plants. The antifeedant inhibited feeding of the insects, while insect growth regulator did not inhibit feeding for the first 48 h of application, but caused the death of beetles and larvae after 2 weeks (Griffiths et al. 1991). The joint action of antifeedant and IGR is the application of antifeedant on the tender leaves of a plant and IGR on the lower leaves of the same plant. Application of antifeedant caused the beetles to move

on the lower parts of mustard plant, but when the insects were in contact with the IGR on the lower leaves of the plant, it resulted in death of the insect pests.

9.6 Phyto-Antifeedants: Prospects for Commercial Use

Till date, in excess of 1000 compounds of plant origin as antifeedants have been isolated and tested against a number of insect species, and more compounds are being added as antifeedants in laboratory conditions (Koul 2005, 2008). At present, the efficacies of the antifeedants in field conditions are very few due to variations in responses among different insect pests and habituation of insect pests towards antifeedants as well as quick degradation of the antifeedant compounds in the field conditions. A major concern is that most of the commercial synthetic pesticides are broad spectrum, and the antifeedants will be broad spectrum in characteristics like synthetic pesticides. Most of the phyto-antifeedants act only on a limited number of insect pests, and when these compounds are applied in the field, these antifeedant compounds can act on specific insect pests, but, on the other hand, the antifeedant compounds may not be effective, and other insects present in the field may be attracted towards the crop plant, which ultimately lowers the crop production. Further, the cost of developing a particular antifeedant for a specific pest is a big question. This is the reason that only neem as antifeedants is commercially available in the market.

Polygodial or methyl salicylate as antifeedants resulted in a reduction in aphid populations, and subsequently, an increase in the production of winter wheat was recorded in IARC Rothamsted. The reduction in aphid population after application of polygodial is equal to that of application of pyrethroid insecticide cypermethrin (Pickett et al. 1997). Another limonoid antifeedant, toosendanin, obtained from the bark of the toosendan and *M. azedarach* has got much attention throughout the world as a commercial biopesticide by the scientists (Chiu 1989; Isman 1994; Chen et al. 1995; Koul et al. 2002). Due to public awareness that botanical pesticides are safer than synthetic ones, the applications of botanical pesticides are increasing throughout the world. The production of biopesticides is estimated ca. 2% of the US $60 billion global pesticide market. However, microbial insecticides, such as products from *Bacillus thuringiensis*, dominate among the biopesticides. At present, the productions of biopesticides are increasing at a rate of 16% per annum, while the synthetic pesticides are increasing at a rate of 5.5% per annum (Miresmailli and Isman 2014). The use of some essential oils as biopesticides without regulatory review by the US Environmental Protection Agency (EPA) provided in the list [25 (b)] of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) has paved the way to commercialize some essential oils. Further research on the effects of antifeedants in the insect sensory systems and formulations of antifeedant compounds in such a way that these compounds could not be degraded in the environmental conditions as well as development of broad-spectrum antifeedant compounds similar to that of synthetic pesticides are needed to get the most effective results of phyto-antifeedants against insect pests in the crop fields.

9.7 Conclusions

Application of antifeedants from plant parts helps us to utilize plant defense mechanisms and subsequently, helps to reduce the use of synthetic pesticides. To get the best results by using phyto-antifeedants, the following criteria should be considered: categorization of the natural sources, maintenance of quality, adoption of standardization strategies and modification of regulatory constraints; if these four criteria are properly addressed, the phyto-antifeedants could be as competitive and successful as the synthetic ones. Limonene at lower concentration acts as an antifeedant, but this compound causes allergic reaction on the human skin at higher concentration. Hence, basic research in combination with field trials of the isolated phyto-antifeedant at different doses is necessary to get environment-friendly safe products for insect pest control. However, most of the research on phyto-antifeedants presents that crude plant extracts could act as antifeedant on a particular insect species in the laboratory. This is the major drawback of basic research on phyto-antifeedants, which should be avoided. It is better to identify the compound from plant sources, which acts as insect antifeedant. If it is not possible to identify the compound of interest, scientists should be in collaboration with farmers for application of plant-based crude extracts for insect pest control in the field, which is more valuable than that of laboratory studies. To obtain the best results of the application of phyto-antifeedants, it is prerequisite that (1) proper technique should be adopted to maintain the integrity of phytochemical mixtures; (2) development of broad-spectrum phyto-antifeedants, which is similar to that of synthetic ones in action and the production cost of phyto-antifeedants, would be lower than that of synthetic ones; and (3) application of advanced technologies and delivery methods, such as nanotechnology, and micro- and nano-encapsulation techniques may provide qualitative and quantitative release of phyto-antifeedants for insect pest control.

Points to Remember

- About 10% of the insect pests are major pests, and insect herbivores cause one-fifth of the world's crop loss per year throughout the globe.
- Four major and 26 minor crops are responsible for ca. 95% of human sustenance, indicating that many of these crop plants are grown for a long time.
- Application of phyto-antifeedants helps us to make use of natural plant defense mechanisms, which is essential to reduce the use of synthetic pesticides. However, it is prerequisite that phyto-antifeedants should have to be broad spectrum, like the available synthetic compounds.
- Most of the phyto-antifeedants are from 43 families of plants. However, four families—Meliaceae, Asteraceae, Labiatae and Leguminosae—are more exploited for identification and extraction of compounds, which are acting as insect antifeedants.
- The known phyto-antifeedants belong to groups, like various terpenes (monoterpenes, sesquiterpenes, diterpenes and triterpenes), flavonoids, alkaloids, coumarins, steroids, etc., and each species of insect may employ these compounds in an idiosyncratic manner, so that the same compound may have

altered fates in different species of insects, implicating that different mechanisms are involved in antifeedant action.
- The four criteria—categorization of the natural sources, maintenance of quality, adoption of standardization strategies and modification of regulatory constraints—are necessary to obtain the best results of the application of phyto-antifeedants.
- The formulation of antifeedant compounds including large-scale field trials would help to encourage farmers to use natural antifeedants.
- Phyto-antifeedants can be combined with natural plant substances, such as physiological toxins, to manipulate insect behaviour in integrated pest management strategy.

Acknowledgements I am thankful to Ms. Swati Das (UGC-SRF), Ms. Anamika Koner (SVMCM, NET-LS), Mr. Syed Husne Mobarak (CSIR-SRF), Ms. Paroma Mitra (Project fellow) and Mr. Rahul Debnath (UGC-SRF) for preparing figures and references of this chapter.

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Chemoecological Responses of Plants

10

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Contents

Abstract

Plants, though immovable, are able to defend themselves from insect pest attack through production of low molecular weight volatile organic compounds (VOCs). They also use these volatiles for crosstalk with other plants and insects for their growth and well-being. The volatile organic compounds (VOCs) help in communication in the trophic system. Three major biochemical routes are involved in the synthesis of VOCs; they are isoprenoid, lipoxygenase and shikimic acid pathways. The volatiles thus released by the plants become the

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Omkar (ed.), *Molecular Approaches for Sustainable Insect Pest Management*,
https://doi.org/10.1007/978-981-16-3591-5_10

'words' in their inaudible dialogues that need to be understood for improving plant defense mechanism.

Keywords

Chemical ecology · Herbivory · Pest management · VOC

Learning Objectives

1. Plants release volatile organic compounds during biotic stress.
2. The VOC released aid the plants to communicate amongst themselves and also provide message to pest and natural enemies.
3. Deciphering the VOC will aid to device better pest management strategies.

10.1 Introduction

Plants are subjected to biotic (herbivory) and abiotic challenges in their environment. Though plants are sedentary, they have excellent dynamic and wide-ranging metabolic capacities that help them to tide over biotic or abiotic stress over spatiotemporal scale. Apart from mechanical protection to combat herbivory attack that acts as structural barriers, plants also release an array of volatile organic compounds, like terpenes, benzenoids, phenylpropanoids and amino acid derivatives when subjected to stress, and this helps them to overcome the pressure and retain their vegetative and reproductive phase. The idea that plants are communicative came to light through the studies of Baldwin and Schultz (1983) who reported that potted poplar and sugar maple trees released airborne cues (volatiles) when their tissues were damaged that stimulated biochemical changes in neighbouring plants, forewarning them of phytophagous insect attack. Volatiles are complex chemical compounds of low molecular masses (<300 Da) possessing low polarity and high vapour pressure (0.01 kPa or higher at 20 °C) that allow them to easily travel through membranes, evaporate and travel over long distances in the atmosphere. Plants contain five or six biosynthetic group of secondary metabolites, and within each group there are structurally related analogues and derivatives that aid in defense. The chemical signals produced by plants facilitate in their interaction with beneficial and harmful organisms. Damage by herbivores and pathogens causes biotic stress in plants, which cause them to adopt a strategy to perceive the biotic interaction and then translate the perception to conducive defense (Heiden et al. 2003; Halitschke et al. 2004; Shiojiri et al. 2006; Allmann and Baldwin 2010).

10.2 Green Leaf Volatiles (GLVs)

Plants when subjected to attack by herbivore emit a blend of volatile organic compounds (VOCs) that are specially designed morphological structures or secondary metabolites, like terpenes, fatty acid derivatives, phenylpropanoids, benzenoids

and green leaf volatiles (GLVs) that are toxic, repellent or antifeedant to herbivores besides possessing antifungal activities (Heiden et al. 2003; Shiojiri et al. 2006; Gouinguené and Turlings 2002). GLVs consist of a family of C6 compounds, including aldehydes, alcohols and esters, which trigger the jasmonate dependent defense reaction. They are released by green plants and in response to damage caused abiotic stimuli (Hatanaka 1993; Halitschke et al. 2004; Gomi et al. 2003; Brilli et al. 2011), by herbivores (Fall et al. 1999; Turlings and Loughrin 1995) or pathogens (Croft et al. 1993; Heiden et al. 2003; Shiojiri et al. 2006). The emission of the volatiles is also influenced by factors like environmental conditions that include humidity, temperature and fertilization (Gouinguené and Turlings 2002). Undamaged plants emit traces of GLVs (Turlings and Loughrin 1995; Allmann and Baldwin 2010), but upon damage/stress their levels can rise (D'Auria et al. 2007). Repetitive wounding by herbivory or other biotic stress can lead to transient or sustained emission of GLVs (Loughrin and Manukian 1995). GLVs act as signals to induce resistance in undamaged neighbouring plants. They influence plant-pathogen interactions at varied levels. In addition to triggering the expression of wound response gene, they act directly by possessing antimicrobial activity. GLVs induce plant defense and trigger 'priming', which prepares the plant to respond to damage inflicted by pathogen or pests (Farmer 2001; Baldwin et al. 2006; Frost et al. 2008). In addition, the volatiles affect jasmonic acid signalling, and this, in turn, affects phytohormone dynamic equilibrium (Liu et al. 2012; Christensen et al. 2013). Plants' response to biotic and abiotic stress is best addressed due to the crosstalk between phytohormones in plants (Scala et al. 2013).

10.3 Green Leaf Volatile Biosynthesis

Metabolites of the lipoxygenase (LOX) pathway are associated with stress signalling in plants. The synthesis route of GLVs is via the hydroperoxide lyase (HPL) branch of the oxylipin pathway and is responsible for regulating the defense and developmental pathways in plants (Scala et al. 2013). Lipids from the membrane are converted to green leaf volatiles through LOX pathway (Blee and Joyard 1996). Membrane lipids when mixed with enzymes liberate the fatty acids, which supply the substrates for GLV biosynthesis (Matsui 2006). But the compounds are also produced upon stress without any mechanical damage as well as systemically from other parts of the plant; this demonstrates that mechanical damage is not necessary for their production and release (Matsui 2006).

10.4 Sampling and Analysing Volatile Organic Compounds from Plant

Understanding of the trophic interactions involves the understanding of biochemistry, physiology, ecology and chemistry of plant VOCs. This can be achieved when robust systems are in place to trap and characterize the volatiles (Millar and Sims

1998). Development of tabletop chemical detectors, like the gas chromatography-mass spectrometry (GC-MS), has enhanced the ability to analyse the volatiles emitted from plants in qualitative and quantitative terms (Tholl et al. 2006). The trapped compounds on elution with the appropriate solvent are concentrated and injected into the GC column through injector port. The compounds are separated based on their molecular weight and are detected. In case of MS, the compounds entering the MS are ionized by electron impact (EI), and the positively charged molecule fragments are selected according to their mass-to-charge (m/z) ratio by entering a quadruple ion trap. The sensitive MS can detect up to picogram range for the full-scan mode. Identification of compounds in GC-MS analysis is done by using the mass spectral libraries, such as Wiley and NIST MS databases (Tholl et al. 2006).

Improvements made in chemical detection have helped to elucidate and characterize the VOCs. This has helped to understand the chemical and biochemical aspects involved in signal-transduction pathways that are involved in the biosynthesis of the induced volatiles. This understanding of VOC has helped to exploit the chemo-ecological approaches to enable development of new crop varieties that have better ability to stand against the stress caused by abiotic and biotic factors.

10.5 Plant Volatile Sampling

In case of plant volatile sampling, the collection is done in situ from whole plants without causing stress to plants. If the collection is to be done in site-specific manner, then sampling is limited to parts of the plant, like vegetative or reproductive parts, as this would aid to differentiate the volatiles released from specific tissues (Tholl et al. 2006).

Volatiles surrounding the airspace (headspace) around the plant parts are subjected to dynamic headspace sampling, and this facilitates in estimating the real-time emission of the compounds from a plant at a particular situation. Dynamic headspace sampling of volatiles is superior to the solvent extraction, as this method would elute all the compounds, forming the matrix that would make it difficult to identify those compounds released due to herbivory that have ecologically relevant applications. Materials, like glass, metal and special plastics such as Teflon that are inert are to be used for volatile trapping (Tholl et al. 2006).

10.6 Sampling Volatile Organic Compounds in Static Headspace

In case of static headspace analysis, the plant or its parts are held in a container, and volatiles released by the plant matrix are collected on an absorbent. In this process, there is no flow of air over the matrix, and only the static air surrounding the plant surface is collected. The emitted volatiles are concentrated in the adsorbent, and this method prevents the addition of impurities from continuous flow to sampling device, which may hinder the detection of VOCs that are present in extremely low

concentrations. The advances in static headspace analysis are the solid phase microextraction (SPME) that is very simple to transport and use for collection of volatiles and then detect them at as low as ppbv (parts per billion by volume) range. In solid phase microextraction the volatiles are exposed to fibre coated with various adsorbents. The activated fibre when exposed to matrix traps the volatile organic compounds on completion of sampling the fiber needle is retracted into the syringe. When the sample is to be characterized, the fibre is pushed out and inserted into the injection port for thermal desorption. The advantage with the system is that there is no need to depend on solvents that contaminate the environment. Thermal desorption of VOCs also helps to do away with the impurities in solvent, which will interfere with sample analysis (Tholl et al. 2006).

10.7 Dynamic Headspace Sampling

Dynamic headspace sampling is a frequently used technique for VOC estimation. In this method, there is a continuous flow of activated charcoal-filtered air, which flows into the container housing the plant matrix to be sampled. In the outlet side, the adsorbent loaded in a glass vial collects the analytes that were released from the plant surface. The carrier gas is let out through the adsorbent tube. Care should be exercised to limit the trapping period as an extended period of trapping could lead to loss of the compounds trapped in the adsorbent. The problems encountered in static headspace system, like buildup of temperature, humidity and accumulation of deleterious volatiles, can be avoided due to continuous flow of clean air (Tholl et al. 2006). Plant volatiles are of low to moderate molecular weights (<250) and low boiling points (20–340 °C). These include alcohols, aldehydes, ketones, acids, esters, etc. The chemical characterization of these compounds is effectively by GC MS (Littlewood 1970; Crippen 1973).

10.8 Gas Chromatographic Separation and Detection of Plant VOCs

For GC analysis of VOCs, samples in solvents are injected into a port in GC having a split or splitless mode or by thermal desorption methods where the analytes are desorbed directly with a rise in temperature from 250 to 300 °C. In a two-stage thermal desorber, the volatiles eluted are concentrated in a cryotrap, which is then let into the injector port to the GC column (Lockwood 2001; Merfort 2002; Handley and Adlard 2005; Tholl et al. 2006). The compounds are separated when introduced in the fused silica capillary columns (e.g. DB-1, DB-5, CP-Sil 5) and the more polar polyethylene glycol polymers, including Carbowax 20M, DB-Wax and HP-20M. The flowrate with carrier gas is maintained at the optimum level to prevent proper elution. The compounds on separation in the column are detected using an array of detectors.

Following separation on a GC column, volatile compounds are analysed by a variety of detectors. Flame ionization detector (FID) is widely used to have a stable response and high sensitivity.

10.9 Role of VOC and C6 GLVs in Plant Defense

From the multitude of volatiles released by plants representing fatty acid derivatives, terpenes, indoles and molecules from other chemical families, ethylene (C_2H_2), a powerful activator of plant defense, was discovered first. Increasing attention to volatiles studies helped in identification of more chemicals released by plants that help them ward off insects and pathogens.

It was found that C6 compounds are the primary volatiles released by plants in response to biotic stress. Emission of (*Z*)-3-hexenal and its isomer (*E*)-2-hexenal by plants inhibits the growth of pathogenic bacteria and fungus, *Botrytis cinerea* (Kishimoto et al. 2008; Prost et al. 2005).

The burst of C6 aldehydes in wounding site acts as a barrier against the invasion by pathogens. Exogenous application of GLVs to undamaged plants results in switching on the defense-related genes followed by induction of secondary metabolites. In maize, treatment of GLVs resulted in accumulation of higher levels of endospermic jasmonates (Choudhary et al. 2008).

Though GLVs are beneficial to plant defense against herbivory, if accumulated it could also be toxic. (*Z*)-3-hexenal is toxic, and it is converted to stable (*E*)-2-hexenal. External application of C6 aldehyde at high concentration causes toxicity in plants. At lower concentration, they are converted to alcohols and esters. Upon wounding the plant releases (*Z*)-3-hexenal followed by (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate. In addition to conversion, the plants also neutralize the toxic compounds by forming conjugates with glutathione *S*-transferase.

There are numerous evidence to prove that biotic stress in plants leads to release of GLVs. Lima beans infected by *Pseudomonas syringae* release (*E*)-2-hexenal and (*Z*)-3-hexenol (Croft et al. 1993). Nicotiana infected with *P. syringae*, emits (*E*)-2-hexenal. The emission of GLVs in both cases starts 18–20 h postinfection (Whalen et al. 1991). The plausible reason for production of GLVs at this site is that they possess antimicrobial activity (Hamilton-Kemp 1992; Nakamura and Hatanaka 2002; Scala et al. 2013). The plant produces the GLVs after being invaded or wounded so as to decrease the infection and inhibit the growth of the pathogen (Hirano and Upper 2000).

MVOCs have influence on herbivores and higher trophic levels (Pieterse and Dicke 2007; Wenke et al. 2010). In a study on the response of maize to fungal pathogen *Setosphaeria turcica*, insect pest *Spodoptera littoralis* and parasitoid *Cotesia marginiventris* in the presence of 2,3-butanediol (2,3-BD)-producing bacteria, *Enterobacter aerogenes*, this also enhanced the resistance in maize to Northern corn leaf blight fungus, *Setosphaeria turcica*.

Herbivory damage to plants leads to release of blend of compounds called herbivore-induced plant volatiles (HIPVs) that have a role for defending the plant

or attracting the natural enemies of the pest or serving as repellents to the pest. HIPVs are volatile compounds released from plant matrix due to herbivory or other abiotic or biotic stresses. The release of compounds varies according to the type of insect damage, and the release pattern has a diurnal variation. The release of compounds is either used by plants to advertise its attack to the neighbouring plants so as to enhance the defense preparedness in the neighbouring plants. These compounds are also used as cues by natural enemies of the insect feeding on the plants. This is an indirect defense measure adopted by the plant to prevent its damage from the insects.

HIPVs range from terpenes, green leafy volatiles (GLVs), ethylene, methyl salicylate and other VOCs. GLVs consist of (*Z*)-3-hexenal, n-hexanal and (*Z*)-3-hexenol, (*Z*)-3-hexen-1-yl acetate and their isomers. GLVs play an important role in attracting the natural enemies; in few cases these GLVs are used as cue by the adult female to avoid laying the eggs from plants that are damaged by its conspecifics.

Methyl salicylate (MeSA) has been a component in headspace volatiles, and it serves as a vital cue for natural enemies like *Geocoris pallens* Stal., ladybird beetle and lacewing, *Chrysopa nigricornis*. Damage by Fall armyworm, *Spodoptera frugiperda*, leads to release of compounds that include methyl benzoate and methyl salicylate and are used as attractants for the natural enemies, like *Cotesia marginiventris*.

Insect herbivory causes systemic changes in the production of plant volatiles, particularly methyl salicylate, making bean plants, *Vicia faba*, repellent to aphids but attractive to aphid enemies, such as parasitoids. Such effects can also occur in aphid-free plants but only when they are connected to aphid-infested plants via a common mycorrhizal mycelial network (Babikova et al. 2013).

10.10 Priming for Plant Self-Defense

Priming aids to induce the defense in plants, and this is triggered by biotic agents, like the plant pathogens or herbivory or by applying molecules like salicylic acid, beta-aminobutyric acid and benzothiadiazole (Zimmerli et al. 2000, 2001; Yi et al. 2009; Conrath et al. 2006). SA and its analogue BTH induce a priming called systemic acquired resistance that is effective against a broad spectrum of pathogens (Hien Dao et al. 2009). The action of SAR differs from ISR in a manner that the priming triggered resistance in the latter type occurs when the beneficial bioagents colonize the root system of a plant, which enables the plant to provide resistance on aerial parts of the plant (Van Loon et al. 1998; Conrath et al. 2002). Priming occurs due to not only biotic challenges but also signals emitted by the conspecifics that are nearby or from the distal part of the same plant that are damaged by pathogens or herbivores (Heil and Kost 2006).

10.11 Conclusions

Volatile organic compounds are produced by all living organisms including plants, animals, human beings and microorganisms termed as biogenic volatile organic compounds (BVOC). Though produced in traces, the volatiles act as powerful communication signals among the interacting organisms, initiating a cascade of metabolic activities that help in their growth, development, reproduction and defense. Plants, though sedentary, employ volatiles without moving from their place to defend themselves from insect pests. Development of recent technologies that help in improved capture of the volatiles and their analysis aided by bioinformatics are helping us to understand the inaudible dialogues between plants and herbivory that aids in better way for strengthening the plant protection technologies that eventually will bolster the food security of the country in the coming years.

Points to Remember

- Volatile organic compounds are produced by all living organisms including plants, animals, human beings and microorganisms termed as biogenic volatile organic compounds (BVOC).
- VOCs released in traces act as powerful communication signals among the interacting organisms, initiating a cascade of metabolic activities that help in their growth, development, reproduction and defense in plants.
- Chemical characterization of the VOC aids to decipher the compounds that are released during biotic and abiotic stress.
- The volatiles released from plants subjected to herbivory aid to defend plants by communicating with its conspecifics by the VOC signal it releases and also by advertising the presence of pest to its natural enemies.
- Development of recent technologies that help in improved capture of the volatiles and their analysis aided by bioinformatics are helping us to understand the inaudible dialogues between plants and herbivory that aids in better way for strengthening the plant protection technologies that eventually will bolster the food security of the country in the coming years.

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Bioinformatics

11

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Contents

Abstract

Insects occupy the largest part of the phylum Arthropoda and manifest tremendous diversity; although some species of insects are beneficial, others are a problem to humans, as they reduce crop production, cause food losses and spread diseases. Therefore, it is important to maintain the pest population below the level of economic threshold to reduce the economic losses. Insect pests are developing resistance against insecticides; it became a challenge to improve understanding of the factors driving pest adaptation and evolution. With the surge of sequence information, researchers are accessing data to infer the biological questions and concentrate on genome sequencing to understand gene expression, gene regulation, quantification, genetic traits and gene disruption. Implementation of

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Omkar (ed.), *Molecular Approaches for Sustainable Insect Pest Management*,
https://doi.org/10.1007/978-981-16-3591-5_11

bioinformatics techniques are providing meta-omic tools for insect-microorganism interactions, synthesizing target-oriented insecticides and establishing pest's evolutionary history. In this proposed chapter, we discuss the efficacy and utility of bioinformatics techniques in insect pest management. For instance: (1) analysis of insecticide resistance proteins using a computational tool (DIRProt), an ACE tool for insect resistance mutations, and using bioinformatics tools to detect gene arrangement accountable for adaptation; (2) OffTargetFinder software provides species-specific RNAi design to manage insect pests, sterile insect technique with RNAi and InsectBase platform for comparative genomic analysis on gene families, pathways and orthologs; (3) ConFind (conserved region finder) is for conserved sequence analysis and interpretation, and CryGetter automates the retrieval of Cry protein; and (4) using the gene disruption techniques, clustered regularly interspaced short palindromic repeats (CRISPR), and population suppression techniques.

Keywords

Bioinformatics applications · CRISPR and RNAi techniques · Insect pest management · Sterile insect technique · Pesticide-target interaction database

Learning Objectives

1. Insect pests occupying the phylum Arthropoda manifest tremendous biodiversity and are of global concern, causing crop losses. Although, a majority of insect species are beneficial, others are a problem to humans because they adversely affect crop quality, cause heavy losses in yield and spread vectors of crop diseases.
2. The massive use of pesticides in pest and crop management adversely affects the environment and leads to resistance in insects involving multiple resistance mechanisms, leading to rapid evolution and adaptation.
3. Biorational approaches have a prospective and significant role in sustainable, eco-friendly safe pest management.
4. Bioinformatics researches provide a platform and offer plenty of information to reduce chances of sequence analysis errors and provide a new way to formulate novel species-specific insecticide.

11.1 Introduction

The prime aspect for sustainable insect pest management is to comprehend the biology and behaviour of pest species to conclude the kind of crop losses they produce and to secure a crop production system that enhances the utility of persistent eco-friendly approaches to protect the crops and achieve maximum yield without generating any aftereffects. Insect pests exhibit a wide range of feeding habits, such as piercing and sucking (e.g. psyllids, mirid bugs, aphids and coccids) and biting and chewing (e.g. caterpillars, sawfly larvae, webworms, leaf rollers, skeletonisers,

cutworms, flea beetles, blister beetles, cucumber beetles, diamondback moth (DBM), caterpillars, beetles, slugs and snails), that affect crop yield and spread diseases.

Biorational insecticides (derived from natural sources) play a significant role in sustainable, environment-friendly and less detrimental and safe pest control. A diverse range of insecticides used to suppress pest populations put some risks to beneficial insects and the environment. Insect pests manifest tremendous diversity, morphologically and genetically, they acquire rapid evolution and adaptation in adverse environmental conditions (Simon and Peccoud 2018). Pélissié et al. 2018 studied spatial and temporal genomics for understanding the mechanism of rapid evolution in insect pests.

The utility of biological methods, such as insect toxins produced by *Bacillus thuringiensis* (Bt) (Bravo et al. 2011), protease inhibitor (gut analysis) (Bapat et al. 2020), α-amylase inhibitors (Kaur et al. 2014), chitinase and cholesterol oxidase, contributed to an efficacious strategy in the insect control by engineering transgenic crops (Carriere et al. 2015; Krishna et al. 2016). In a study that was done on DBM, *Plutella xylostella* is one of the economically important lepidopterous pests that cause loss in vegetables including cabbage, cauliflower, broccoli, brussels sprouts etc. It is a global concern, mainly due to its acquired resistance to almost all chemical groups of insecticides applied for its control under continuous insecticide stresses. The development of biorational insecticides for insect pests to reduce crop losses and to discover sustainable and novel methods is great challenge for researchers. With advanced technologies, scientists are focusing on eco-friendly strategy for insect pest management.

Recently developed techniques emphasise the integration of molecular techniques and bioinformatics approaches (Singh et al. 2011; Iquebal et al. 2015) in the agricultural field to study the various aspects of insect resistance proteins, gene expression, sequence pattern of gene mutations, qualitative and quantitative analysis of proteins and their interactions at the genomic level. Moreover, bioinformatics approaches advance an intensive understanding of the structural and functional mechanism of biological processes. It encompasses statistical and mathematical techniques with computational algorithms, assists to retrieve genomic data information, an aids to manage the heap of biological data, visualisation and interpretation of wet bench outcomes.

A large number of genomes are sequenced, and more are in the pipeline; utilising genomic information along with synthesising it to discover new knowledge has become a key subject of advance biological research. Accessibility of genome sequences, expressed sequence tags (ESTs), genetic linkage maps and insect transgenesis provided new dimensions to detect and quantify selection in insect pest populations, to obtain the answers about the mechanisms behind rapid evolutionary changes for fundamental research in entomology.

Sachidanandam (2005) discussed the perspective of RNA interference (RNAi) pathways in transcription, post-transcription silencing, epigenetic silencing, related proteins and scrutinised occurring bioinformatics challenges. It is a universal mechanism in biological system and is friendly to use as a tool for forward genetics. RNAi

technique is sequence-specific; post-transcriptional gene silencing induced by double-stranded RNAs (dsRNAs) and small interfering RNA (siRNA) degrade the messenger RNA (mRNA) to inhibit gene expression. *Lasioderma serricorne* (cigarette beetles) are pests of stored tobacco. Knockdown of LsNAG2 (β-*N*-acetylglucosaminidase 2) in the fifth instar larvae of cigarette beetles led to the reduced expression of genes involved in chitin synthesis and impaired moulting and wing development (Yang et al. 2019; Christiaens et al. 2020).

Moreover, the sterile insect technique (SIT) is also useful in pest control involving mass rearing of reproductive gene silencing using RNAi approaches. Luo et al. (2017) studied the approachability and efficacy of RNAi technique in uncovering *Bemisia tabaci* (whitefly), a phloem feeder by dsRNA ingestion, to suppress the activity of RNAi-suppressing nuclease genes. Despite these genetic-based approaches used to design insecticides, bioinformatics tools are useful in engineering pest-specific insecticides to predict insect resistance proteins.

An emerging gene editing technique, i.e. clustered regularly interspaced short palindromic repeats (CRISPR), is utilised to convert susceptible insect alleles to insect resistance alleles and combat the evolving pests (Sun et al. 2017; McFarlane et al. 2018). Current progress in genome editing, especially with the emergence of CRISPR (Cong and Zhang 2015), enables the application of reverse genetics (work reverse from DNA or protein to synthesise a mutant gene) (Gurumurthy et al. 2016). However, the procedure requires time and effort to conclude suggestions of the linkage between genotypes with phenotypes. Cui et al. (2017) rigorously reviewed the applications of the CRISPR gene editing technique and reported the gene function and its interactions in insects.

In this chapter, we review a comprehensive exploration, utility and significance of bioinformatics approaches, used to rectify the problem of insect pests along with species-specific gene modification and pest-specific insecticide designing tools. Furthermore, we will discuss genomic, proteomic databases and gene expression profiles analyses, which play an important role in developing transgenic crop varieties and increasing crop productivity (Ives et al. 2011). Bioinformatics tools, such as DIRPROT (a web server) and acetylcholinesterase (ACE) (Guo et al. 2017) are applicable in detecting, retrieving and designing species-specific insect resistance proteins. It is necessary to understand pathways of resistance mechanisms involving insect resistance mutations, to detect gene arrangement accountability for adaptation. One such bioinformatics approach is CryGetter (Buzatto et al. 2016), a web tool to automate the retrieval of Cry protein (crystal protein) produced by *Bacillus thuringiensis*, which is lethal to the insects. The OffTargetFinder (Good et al. 2016, 2017) web tool provides nucleotide stretches that can be used to design species-specific RNAi to manage insect pest; InsectBase platform for comparative genomic analysis on gene families, pathways (Zhang et al. 2014) and orthologs; and ConFind (conserved region finder) (Smagala et al. 2005) for conserved sequences analysis and interpretation, using the gene disruption techniques. Lester et al. (2020) examined a potential genes drive to spermatogenesis in common wasp (*Vespula vulgaris*) which is an invasive species. CRISPR technique was used

as outcome depicts that gene drive could effect viable supression in wasp and other haplodiploid insects.

11.2 Physiological and Biochemical Pathways to Understand Mechanism of Insect Pests

Insect pests vigorously interact with abiotic and biotic factors. Thus, a better understanding of pest interactions and association with host plants may help in creating more effective pest management systems. Generating an understating of physiological, biochemical and signalling pathways involved during host plant-prey-predator interactions may help design more effective pest management pathways.

For example, PathCase (http://nashua.case.edu/pathways) (Elliott et al. 2008) provides an interface to store, analyse and visualise the metabolic pathways. It contains information at the genetic level, molecular level and biochemical level. MetaCyc (https://metacyc.org/) (Karp et al. 2002) provides a searching platform to access enzyme pathways and also contains a catalytic function of enzymes and substrate regulation. It adds information that can be used for genetic engineering, i.e. inserting, replacing and removing an enzyme in a pathway. Braunschweig Enzyme Database (BRENDA) (https://www.brenda-enzymes.org/) (Jeske et al. 2019) provides a broad range of enzyme-specific parameters. The annotated information of gene, protein and enzyme sequences can be obtained to retrieve targeted insect pest information. Improved tools and online servers are available for construction of pathways, viz. Insect Pathway Construction (iPathCons) (http://www.insect-genome.com/ipathway/ipathcons/ipathcons.php) (Zhang et al. 2014), Protein Analysis Through Evolutionary Relationships (PANTHER) (http://pantherdb.org/) (Mi et al. 2019), Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg/) (Du et al. 2014) and Gene Ontology (GO) (http://geneontology.org/) (Gene Ontology Consortium 2017), to decipher a molecular interaction from insect genome for gene function analysis.

InsectBase database (Yin et al. 2016) provides iPathCons interface which redirects the query from KEGG database to develop a pathway. Zhang et al. (2014) have constructed pathways of 52 molecular interactions in insects, also containing 37 sequences of genome and 15 sequences of transcriptome. All the above-mentioned pathways can be retrieved from iPathDB (Zhang et al. 2014), which enables searches on web server to access data. The database provides a great degree of information for entomology researchers.

Insect pathways involving disease-related insect hormones (juvenile and moulting hormones) (Yin et al. 2020), xenobiotic metabolism and wing development provide information about resistance mechanisms. RNAi pathways have multiple applications in crop protection via knocking down enzymes and proteins (Kola et al. 2015). siRNA pathway (siRNA-mediated), miRNA pathway (miRNA-mediated) and piwi-interacting RNA pathway (piRNA-mediated) play an important role in pest management. Defence against viruses and transposable elements is mediated via

siRNA pathways (McCue and Slotkin 2012), regulation of gene expression via miRNA pathways and suppression of germ line transposon expression via the piRNA pathway (Ozata et al. 2019). The primary function of these RNAi pathway genes may also vary among different insect species. In some coleopteran and dipteran species, insect pests favour the take-up dsRNA rather than siRNA, via clathrin-dependent endocytosis. In other species siRNA works significantly in other insect species (Pinheiro et al. 2018).

11.3 Comparative Genomics and Proteomics Analysis Tools in Insect Pest Management

Insect pests display extensive diversity at morphological level; however, it is preserved at genetic level. Worthwhile sequence analysis is an approach to introduce a genome, transcriptome and proteome to a wide range of analytical methods applied to understand its characteristics, such as comparative sequence analysis, conservative sequence patterns, evolutionary topology etc., to conclude structure, function and evolution of insect pests (Mitter et al. 2017). Dawkar et al. (2013) highlighted species particular metabolic pathways, which are exerted by insects to convert adulterants into less toxic substances or the flushing pathways from the insect body system. In studying insect proteins and its interaction and modifications with reference to insecticides, resistant insects can be characterised to decipher the molecular networks taking part in metabolism of detrimental compounds.

Sequence-based transcript expression profiling studies have revealed that during interaction of *Helicoverpa armigera* with various host plants (Celorio-Mancera et al. 2012), differential expression was identified for genes involved in primary and secondary metabolism, environmental response, cellular processes, xenobiotic metabolism and extracellular matrix receptor pathways (Celorio-Mancera et al. 2012). One approach studied the genome-wide response of cotton bolls infested with bollworm using transcriptomics and proteomics (Kumar et al. 2016). Comparative analysis suggests that both the proteome and genome were regulated differentially during bollworm infestation (Kumar et al. 2016). Genome tilling arrays and differential proteomics of *Tribolium castaneum* challenged with diflubenzuron revealed that UDP-*N*-acetylglucosamine, pyrophosphorylase and glutathione synthetase were significantly upregulated (Merzendorfer et al. 2012). The protein profiling studies in sweet potato whitefly, *B. tabaci*, have revealed a molecular basis for thiamethoxam resistance (Yang et al. 2013). Saadati and Toorchi (2017) studied plant protein accumulation in the gut of *Eurygaster integriceps* (sunn pest), using proteomics approaches to unravel plant-animal proteins, which provides a new opportunity for using insecticidal proteins as insecticidals in the transgenic wheat and barley production, and it has no adverse effects on other organisms. Sunn pest is a serious pest of wheat and barley crops.

Researchers have focused on the selection of insecticide resistance genes by analysing protein, genomic and proteomic databases. These databases provide enormous sequence datasets for comparative analysis of evolving genes, which are

accountable for resistance and adaptation. Databases accommodate a large number of biological data and also classify and organise related information about phenomics, genomics and transcriptomics of agriculturally important pest insects. Several generalised and specialised databases are available in public domain containing immense information used in mainly three areas of genomic and molecular research outcomes: molecular sequence analysis, molecular structural analysis and molecular functional analysis. The GenBank established in the 1980s and fast database searching algorithms, i.e. FASTA by William Pearson (Pearson 2016) and BLAST (Basic Local Alignment Search Tool) were developed by Stephan Altschul (Shah et al. 2018).

Several lepidopteran insect databases are publicly available, such as SilkDB (Wang et al. 2005; Duan et al. 2010; Lu et al. 2020), KAIKObase (Minami et al. 2009; Shimomura et al. 2009) and MonarchBase (Zhan and Reppert 2012).

1. **DBM-DB (Diamondback moth Genome Database)** (Tang et al. 2014) provides comprehensive search tools and datasets and accessible platform for researchers to study comparative genomics, biological gene interpretation and gene annotation of DBM insect pest. It contains assembled transcriptome datasets from multiple DBM strains and developmental stages and the annotated genome of diamondback moth (DBM), *Plutella xylostella*. The database also provides integrated datasets from publicly available ESTs from the NCBI, and another database, i.e. **KONAGbase** (Jouraku et al. 2013), enables access to DBM genome and putative gene sequences for comparative studies. Through sequencing, the DBM genome and stage-specific transcriptomes provide new mechanisms to control DBM along with a better understanding of its biology. Baxter et al. (2011) constructed a sequence-based genetic linkage map of the DBM genome using RAD-seq (restriction site-associated DNA sequencing). RAD sequencing facilitates genetic variant discovery by sequencing only the DNA flanking specific restriction enzyme sites, allowing orthologous sequences to be targeted in multiple individuals.
2. KONAGbase (Jouraku et al. 2013) also provides genomic and transcriptomic information of *P. xylostella*. It provides comprehensive transcriptomic and draft genomic sequences with useful annotated information with easy access web interface. It enables researchers in time-efficient manner to search for target sequences, such as insect resistance-related genes. Due to continuous update and additional genomic/transcriptomic resources, analysis tools are providing interface for further efficient exploration of the mechanism of insecticide resistance and the development of effective insecticides act with a novel mode of action for DBM control. Information provided by database is listed into four sets, i.e. (1) transcriptomic sequences of ESTs/mRNAs (37,340) and RNA-seq (147,370) contigs, which were clustered and assembled into unigenes (84,570 sequences), contig (30,695), pseudo singleton/RNA-seq contig (50,548) and singleton (3327) predicted proteins (84,562 sequences); (2) genomic sequences of (88,530) WGS (whole-genome sequences) contigs with (246,244) degenerate contigs and singletons from which de novo identified repeat sequences and

(34,890) predicted gene-coding sequences were extracted; (3) the unigenes and predicted gene-coding sequences were clustered, and (32,800) representative sequences were extracted as a comprehensive putative gene set; and (4) repeat sequences generated from the assembled and de novo sequences were identified from the WGS sequences by RepeatScout (6310 sequences) (Price et al. 2005). Some specific databases including SPODOBASE (Negre et al. 2006) an EST database, provide detailed information related to lepidopteran pest, *Spodoptera frugiperda*, as it affects up to 350 plant species accountable for extensive economic damage. The ESTs are withdrawn from five potent cDNA libraries, prepared from three different *S. frugiperda* tissues such as haemocytes, midgut and fat body. The Sf21 and Sf9 cell lines are deposited in the database. Sf9 cell line is also used to study pesticide resistance and produce heterologous proteins (Negre et al. 2006). The study of mentioned tissues scopes the significance of biological processes in immune responses and plant-insect interactions. SPODOBASE database accommodates 29,325 ESTs, which are annotated and clustered into non-redundant sets with 2294 clusters and 6103 singletons. Available database information can be used for better understanding of the functional genomics (gene function and interaction) and physiology and identification of new molecules targeted against lepidopteran pests that could be used as safe biopesticides for sustainable agriculture (Singh et al. 2011).

3. **Lepbase** (Challi et al. 2016) is a genomic resource database for Lepidoptera that supports genomic approaches to understand evolution (Dasmahapatra et al. 2012; Ahola et al. 2014; Derks et al. 2015; Zhang et al. 2016), speciation (Martin et al. 2013; Cong et al. 2016; Cheng et al. 2017), olfaction (You et al. 2013; De Fouchier et al. 2017), behaviour (You et al. 2013; Chardonnet et al. 2014; Knight 2014; Derks et al. 2015; Uiterwaal et al. 2018) and pesticide resistance (You et al. 2013) in a broad range of target species. Lepbase offers a core set of tools to make genomic data widely accessible including an Ensembl genome browser (Stalker et al. 2004; Fernández and Birney 2010; Fernández-Suárez and Schuster 2010; Zerbino et al. 2018), text and sequence homology searches and bulk downloads of consistently presented and formatted datasets.
4. **LepidoDB** (d'Alençon et al. 2017) is a centralised bioinformatics resource that was developed to facilitate the comparative genomics of two major lepidopteran pests, the noctuid moths *H. armigera* and *S. frugiperda*, by analysing synthetic relationships and genome arrangements. This database information system was designed to store, organise, display and distribute various genomic data and annotations of the above-mentioned three species. For example, LepidoDB provides automatic annotations with KAIKOGAAS (Shimomura et al. 2004) and comparisons to insect proteomes and UniProt (UniProt Consortium 2015). The alignments of transcript sequences, transposable elements predictions or results of the different cross-comparisons process to emphasise conserved regions and orthologous genes. The system was constructed using open-source software tools from the GMOD (Generic Model Organism Database) (O'Connor et al. 2008) including a Chado database (Mungall et al. 2007); GBrowse (Wang et al. 2006; Donlin 2009; Stein 2013), a simple but rapid

genome browser; Comparative Genetic Map Viewer (cMAP) (Fang et al. 2003), a graphical tool which facilitates the navigation within multiple maps of genome sequences; and Apollo (Lewis et al. 2002), an application for the genome annotation editor.

5. **ChiloDB** (Yin et al. 2014) is a database that provides explicit information of genome and transcriptome related to rice pest, *Chilo suppressalis* (Luo et al. 2016a, b). Recently obtained information of genomic and transcriptome sequence data are integrated with protein coding genes, RNA-seq microRNA and piwi-interacting RNAs (piRNAs), to store on the database. Moreover, it provides comprehensive search tools and downloadable datasets for comparative genomics and gene annotation of this important rice pest. ChiloDB contains the first version of the official SSB (striped stem borer) gene set, comprising 80,479 scaffolds and 10,221 annotated protein-coding genes. Additionally, 262 microRNA genes of SSB predicted from a small RNA library, 82,639 piRNAs identified using the piRNA predictor software, 37,040 transcripts from a midgut transcriptome and 69,977 transcripts from all the samples are mixed and integrated into ChiloDB. This is an open-source database that enables a continuous service, such as biology, evolution and control of SSB pest for researches. This is the very first database as per knowledge, which exclusively contains genomic and transcriptomic information of rice pests. RNAi technique revealed that salivap-3 is a key protein factor in forming the salivary sheath, while annexin-like5 and carbonic anhydrase are indispensable for *Nilaparvata lugens* (brown plant hopper) survival. These novel findings will significantly help to clarify the complex functions of salivary proteins in the physiological process of *N. lugens* and elucidate the interaction mechanisms between *N. lugens* and the rice plant, which could provide important targets for the future management of rice pests (Huang et al. 2016). Du et al. (2020) elucidated molecular control via genomic and genetic levels of insect resistance in rice.
6. **MODII (Molecular Database on Indian Insects)** (Pratheepa et al. 2018) has been designed based on three-level architecture of the client-server technology. This database gives sequence information collected from the NCBI (National Center for Biotechnology Information) (Brown et al. 2015; NCBI Resource Coordinators 2016; O'Leary et al. 2016; Winter 2017; Sharma et al. 2018) and the sequences from the Division of Genomic Resources, Indian Council of Agricultural Research (ICAR)-National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru, India, and other public domains. This database is available online at http://cib.res.in, the local server of ICAR-NBAIR, and is updated regularly. MODII has been listed (Fig. 11.1) into specific online databases. Some of the online data bases are:
   (a) The InsectPestInfo is a database on insects and other arthropods including invertebrates covering taxonomy, distribution, field identification, damage, natural enemies and sequence data developed under the National Agricultural Bioinformatics Grid (NARG). The database presently contains information on wheat, rice, millets, sugarcane, oilseeds, fibre crops, pulses, vegetables, fruits, tuber crops, plantation crops, spices and condiments,

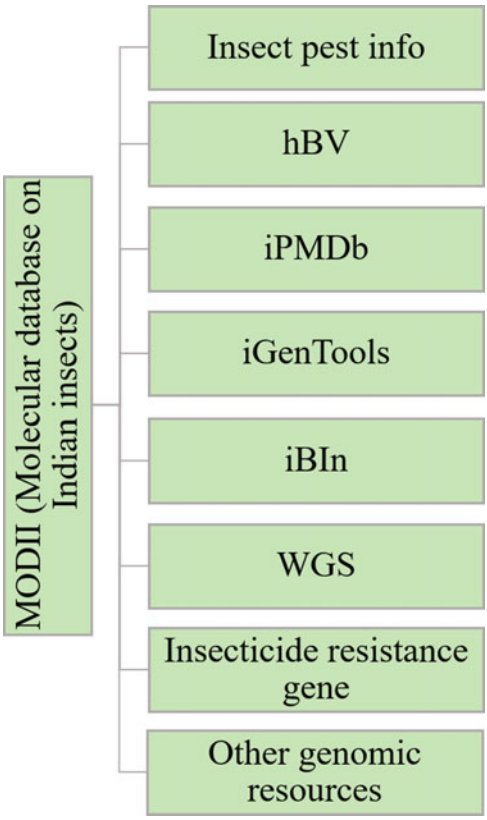


Fig. 11.1 MODII (Molecular Database on Indian Insects), containing eight interfaces including insect-related information

tobacco, ornamental, jatropha, mulberry and green manure on 358 species belonging to different ecosystems.

(b) The Insect Barcode Information System (IBIn) is an online database, it contains DNA-based species descriptions that could enable us to catalogue insects exsisting on earth quicker. It will be very useful especially to ecologists, conservationists and diverse agencies in charge of controlling pests and invasive species.

(c) Whole Genome Sequencing (WGS) database along with metadata and links have been established for 20 WGS of agriculturally important insects of different orders like Coleoptera, Diptera, Hemiptera, Hymenoptera and Lepidoptera to the NCBI website.

(d) iPMDbInsect Protein Model Database is under progress, which gives the 3D structure of insect protein prediction models. This helps to understand the insect protein structures, the target sites for the insecticides and the mutations in these proteins that caused the resistance towards insecticides.

(e) Honey Bee Viruses (hBV) database contains WGS of viruses that cause problems in honeybee development and production. This database hosts the complete genomic information on honeybee viruses, which infect different

species and honeybee populations in India. This is an important database, which initiated honeybee viral disease identification and management. Presently, this database contains seven Sacbrood virus WGS along with the metadata and WGS of acute bee paralysis virus, black queen cell virus, deformed wing virus, Kashmir bee virus, Sacbrood virus and Thai Sacbrood viruses.

(f) Insecticide Resistance Gene Database (IRGD). Managing insect pests is a challenge nowadays since agricultural pests are developing resistance against insecticides like organophosphates, synthetic pyrethroids, organochlorinates and other new groups. Insecticide resistance is a widespread phenomenon and leads to frequent and bulk use of pesticides that impact risk to the environment and organisms. The Insecticide Resistance Gene Database (IRGD) for important pests is essential to carry out molecular studies on insecticide-resistant genes, like cytochrome P450, acetylcholinesterase (AChE), knockdown resistance (KDR) and resistant to dieldrin (Rdl) gene. Hence, the Insecticide Resistance Gene Database (IRGD) has been developed, and this database helps researchers in designing novel molecules for overcoming insecticide resistance in agricultural pests. Presently, IRGD contains 851 sequences for the pests *Aphis gossypii* Glover, 1877 (Herron and Wilson 2011), *Acyrthosiphon pisum* Harris, *Bemisia tabaci* (Gennadius), *Helicoverpa armigera* (Hubner), *Plutella xylostella* (Linnaeus), *Spodoptera exigua* (Hubner), *Spodoptera litura* (Fabricius), *Nilaparvata lugens* (Stal), *Myzus persicae* (Sulzer), *Tribolium castaneum* (Herbst) and *Lucinodes orbonalis* Guenee with key features, like search, view, ORF Finder etc., and this database is updated regularly.

(g) Genomic tools (iGenTools) are necessary to carry out analysis on the sequence data, and hence some of the tools like calculation of GC and AT percentage, DNA to protein sequence (translation), reverse compliment and protein parameter analysis tool have been developed and included into MODII.

(h) Other Genomic Resources (OGR) of the National Bureau of Agricultural Insect Resources (NBAIR) has been developed for microbes for which genome sequencing has been done from the institute ICAR-NBAIR. Presently, it contains 203 accessions along with metadata. Links have been established for these accessions to the NCBI website. The metadata comprises metadata information, voucher information and organism classification. The biological database in agriculture (Lal et al. 2013) has been designed, and the sequence information is available in the local server of ICAR-Indian Agricultural Statistics Research Institute. Entomologists involved in molecular research can use this information for their research work. Different databases of MODII have been given, and the brief description of MODII is explained in this chapter.

7. **InsectBase** (Yin et al. 2016) intends to provide a comprehensive platform for researchers interested in analysing insect genes. The database contains more than 12 million of sequences, encompassing the genomes of 138 insects,

transcriptomes of 116 insects, gene sets of 61 insects, 36 gene families of 60 insects, miRNAs of 69 insects, piRNAs from 2 insects and pathways (22,536) of 78 insects, 679,881 untranslated regions (UTR) of 84 insects and 160,905 coding sequences (CDS) of 74 insects.

8. **AphidBase** (Legeai et al. 2010) previously was a web application for the analysis of aphid ESTs; now it has been upgraded to all-inclusive genome information resource related to aphids. Integrating the best attributes of different eukaryotic model organism databases, i.e. WormBase, FlyBase and VectorBase, it provides descriptive knowledge of aphids. It includes a genome browser for visualising genome annotation and robust search capabilities to retrieve the metabolic networks of aphids, their symbionts and phylogenomics for pea aphid, *Acyrthosiphon pisum*.
9. **WaspBase** (Chen et al. 2018) database helps in understanding the interactions of tritrophic systems and parasitic mechanism of wasps. However, the genomic resources for this tritrophic system (Francati 2018) are not well organised. WaspBase database contains information associated with transcriptomes (573) of parasitic wasps (35) and genome sequences of 12 parasitic wasps, 5 insect hosts and 8 plants. In addition, along non-coding RNA, untranslated regions and 25 widely studied gene families from the species genome and transcriptome data have been identified. WaspBase provides conventional web services, such as BLAST, search and download, together with several widely used tools, such as profile hidden Markov model (Skewes-Cox et al. 2014), multiple alignment using fast Fourier transform (FFT), automated alignment trimming and JBrowse (Buels et al. 2016; Hofmeister and Schmitz 2018).
10. **FlyBase** (Ashburner and Drysdale 1994; FlyBase Consortium 2003; Drysdale and FlyBase Consortium 2008; McQuilton et al. 2012; Thurmond et al. 2019) has provided a freely available online database of biological information about *Drosophila* species, focusing on the model organism *Drosophila melanogaster*. The need for a centralised, integrated view of *Drosophila* research has never been greater, as advances in genomic, proteomic and high-throughput technologies add to the quantity and diversity of available data and resources (Marygold et al. 2016).
11. **InSatDb** (Insect Microsatellite Database) (Archak et al. 2007; Archak and Nagaraju 2007) database unlike many other microsatellite databases that cater largely to the needs of microsatellites as markers presents an interactive interface to query information regarding microsatellite characteristics of five fully (fruit fly, honeybee, malarial mosquito, red flour beetle and silkworm) sequenced insect genomes. This database allows users to access microsatellites annotated in base pair size and sequence repeat units; genomic locations, i.e. exon, intron, upstream and transposon; nature and sequence base composition based on motif repeats; and % GC content. One can access microsatellite cluster information and a list of microsatellites with conserved flanking sequences. InSatDb accommodates complete information related to insects and also connects interface links to retrieve various details. A section could be used for sequence comparisons to illustrate the comparative genomic analysis of insect pests.

11.4 RNAi as a Bioinformatics Client

Sustainable agriculture depends on the approach and technology that integrates effectively with the least environmental aftereffect. RNA interference (RNAi), a eukaryotic process in which transcript expression is reduced in a sequence-specific manner, can be incorporated to suppress plant pests and pathogens. The application of RNAi to pest control is an attractive substitute to conventional chemical control, as it affects only target pest species. The pests for which this technology is being developed are beetles, moths, locusts and various phloem feeders including aphids (Fig. 11.2). Choosing a suitable method for delivering the dsRNA into insects depends upon the target gene. Consideration of the feeding behaviour of insects can provide an appropriate strategy for transferring RNAi inducing molecules. For non-autonomous cell, RNAi has high applicability, and its effects are of two types: environmental RNAi (eRNAi) (Ivashuta et al. 2015) and systematic RNAi (Cao et al. 2018). Spraying, soaking and microinjection are efficient processes to internalise the dsRNA in insects (Jacques et al. 2020).

Given the probability that RNAi-based technologies may be entering our agricultural landscapes soon, it is critically important to establish the species specificity of such RNAi-inducing molecules (Burand and Hunter 2013). Researchers have developed a bioinformatics tool that searches transcriptomic databases to assist RNAi-technology developers in increasing the pest specificity of RNAi molecules and provide information for regulatory authorities and the public on the relative environmental risks that these molecules have on non-target organisms. This web-based tool is called 'OffTargetFinder' (Good et al. 2016) (Fig. 11.3), and via various outputs it enables users to refine the regions within a gene of interest and to remove significant off-target effects in another arthropod species. It assists in recognising target species to be tested experimentally in the ecological risk assessment process (Romeis et al. 2013). SeedSeq (Das et al. 2013) is an off-target transcriptome database. Online Genome-wide Enrichment of Seed Sequence matches (GESS) tool extracts the seed sequences from active and inactive RNAi reagent sequences and then searches the transcript sequences for perfect matches. This software predicts miRNA off-target effects in large-scale RNAi screen data by seed region analysis (Yilmazel et al. 2014).

pssRNAit (Ahmed et al. 2020) is a web server for designing practical and specific plant siRNAs with Genome-Wide Off-Target Assessment (Ahmed et al. 2020). PFRED (Sciabola et al. 2020) is a computational platform for siRNA and antisense oligonucleotides design. MysiRNA provides an interface to construct a workflow for efficient siRNA design (Mysara et al. 2011).

siDirect 2.0 (Naito et al. 2009) provides functional, target-specific and off-target minimised siRNA design for mammalian RNAi. siRNA-Finder (si-Fi) (Lück et al. 2019) is an open-source software for design optimisation of RNAi constructs necessary for specific target gene knockdown. It extends the expertise in predicting RNAi datasets and off-target search, essential for the constructive applications of RNAi. si-Fi software can be used to customise sequence databases in standard FASTA format. Strand analysis (SA) is a free online software (Pereira et al. 2007)

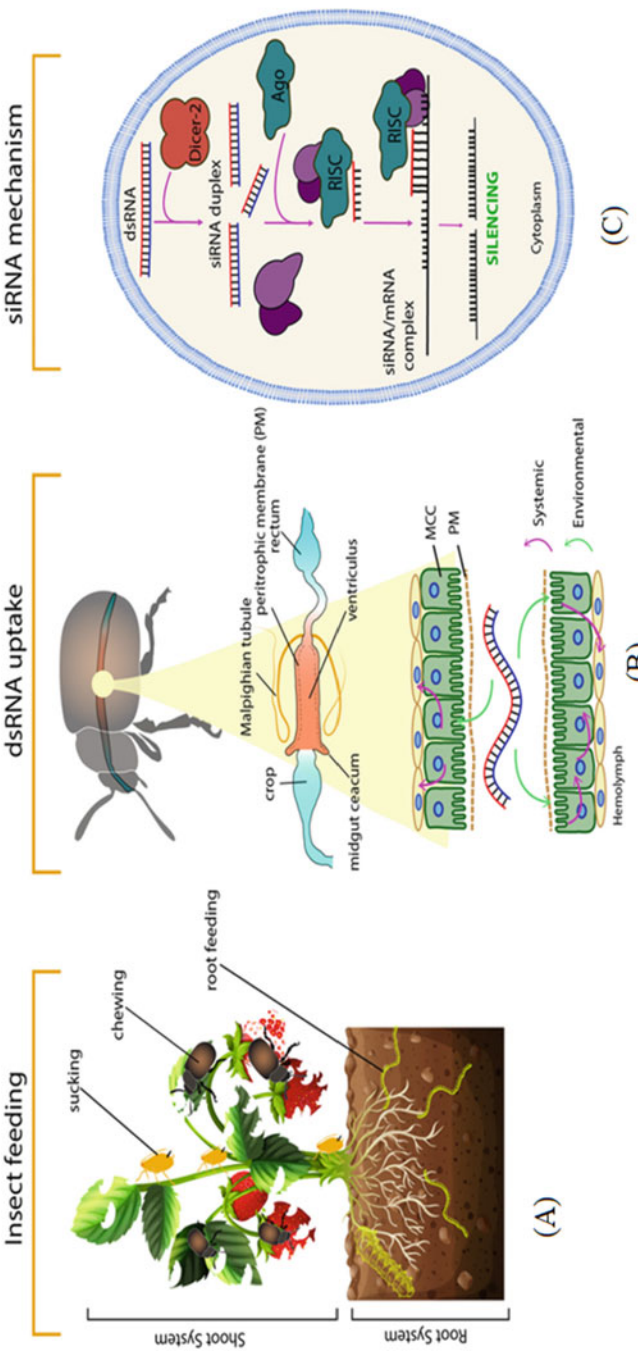
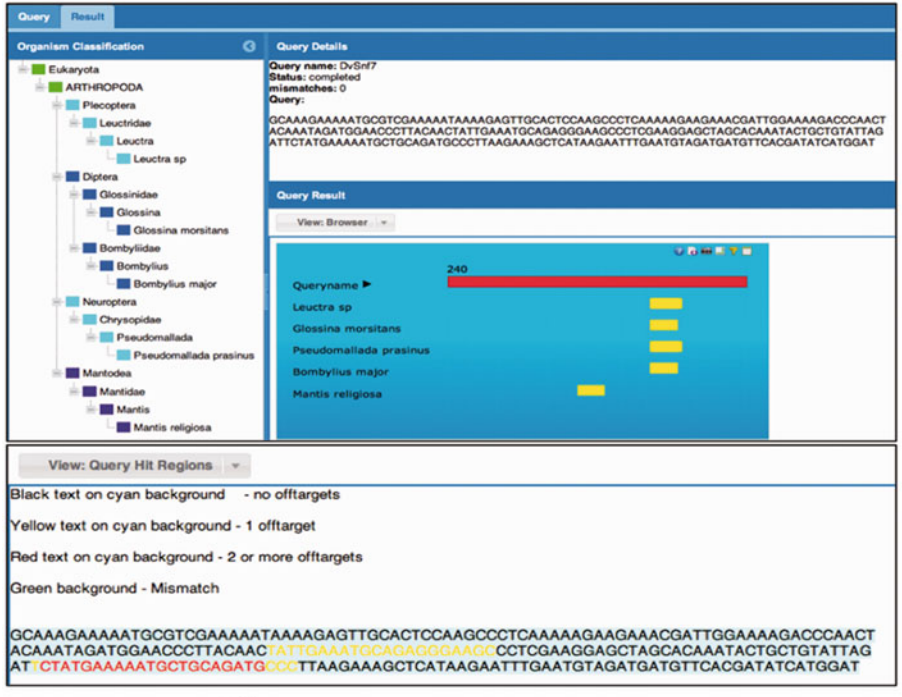
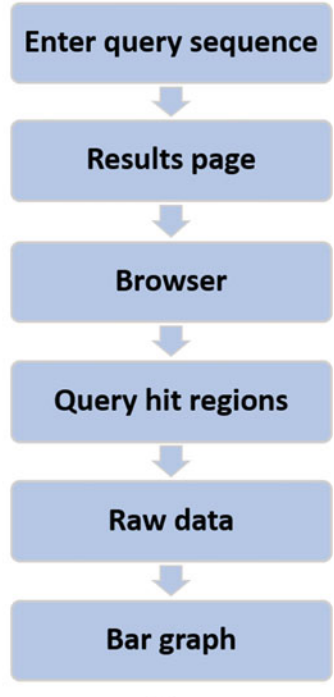


Fig. 11.2 Basic steps of RNAi technique in insect pest control (adopted from Joga et al. 2016). (**a**) Depicting the types of insect pests (i.e. sucking, chewing and root feeding), (**b**) effect of dsRNA uptake on insect, (**c**) siRNA mechanism of gene silencing



(A)



(B)

Fig. 11.3 Depiction of OffTargetFinder tool. (**a**) An example of *Snf7* gene from the beetle *Diabrotica virgifera* executed and displaying the hit in result section analysed for cross-species by Bachman et al. (2013). Along with five more species, Plecoptera (a stonefly), Diptera (specifically a tsetse fly and a bee fly), Neuroptera (a lacewing) and Mantodea (a praying mantis) hits were observed in query result section. (See cladogram on the left panel). (**b**) General algorithm in executing a query sequence and analysing results

for the identification of the best RNAi targets based on thermodynamics features (Pereira et al. 2007). siRNA Sequence Probability-Off-Targeting Reduction (siSPOTR) (Boudreau et al. 2013) is an easy interface and provides the user to paste or upload a template mRNA sequence (plain sequences/FASTA format) intended for knockdown and produces an output of candidate siRNAs/shRNAs arranged with the least off-targeting potential (POTS) at the top. The result provides the actual sense and antisense sequences to order, creating sequence modifications to improve loading of the suitable strand.

Sfold software is a user-friendly web server that enables access for the rational design of RNA-targeting nucleic acid, which includes siRNA, antisense oligonucleotides and trans-cleaving ribozymes for gene knockdown studies (Ding et al. 2004). Upon delivery into the cells, siRNAs are incorporated into the RNA-induced silencing complex (RISC) as a double-stranded RNA. RISC is the effector complex containing argonaute protein (Ago) with silencer activity (Naito and Ui-Tei 2012). It is essential to reduce the effectiveness for off-target effects (OTEs) by the meticulous design of dsRNAs. E-RNAi (Horn and Boutros 2010) and SnapDragon (http://www.flyrnai.org/) are examples of software that automatically design dsRNAs for use with RNAi and search for OTEs in a selection of well-referenced genomes. To design dsRNAs manually, dsCheck (Naito et al. 2005) can be used to predict potential OTEs.

1. **Sterile insect technique (SIT)** (Knipling 1955; Darrington et al. 2017) can also be implemented through RNAi technique (Whyard et al. 2015). The SIT depends upon the mass multiplication of sterile insects for release (conventionally males) that consequently mate with wild female individuals, which results in sterile mating and reduced offspring production (Knipling 1998; Krafsur 1998). Traditionally sterility is induced via irradiation, the results of which are additionally detrimental to insect fitness (Guerfali et al. 2011). A recently developed approach is the production of self-limiting (Kandul et al. 2019) genetically engineered insects which can be highly effective (Harris et al. 2011; Gorman et al. 2016; Carvalho et al. 2020). Genes that can induce sterility when knocked down can be targeted in adult insects for use with SIT. The principles by which RNAi might offer an alternative route for the induction of sterility, as well as other potentially useful manipulations for insect control, were recently investigated in a study using *Aedes aegypti* (Linnaeus) (Whyard et al. 2015).
2. **iBeetle-Base** is the database for RNAi phenotypes in the red flour beetles (Donitz et al. 2015) and provides access to sequence information and links for all *Tribolium castaneum* genes. The iBeetle-Base contains the annotations of phenotypes of several thousand genes knocked down during embryonic and metamorphic epidermis and muscle development in addition to phenotypes linked to oogenesis and stink gland biology. The phenotypes are described according to the entity, quality and modifier system using controlled vocabularies and the *Tribolium* morphological ontology (TrOn). It is used for studying insect typical development, the evolution of development and for research on metabolism and pest control.

11.5 Specific Tools to Design Insecticides

Agrochemical products are designed to protect plants from the invasion of insect pests, weed and fungi. However, the persistent use of pesticides causes development of insecticide resistance in insect population (Casida 2009). Moreover, most of these pesticides are broad spectrum with concerns for environmental safety and toxicity. In this advancing scenario, the requirement of efficient and ecological protection and the development of new agrochemicals approaches are essential.

1. **PTID (Pesticide-Target Interaction Database)** (Gong et al. 2013b) is an integrated web resource and computational tool for agrochemical discovery (Gong et al. 2013b), which comprises a total of 1347 pesticides with annotation of ecotoxicological and toxicological data as well as 13,738 interactions of pesticide target and 4245 protein terms via text mining. Besides, through the integration of ChemMapper (Gong et al. 2013a), a collaborative computational approach to polypharmacology, PTID can be used as a computational platform to identify pesticide targets and design novel agrochemical products. In addition to these data, several computational tools for target exploration and virtual screen were also integrated into PTID. A potential application of PTID includes identification of pesticides by structures or properties interest, prediction of potential targets or assessment of toxicity and environmental effect. As per knowledge, PTID is the first attempt to establish a pesticide database, which is integrated with the understanding of protein-protein interactions. It is expected that PTID will serve as a useful resource for the development of agrochemicals.
   Insecticide resistance and adaptation is a significant challenge discriminating the insecticide-resistant proteins from non-resistant proteins. DIRProt (http://cabgrid.res.in:8080/dirprot) (Meher et al. 2017) is a free, available online computational application to find resistance and non-resistance proteins. An online prediction server DIRProt has also been constructed for computational prediction of insecticide-resistant proteins. The algorithm uses a non-parametric approach and utilises support vector machine (SVM) approach, which is often used to recognise specific patterns. A query sequence of the protein in FASTA format used as input, an algorithm of DIRProt resulted from test patterns (Fig. 11.4) indicate that out of ten proteins only two have probability >0.5, then the predicted test protein will be considered as insecticide resistance else will be non-resistance.
2. **AChE (Acetylcholinesterase)** (Guo et al. 2017) is also used for detecting resistance mutations from genome re-sequencing data. The AChE approach is used to analyse RNA-seq datasets related to seven insect pests. This interface demonstrates that AChE can successfully identify resistance mutations from millions of reads (Fig. 11.5). The mechanism of AChE have been developed to find out the resistance mutations occurring in insect RNA-seq data. Mutations cause target insensitivity. AChE is the target of organophosphate (OP) (Houndété et al. 2010) and carbamate insecticides, which are used to control nearly all notorious agricultural and medical pests such as rice stem borers, Colorado potato

Fig. 11.4 Depicting the DIRProt web server. (**a**) Query protein sequences in FASTA format. (**b**) Example protein data sequences has been loaded. (**c**) Retrieved result of proteins on the basis of probability

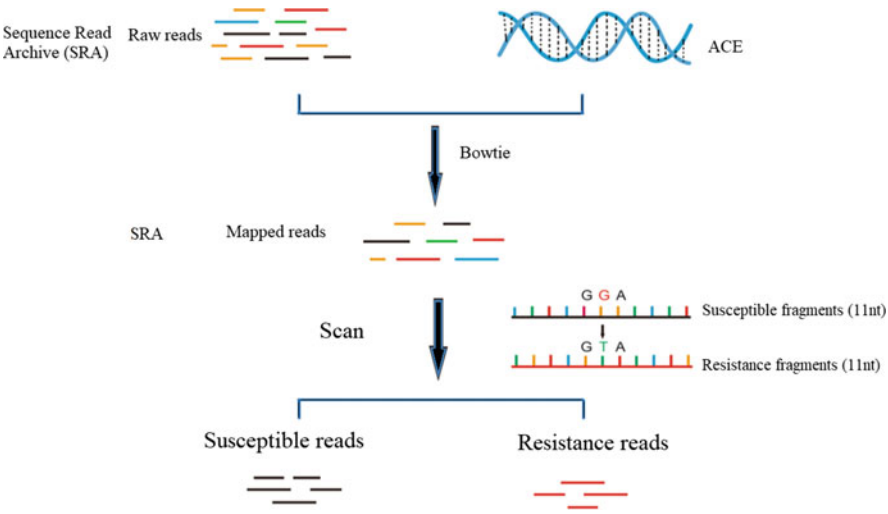


Fig. 11.5 Representing the mechanism of AChE (acetylcholinesterase) to detect susceptible reads and resistance reads. (Adopted from Guo et al. 2017)

beetles, mosquitos and houseflies. Two AChE proteins, i.e. Ace1 and Ace2, are identified in all the insects except the Cyclorrhapha suborder of Diptera.

3. **CryGetter**. *Bacillus thuringiensis* (Bt) is a bacterium, which is naturally present in soil and economically significant, as it produces crystal protein (Cry protein) toxic to insects (Fig. 11.6a). A software tool called CryGetter (Buzatto et al. 2016) is capable of retrieving data related to these proteins, storing it and presenting it in a user-friendly manner. This bioinformatics tool aligns a query protein sequence to detect and describe a statistical attributes-based alignment. It allows users to generate more accurate results since using it may prevent the error-prone task of manually getting all the necessary data and processing them in various software interfaces to retrieve the exact result generated by CryGetter in

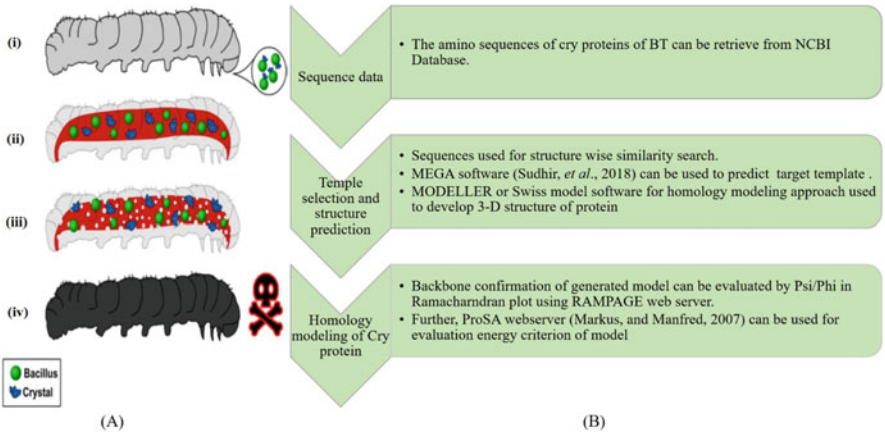


Fig. 11.6 (**a**) Cry protein mode of action (adopted from Buzatto et al. 2016). (i) ingestion of Cry proteins by target insect (ii) binding to specific receptor (iii) gut perforated by Cry proteins (iv) death of insect. (**b**) Algorithm for homology modelling of Cry protein and used bioinformatics softwares

an automatic environment. Since proteins are important and play a significant role in agro-industry, hence it is important to develop such type of computational tools to study the evolution of Bt Cry toxins and insecticidal activity (Bravo et al. 2011). Here are the general steps of methodology (Fig. 11.6b) for in silico modelling and functional interpretations of Cry proteins that enable to retrieve the required Cry protein sequences from databases along with protein sequence template selection web tools to derive three-dimensional models and homology modelling of achieved protein model (Wiederstein and Sippl 2007). Appropriate Cry protein sequences can be retrieved from the National Center for Biotechnology Information (NCBI) database, a public domain. Currently, 13,136 Cry protein sequences of Bt are available on the database. Homologous protein is used as a template designed by executing protein sequences with IntFOLD (McGuffin et al. 2019), an integrated server for protein structure modelling. Alignment software, i.e. between the template and target homology detection and structure prediction by HMM-HMM comparison (HHPRED), can produce pairwise query-template alignments, multiple alignments of the query with a set of templates selected from the search results, as well as three-dimensional structural models that are calculated by the MODELLER software (Webb and Sali 2016) from these alignments. The midgut aminopeptidase N (APN) of pest insect is a receptor for *Bacillus thuringiensis* Cry1 toxin (Pardo-Lopez et al. 2013). The development, design and synthesis of novel Cry toxins and improvement in harmful activities depending on the conserved structures, may contribute to the management of insect resistance in the field (Shokry et al. 2012).

4. **CryProcessor** (https://github.com/lab7arriam/cry_processor) (Shikov et al. 2020) is an open-source platform that allows precise mining of 3D Cry toxins.

CryProcessor allows to search for sequences of Cry toxins proteins directly and also predict the domain layout of arbitrary sequences. One strategy to overcome this difficulty is to extend the diversity of Cry toxins used in agriculture, by a comprehensive search for new toxins. Another approach implies designing artificial toxins. Bt toxin scanner allows searching and extracting a new Bt toxin from a set of biological sequences, though it has limitations for a large number of sequences. It does not provide information about domain structures. CryProcessor uses two modes of search, i.e. find domains and domain only, which retrieve results using HMM algorithm to extract complete toxins with domain structure, and predict protein-specific domain. The tool can be launched with FASTA format by default with PathRacer mode, and SPAdes implies genome assembly.

11.6 Pest-Specific Insecticide Design with In Silico Tools

The approachability of high-throughput screening (HTS) data from easily accessible biological databases makes it possible to utilise in silico target prediction mechanism to suggest the mode of action of a compound via mining of bioactivity data.

Docking programs and their algorithm (Fig. 11.7) detect new sights of insecticide resistance. In silico bioinformatics tools draws focus towards the biochemical mechanism of insecticide resistance. As discussed above AChE is an important protein in inducement of insecticide resistance. Houndété et al. (2010), Tilve et al. (2014) and Herron and Wilson (2017) conducted a study on *A. gossypii* and *B. tabaci*

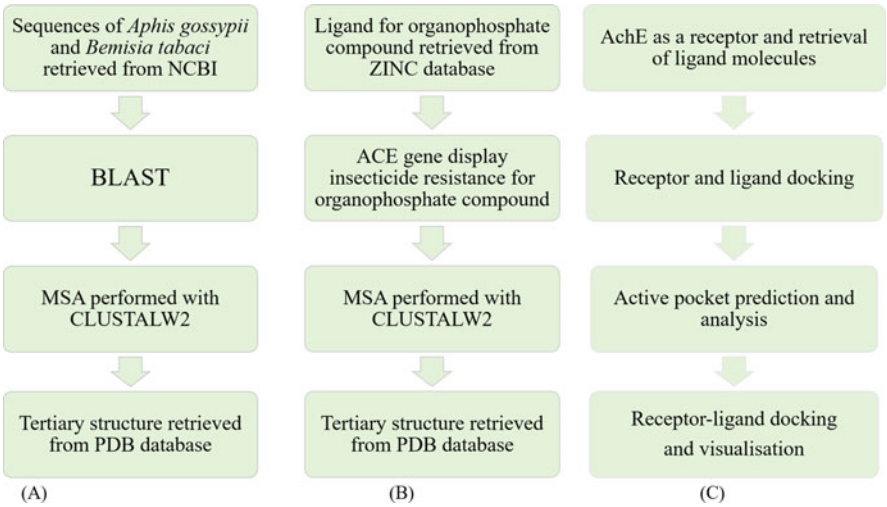


Fig. 11.7 Algorithm of In silico design of insecticide (**A**) Acetylcholinesterase (AchE) gene sequence retrieval (protein) (**B**) Organophosphate compound retrieved from ZINC database (**C**) As a result ACE gene display insecticide resistance for organophosphate compound

to evaluate AChE mode of action in resistance. AChE genes were checked for in silico docking with eight insecticide compounds (monocrotophos, acephate, mevinphos, chlorfenvinphos, dicrotophos, crotoxyphos, dichlorvos and heptenophos). AChE is a receptor *A. gossypii* and *B. tabaci*, which helps to directly consider the protein structures as a receptor molecule for this study. Here are the algorithm of insilico design of insecticide comes under three steps (Fig. 11.7a): (A) AchE gene sequence retrival from database (B) Ligand retrival from database (C) Result interpretation. Acetylcholinesterase (AChE) gene sequence retrieval (protein), AChE sequences of *A. gossypii* and *B. tabaci* were retrieved from public domain NCBI. BLAST was used to find regions of similar sequences, as the programme compares sequences against query to get the results. CLUSTALW2 was implemented to find similarity and conservation with MSA (multiple sequence alignment) approaches. Tertiary protein structures of *A. gossypii* and *B. tabaci* were obtained from PDB (Fig. 11.7b). Ligands, i.e. organophosphates, were retrieved from ZINC database that enables access to compounds for structure-based virtual screening. As a result, AChE gene displays insecticide resistance for organophosphate compound. Retrieval of ligand and receptor molecule, Protein-ligand interaction/docking, Discovery studio tool (Studio Discovery 2008), visualisation tools were used to inspect the protein-ligand interaction and prediction of active pocket (Fig. 11.7c). Here this study examined AChE genes of *A. gossypii* and *B. tabaci* insects and conducted the in silico docking with the eight insecticide compounds and found that two compounds (tetrachlorvinphos and dicrotophos) based on affinity to the receptor are significantly docked.

11.7 DNA Barcoding in Invasive Insect Pest Identification

Invasive insects draw attention as they affect ecosystem stability of native species. Conventionally, species identification relies on morphological traits (Khamis et al. 2012). However, taxonomic identification in itself is not worthwhile in some cases of invasive species as life history details are required (Gariepy et al. 2014). DNA barcoding and implementation of small genomic sequences as markers can be used for species identification. DNA barcoding is a standardised molecular identification method with numerous applications that have been used extensively to identify immature life stages of insects. The Barcode of Life Data Systems (BOLD) (Ratnasingham and Hebert 2007) is a publicly accessible domain providing a reference library and analytical capabilities for DNA barcode projects. The robustness of the BOLD database system is in the integration of information along with metadata chromatogram catalogue, the position of specimen voucher and the occupancy and location of the long-lasting depository of DNA. A significant characteristic of the BOLD system is the barcode index number (BIN). BINs are molecular operational taxonomic units (MOTU) generated by the refined single linkage (RESL) algorithm, based on available BOLD data. BINs contribute interim taxonomic identifications or species classification, established on a molecular barcode. The efficiency of DNA barcoding assist in the identification of specimens can be

estimated by providing taxonomic resolution (Federhen 2012). This can include specimens that can be identified to the species level or specimens that are grouped into an interim taxonomic framework (i.e. BINs).

To test the use of DNA barcoding in the identification (Kress and Erickson 2012) of insect specimens, a set of predominantly immature microlepidoptera from the superfamilies Tortricoidea and Gelechioidea was examined. Both Tortricoidea and Gelechioidea were found to be diverse, containing numerous regulated and economically important species, many of which are represented in BOLD (Madden et al. 2019). The first approach is to conclude the similarity between morphological characteristics and molecular-based identifications for intercepted microlepidoptera. The second objective is to develop a framework for the use of DNA barcoding and BIN interim taxonomy with respect to border identification protocols for intercepted insect specimens (Madden et al. 2019). Garzón-OrduñaI et al. (2020) identified the larval and pupae of tephritid fruit flies with Sanger DNA sequencing and single-molecule real-time sequencing (SMRT) approach along with bioinformatics tools to compare generated sequences. DNA barcoding based on cytochrome oxidase I (COI) sequences has shown that *Eurygaster integriceps* differs significantly from these closely related species, which enables its rapid and accurate identification (Syromyatnikov et al. 2017). Kang et al. (2019) conducted a study with 581 samples of quarantine insects via random searching on containers of foreign shipping vessels to identify lepidopteran insects using DNA barcoding. Barcoding of *Spodoptera* species assignment is an effort to expand the barcoding database to become broader and representative of the relevant domestic and exotic species. It is challenging to distinguish *Spodoptera* by morphological characteristics as it is possible to consider non-*Spodoptera* species, e.g. members of the *Mythimna* and *Helicoverpa* species complexes, whose juvenile stages feed on many of the same hosts, such as *Spodoptera litura* (tobacco cutworm) and *Spodoptera littoralis* (*Egyptian cotton leafworm*). The barcoding database has especially been useful in monitoring invasive *Spodoptera* and other lepidopteran pests in the United States (Nagoshi et al. 2011). A major feature of DNA barcoding is that it allows prompt identification of pest young instars, as well as of fragmentary cuticular segments. Recognising the early signs of pests in order to deal with the problem is crucially important. Therefore, the accurate taxonomic identification is a pivotal issue in biological research, in order to allow the implementation of agricultural importance. Moreover, misidentifications could lead to unsuccessful control measures that efficiently increase the impact caused by a particular pest species (Karthika et al. 2016). Accurate species identification is necessary for cost-effective pest control strategies. Conflitti et al. (2013) tested the utility of COI barcode for identifying members of the black fly genus *Cnephia* Enderlein (Diptera: Simuliidae).

11.8 Transgenerational Effects of Insecticide and Implication for Rapid Pest Evolution

Evolutionary processes that give rise to insecticide resistance drive the evolution of insect pests. Insecticide resistance has been widely observed to increase with frequent and intense insecticide exposure, but can be lost following the relaxation of insecticide use. One such approach to understand insecticide resistance is associated with epigenetic modifications, as it impacts the gene expression patterns without altering the base composition of DNA (Chari et al. 2010). Epigenetics is the field of study that examines how environmental factors influence heritable change in gene expression. Several epigenetic mechanisms are heritable and could underlie the transgenerational effect of insecticides. Epigenetic modifications, such as DNA methylation, histone modifications and small RNAs, have been observed to be heritable in arthropods (Herman et al. 2014), but their role in the context of the rapid evolution of insecticide resistance remains poorly understood. It is likely that (1) insecticide-induced effects can be transgenerationally inherited (Brevik et al. 2018), (2) epigenetic modifications are heritable (Collotta et al. 2013) and (3) epigenetic modifications are responsive to pesticide and xenobiotic stress. Therefore, pesticides may drive the evolution of resistance via epigenetic processes (Burggren 2016). Resolving the role of epigenetic modifications in the rapid evolution (Mendizabal et al. 2014) of insect pests has the potential to lead to new approaches for integrated pest management and improve our understanding of how anthropogenic stress may drive the evolution of insect pests. O'Neal et al. (2018) described insecticide resistance as an example of (1) adaptability of insect pests, (2) in the design of resistance pest management programme and (3) a significant application of evolutionary biology. Epigenetic modifications, such as DNA methylation, histone modifications, and small RNAs, have been observed to be heritable in arthropods, but their role in the context of the rapid evolution of insecticide resistance remains poorly understood.

11.9 Conclusions

Bioinformatics with its comprehensive approaches and techniques has intervened in all the branches of science, i.e. biomedical research (Luo et al. 2016a, b), clinical medicine, drug discovery and development (Macarron et al. 2011), evolutionary studies, crop improvement (Arora and Narula 2017), microbial applications (Young et al. 2012), comparative studies and insect resistance and pest management (Valadez-Lira et al. 2012; Huang et al. 2017). Development and implementation of bioinformatics techniques provide a structural and functional understanding of the biological processes. In this chapter, we discussed the implementation and uses of bioinformatics in pest management. Strategies to prevent such damage and losses caused by insect pests can increase production and substantially contribute to food security. Concluding remarks of this chapter are as follows: (1) DNA-based technologies are likely to greatly increase pest detection speed, sensitivity and

accuracy. (2) Biological and physiological pathways coordinated by various hormones and neuropeptides (Schoofs et al. 2017), such as moulting and metamorphosis, are regulated by steroid and juvenile hormones (Cheong et al. 2015), respectively. Hence, alteration in hormone and neuropeptide responsible for the growth and development of insect pest can prove a view to suppressing the population (Kyrou et al. 2018). (3) Biomarkers of crop damage and disease, such as volatile chemicals, may be also useful in detecting pest outbreaks (Runyon et al. 2020). (4) Utilising the bioinformatics techniques to understand pest's evolutionary history, synthesising target-oriented insecticides to control pests and meta-omics tools to understand insect-microorganism interactions. (5) With the flood of sequence information, researchers retrieving data to access the biological answers much concentrate on genome sequencing to understand gene expression, gene regulation, quantification, genetic traits and gene disruption. (6) RNAi and CRISPR techniques are prime, efficient, eco-friendly approaches in insect pest management. (7) Evolution of insect pest via epigenetic processes. (8) In silico tools are applicable in detecting the insecticide interaction with the protein molecule. (9) DNA barcoding technique is useful in the identification of invasive pest species. Thus, bioinformatics approaches providing dimensions in insect pest management are time-efficient and less error-prone. These bioinformatics tools and techniques can develop a firm platform to develop an efficient strategy against insect pests.

11.10 Future Prospects

The study of insect pest management and understanding the physiology and biochemical mechanisms are vital to develop an ecofriendly cure. The utility of pesticide successfully suppresses the pest, but it negatively affects other organisms. Researches working on an eco-friendly cure to suppress the pest population, biopesticides, focus on the mode of action, including mechanisms to replace the use of chemical pesticides. Synthetic pesticides are highly specific and have less adverse effects on the environment and organism diversity. Recent tools, including semiochemicals and plant-incorporated protectants as well as botanical and microbiologically derived chemicals, are playing an increasing role in pest management, along with plant and animal genetics, biological control, cultural methods and newer synthetics. A biological tool such as developing Bt crops can be successfully established in agriculture, which may result in the reduction of the use of pesticides, and this is an eco-friendly technique. Hence, it is preferable to concentrate on utility and development of biological method or to integrate them with conventional methods. Biopesticides are dragging attention because of usability and improved application methods and eco-friendly and cost-effective formulations. Therefore, biopesticides are a rational choice for pest management, especially as an improved balance between cost and efficiency becomes a reality in the near future. Developing a new required approach for insect pest control that has less impact on the environment, here are some genome editing and gene silencing techniques on which researchers are continuously working on: CRISPR and RNAi (Kola et al. 2015).

These strategies are efficiently useful. CRISPR is a gene editing system; it can upgrade the inheritance of the gene drive implemented through sexual reproduction and thus can be spread shortly through the population. RNAi, a gene silencing technique, is a sequence-dependent approach with high target specificity. It is developing new eco-friendly methods for insect control that reduced negative impact. SIT and genetic elimination methods, e.g. RIDL techniques, are gene driving procedures; they help in population suppression and population replacement. Understanding the pest's evolutionary biology can be an effective approach in pest management and insect resistance and may drive useful insights to reduce potential problems. Evaluation of the topology and interaction between pests and host plants possibly can help to find a solution in pest suppression. Insect resistance is a prime factor that slows down the insecticide mechanism; hence, it is essential to infer the topology of insecticide resistance. One such approach to designing species-specific insecticide is the sequence-dependent approach; bioinformatics tools, such as PTID, DIRProt, CryGetter and AChE, are providing an efficient strategy to develop a new insecticide. In silico docking method may be considered to detect the interaction between protein and pesticide compounds to check the compatibility and significance.

Points to Remember

- Insects possess a tremendous diversity, such as size, structure and behaviour, and are considered as most successful due to their biological characteristics.
- Biopesticides have the merits to act and play an important role in sustainable, eco-friendly, less effective, safe pest control. A diverse range of insecticides use, chemical mode of action, may help manage the pest populations while causing less risk to eco-friendly insects and the environment.
- The utility of biological methods, such as insect toxins produced by *Bacillus thuringiensis*, protease inhibitor (gut analysis), α-amylase inhibitors, chitinase and cholesterol oxidase, contribute a productive strategy in insect control by engineering transgenic crops.
- Transgenic crops expressing insecticidal toxins are widely used. The economic benefits of these crops would be lost if toxin resistance spread through the pest population. The primary resistance management method is a high-dose/refuge strategy, requiring toxin-free crops, as toxin doses sufficiently high kill wild-type insects and insects heterozygous for a resistance allele.
- Recently developed techniques emphasise the integration of molecular and bioinformatics approaches in the agricultural field to study the various aspects of insect resistance proteins, gene expression, sequence pattern of gene mutations, qualitative and quantitative analysis of proteins and their interactions on a genomic level.
- RNAi technique is sequence-specific, post-transcriptional gene silencing induced by dsRNAs and siRNA that degrade the mRNA to inhibit essential gene expression.
- Aside from these genetic-based approaches used to design insecticides, bioinformatics tools are also useful in designing pest-specific insecticides to predict insect

resistance proteins. CRISPR gene editing is an emerging technique to convert insect sensitive alleles to insect resistance alleles to combat the evolving pests.

- Engineered strains of agricultural pest species including moths such as the diamondback moth, *Plutella xylostella*, and fruit flies, such as the Mediterranean fruit fly *Ceratitis capitata*, have developed lethality that only operates on females.
- Sterile insect technique is an area-wide pest control method that reduces agricultural pest populations by releasing mass-reared sterile insects, which then compete for mates with wild insects. Contemporary genetic-based technologies use insects that are homozygous insects for a responsible dominant lethal genetic construct rather than being sterilised by irradiation. These transgenic insect technologies could form an effective resistance management strategy.

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Nanotechnology in Insect Pest Management

12

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Omkar (ed.), *Molecular Approaches for Sustainable Insect Pest Management*,
https://doi.org/10.1007/978-981-16-3591-5_12

Abstract

Nanotechnology has a wide range of applications in the stream of agriculture, medicine, pharmaceuticals, etc. In recent decades, it has become one of the most important technological interventions, especially in agriculture. The present-day agriculture is facing several bottlenecks in maintaining productivity in shrinking resources. On the other hand, crop losses from insect pests are increasing. Different chemical-based pesticides used in crop protection are directly or indirectly affecting living beings and the environment. Different ways were worked out to solve this problem, and nanotechnology is one of the most effective methods in the management of insect pests in agriculture. This technology helps in increment in crop yield along with plant protection against a variety of biotic and abiotic stresses. Different nanoparticles (NPs), such as Ag NPs, Au NPs, $Mg(OH)_2$ NPs, magnetite NPs, and essential oil NPs, are being used for insect pest control. These nanoparticles are formulated in lipid, polymer, clay, metal, and other nanoformulations for better delivery of active ingredients. Key benefits of nanopesticides are low dose, high active ingredient loading, slow and controlled release, biodegradable, reduced losses, protection against photodegradation, etc. There is a long way to go in nanopesticide research in pest management, and different ways are looked upon for reducing off-target effects and other demerits of nanopesticides. Therefore, to sustainably protect plants from insect pests, the use of bioconjugated nanomaterial-based insecticides and pesticides could be a viable option that would be desirable in precision farming.

Keywords

Nanotechnology · Nanopesticides · Nanoformulations · Nanocarriers · Insect pest control

Learning Objectives

1. Exploring potential roles of nanotechnology in insect pest control, especially when crop losses from insect pests are one of the key bottlenecks in maintaining productivity and the use of conventional pesticides has environmental concerns.
2. Understanding nanotechnology as an alternative to conventional pesticides in terms of pest control efficacy, cost of operation, and environmental sustainability.
3. Delving into pesticidal properties of different nanoparticles (NPs) such as Ag NPs, Au NPs, $Mg(OH)_2$ NPs, magnetite NPs, and essential oil NPs.
4. Elaborations on lipid, polymer, clay, metal, and other nanoformulations for better delivery of active ingredients.
5. Highlighting advantages of nanopesticides such as low dose, high active ingredient loading, slow and controlled release, biodegradable, reduced losses, and protection against photodegradation, along with key demerits.

12.1 Introduction

Feeding billions of mouths with continuously reducing resources is a major challenge to mankind. Novel technologies and ideas for increasing crop productivity per unit resources are of utmost need. Technological advances have made input efficient systems in agriculture production, which could grow more crops in limited resources while maintaining food quality and environmental sustainability (Fuglie et al. 2019). Apart from maintaining soil fertility in an input-intensive cropping pattern, increasing the socioeconomic status of the farming community by reducing the cost of cultivation is also a social responsibility and need of the hour (Show 2018). Maintaining the environmental sustainability is another crucial aspect to be taken care of while balancing the productivity and cost of cultivation (Grzelak et al. 2019). The challenge is not limited to productivity per se (Thornhill et al. 2016), but also maintaining the environmental sustainability and socioeconomic status of the farming community are crucial. Thus technological advancements on smart inputs are prerequisites for economic crop production by increasing efficiency of every particle of applied input so that crop productivity could be maintained with environmental sustainability along with the reduction in the cost of cultivation (Sekhon 2014; Liu and Lal 2015; Grzelak et al. 2019).

One such technology is nanotechnology. The term nanotechnology was first used by Norio Taniguchi in 1974 (Bulovic et al. 2004). The applied usage enhanced exponentially in the last two to three decades due to the technological advancements made in generating and handling nanosized materials (Gibney 2015). The term "nano" is developed from the Greek word meaning "dwarf" (Bhattacharyya et al. 2010). Nanoparticles have size ranging from 1 to 100 nm (Salata 2004). As per the definition of the US Environmental Protection Agency (2007), the term nanotechnology is defined as "the science of understanding and control of matter at dimensions of roughly 1–100 nm, where unique physical properties make novel applications possible." However, from the agricultural perspective, the dimension has been defined between 10 and 1000 nm (Scott and Chen 2013).

Reducing size to nano-dimensions has a quantum confinement effect on the properties of the material (Sun 2007). Higher surface area and active nature coupled with magnetic behavior give nanoparticles some unique electronic and optical properties (Sun 2007; Pokropivny et al. 2007; Aziz et al. 2015; Prasad et al. 2016). In the recent past, the term green nanotechnology is used for less harmful NPs synthesized from plants to overcome the toxic nature of chemically synthesized NPs (Prasad 2014; Kandasamy and Prema 2015). The nanotechnology-based market was increased by more than eight times from 2002 to 2015 (reviewed in Prasad et al. 2017; source http://www.hkc22.com/). Nanotechnology also helps in delivering nutrients, insecticides, fungicides, herbicides, etc. (Scott 2007; Bharani et al. 2014). In the case of nutrient management, nanotechnology could help in delivering nutrients to the plant-available forms. It also helps in the slow and controlled release of active ingredients, which reduces loss and cost. Varied nano-based initiatives are being taken for restoring agroecosystem along with boosting agriculture production

(Mukhopadhyay 2014). Nanofertilizers, nanopesticides, nano-based pest surveillance, nanotracking of insect-plant interactions, nanocarriers, nanomaterials for food preservation and packaging, nanosolutions for removal of soil and water contaminants, nanosensors for precision water management, nano-based soil reclamation, nano-based improvement of shelf life, etc. are few important nano-based initiatives in agriculture (Mukhopadhyay 2014).

In the conventional insect pest management, chemical pesticides like organochlorines, organophosphorus, carbamates, and pyrethroids are used. These pesticides are harmful to living beings apart from causing adverse effects on soil fertility. The Ministry of Agriculture and Farmers' Welfare, the Government of India, has banned 27 such pesticides vide Gazette Notification of May 2020 (http://egazette.nic.in/WriteReadData/2020/219423.pdf) for being potentially harmful against humans and animals. Nowadays, nanotechnology is being embraced in the world of pesticides, which has the potential to revolutionize modern-day agri-pest control strategies in different groups of nanopesticides, like insecticides, fungicides, and herbicides (Matsumoto et al. 2009; Peteu 2010). Nanotechnology is used in the field of agriculture for insect pest management, such as the use of silicon nanoparticles. The silver nanoparticles coupled with leaf extract of *Tinospora cordifolia* were found to be larvicidal and pediculocidal for head louse and mosquito (*Culex quinquefasciatus* and *Anopheles subpictus*) (Jayaseelan et al. 2011). Different nanoparticles, being used as pesticides, can also be used as nanocarriers in the nanoformulations, and it is a safe way of delivering active ingredients (AI) inside the plants (Benelli et al. 2017).

12.2 Nanomaterials as Nanopesticides

Different types of nanomaterials are being used in insect pest control research. The most common nanomaterials are Ag NPs (Fouad et al. 2018; Ga'al et al. 2018), Au NPs (Small et al. 2016), magnetite NPs (Chen et al. 2015), $Mg(OH)_2$ NPs (Pan et al. 2017), PEG NPs (Campolo et al. 2017), silica NPs (Arumugam et al. 2016), TiO_2 NPs (Tian et al. 2016; Xue et al. 2018), ZnO NPs (Malaikozhundan et al. 2017; Abinaya et al. 2018), etc. of nanoscale dimensions (1–100 nm or less). These nanomaterials vary in sizes and targets in insect systems with variable insecticidal properties.

Silver nanoparticles (Ag NPs) have specific insecticidal properties such as the reduction in resistance to ROS, impaired movement, impaired locomotory function, distorted sex ratio, impaired ovary and egg development, impaired pupation, damage to cuticular layer, inhibition of gut protease activity, reduced acetylcholinesterase activity, etc. (Meng et al. 2017; Kantrao et al. 2017; Mao et al. 2018; Ga'al et al. 2018). Gold nanoparticles (Au NPs) are reported to lower the viability of ootheca, delay nymph emergence, delay sexual maturity, cause abnormal reproductive and digestive system, reduce life span, interfere with nervous system proteins, etc. (Pompa et al. 2011; Small et al. 2016). Magnetite NPs cause defects in the ovary and egg development (Chen et al. 2015). $Mg(OH)_2$ NPs with Cry proteins damage

the midgut of insects by damaging gut epithelial cells (Pan et al. 2017). Polyethylene glycol (PEG) with citrus peel EO NPs cause damage in the early stages of egg hatching causing larval mortality (Campolo et al. 2017). Silica NPs damage the midgut in the microvillar zone and enterocytes (Mommaerts et al. 2012) and retard egg-laying when applied with pulse seeds (Arumugam et al. 2016).

Nanoparticles have a different mode of action in insect control, such as damage to the cuticle layer, interference with biochemical reactions, reduced mobility, disruption of the midgut, genotoxicity, etc. (Shahzad and Manzoor 2019) NPs, like non-structural alumina, disrupt the insect cuticle by dehydration (Stadler et al. 2017). Strong abrasive behavior of such NPs causes splits and scratches to the target insects (Arumugam et al. 2016). Essential oil-based NPs also cause contact toxicity as reported against *Rhyzopertha dominica* and *Tribolium castaneum* (González et al. 2014). NPs also affect insect reproduction and development. Exposure of insects to nanoparticles affects female fertility apart from reducing egg hatching rate (Wu and Uskoković 2017) and reduced fecundity in *Chironomus maculatus* (Malaikozhundan et al. 2017). Silver NP-based nanoinsecticides in *Chironomus riparius* interfere with sex ratio (Nair et al. 2011). BT-ZnO nanoparticles increased the larval and pupal durations. TiO_2 and ZnO NPs are reported to lower sperm production rates in *Agrius convolvuli* (Kubo-Irie et al. 2015). Some of the NPs affect the midgut of insects. Internalization and penetration of the cells lead to cell death and loss of membrane stability (Shahzad and Manzoor 2019). Few NPs, like silver NPs, cause DNA damage after ingestion in the gut and brain tissues (Mao et al. 2018). It is also reported to downregulate CrL15 responsible for assembly of the ribosome and affect protein synthesis (Nair et al. 2011).

NPs alter the biochemical functions of insects leading to reduced mobility or death. At the cellular level, NPs, like Ag NPs, cause stress followed by the generation of cytokines and reactive oxygen species, reduction in lipid droplets, damage to the respiratory chain, and membrane potential (Ma et al. 2015; Mao et al. 2018). The mobility of insects is also affected by ingestion of Ag NPs, as it causes impairment in crawl and climbing ability in larvae and adults (Raj et al. 2017). Ag NPs also interfere with membrane stabilizing calcium-binding protein calexcitin (Meng et al. 2017). Gold nanoparticles (Au NPs) have protease-inhibiting properties, especially to trypsin in the serum of *A. aegypti* mosquito larvae and also to other pests, such as *Helicoverpa armigera*, *Callosobruchus maculatus*, *Callosobruchus chinensis*, etc. (Patil et al. 2016). Monoterpenes of essential oil-based nanoparticles interfere with neural functioning by inhibiting compounds, like octopamine, cytochrome P450-dependent monooxygenases, GABA-gated chloride channels, etc. (Ziaee et al. 2014).

New development in the field of nanotechnology as the nanomaterial-based target delivery of CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 in the genetic modification in GM crops is a big achievement in the field of agriculture and insect pest management (Ran et al. 2017). Nanotechnology provides a green and efficient way of nature by managing insect pests without harming the environment.

12.3 Nanoformulations of Pesticides

Conventionally, the pesticides come in formulations as granular, solution concentrates, emulsifiable concentrates, wet table powders, and suspension concentrates. Further development in pesticide research was targeted to overcome the shortfalls in these formulations, like safety in manufacturing and use, convenience in manufacturing and use, easy disposal, reduction in the quantities to be applied, decrease loss to the environment, off-target effects, etc. Thus several new formulations are developed, such as suspoemulsion, o/w emulsion, microemulsion, multiple emulsion, microcapsules, etc. In the last few years, the research interest is driven towards nanotechnology in agriculture, especially on nanopesticides. Nanopesticides aim at two major issues: (1) increasing solubilization of poorly soluble active ingredients and (2) slow and targeted release of active ingredients. Different types of nanoformulations are discussed below:

12.3.1 Polymer-Based Encapsulation

Polymers are one of the most commonly used nanomaterials for encapsulation of active ingredients (AI). The key advantages of such formulations are low cost, biodegradable nature, and non-production of harmful byproducts (Kumar et al. 2017). Polymers, like chitosan, polyethylene glycol, alginate, gelatin, and poly-e-caprolactone, are routinely used in encapsulating nanopesticides (Kulkarni et al. 2000). Imidacloprid encapsulated in nano-dispensers has shown to have effects on pathogen vector at 200 times less AI concentration than conventional formulations (Meyer et al. 2015). Since the AI quantity used is very less, it is relatively safe for humans and the environment (Kumar et al. 2019). In this formulation controlled release of AI can be done by adjusting the type of polymeric nanoformulation used, e.g., microcapsule, microemulsion, etc. A high degree of control can be achieved through nanocapsulation. Xu et al. (2017) reported imidacloprid nanoformulation in poly(*N*-isopropylacrylamide) capped polydopamine microsphere having temperature and NIR light-sensitive drug release profile apart from having a high loading capacity. Thus, nanoformulations do not release AI below 15 °C, a 20% AI release at 25 °C, and ~65% AI release at 40 °C after 5 h. Similarly, under NIR light ~15% AI release was achieved in 30 min as compared to a release of ~5% without irradiation. Thus, the controlled release of AI based on insect feeding behavior could save the quantity of the AI required for effective pest management.

12.3.2 Lipid Nanomaterial-Based Encapsulation

These are colloidal nanocarriers with benefits, like stability, nontoxicity, high drug loading, ease of target-specific release, etc. (Zheng et al. 2013). Various kinds of lipid nanocarriers, such as solid nanocarriers, nanostructured lipid carriers, and nanoemulsions, are being used (Gaber et al. 2017). The specific benefit of lipid

nanoformulation is the protection from photodegradation (Nguyen et al. 2012a). It can incorporate hydrophilic and hydrophobic AIs as well apart from high AI stability from chemical degradation (Li et al. 2018). The lipid-based NPs are effective in plant absorption and further movement of AI to the target site (Kumar et al. 2019). These nanoformulations are not having any phytotoxic effects on seed germination and early plant growth (Nakasato et al. 2017). It also provides high photostability as seen in the case of UV exposure to deltamethrin (Nguyen et al. 2012b). Further development in lipid-based NPs is the use of renewable sources, such as rice bran oil, which have low side effects, and the capability of loading two AIs (Niculae et al. 2014). However, their toxicity to different off-targets is to be studied.

12.3.3 Clay Nanoparticles-Based Encapsulation

The clay-based nanomaterial is used for the slow release of active ingredients. It has qualities like slow release and high AI loading capacity due to its larger surface area (Ianchis et al. 2017). About 20% higher pesticide AI loading is reported than that of the conventional one (Cao et al. 2017). It has high photothermal stability providing high bioactivity to the pesticides (Kumar et al. 2019). Pectin cross-linked silica nanocapsule-based kasugamycin have been reported to have high photothermal stability along with high AI loading (Fan et al. 2017). Mesoporous silica nanoparticle-based chlorantraniliprole pesticide exhibited high AI loading, high resistance of AI, and high larval mortality of *Plutella xylostella* for a longer time (Kaziem et al. 2017). The clay-based nanoparticles are also useful in the controlled release of AI (Rani et al. 2014), such as neem oil in biogenic silica nanostructures showing an efficient and controlled release profile (Mattos et al. 2017). These neem oil NPs showed enhanced stability, antioxidant properties, and excellent mortality to *Acromyrmex crassispinus* ants. Further development in smart AI release in NPs is taking place by making it stimuli-responsive, such as pH, redox, enzymes, and temperature (Chen et al. 2017).

12.3.4 Metal-Organic Framework-Based Encapsulation

Metal-organic frameworks (MOFs) are known as efficient carriers for the target delivery of AIs (Kumar et al. 2019). Efficient AI adsorption, multiple active sites, well distribution of AI, presence of multiple topologies, high ion exchangeability, etc. are a few of the qualities of MOFs, which overcome limitations of conventional formulations (Brozek and Dincă 2014; Nehra et al. 2019). It also prolongs the effective lifetime of the AIs. However, the non-biodegradability of these NPs is still a problem and needs to be addressed (Glaser 2015). In the recent past, easily degradable alternatives, like Ca and Fe, are being used as green MOFs. These NPs degrade to their components in soil and also help in nutrition (Yang et al. 2017). To solve the problem of metal toxicity, the use of green MOFs could be viable

alternatives, like in the case of Ca and lactate-based nanofumigant *cis*-1,3-dichloropropene having a 100 times slower release (Yang et al. 2017).

12.3.5 Green Nanoformulations

Nanomaterials, like TiO_2, are arising as a potential environmental threat (Conway et al. 2015). Studies on the effects of TiO_2 on microorganisms and soil enzymes indicated strong negative impacts on ammonia-oxidizing microbes and nitrification enzymes (Simonin et al. 2016). Therefore, the use of nontoxic, biodegradable nanomaterials, like starch, chitin, clay, etc., is being explored (Narayanan et al. 2015). Some workers have reported microorganisms (cyanobacteria) as nanocarriers due to their surface properties, biodegradability, abundance, plant beneficial, and environment-friendly nature (Giessen and Silver 2016). The study by Yan et al. (2013) indicated the success of cyanobacteria-based nanopesticides containing avermectin. This formulation has high photostability and stimuli-controlled AI delivery as compared to free avermectin. Spinosad produced from actinomycetes bacterium *Saccharopolyspora*, which belongs to the family spinosyn consists of spinosyn A and spinosyn D (Sparks et al. 2012). It is found to be more effective in cotton and other pest-affected crops on large scale, but it has shown good result in resistance against *Liriomyza trifolii* (Ferguson 2004), *Plutella xylostella* (Zhao et al. 2006), *Musca domestica* (Khan et al. 2014), *Heliothis virescens* (Young et al. 2003), *Frankliniella occidentalis* (Loughner et al. 2005), and *Spodoptera litura* (Rehan and Freed 2014).

12.3.6 Nanoparticles as Active Ingredients

The use of nanoparticles themselves as a pesticide is a very promising field of research and has great potential. Different kinds of nanoparticles are reported as nanopesticides, such as copper NPs, silica NPs, non-structured alumina, etc. (Stadler et al. 2010; Arumugam et al. 2016; Le Van et al. 2016). Copper NPs are being widely utilized in different crops, such as Bt cotton (Le Van et al. 2016), and also have antifungal and herbicidal activity. The silica nanoparticles are reported to be effective against stored gains pest, *Callosobruchus maculatus*, in seeds of various pulses, such as *Cajanus cajan*, *Cicer arietinum*, *Vigna radiata*, *Vigna mungo*, *Vigna unguiculata*, etc. (Arumugam et al. 2016). The insecticidal activities of nanostructured alumina (NSA) were reported by Stadler et al. (2010) against *Sitophilus oryzae* and *Rhyzopertha dominica*. These inorganic nanomaterials are found to have high pest control comparable to commercially available pesticides (about 95%) apart from being cheaper and environmentally safe. NSA is also effective against leaf-cutting ants, *Acromyrmex lobicornis* (Buteler et al. 2018).

12.3.7 Other Nanoformulations

Various other kinds of nanopesticide formulations were worked out, like Bt nanoformulation with $Mg(OH)_2$. Coating of Cry11Aa toxins of *Bacillus thuringiensis* with $Mg(OH)_2$-based nanoparticles was found to increase their bioactivity as well as stability under UV light (Pan et al. 2017). $Mg(OH)_2$ nanoparticles also protected the Cry proteins and provided about four times slower degradation as compared to control apart from increasing toxicity to *Culex quinquefasciatus* (Sarlak et al. 2014). Graphene oxide (GO) was used as a nanocarrier of the pesticides, e.g., chlorpyrifos, endosulfan, and malathion, providing them favorable hydrophobic interaction and controlled release profile (Maliyekkal et al. 2013).

12.4 Merits and Demerits of Nanopesticides

Nanotechnology is a growing field in agriculture, and continuous efforts are being taken for increasing its suitability and reducing off-target effects. Some of the key merits and demerits of nanotechnology in insect pest management are discussed below:

12.4.1 Merits of Nanopesticides

Some of the key merits of nanopesticides are listed below:

High surface area: The nanomolecules show many extraordinary properties not shown by the bulk material. It attains a high surface area with higher numbers of active atoms on the surface (Maurice and Hochella 2008) giving it different densities and reactivity with varied surface composition.

Controlled release: Microencapsulation of pesticides provides controlled release by controlling the degradation of NPs by the type of surfactant use (Katagi 2008).

Higher retention of a microemulsion of emamectin benzoate on leaf surface provides higher residues on leaf and thus higher protection against moths in rice (Fan et al. 2010).

Increase stability: Nanoemulsion reduces hydrolysis and increases the stability of active ingredients and is useful in increasing effectiveness and reducing the dose of AI (Song et al. 2009). Nanoemulsion of β-cypermethrin is reported to have high efficacy due to increased stability and thus the bioavailability (Zeng et al. 2008).

Reduced volatilization losses: Nanoemulsion of garlic essential oil has reduced volatilization as compared to free garlic essential oil (Yang et al. 2009).

High biocidal activity: Nano-dispersion of triclosan has very high biocidal activity as its MIC over ethanol/water system is eightfold lower (Zhang et al. 2008). Nanometal with active ingredients of imidacloprid caused higher toxicity of insects as compared to their aqueous formulations (Guan et al. 2008).

Reduced leaching loss: Polymer-based nanopesticide, tebuconazole, has reduced leaching from treated wood as compared to their aqueous solution (Salma et al. 2010). Nanopolymer-based ethiprole has enhanced penetration to the plants (Boehm et al. 2003). In these nanopolymers, the release of AI can be controlled by changing the molecular weight and ratio of polymers (Shakil et al. 2010). Nanoformulation based on layered double hydroxides and clays provides higher persistence and reduced leaching with similar bioavailability.

Reduction in harmful effects of pesticides: Pesticides have severe effects on off-targets, like endocrine disruptors, neurodevelopmental toxicants, immune toxicants, and carcinogens, especially affecting the nervous system (Kalliora et al. 2018). Therefore, slow-release nano-encapsulated pesticides reduce the loss of pesticides to the environment and reduce possible off-target effects (Agrawal and Rathore 2014). Metal and organic nanoformulations fasten the degradation of AI in soil (Guan et al. 2010).

Maintaining soil health: There are reports of remediation of pesticide residues in contaminated sites using zerovalent iron nanoparticles since they have high adsorption affinity. It further improves soil health by improving soil binding properties as in the case of calcium carbonate nanoparticles, helping in the formation of microaggregates and macroaggregates in soil (Liu and Lal 2012).

Lower toxicity to off-target: Solid lipid nanoparticles of γ-cyhalothrin provide lower toxicity to non-target organisms (Frederiksen et al. 2003). Solid lipid nanoparticles of *Artemisia arborescens* essential oil have lower evaporation loss than emulsion (Lai et al. 2006).

Protection from photodegradation: In porous hollow silica-based nanoformulation, UV shielding provides slower degradation as compared to free AI (Li et al. 2007).

Increase solubility: Nanoformulation causes increased solubility of poorly soluble particles and high stability than EC (Kah et al. 2013).

Tracking system: The nanomaterials, like fluorescent photoresponsive nanocarriers, could also be used as a tracking system of AI movement and release profile to make a more efficient delivery module (Zheng et al. 2016). This technique has attracted pesticide delivery research by providing the ability to assess the changes induced inside the plant by pesticides and their possible mode of interaction. These photoresponsive nanocarriers can be coupled with suitable imaging techniques for improvement in the pesticide activity and its release profile.

12.4.2 Demerits of Nanopesticides

Despite several known benefits, nanoparticle-based pesticides are reported to have a few demerits. Some of them are listed below:

Effect on antioxidant mechanisms of plants: Significant reduction (29–85%) in antioxidants and defense-related metabolites were reported in spinach leaves by $Cu(OH)_2$ nanopesticides (Zhao et al. 2017). The application was done on 4-week-old spinach plants by $Cu(OH)_2$ nanopesticide foliar spray (0.18 and 18 mg/plant),

and GC-TOF-based metabolomics approach was used to assess metabolic alterations. Similar results were obtained from lettuce by $Cu(OH)_2$ nanopesticide (Zhao et al. 2016). However, in this study, tricarboxylic (TCA) cycle and some amino acid-related pathways were affected apart from reducing antioxidants, like *cis*-caffeic acid, chlorogenic acid, 3,4-dihydroxycinnamic acid, and dehydroascorbic acid. In wheat, Silva et al. (2020) reported sugar metabolism impairments and shift in metabolic pathways towards amino acid metabolism due to TiO_2 nanoparticles.

Effect on soil organism: The polymer-encapsulated and lipid nanoformulations of atrazine were found to have toxicity against soil nematode, *Caenorhabditis elegans* (Jacques et al. 2017).

Effect on developmental processes of other organisms: Aksakal and Sisman (2020) have shown induction of developmental toxicity by $Cu(OH)_2$ nanopesticide in embryos of zebrafish including mortality, reduced hatching rate, heart rates, malformations, etc.

Effects on the environment: Some nanoparticles, like TiO_2, are reported to have serious implications on the environment (Conway et al. 2015). It has emerged as pollutant in agriculture and other parts of the ecosystem. Impact study on the 90-days exposure of TiO_2-based nanoparticles to microorganisms showed to have strong negative effects on ammonia oxidizers and nitrifying enzymes of the soil (Simonin et al. 2016). The serious impacts of this kind of nanoparticles need thorough studies for modification of the soil and ecosystem functioning.

Slow degradability: Metal nanoparticles are non-biodegradable.

Lack of information on toxicity: The information on the effects of nanoformulations on the ecosystem is sparse (Kumar et al. 2019).

Difficult to operate at field condition: The stimuli-responsive release behavior of the nanopesticides is difficult to be maintained in the field conditions; as a result, the efficacy of AI reduces (Kumar et al. 2019).

12.5 Conclusions

Nanoformulations have great potential in insect pest management. Development of efficient nanocarriers, nanotrackers, nano-AI, control release, higher AI loading, lower toxicity to ecosystem, etc. are required for increased dissemination and utility of nanopesticides. Different nanomaterials have varying properties based on the type of material used. These nanomaterials can be efficiently utilized for various target insects at low cost and high specificity. However, short- and long-term toxicological studies on the effects of nanopesticides on the ecosystem are the need of the hour. Efficient alternatives of non-degradable nanoparticles widen the scope of nanopesticides in agriculture. Nevertheless, the untapped potential of nano-dimensions could be a boon in plant protection.

Points to Remember

- The nanosize range is 10–100 nm, but in agriculture, dimension of 10–1000 nm is considered.
- At nanoscale, material attains extraordinary properties.
- Key uses of nanomaterials are slow release, less quantity required, active particles with high activity, reduced input cost, resistance to photodegradation, and reduced loss to the environment.
- Some nanoparticles, like TiO_2, have toxic effects on the ecosystems.
- Metal nanoparticles are non-biodegradable.
- Green NPs are safer and biodegradable alternatives.
- Toxicological studies on the effects of nanoformulations on the ecosystem need to be taken up.

Acknowledgments Authors gratefully acknowledge Amity Institute of Biotechnology, Amity University Uttar Pradesh, Lucknow Campus, Lucknow, UP, India, and ICAR-National Bureau of Agriculturally Important Microorganisms, Kushmaur, Maunath Bhanjan, UP, India, for all the support rendered.

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Ethics and Safety Concerns

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Contents

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Omkar (ed.), *Molecular Approaches for Sustainable Insect Pest Management*,
https://doi.org/10.1007/978-981-16-3591-5_13

Abstract

Biopesticides have been an IPM tool for several decades for crop health management. Both phytochemicals and microbial pesticides are two biopesticide groups that have been regulated in India. This knowledge-intensive technique in pest management needs frequent updating of scientific information by the manufacturers of microbial pesticide (MP) formulation in tandem with government and private extension system to popularise these products in integrated pest management in crops. Quality review management of MP products is essential to sustain the shelf life and field bioefficacy of the products. The biowaste management of MP production units should follow GLP and GMP standard operating procedures to prevent undesirable leakage of harmful microorganisms following the relevant national guidelines and international conventions. Legal compliance of label expansion of MP formulations across various crops as per good agriculture practice to manage target pests would provide farmers higher economic benefits. Risk assessment based on the perceived hazards in handling microbial biocontrol agents as MP has to be based on the global conventions and norms with regard to biological substances.

Biotechnology tools and techniques to deploy genetically modified crops as well as gene editing technology (Crispr-Cas9) for attaining pest resistance and higher commodity output with better quality parameters are promising. Ethical and practical considerations for commercialisation of GM crops from cisgenic, transgenic or subgenic products need careful analysis for science-based assessment or 'decision tree-based' evaluation of the potential hazards. Risk assessment protocols for GM and GE crops are significant to alleviate the perceived hazards from them to humans and the environment in accordance with relevant laws and rules in India. The socio-economic evaluation studies on the benefits over the possible environmental risk shall make the consumers aware of the GM and GE agriculture commodity to enable them to make informed choice for consumption of those commodities.

Keywords

Biopesticides · Microbial pesticide formulations · Quality review management · Biowaste management · Biotechnology tools · Gene editing technologies · Decision-tree-based risk assessment · Socio-economic evaluation

Learning Objectives

1. Biopesticides have been an IPM tool for several decades for crop health management. Both phytochemicals and microbial pesticides are two biopesticide groups that have been regulated in India under the Insecticide Act, 1968. It is a knowledge-intensive technique in pest management and needs frequent updating of information by the manufacturers of microbial pesticide (MP) formulation in tandem with government and private extension system to popularise integrated pest management in crops.

2. The research and development for isolation, identification, evaluation and finalising the bioefficacy-based dosage of the candidate microbial pesticide needs adequate data generation under various agroclimatic conditions for managing target crop pests either individually or in integration with other pest management tools. The MP formulations may have label claim for the respective crops against given pests on which evaluation data are submitted towards registration for commercial production under Section (3) of the Insecticide Act, 1968. However, the Rules under this act may be modified to extend label use against the same pests in other crops too.
3. The appropriate formulation technology has to be used for developing the MP formulations by adopting the good lab practices and good manufacturing practices for their manufacture within the global code of conduct for this purpose for making the MP manufacture industry viable. Risk assessment based on the perceived hazards in handling microbial biocontrol agents as MP has to be based on the global conventions and norms with regard to biological substances.
4. Genetic modification has been one of the latest technologies deployed for crop pest management by incorporating alien genes that express insecticidal proteins, such as delta endotoxin, of soil bacterium, *Bacillus thuringiensis*, Crystal (Cry) gene family expressing delta endotoxins. Other biotechnology products using alien genes, such as Tm12 gene, to impart resistance to whitefly in cotton crop, are in progress. Recent spurt in research on the gene editing (GE) using clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated protein (Cas) (Crispr-Cas9) techniques for crop resistance against pests is in progress. Risk assessment protocols for GM and GE crops are significant to alleviate the perceived hazards from them to humans and the environment in accordance with relevant laws and rules in India.
5. The socio-economic evaluation studies on the benefits over the possible environmental risk shall make the consumers aware of the GM and GE agriculture commodity to enable them to make informed choice for consumption of those commodities.

13.1 Introduction

Following various global experiences, the designing of integrated pest management (IPM)[1] in India made IPM the national norm in plant protection of crops since 1992. Options to integrate biological control agents in integrated pest management (IPM) were explored once the research in this direction provided valuable knowledge and techniques to mass-produce these naturally occurring organisms for use in crops in alternation with chemical pesticides, especially in perennial crops as well as in long-duration annual crops (Radcliffe et al. 2009). Out of the various biocontrol agents for crop pest management, microbial natural enemies were found to be potent weapon to

[1] http://ppqs.gov.in/divisions/integrated-pest-management/ipm-glance

suppress crop pests that cause extensive crop losses. It is a knowledge-intensive technique in pest management and needs frequent updating of information by the manufacturers of MP formulation in tandem with government and private extension system to popularise integrated pest management in crops. Farming of crops for food, fibre, fodder and feed has been part of human civilisation. The crops that are chosen to be cultivated in definitive seasons have certain packages of practices to be followed in order to achieve maximum harvest of their genetic potential. The components of agroecologies bring about biotic stresses to crops at different phenological stages. Pests, including insects, mites, nematodes, vertebrate animals, plant diseases, weeds etc., cause various metabolic stresses in crops. Farmers are guided to follow certain management practices to contain and suppress damage to their crops due to herbivory from a host of organisms.

Alternate options of crop pest (insects, mites, plant pathogens, nematodes, weeds etc.) management have been researched upon for over the last five decades to replace/supplement chemical insecticides through IPM for optimal crop production. For effective pest suppression in farmlands, intensive research on phytochemicals, microbial pest control agents and the use of advances in molecular techniques for pest suppression have contributed substantially in India as much as in the rest of the world. Reduction in chemical pesticides was aspired for while utilising alternate pest management strategies.

This chapter delves around the microbial biocontrol agents that are presently manufactured as specific formulations for application in crops. The regulatory and ethical processes for utilising microbial pesticides (MP) are significant aspects while planning for the research and development including commercial production. Their use in agriculture is regulated through various laws and rules that guide the risk-free production and use in farms. Genetically modified (GM) crops are products from various biotechnological research and have offered better chance for growing pest-free crops. The result of introduction of GM crops to create insect-free crops, such as cotton, soybean and maize, has been impressive in the first few years. However, both these sectors, i.e. MP formulations and GM crops, have been regulated in all countries due to the perceived hazards to humans and the environment. Gene editing to impart resistance to pests of crops using clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated proteins (Cas) (Crispr-Cas9) techniques has been intensely pursued currently. Regulatory environment for these biotechnology products also would succeed such inventions for their environmental release.

13.2 Biotic Stresses in Crops and IPM Strategies

Biotic stresses in crops have made the intensive agriculture in various cropping systems highly dependent on the use pesticides. Out of the agrochemicals that are needed to sustain crop production, synthetic chemical pesticides have about 39% share (Subhash et al. 2017) in 2016–2017 in India. There has been immense introspection about the continuing use of chemical pesticides for crop production

in India as well as in many other nations. The national production of biopesticides is about 3000 MT in 2005–2006 and has grown to 7890 MT, as seen in the state-wise consumption data between 2014 and 2019 (Table 13.1).

Biocontrol agents are an immense discovery for nature-friendly mitigation of biotic stresses in crops. The pest management using these naturally occurring organisms in crop fields has been a tremendous step to reduce the overdependence on chemical synthetic pesticides. The conservation and/or augmentation of natural enemies of pests could be achieved by reducing the impedance of chemical pesticides that have a number of nontarget adverse impacts on natural enemies and many other nontarget organisms. National policy on agriculture, 2000[2] and 2007,[3] have pronounced the need for implementing integrated pest management with emphasis on alternate pest management approaches that would conserve natural enemies in crop fields and suppress all pests including disease-causing organisms. Crop protection has attained very decisive and pragmatic integrated pest management approach and has resulted in the economically beneficial harvesting of crop commodities from avoidable crop losses due to pestilence. Pests including plant pathogens are being managed presently using chemical synthetic pesticides along with biological control agents in crops by integrating MP formulations.

13.2.1 Biological Pesticides for Biotic Stress Management in Crops

Botanical-origin pesticides, such as azadirachtin and other alkaloids from neem seeds, *Pongamia* spp. (*Karanj*), pyrethrum, rotenone etc., have been regulated under the Schedule of Insecticide Act, 1968, and Rules, 1971. The augmentation and conservation of natural enemies of crop pests are the basic approach to manage pests by utilising their natural enemies, such as parasitoids, predators and microbial pathogens. Immense advances in the knowledge on these organisms have led to robust package of practices for integrated pest management in crops (Chandler et al. 2011; Ranga Rao et al. 2007; Sinha and Biswas 2008).

Along with these augmentations of biological control agents against insects and mite pests are parasitoids/predators/microbial pathogens of various pests as well as antagonists of plant pathogens. All these organisms are picked up from farms and from agroecological situations and identified for their specific use against crop pest spectrum for achieving crop protection. The social and environmental costs for farmers have been rationalised due to the discreet and judicious pest management plan as a national policy in the deployment of such tools in crop IPM in the country.

[2]National Policy on Agriculture (2000) Department of agriculture and cooperation. Ministry of Agriculture, Government of India. http://agricoop.nic.in/sites/default/files/npff2007%20%281%29.pdf. Accessed 22 Oct 2019.

[3]National Policy for Farmers (2007) Department of agriculture and cooperation. Ministry of Agriculture, Government of India. http://agricoop.nic.in/sites/default/files/npff2007%20%281%29.pdf. Accessed 22 Oct 2019.

Table 13.1 Consumption of biopesticides in various states from 2014 to 2019 (as of 13 April 2020) (MT). http://ppqs.gov.in/statistical-database

S. No.	States/UTs	2014–2015	2015–2016	2016–2017	2017–2018	2018–2019	2019–2020 (Prov.)
1.	Andhra Pradesh	53	25	9	5	10	0
2.	Bihar	252	286	314	320	350	560
3.	Chhattisgarh	284	370	380	405	505	550
4.	Goa	12	14	3	5	6	6
5.	Gujarat	279	273	305	354	306	307
6.	Haryana	330	340	380	390	410	400
7.	Himachal Pradesh	15	16	2	1	2	*NR*
8.	Jharkhand	3	7	11	38	41	91
9.	Karnataka	530	505	473	544	544	530
10.	Kerala	631	606	662	717	862	717
11.	Madhya Pradesh	309	395	1063	326	322	336
12.	Maharashtra	486	1173	1454	1271	1164	1082
13.	Orissa	267	271	310	310	310	220
14.	Punjab	136	138	134	259	246	242
15.	Rajasthan	157	9	9	10	15	209
16.	Tamil Nadu	286	286	294	630	500	813
17.	Telangana	82	94	85	77	84	102
18.	Uttar Pradesh	43	46	46	46	47	48
19.	Uttarakhand	22	30	31	50	52	116
20.	West Bengal	680	950	838	951	997	1017
Subtotal		4855	5834	6802	6710	6772	7345
North-Eastern							
21.	Arunachal Pradesh	*NR*[a]	*NR*	*NR*	*NR*	17	18
22.	Assam	130	150	188	217	234	243
23.	Manipur	0.75	0.85	1	1	*NR*	*NR*
24.	Meghalaya	16	23	24	75	*NR*	*NR*
25.	Mizoram	*NR*	*NR*	*NR*	*NR*	*NR*	*NR*
26.	Nagaland	12	12	12	14	18	19
27.	Sikkim	*NR*	*NR*	*NR*	*NR*	*NR*	*NR*
28.	Tripura	122	95	146	142	138	167
Subtotal		281	280	372	449	406	447
Union Territories							
29.	Andaman & Nicobar	0.70	*NR*	*NR*	*NR*	*NR*	*NR*
30.	Chandigarh	*NR*	*NR*	*NR*	*NR*	*NR*	*NR*
31.	Dadra & Nagar Haveli	*NR*	*NR*	*NR*	*NR*	*NR*	*NR*
32.	Daman & Diu	*NR*	*NR*	*NR*	*NR*	*NR*	*NR*

(continued)

Table 13.1 (continued)

S. No.	States/UTs	2014–2015	2015–2016	2016–2017	2017–2018	2018–2019	2019–2020 (Prov.)
33.	Delhi	*NR*	*NR*	1.30	*NR*	13	*NR*
34.	Jammu & Kashmir	0.05	0.50	1	1	2	2
35.	Ladakh	*NR*	*NR*	*NR*	*NR*	*NR*	*NR*
36.	Lakshadweep	*NR*	*NR*	*NR*	*NR*	*NR*	*NR*
37.	Pondicherry	16	33	14	14	11	10
Subtotal		16	34	16	16	25	12
Grand total		5152	6148	7190	7174	7203	7804

Source: States/UTs Zonal Conferences on Inputs (Plant Protection) for Rabi & Kharif Seasons
[a]*NR* not reported

The environmental sustainability of agriculture farms has been improved through such smart solutions (Arora et al. 2018).

13.2.2 Microbial Pesticides for Pest Management

The entomopathogenic fungi, such as *Metarhizium anisopliae*, *Metarhizium* (*Nomuraea*) *rileyi* and *Beauveria bassiana*; bacteria, such as *Bacillus subtilis* and *B. thuringiensis*; viruses, such as nuclear polyhedrosis viruses and cytoplasmic polyhedrosis viruses; protozoan diseases; and entomopathogenic nematodes have been evaluated successfully and integrated appropriately in IPM of crop insect pests occurring in soil and aerial plant parts. In respect to the antagonistic organisms that are deployed in the augmentative biocontrol of plant pathogens, fungi, such as *Trichoderma* spp., and bacteria, such as *Pseudomonas fluorescens*, have been utilised as biological pesticides to manage various fungal and bacterial diseases in crops season after season.

Microbial natural enemies of crop pests became fascinating component in the biological control of crop pests (Swati and Adholeya 2008). These could be augmented easily using various microbiological production techniques including the use of fermenters. This knowledge-intensive technique in pest management needs frequent technical knowledge updation by the manufacturers of MP formulation regarding the use and in tandem with government and private extension system for farmers to comprehend and accordingly utilise these products in their farms. The access of desirable MP formulations by farmers for use in their farms is required either from market shelves or from their own production facility. The farmers can access MP formulation technology from research institutions and go for 'own' production of the relevant microbial species under the technical supervision of the research institutions. Such production is exclusively for use in farms and cannot be for doing business. The Insecticide Act, 1968, and Rules thereon, 1971, do not prohibit farmers from producing their own MP formulations for use in their farms.

There has been considerable interest amongst scientists to isolate microbial control agents from agroecosystems, identify them and multiply their pure cultures for use against insect pests in crops and other relevant systems (Gupta 2006; Rabindra 2001). Many research institutions in the country have commercialised their discoveries of candidate microbial control agents (fungi, bacteria, viruses, protozoa, nematodes and the like) along with the technology for manufacture of these microbial pesticides (MP). Pest management in crops under various cropping systems, such as paddy, wheat, maize, pulses, oilseeds, cotton, jute, spices, condiments, vegetables, orchard crops etc., is achieved by utilising, amongst other tools, the microbial control agents (Koul et al. 2003; Mishra et al. 2020; Rabindra 2005; Kumar et al. 2019). The MP formulations that are deployed for biological management of biotic stresses is viewed as the safety system to reduce or prevent all perceived risks due to their large-scale use of chemical pesticides (FAO 1988; Chandler et al. 2008).

Business models that offer entrepreneurships for the production of microbial biopesticides in rural India have been designed and developed (Amin 2013). There are many examples that lead village youths into technopreneurship opportunity in mass-producing the microbial control agents (Kumar et al. 2019). The grain-based (sorghum, rice etc.) dry fermentation mass production system has been part of the technology package offered along with the candidate MP species and strain of NARS and CSIR institutions. The commercialisation of these MP production technologies from these institutions needs in-built follow-up regarding the quality insurance of the standard operating procedures laid out by the institution for their mass production.

Many public institutions under the National Agricultural Research System (NARS) and under the Council of Scientific and Industrial Research (CSIR) by the National Chemical Laboratory, Pune-National Collection of Industrial Microorganisms (NCIM) have discovered many candidate microbial bioagents for crop pest management. Further research proceeded to find out formulation technology using these strains for their commercial manufacture for use in agriculture farms to protect crops from various biotic stresses. However, there are no patents that have been registered in India or any other country for the manufacture process of MPs.[4] The science, technology and innovation (STI) of MP formulations in terms of research/innovation and commercial manufacture have not attained the equivalent expertise and capacities as in the case of microbial pharma processes. The critical mass that is essential in the country for this purpose is yet wanting to attain perfect manufacturing posture. This is one of the reasons for the poor spread of this technique, as an essential coordinate of IPM in crops. The convincing stand of the MP formulations for effective suppression of pests even under organic farms is shaky due to the variation in bioefficacy in the same crop season. With comparative bioefficacy of insecticides that farmers generally deploy to get 'quick-kill' effect, the acceptance of microbial pesticides becomes limited. Herbivory management using

[4] http://www.ipindia.nic.in/advanced-search.htm. Accessed 27 Aug 2020.

Table 13.2 Data on number of registrants of microbial entomopathogen biopesticides under section 9(3) in CAB & RC database and Kumar et al. (2019)

S. No.	Product name	No. of registrations	Number and type of commercial formulations[a] (AS, SC, WP)
1.	*Beauveria bassiana*	87	46 [b]AS, SC, WP
2.	*Beauveria brogniartii*	01	01 WP
3.	*Hirsutella thompsonii*	01	AS, WP
4.	*Isaria* (=*Paecilomyces lilacinus*) *fumosorosea*	03	03 AS, WP
5.	*Pochonia chlamydosporia* (=*Verticillium chlamydosporium*)	04	02 WP
6.	*Purpureocillium lilacinum* (=*Paecilomyces lilacinus*)	35	20 AS, WP
7.	*Metarhizium anisopliae*	33	26 AS, SC, WP
8.	*Lecanicillium (Verticillium) lecanii*	62	42 AS, WP
9.	*Lecanicillium (Verticillium) lecanii* + *Hirsutella thompsonii*	01	01 AS
10.	*Bacillus thuringiensis kurstaki*	35	25 AS, WP
11.	*Bacillus thuringiensis israelensis*	12	12 AS, WP, DG
12.	*Bacillus thuringiensis galleriae*	01	01 FC
13.	*Lysinibacillus sphaericus*	03	01 WP
14.	*Bacillus firmus*	01	01 WP
15.	*Helicoverpa* NPV	22	11 AS
16.	*Spodoptera litura* NPV	05	5 AS
	Total	306	196

http://ppqs.gov.in/statistical-database as on 22/09/2020

[a]Not necessarily a complete list of All products

[b]*AS* aqueous suspension, *DG* dispersible granules, *FC* flowable concentrate, *SC* suspension concentrate, *WP* wettable powder

MP formulations may not be on firm footing in the absence of assured quality products. The national requirement of MP formulations is met with the Central Insecticide Board-Registration Committee (CIB-RC)-registered MP formulations (Table 13.2). Rabindra and Grzywacz (2010) illustrated the regulatory process in India, as of 2009, for registering three fungal entomopathogens; three fungal nematicides; three bacterial entomopathogens; two fungal antagonists and one

bacterial antagonist against plant pathogens. Kumar et al. (2019) provided comprehensive data on the 306 registered microbial pesticides of 16 MP organisms and their 196 commercial formulations. It appears that there is significant variation in the database of Directorate of Plant Protection, Government of India, in regard to the details of registered MP formulations and their manufactured quantity of the formulations. New-age innovations in formulation technology including the use of nanomaterials have intensified the vistas on improving efficiency of pest control in crops (Chhipa and Joshi 2016; Koul 2019). The regulatory machinery will then be challenged with novel registration guidance documents for such MP formulations using nanotechnological processes and substances.

India adopted the organic farming policy in 2005.[5] The organic means and methods of agriculture became a 'reinvented' wheel in the wake of increasing consumer awareness about the health advantages assumably with the consumption of organically grown commodities. The biological pesticides became strong candidates in managing pests in organic farms, and Technical Bulletins on organic farming, such as that of ICAR-Central Institute of Cotton Research, promoted their use (Rajendran et al. 2000). Organic cultivation in India is in an area of 3.67 m ha,[6] and the organic certification area (registered under National Programme for Organic Production) is about 2.3 m ha cultivable area. The state of Madhya Pradesh has covered the largest area under organic certification followed by Rajasthan, Maharashtra, Gujarat, Karnataka, Odisha, Sikkim and Uttar Pradesh. The assessment of annual requirement of MP formulations for organic farms in the country is worthwhile to project the annual manufacturing requirement. The requirement for MP formulations in at least 10% of the 2.3 m ha of organic farmed area in the country can be around at the rate of 5 kg/ha of any one MP organism sprayable/wettable powder (the most common formulation in use) shall be 11.5 million kg, far lower than the total production quantity of biopesticides in Table 13.1.

13.3 Regulatory Process of Biopesticides in India

The regulatory framework is for registering any MP formulation product for commercial production by micro, small and medium enterprises (MSMEs) and registered companies for manufacturing in factories. The international guidance document (FAO 2012) for regulatory management of biopesticides is the one that can be used as harmonised steps. Kabulick et al. (2010) provide the global glimpse of the regulatory situation of microbial pesticides. Regulatory requirement for the commercial manufacture and marketing of MP formulations in the country was identified in the late 1980s. Mensink and Scheepmaker (2007) concluded that plant protection products with active microorganisms are allegedly less hazardous to the environment

[5] https://ncof.dacnet.nic.in/Policy_and_EFC/Organic_Farming_Policy_2005.pdf

[6] http://apeda.gov.in/apedawebsite/organic/Organic_Products.htm#:~:text=As%20on%2031st%20March%202020,Hectare

and wildlife than synthetic chemical pesticides. In order to alleviate environmental safety concerns of possible contaminant microbials in the MP formulation, their potential toxicity and pathogenicity tests may be relevant. They also point out a 'decision tree model' as followed in the European Union through the scientific scrutiny steps of characterisation, identification and efficacy and also emission, exposure, environmental effects and the environmental risk assessment. It is advisable to take up such technical scrutiny by regulators on a case-by-case basis using scientific judgement for assessing the microbial ecology, limited experience with regulatory test protocols and taxonomic status in relation to the indigenousness of active microorganisms from the data package of the applicant. The decision tree offers regulatory guidance on the environmental safety evaluation of microbial plant protection products.

The NARS and CISR institutions that were involved in the pioneering research on identification of suitable microbial agent strains empowered with local adaptation were chartered to develop guidance document for the Central Insecticide Board to suitably incorporate in the Insecticides Rules, 1971, and Guidelines[7] for registration of the candidate formulations under the Insecticide Act, 1968. The NARS institution that developed MP formulations commercialised them to private individuals and companies for large-scale production and marketing. These entrepreneurs including big companies have sought the registration[8] of their specific microbial strains of those fungal and bacteria species in the pesticide formulation(s) for specific crop labels in Form I after following the relevant Guidelines for microbial biopesticides as provided by the Registration Committee of the Central Insecticide Board in regard to the data requirements on bioefficacy, toxicology and packaging in addition to depositing the formulated microbe strain in any of the designated and notified national microbe depositories. The MP formulations may have label claim for the insect pests in the respective crops where evaluation data are submitted towards registration for commercial production under Section (3) of the Insecticide Act, 1968. However, the Rules under this act may be modified to extend label use against the same pests in other crops too, based on scientific study. The registration certificate of microbial pesticides may carry the manufacture process that is fit for the production of respective MP formulations.

In India, there is no legal restriction to mass-produce MP formulations in agriculture farms for their own use. Farmers and farm producer groups and farm producer organisations can produce any MP formulation on no-profit-no-loss basis under the guidance of the technology discovering research institution. Innovative fermenter techniques are deployed by such groups (Plate 13.1) for mass production of MP formulations for application on crops for pest management.

[7] https://pesticides-registrationindia.nic.in. Accessed 10 Jul 2020.

[8] https://pesticides-registrationindia.nic.in. Accessed 10 Jul 2020.

Plate 13.1 Innovative initiative by farmer groups for on-farm mass production of microbial pesticides. (With permission to reproduce Courtesy: Foundation for Agriculture Resource Management and Environmental Remediation (FARMER), Ghaziabad, Uttar Pradesh)

13.3.1 Ethical and Regulatory Concerns in the MP Formulation Sector

There are various ethical and safety issues of major concerns in the use of microbial products and molecular tools and techniques in insect pest management. Therefore, when the proper safety precautions are taken, colonies of microorganisms can be safely isolated from homes, yards, gardens, etc. The majority of microorganisms are pathogenic,[9] but bacterial cultures or Petri plates that contain any type of bacterial colonies should be treated with general safety precautions (Anonymous 2007; EC 2005; Hauschild 2012; James 2008). The GLP and GMP (as described in the following section) dossiers of the licensed manufacturing firm should have all the biosafety protocols recorded, and those are to be meticulously followed. India being signatory to the Biological Weapons Convention[10] (that came into force on 26 March 1975) the states to ensure that the abiding principles and protocols of the Convention need to be put in place and practiced. The states may have to provide

[9] https://www.sciencebuddies.org/.../references/microorganisms-safety. Accessed 12 Aug 2020.

[10] https://en.wikipedia.org/wiki/Biological_Weapons_Convention came into force w.e.f. 26th March 1975. Accessed 12 Aug 2020.

"Each State Party to this Convention undertakes never in any circumstances to develop, produce, stockpile or otherwise acquire or retain: (1) Microbial or other biological agents, or toxins whatever their origin or method of production, of types and in quantities that have no justification for prophylactic, protective or other peaceful purposes; (2) Weapons, equipment or means of delivery designed to use such agents or toxins for hostile purposes or in armed conflict."

the necessary undertaking to the Ministry of Home Affairs (MHA), Government of India, periodically, in accordance with MHA guidelines on this.

In the interest of assuring farmers of the expected bioefficacy of the MP formulation, there is need to establish and assure quality in terms of international norms as guided by the UN Forum for Sustainability Standards (UN-FSS) and as recommended by the Quality Council of India (QCI). The ethics in the business of microbial pesticides need the following considerations.

Ethical considerations matter in the use of MP formulations that are marketed for pest management shall be:

(a) Absence of the consistent concentration (in terms of colony-forming units), as prescribed microbial species/strain content of the target MP formulation.
(b) Ensuring the absence of known/unknown hazardous, dubious and dangerous microbes including the non-culturable ones.
(c) Lack of consistent bioefficacy against the target pest(s) in the crops with label claim for the MP formulation.
(d) Absence of quality regulatory management (QRM) as laid out by the Quality Council of India for the manufacture, transport, storage and use of MP formulations.
(e) Use of formulants of dubious quality used in the manufacture of the MP formulations resulting in the harming of target crops and agroecology.
(f) Release of untreated objectionable effluents and laboratory/factory wastes into the environment.

In order to obviate the most of the above ethical issues in the marketing and use of MP formulations, suitable guidelines and code of conduct have been placed in public view for compliance and for confirming the manufacture and marketing of absolutely high-quality MP formulations for pest management use in farms. As described elsewhere GMP and GLP are essential protocols for compliance by licensed manufacturers of MP formulations.

The MP formulations for farm pest management are manufactured in unorganised sector as well as in organised factories of big industrial factories. In both situations, there is a strong need for the quality review management (QRM)[10] protocol in place. This ensures the systematic assessment and control of risks during manufacture of microbial agents. In this context all manufacturing facilities shall have a state-of-the-art microbiology laboratory manned by technically experienced and talented scientific personnel. The in-house QRM documentation for batchwise production of the microbial pesticides products would be further audited by the National Accreditation Board for Testing and Calibration Laboratories/International Standardisation Organisation (NABL/ISO) system. As the microbial pesticides have become common IPM component in crop pest management, there have been regulatory issues in the manufacturing of formulations. The major concern is about the MP formulations with dubious quality (NAAS 2013) bereft of good manufacturing practices (GMP).

13.3.2 Management of Quality Compliance

The formulation research for MP terminates in NARS institutions with the development of techniques for culturing the specific strains of microbial species for known bioefficacy against the target insect/mite pests in crops and using ingredient recipe for dust formulation, wettable formulation or the liquid formulation. The quality management protocols have to be set into operation for the MP formulations (Anonymous 2020; Van Lantern 2003). The step ahead for scaled up production plan and packaging did not receive much research attention. The result was that the technology to manufacture got restricted to the sharing of MP strain and the MP formulation. Their packaging, storage and transport were not given due attention, and due diligence on the regulations for these aspects was not heeded to. It is also significant to observe that very few private companies that came up with their own R&D setup to develop their own microbial pesticide strains and formulations thereon; these technology packages were neither acquired from any foreign collaborators nor developed with the help of global leaders in this field.

The quality review management (QRM) of MP formulations needs greater attention in regulatory management. The regulatory entities of the states can apply the QRM principles that are put in place for drug manufacture for assuring quality production of MP formulations. Satpathy (2018) brought out the existing regulatory norms in the country and the absence of desirable quality in the MP formulations that are marketed, as prescribed by the Central Insecticide Board in its guidelines for microbial biopesticides. The use of substandard MP formulations in IPM of crops cannot only face ineffective pest suppression but also bring in undesirable exposure to humans with health challenges from contaminant microbials.

Ultimately the most commonly formulated MP products, as dust formulation or wettable powder formulation, are filled in polybags of suitable size that are held in carton boxes before being stacked and transported to the designated markets. In case of liquid formulation, packaging principles to adopt polypropylene (PP) bottles need to be followed. Ideally glass bottles should be preferred. The storage stability of the spores in the shelf life studies of the MP formulation in PP bottles in comparison to glass bottles needs to be studied.

13.3.3 Model Format for Quality Management Protocol for Microbial Pesticide Manufacture Factory

This procedure is applicable at the microbial pesticide manufacture factory. The objective of quality review management is to conduct routine reviews of the whole quality system in the production line according to a planned schedule. This includes the review of both operational and quality system review.

Operational review based on the manufacture plan for every month as well as the capacity utilisation plans based on raw material supply and production process for each batch of MP formulation.

Quality system review shall be done by team headed by responsible personnel of the company. The quality review shall include data analysis of batchwise production, raw material mobilisation and their quality reports, instances of arising problems and their resolutions.

Forms and records shall be maintained and audited periodically—calendar for operation review and quality review meetings (monthly).

13.3.4 Good Manufacturing Practices (GMP)

Good manufacturing practices for the production of microbial agents have been enunciated by various international agencies (WHO,[11] FAO-OECD[12]). However, there is no agency that can oversee the QRM of the products that are marketed as microbial biopesticides for crop pest suppression. The desirability of empowered institutions, such as the Quality Council of India (QCI), to undertake QRM of MP formulations in the country is to be introduced. Adulterants and contaminants are important parameters for the QRM of MP formulations.

As the existing manufacture of microbial pesticides and their formulations, especially using dry-fermentation techniques are yet to follow any national Guidance Document for certification of both processes and the product. There is concern about potentially harmful and hazardous microbial contamination that is to be resolved. Certified GMP batch production and their marketing would enable quality managed products for effective and efficient crop pest management. The cost of using MP formulations with poor quality is equivalent to the cost of crop loss due to pestilence. Hence, the farmers suffer double loss since they lose the crop even in spite of investing in MP formulations. The biohazard due to microbial contaminants to farm families and consumers of farm commodities arising out of the sub-standard MP formulations and hazardous contaminant microbes needs strong regulatory audit for verification of self-certified MP products. Self-certification shall be made mandatory for all MP production units to guarantee the absence of harmful microbes and other additives in their products. The WHO guidelines (1996) provide significance of reducing bioburden and biohazard by following GMP in the manufacturing process. The MP formulation industry in the country shall establish inter-factory audit system in which multifactor analysis of perceived risk factors can be assessed annually. India is signatory to the Biological Weapons Convention (BWC) and has the obligation to report to the Convention about the peaceful purposes to handle all microbes for health, agriculture and any other national purposes and needs.

[11] https://www.who.int/biologicals/areas/vaccines/Annex_2_WHO_Good_manufacturing_practices_for_biological_products.pdf?ua=1. Accessed 10 Jul 2020.

[12] OECD/FAO (2016). OECD-FAO guidance for responsible agricultural supply chains. Paris, Organisation of Economic Co-operation and Development (https://mneguidelines.oecd.org/OECD-FAO-Guidance.pdf)

13.3.5 Good Laboratory Practices (GLP)

India has institutionalised GLP through the Quality Council of India (QCI). However, the MP manufacturing sector needs to align and establish GLP norms for microbial agents that are used for the manufacture of MP formulations. Such back-end laboratory would be the pivotal setup for the maintenance and management of microbial pure cultures without microbial contamination as well as invasion by laboratory mites (Onions 1990). It is significant to note that only about 1% of the reported microorganisms are culturable. The rest of the uncultivable microorganisms can still be the contaminants in the fungal and bacterial cultures. Molecular tools provide certain degree of tests for the obvious contaminations. However, still cautious, systematic QRM procedures need to be set up in the laboratories to obviate any possible contaminations of MP formulations. Careful consideration on the GLP practices can reduce such possibilities. Anticipatory research on the potential contaminant microbes under different fermenter operation conditions, media composition and any other extraneous factors is needed.

13.4 Hazard Perception in the Use of Microbial Biocontrol Agents

The perceived threats in the form of hazards, such as allergenicity (Ward et al. 2011; Darbro and Thomas 2009), emanating from the production and subsequent use of microbial biopesticide formulations in terms of the primary microbial species as well as potential contaminant microbials (including non-culturable) that could end up in the product during manufacture, packaging, transport and storage need clear understanding by the manufacturers. The conscious effort to avoid such introduction of hazardous microbials in the formulation product is one of the quality management protocols to be stringently followed.

Biowaste management under the Basel Convention (1992) regulates transboundary movement of hazardous wastes including biological wastes (Annexure-I). Further, the GMP protocols of the manufacturing site for MP pesticides and the GLP protocols of its laboratory also stipulate the waste management and waste processing of the spent cultures/media and labware. MP formulation industry can be the self-regulatory mechanism in the country for both overseeing the compliance obligation under the National and State Pollution Control laws and Biological Weapons Convention (BWC) through self-governance protocols aligned to national and international codes of conduct.

As in the case of the UN Environment Program (UNEP) London Guidelines for the Exchange of Information on Chemicals in International Trade, there is the exchange of information under BWC. The national code of conduct on microbial research and manufacture shall have the database on the firms and entities who are involved in handling various live microbes for any of the microbial pesticide development and manufacture for marketing. The bioburden of microbial flora (either hazardous or otherwise) present (WHO Guidelines, 1996) in raw materials

for formulating MP products, in the production media, intermediate finished goods, etc., as the case may be, is to be evaluated by licensing authority of states in tandem with the State Pollution Control Board in all states and union territories.

Another major concern is the effluents that are released from these production units (OECD/FAO 2016[13]). The pollution control boards shall ensure oversight of the quality of effluents that are permitted to be released into the environment. The GMP for manufacture of MP formulations also covers the management of factory effluents. In the case of factories handling microbes, the spent culture broth and other chemicals used in fermenters and laboratory culture systems have to be treated before being flown out into public drains. Licenses that are required in various states from respective state pollution control board to run the factory for the manufacture of microbial agents relate to effluent management: air/water/soil pollution control for chemicals and microbials. The regulatory framework includes Water (Prevention and Control of Pollution) Act,[14] 1974, and Air (Prevention and Control of Pollution) Act, 1981.

The precise and succinctly defined hazard perception is essential for each microbial pesticide formulation product, in order to attain risk assessment of this class of biological pesticides. The required hazard definition for MP formulations on the manufacture, transport, use and disposal of microbial pesticides has to be firmed up. However, the present regulations of these products do not visualise and update the regulatory requirements in this context.

An emerging issue in the context of MP formulation is the deliberate contamination with synthetic chemical pesticides in Indian pesticide market with poor quality assurance on the colony-forming unit count of microbial agent. The 'quick kill' effect of such formulations may attract the customership of farmers; however, such of these spurious and contaminated MP formulations deceive and mislead farmers on the bioefficacy and toxicology of such products and may leave undesirable chemical pesticide residues in the crop commodities. Moreover, these formulations become ill defined in hazard perception and risk mitigation assurance.

13.5 Packaging and Container Compatibility

The code of conduct (FAO 2015)[15] defines packaging as 'the container together with the protective wrapping used to carry pesticide products via wholesale or retail distribution to users', whereas repackaging refers to 'the transfer of a pesticide from any authorised commercial package into any other, usually smaller, container

[13]OECD/FAO (2016). OECD-FAO guidance for responsible agricultural supply chains. Paris, Organisation of Economic Co-operation and Development. https://mneguidelines.oecd.org/OECD-FAO-Guidance.pdf

[14]http://moef.gov.in/about-the-ministry/organisations-institutions/boards/central-pollution-control-board/ and https://cpcb.nic.in/

[15]http://www.fao.org/3/a-i5008e.pdf. Accessed 12 Aug 2020.

for subsequent sale'. National laws, rules and guidelines have been aligned to this FAO document including the code of conduct. Accordingly, packaging of pesticides for MP formulations should conform to the safety for transport, storage, handling and use without allowing the degradation of pesticide. Further, the packaging should not create danger to human health and the environment. The packaging of pesticides should not resemble common packaging of consumable goods. The label for use and risk reduction measures has safety mechanism that would avoid inadvertent handling by children. Reuse of pesticide packaging containers should be banned and punishable under the relevant national law. Packaging and repackaging of pesticides can be undertaken only at the licensed premises under supervision of competent personnel. It is advised to store in cool and dry ambient conditions. However, MP formulations need stringent temperature management of the rooms, where they are stored with the shelf life prescribed on the label to be sustained for achieving very good shelf life that permits effective pest suppression due to the presence of active and live propagules under storage.

13.5.1 Packaging, Storage and Transport

It is realised that there is no globally recognised regulatory model that would obviate possible hazards in the manufacturing, packaging and transport of MP formulations for use in crop pest management (Arora et al. 2016). Continuing efforts by global agencies, such as the Codex Alimentarius,[16] International Organization for Biological Control (IOBC), European and Mediterranean Plant Protection Organization (EPPO) and Organization for Economic and Co-operative Development (OECD), to develop standards for developing global standards and models for packaging and transport of biological substances and prevent any arising handling hazards in transport and storage.

13.6 Utilisation of Biotechnology Procedures for Pest Management

From the late 1990s, the scientific developments in GM technology trickled in for their wide variety of applications. Progress in agriculture has been immensely benefited because of the advances in various component science and technology areas (Parekh 2004). There have been several advances in the scientific pursuit of biotechnology in agriculture and other sectors for human benevolence. The associated understanding of ethical, safety and intellectual property issues of every discovery is under constant debate in recent decades (Nambisan 2017).

[16] http://www.fao.org/fao-who-codexalimentarius/news-and-events/news-details/en/c/1189277/. Accessed 13 Aug 2020. **Codex looks to harmonise regulation of biopesticides (6 April, 2019)**.

The biotech products ultimately undergo appropriate risk assessment within the existing knowledge sphere, resulting in the labelling of the product for offering informed choice for consumers. However, the global debate on the worthiness and goodness of fit of the biotechnology products in agriculture has entered devious arguments related to matters other than science too (Kinderlerer and Adcock 2003). This crystal protein is found to be present in the specific strains of *Bacillus thuringiensis*, a soil-inhabiting bacterium and can kill various insect pests that affect crops, such as caterpillars, maggots, grubs and so on. In the quest for tangible pest management, the idea of toxifying crop plants with the alien gene-expressed insecticidal protein, such as delta endotoxin, was explored and commercialised globally in many crops.

Although many other biotechnological interventions were lined up for improving the quality and quantity of farm commodity output, the most favoured technology was Bt gene technology in crops to thwart insect pestilence. As a model crop, cotton became the global example. Other crops, such as soybean, maize and rice, have also been genetically modified with the target genes that express Bt delta endotoxin at given phenological stage of the crops.

India took Bt cotton regulatory approval under the Environment (Protection) Act,[17] 1986, and Rules[18] of the Ministry of Environment, Forest and Climate Change, Government of India, to manage the environmental release of the alien Bt delta toxin Cry gene(s) in cotton since 2002. Many research institutions under CSIR, NARS and others undertake development research to get biotech crops with various features and traits. All these have the regulatory protocols under the Review Committee on Genetic Manipulations (RCGM) and Genetic Engineering Appraisal Committee (GEAC) guidance documents-based evaluations before being approved for release to the environment for cultivation.

There has been a strong interest to alter genetic virulence of microbial pesticide organisms to improve their virulence, and tools such as Crispr-Cas9 techniques for gene editing and improving the existing strains for virulence are contemplated. Such gene-edited organisms (GEOs) have so far not been commercialised. There is presently a government ban on the environmental release of GM microbial biocontrol agents for pest management, while the environmental release of GEOs is under policy discussion by the Department of Biotechnology, Ministry of Science and Technology (NAAS 2020).

13.6.1 Biotechnology Advancements in Crop Pest Management

Over the last 30 years, the ability to modify specific genes in microorganisms has revolutionised numerous fields of the biosciences, including medicine, agriculture

[17] http://moef.gov.in/rules-and-regulations/environment-protection/. Accessed 17 Aug 2020.

[18] http://moef.gov.in/wp-content/uploads/2018/03/THE_ENVIRONMENT.pdf. Accessed 17 Aug 2020.

and basic research into life processes. Molecular tools and techniques under the modern branch of biotechnology led to the utilisation of genetic transformations (both cis and trans) for integrating alien pest resistance genes and other such useful genetic trait expressing genes into certain crop species to thwart insects and pathogens. In the wake of increased consumer consciousness on the potential risks and hazards to terrestrial biomes, agroecologies and to human health due to the introduction of genetically modified (GM) crops as well as their impact on food webs including nontarget organisms, there has been stringent enforcement of various regulatory protocols to reduce such perceived risks while using such crops for food, fibre and feed production.

The scientific research in this area is carried on under public and private funding in NARS/CSIR institutions. The Bt brinjal with resistance to fruit and shoot borer (*Leucinodes orbonalis*) was the last instance where the regulatory moratorium was applied for the release for cultivation in the country. In the scientific research front in this realm, the latest publication is on the identification of Tma12 protein (Yadav et al. 2019) that is reported to toxify whitefly in GM cotton plants (Shukla et al. 2016). However, the common thread of scientific discussion is about the cost/benefit ratio of crop biotech products with insecticide-expressing traits that are expected to be overpowered by target insect pest species due to biological adaptation prowess. Ultimately such GM biotechnology products cannot sustain the strong adaptations of both oligophagous and polyphagous pests in crops. This has been the experience in Indian cotton crop fields.

In the present millennium, intensive application of biotechnological products globally became significant for pest management in crops. The prominent amongst these was the crop genetic modification by incorporating the alien gene expressing the delta endotoxin from the prominent MP bacteria, viz. *Bacillus thuringiensis*, managing predominantly caterpillar pests in crops, such as cotton, maize etc. Gene editing technology has become a new tool for crop pest protection. The regulatory and ethical components for managing such developments have been taken up in various countries in order to manage the perceived hazards and risks for humans and nature once the biotechnological products were commercialised and released into environment.

13.6.2 Risk Assessment Protocols of Genetically Modified (GM) Biocontrol Agents

Novel technological discoveries and their applications in agriculture have influenced the modern crop production globally. The issues on the absorption and acceptance of the technologically driven agricultural commodities are to be brought under the category of ethics that consider the risk evaluation and hazard mitigation. CAST (2005) advocated the institutionalisation of ethics in agriculture in order to evaluate and independently bring out transparent benefits and attendant hazard level for the environment and consumers.

Risk assessment as done in the regulatory system of the European Union is to bring out the potential perceived risk due to GM technology. Comparative risk assessment has been undertaken by Steinhauser (2001) between chemicals and GM products. The US Food and Drug Administration relies on the hazard identification of GM products and finalise the risk perception based on the hazards. An essential element in the ethical evaluation of biotechnology is the analysis of the possible harms and weighing these risks against the probable benefits.

Risk evaluation protocols for genetic modifications have become a major field that has implication in the microorganisms that are immensely used in the development of more efficient products. However, the global opinion has not been unanimous on the commercial release of such GM microorganisms for agriculture purposes. In India too, there has been no acceptance of GM microbial pesticides due to evident concerns regarding the unknown effects to humans and the environment. The stringent guidelines in research laboratories with Biosafety Level (BSL) 4 level facility make the costs and elaborate infrastructure very high.

Genetically modified microbial biocontrol agents have been undertaken to sharpen the targeted bioefficacy as well as to improve the non-competitive performance in various agroecologies. The environmental impact and risk assessment thereon (Anonymous 2000; Migheli 2001a, b) are mandatorily undertaken in order to permit the release of such GM bioagents for use in crops. Biosafety and ethical concerns are important considerations are to be imposed in the regulatory framework (Zadoks 1998; Stemke 2004). However, this capability raises concerns about the potential hazards posed by the technology. In response to these concerns, specific protocols (Stemke 2004) have been developed to safely monitor the use of genetically modified microorganisms (GMMs). In case of approval for environmental release of GM crop plants with traits for biotic stresses, such as pests and diseases, the regulatory body under the Ministry of Environment, Forest and Climate Change, Government of India, is vested with the Environmental Protection Act (EPA), 1986, has placed moratorium presently. Recent scientific advancements in gene editing technology—clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated protein (Cas) (Crispr-Cas9) techniques—have stimulated research interest to develop biotic stress-tolerant crop plants (NAAS 2020).

13.6.3 Ethical and Regulatory Concerns in the Biotechnology of Crops for Pest Management

CAST (2005) advocates institutionalised agricultural ethics in which both farms and food systems need to resolve ethical conflicts and steer socio-economic advantage of the new biotechnological tools and inventions that claim better crop productivity, improved farm commodity quality, better management of biotic and abiotic stresses. Environmental ethics, socio-economic benefits and regulatory policies have been strengthened as an important component in the debates on the advancement in modern biological sciences (Anonymous 2015; Southgate 2002; Kinderlerer and Adcock 2003; Gupta and Chandak 2005; Shukla et al. 2018). The anxiety for seeking answers to unknown concerns has increased over the last few decades

arising out of the explosion in the information flow. While derisive about such anxieties, validation of science-based analytical processes of any new information that floats around is desirable.

Clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated proteins (Cas) (CRISPR-CASPER-9) system is a good tool for modifying crop genome in order to generate gene-edited crop plants to impart resistance to pests. Globally, there has been extensive debate on the ethical and regulatory policy requirements to apply this nascent and potent tool for imparting biotic stress resistance in crop plants. The National Biotechnology Development Strategy Document (http://dbtindia.gov.in/about-us/strategy-nbds) does not contain policy statement on this novel technology for agriculture. The National Academy of Agriculture Sciences (NAAS) has published Policy Brief No. 7 after a consultation on the Draft Guidelines on Gene Edited Organisms (GEOs) from the Department of Biotechnology, Government of India (NAAS 2020). The Indian research scenario in pest resistance using Crispr-Cas9 technique is stated to be at its infancy. NAAS recommends that in the case of GEO too, in line with the EPA (1986) along with the existing Seed Act (1966), the new plant varieties developed through genome editing need to go through the regulatory processes where required, for risk analysis of biosafety and environmental safety, so that the technology applications are in compliance with the protocol. A policy perspective on GEO techniques is in the making by the Department of Biotechnology.

The currency of perceptions has to be modified after acute effort to bring new evidences for and against any perceived threat borne out of the introduction of new technology into farming. Regulatory process of countries strives hard to undertake such intellectual steps to arrive at well-debated clarity of thoughts. In general, open debates involving every logically thinking argument would alleviate most of the apprehensions within the existing scientific and socio-economic realm. There is a tendency to approximate certain potential hazards without looking for evidence-based conclusions. Such instances leave the analysts with inconclusiveness about the technology. The major plant protection concern is the non-uniform expression of the transgenic gene expressing Bt toxin gene(s). The farmer in such cases tends to lose his investment on seeds 'with promise to suppress biotic stress' due to inconsistent protection of target pests and consequent severe crop losses.

The basic evaluation of genetically engineered products from biotechnology[19] utilising transgenic, cisgenic or subgenic tools to derive crops with alien genes to express insecticidal proteins is the science-based analysis of the possible harms and their most likelihood of occurrence, for weighing the risks across the anticipated benefits. Each sovereign nation introducing such GM crop needs to transparently examine the home-generated data on all aspects of safety leading to hazard definition of the GM event and the gene product in the crop plant. Biosafety assessment protocols need to be laid out for each instance of introducing GM crop bearing the genes expressing insecticidal entities in the plant, their metabolites and degradation

[19]https://en.wikipedia.org/wiki/Genetically_modified_crops. Accessed 10 Sept 2020.

products that may have impact to all components of agroecology and other environmental entities as defined by the regulatory system of the country.

The following ethical matters are perceived during the commercialisation of GM cotton delta endotoxin gene (the only crop) permitted for environmental release in India.

1. Whether the endotoxin expressing gene(s) technology is any more relevant for pest management in crops.
2. Need for transparent regulatory mechanism to oversee the claims from GM technology of crop including crop yield (Quain and Ziberman 2003) after environment release of GM crop cultivar into the environment as well license to produce and market their seeds to farms.
3. Well-defined roles and responsibilities of the government agencies that deliver to farmers of all states the information on performance of the GM crop variety as well as perceived hazards due to them from time to time.
4. Overseeing the quality including genetic purity of seeds of the relevant traits that are marketed for cultivation.
5. In case of cotton crop whether lint yield and fibre quality are commensurate with the label claim of the marketed seeds and acceptable for the best market price appreciation, as the crop is to produce cotton lint as industrial raw material.
6. Non-availability of non-GM cotton variety seeds due to non-production and marketing of these as an alternative for farmers for opting those for cultivation. The government has not made adequate provision to provide seeds of cotton varieties that are developed in public institutions. The raw material consumer industry may also support the seed availability through corporate social responsibility (CSR) programmes.
7. All private seed companies have GM technology with monopolistic trends through hybrid seeds, as in the case of cotton crop, with which every year farmers have to depend on those seeds from them. Open-pollinated cotton crop seeds have vanished from market completely. Farmers are compelled to cultivate only GM hybrid cotton seeds.
8. The assurance that insect pest management would be easier with low use of pesticides has not been proven as a faithful technological advantage in India.
9. Transparency of information on the given genetic modification and the gene product(s) expressed in host crops.
10. The potential hazard perception to environment including agroecology.
11. Potential hazard for the target pest species to develop resistance to the given toxin that is expressed in crop plants.

13.7 Conclusions

The microbial pesticide formulations have been registered under the Insecticide Act, 1968, in order to regulate their manufacture and use in farms with respect to crops and target pests as well as to sustain their biological quality. There has been

increasing concerns about the standards and practices in their manufacture, packing, storage, transport and handling in order to reach them to farms for application in crops. Recent spurt in low quality of these formulations as well as their contamination with chemical synthetic pesticides has alerted the regulatory system, consumers and farmers alike. Quality review management of microbial pesticide formulations needs special attention for both manufacturers under GLP/GMP regime. The effluent management of the manufacturing units also needs intense environmental audit to safeguard from perceived hazards.

Genetically modified crops are the best biotechnology-derived products that target increase in both yield and quality of farm commodities. The risk assessment of these commodities in the context of hazard perception and their mitigation has grown into specialised regulatory paradigm.

New biotechnology tools and techniques such as Crispr-Cas9 in crops for pest resistance and higher commodity output with better quality parameters along with options to reduce risks to human and environment are promising. Ethical and practical considerations for commercialisation of genetic modification of crops from cisgenic, transgenic or subgenic products need careful analysis for science-based assessment of the potential hazards. Combination of microbial pesticide formulations and biotech farm crop varieties can be integrated in the overall crop health management architecture. The question of availability at farm gate of these IPM components has deeper introspective policy requirement. The agriculture farms cannot become exploitation grounds and make crop loss to bear year after year for farmers due to inefficient performance of MP formulations as well as GM varieties severe.

Points to Remember

- The microbial pesticide formulations are useful tools for invertebrate pest management of crops in all agroecologies. Their widespread use in pest management and benefits accrued in terms of clean commodities alongside clean farm agroecology and safeguarding consumers' health have futuristic implications in regulatory principles and practice.
- Quality review management of MP products is essential to sustain the shelf life and field bioefficacy of the products. The biowaste management of MP production units should follow GLP and GMP standard operating procedures to prevent undesirable leakage of harmful microorganisms following the relevant national guidelines and international conventions. The critical gaps in ethics and regulatory needs of MP formulations manufactured within GLP/GMP norms can be addressed through quality review management (QRM).
- Biotechnology tools and techniques to deploy genetically modified crops as well as gene editing technology (Crispr-Cas9) for attaining pest resistance and higher commodity output with better quality parameters are promising. Ethical and practical considerations for commercialisation of GM crops from cisgenic, transgenic or subgenic products need careful analysis for science-based assessment or 'decision tree-based' evaluation of the potential hazards.

- Combination of microbial pesticide formulations can be integrated in the pest management architecture after appropriate regulatory approval.

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