Pesticides in Crop Production

Pesticides in Crop Production

Physiological and Biochemical Action

Edited by

Prabhat Kumar Srivastava
Department of Botany, KS Saket PG College
Ayodhya
Uttar Pradesh, INDIA

Vijay Pratap Singh
Department of Botany, C.M.P. Degree College
A Constituent Post Graduate College of University of Allahabad
Prayagraj
Uttar Pradesh, INDIA

Anita Singh
Centre of Advanced Studies in Botany, Banaras Hindu University
Varanasi
Uttar Pradesh, INDIA

Durgesh Kumar Tripathi
Amity Institute of Organic Agriculture
Amity University, Uttar Pradesh, Noida
Uttar Pradesh, INDIA

Samiksha Singh
Ranjan Plant Physiology and Biochemistry Laboratory
Department of Botany, University of Allahabad, Prayagraj
Uttar Pradesh, INDIA

Sheo Mohan Prasad
Ranjan Plant Physiology and Biochemistry Laboratory
Department of Botany, University of Allahabad, Prayagraj
Uttar Pradesh, INDIA

Devendra Kumar Chauhan
D.D. Pant Interdisciplinary Laboratory
Department of Botany, University of Allahabad, Prayagraj
Uttar Pradesh, INDIA

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Contents

List of Contributors

S.B. Agarwal
Department of Botany
Institute of Science
Banaras Hindu University
Varanasi
India

Madhoolika Agrawal
Department of Botany
Institute of Science
Banaras Hindu University
Varanasi
India

Bilal Ahmad Mir
Department of Botany
School of Life Sciences
Satellite Campus Kargil
University of Kashmir
Jammu and Kashmir
India

Saroj Arora
Plant Stress Physiology Lab
Department of Botanical and
Environmental Sciences
Guru Nanak Dev University
Amritsar
India

Palak Bakshi
Plant Stress Physiology Lab
Department of Botanical and
Environmental Sciences
Guru Nanak Dev University
Amritsar
India

Shagun Bali
Plant Stress Physiology Lab
Department of Botanical and
Environmental Sciences
Guru Nanak Dev University
Amritsar
India

Goutam Banerjee
Department of Biochemistry
University of Calcutta
Kolkata, West Bengal
India

Renu Bhardwaj
Plant Stress Physiology Lab
Department of Botanical and
Environmental Sciences
Guru Nanak Dev University
Amritsar
India

Dinesh C. Bilehal
Department of Chemistry
Reva University
Rukmini Knowledge Park, Kattigenahalli
Yalahanka, Bangalore, Karnataka
India

Satpal Singh Bisht
Department of Zoology
D. S. B. Campus, Kumaun University
Nainital, Uttarakhand
India

Magnoli Carina
Departamento de Microbiología e Inmunología
Facultad de Ciencias Exactas Físico Químicas y Naturales
Universidad Nacional de Río Cuarto
Río Cuarto, Córdoba
Argentina

Barberis Carla
Departamento de Microbiología e Inmunología
Facultad de Ciencias Exactas Físico Químicas y Naturales
Universidad Nacional de Río Cuarto
Río Cuarto, Córdoba
Argentina

Carranza Cecilia
Departamento de Microbiología e Inmunología
Facultad de Ciencias Exactas Físico Químicas y Naturales
Universidad Nacional de Río Cuarto
Río Cuarto, Córdoba
Argentina

Pritam Chattopadhyay
Department of Biotechnology
Gauhati University
Guwahati, Assam
India

Nivedita Chaudhary
Department of Vegetable and Field Crop Research
Agricultural Research Organization
Gilat Research Center
M.P. Negev
Israel

and

Department of Botany
Institute of Science
Banaras Hindu University
Varanasi
India

Mahadev B. Chetti
Indian Council of Agricultural Research
Pusa, New Delhi
India

Krishna Kumar Choudhary
Department of Botany
School of Basic and Applied Sciences
Central University of Punjab
Bathinda
India

Christos A. Damalas
Department of Agricultural Development
Democritus University of Thrace
Orestiada
Greece

Neeraj Kumar Dubey
Department of Biotechnology
School of Life Sciences
Central University of Rajasthan
Bandar Sindri, Dist-Ajmer
India

Géraldine Giacinti
Laboratoire de Chimie Agro-Industrielle (LCA)
Université de Toulouse INRA, INPT, Toulouse
France

and

Centre d' Application et de Traitementdes AgroRessources (CATAR)
INPT, Toulouse
France

Aditya Kumar Gupta
Department of Biotechnology
School of Life Sciences
Central University of Rajasthan
Bandar Sindri
Dist-Ajmer
India

Kapil Gupta
Department of Biotechnology
School of Life Sciences
Central University of Rajasthan
Bandar Sindri
Dist-Ajmer
India

Shubhra Gupta
Department of Biotechnology
School of Life Sciences
Central University of Rajasthan
Bandar Sindri
Dist-Ajmer
India

Rohit Joshi
Division of Biotechnology
CSIR-Institute of Himalayan Bioresource Technology
Palampur
Himachal Pradesh
India

Vijay Juyal
Department of Pharmaceutical Sciences
Kumaun University
Campus Bhimtal, Uttarakhand
India

Parminder Kaur
Plant Stress Physiology Lab
Department of Botanical and Environmental Sciences
Guru Nanak Dev University
Amritsar
India

Ravdeep Kaur
Plant Stress Physiology Lab
Department of Botanical and Environmental Sciences
Guru Nanak Dev University
Amritsar
India

Tajinder Kaur
Plant Stress Physiology Lab
Department of Botanical and Environmental Sciences
Guru Nanak Dev University
Amritsar
India

Anjali Khajuria
Department of Zoology
Guru Nanak Dev University
Amritsar
India

Kanika Khanna
Plant Stress Physiology Lab
Department of Botanical and Environmental Sciences
Guru Nanak Dev University
Amritsar
India

Mohammad Saghir Khan
Department of Agricultural Microbiology
Faculty of Agricultural Sciences
Aligarh Muslim University
Aligarh, Uttar Pradesh
India

Mahadev C. Khetagoudar
Department of Chemistry
Reva University
Rukmini Knowledge Park, Kattigenahalli
Yalahanka, Bangalore, Karnataka
India

Sukhmeen Kaur Kohli
Plant Stress Physiology Lab
Department of Botanical and
Environmental Sciences
Guru Nanak Dev University
Amritsar
India

Spyridon D. Koutroubas
Department of Agricultural Development
Democritus University of Thrace
Orestiada
Greece

Aluffi Melisa
Departamento de Microbiología e
Inmunología
Facultad de Ciencias Exactas Físico
Químicas y Naturales
Universidad Nacional de Río Cuarto
Río Cuarto, Córdoba
Argentina

Benito Nicolás
Departamento de Microbiología e
Inmunología
Facultad de Ciencias Exactas Físico
Químicas y Naturales
Universidad Nacional de Río Cuarto
Río Cuarto, Córdoba
Argentina

Puja Ohri
Department of Zoology
Guru Nanak Dev University
Amritsar
India

Subramani Pandian
Department of Biotechnology
Science Campus, Alagappa University
Karaikudi, Tamil Nadu
India

Jogeswar Panigrahi
Department of Biotechnology
School of Life Sciences
Central University of Rajasthan
Bandar Sindri
Dist-Ajmer
India

V. Raghu
Department of Food Technology
Jain University
Kanakapura, Bangalore, Karnataka
India

Manikandan Ramesh
Department of Biotechnology
Science Campus, Alagappa University
Karaikudi, Tamil Nadu
India

Mahendra Rana
Department of Pharmaceutical Sciences
Kumaun University
Campus Bhimtal, Uttarakhand
India

Mohammad Shahid
Department of Agricultural Microbiology
Faculty of Agricultural Sciences
Aligarh Muslim University, Aligarh
India

Anket Sharma
Plant Stress Physiology Lab
Department of Botanical and Environmental Sciences
Guru Nanak Dev University
Amritsar
India

and

Department of Botany
DAV University
Sarmastpur, Jalandhar
India

and

State Key Laboratory of Subtropical Silviculture
Zhejiang A&F University
Hangzhou
China

Shilpi Sharma
Department of Biochemical Engineering and Biotechnology
Indian Institute of Technology Delhi
Hauz Khas, New Delhi
India

Valérie Simon
Laboratoire de Chimie Agro-Industrielle (LCA)
Université de Toulouse INRA, INPT, Toulouse
France

Upma Singh
Department of Biochemical Engineering and Biotechnology
Indian Institute of Technology Delhi
Hauz Khas, New Delhi
India

Ashwani Kumar Thukral
Plant Stress Physiology Lab
Department of Botanical and Environmental Sciences
Guru Nanak Dev University
Amritsar
India

Jyoti Upadhayay
Department of Pharmaceutical Sciences
Kumaun University Campus
Bhimtal, Uttarakhand
India

and

School of Health Sciences
University of Petroleum & Energy Studies
Dehradun Uttarakhand
India

Varsha Ashok Walvekar
Department of Biochemical Engineering and Biotechnology
Indian Institute of Technology Delhi
Hauz Khas, New Delhi
India

Pawan Yadav
Department of Biotechnology
School of Life Sciences
Central University of Rajasthan
Bandar Sindri
Dist-Ajmer
India

Almas Zaidi
Department of Agricultural Microbiology
Faculty of Agricultural Sciences
Aligarh Muslim University
Aligarh, Uttar Pradesh
India

Preface

Pesticides have had a tremendous role in enhancing productivity and yield of crops prominently after the second half of the twentieth century. Most of the countries across the world are observing newer heights in total as well as specific crop production despite the fact that the agricultural fields are being used in non-agrarian tasks like the construction of roads, railways, industries and buildings for human settlements. A massive extent of credit goes to use of agrochemicals in general and pesticides in particular. Increasing human population and constricting agricultural lands do not permit us to give up the use of pesticides and to switch over completely towards organic farming. Additionally, development in industries and agriculture are taken as a general criterion for development of any country. This has resulted into imprudent and unlimited usage of agrochemicals in our farmlands leading to disturbance in abiotic as well as biotic components of soil and water ecosystem and culminating into ecological imbalance.

Pesticides are the only toxic chemicals deliberately released into the environment in large amounts. Some of the pesticides (organochlorines) are biomagnified in the terrestrial ecosystems, so they were banned worldwide. The organophosphorus pesticides were introduced in the 1970s as replacements for the persistent organochlorines. The increased use of organophosphorus pesticides originally seen as lesser threat to the environment but by the time organophosphorus pesticides have become a serious environmental concern due to their high acute toxicity despite their low persistence. Since most of the pesticides are non-biodegradable, they have long residence time in water and soil and thus may enter and magnify at various trophic levels. Excessive and imprudent usage of pesticides not only saturates the soil but also intoxicates the crops by harming their overall physiology and biochemistry. In addition to this, non-target organisms that are important components of the soil ecosystem like soil microbes, bacteria, fungi and blue green algae (privileged to be associated with atmospheric nitrogen fixation, fertility of the soil and nutrient recycling) may be harmed, which may indirectly affect the productivity and food security.

This book titled 'Pesticides in Crop Production: Physiological and Biochemical Action' is an important contribution towards understanding mode of pesticide action in plants, pesticide metabolism in soil microbes, plants and animals, bioaccumulation of pesticides, sensitiveness of microbiome towards pesticides and consequent risk assessment, development of pesticide resistance in pests, microbial remediation of pesticide intoxicated legumes, pesticide toxicity amelioration in plants by plant hormones. This book also encompasses eco-friendly pest management, transgenic strategies to develop resistant plant against the pathogen and pest and impact of pesticide on food stuffs and human health. Analysis of pesticide by GC-MS/MS (Gas Chromatography tandem Mass Spectrometry) is a reliable method for the quantification and confirmation of multiclass pesticide residues in cabbage and cauliflower as case studies has well been included.

Writing an authoritative book that remains relevant over the coming years cannot easily be done by an individual, but rather requires the concerted effort of a team of expert scientists. This book is a concerted task of an assemblage of scholars working in different parts of India and the world along with all the six editors. All editors thankfully acknowledge their contributions. All editors also gratefully acknowledge the team at John Wiley & Sons Limited which made possible the proposed book in its present form.

Editors

Prabhat Kumar Srivastava
Vijay Pratap Singh
Anita Singh
Durgesh Kumar Tripathi
Samiksha Singh
Sheo Mohan Prasad
Devendra Kumar Chauhan

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Development of Pesticide Resistance in Pests: A Key Challenge to the Crop Protection and Environmental Safety

Subramani Pandian and Manikandan Ramesh

Department of Biotechnology, Science Campus, Alagappa University, Karaikudi, 630 003, Tamil Nadu, India

1.1 Resistance: The Introduction

Resistance is the micro-evolutionary course of action by which genetic adaptation through pesticide selection has resulted in increased arthropod populations for which management is more difficult (Whalon and McGaughey 1998). The outcome of resistance is the malfunction of plant protection tools, strategies to limit economic injury of pest populations where failure is due to a genetic adaptation in the pest.

Resistance to pesticides is a complicated and substantial problem in circumstances where chemicals are used to eradicate pest populations. On the other hand, against the economic, communal, and ecological costs linked with this problem, resistant insects are a physiological marvel. Certain populations have become highly resistant to a specified insecticide, which can survive exposure to almost any dose. More than 440 species of pest which have developed resistance to one or more pesticides have been documented. One of the most amazing things in evolutionary adaptation is pesticide resistance due to environmental changes, especially when this has occurred relatively quickly in terms of evolutionary time. Prevalent distribution of resistance in crops and livestock pests is the major threat to the agricultural productivity and many of the serious resistance problems are also documented.

Understanding the molecular mechanisms and resistance adaptations in pest populations is a significant problem. However, the molecular mechanisms of pesticide resistance have continued and the understanding of these resistance mechanisms plays an important role in improving the integrated management and in identifying new targets for the vaccine development which is useful for eradicating the pesticide-resistant pests on agriculture and for public health. Knowledge about resistance will pave the way for the fundamental perceptions into evolution, genetics, physiology, and ecology. Resistance can also make a severe economic loss with social disruption.

Over the past 15 years, the global area allotted to transgenic crops is more than 69 million hectares for reducing insecticidal toxins resultant of the bacterium *Bacillus thuringiensis* (Bt) which has emerged quickly (James 2008). Among these, Bt cotton and Bt maize were the most cultivated plants in this area (James 2008). Effective control of target pests, diminished use of conventional insecticides, and reduced harm to

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non-target organisms are the important benefits of the use of Bt crops (Huang et al. 2005; Cattaneo et al. 2006; Marvier et al. 2007; Hutchison et al. 2010). Another theme is, giving greater importance to field trials and assessment of resistance in field populations will improve resistance management from concept to practice. The final theme is the next generation methodology of pest control which may greatly depend on microbial toxins, mostly through the expression of *Bt* toxin genes in genetically engineered crop plants and microorganisms. The remarkable usefulness of *B. thuringiensis* in killing some pests but which are not applicable for all the species is one of the drawbacks of this technology.

1.2 Pesticide Resistance: A Global Analysis

The evolution of resistance against pesticides is a fundamental problem of modern agriculture (Takahashi et al. 2017). The Analysis of Global Pesticide Resistance arose because of the exponential increase in the cases of resistance worldwide during the second half of the twentieth century and also the recognition by industries of new chemistries ended up with novel modes of action which are a precious resource that should be conserved. International Insecticide Resistance Action Committee (IRAC) mainly worked on different aspects of resistance management, such as detection and monitoring programs, and even more helpful is IRAC's utmost development, which is the effort to develop resistance reporting by mode of action (MOA) classification of pesticides. Based on that, the agrochemical industries have often put the effort to understand, define, monitor, and manage pesticide resistance (www.irac-online.org). The pesticide industry formed IRAC and other resistance action committees after scientific, public, and new regulatory pressures.

1.3 Molecular Genetics and Biochemical Basis of Pesticide Resistance

For the last three decades, incredible advancements have been made in understanding resistance of pesticides in arthropods, initially biochemical and physiological mechanisms, and more recently at the level of molecular genetics and genomics. The greatest improvement in molecular genetic studies has exposed many details about the resistance mechanisms, both at individual and population levels. That improvement has provided new perceptions on the microevolutionary processes that have been produced by them; it has also revealed unforeseen complexities that are very complicated to unravel. There are several mechanisms available for pesticide resistance which has been discussed below (Figure 1.1).

1.4 Changes in Pesticide Binding Sites

Every potent pesticide has one or more specific binding sites on macromolecules within the insect except mitochondrial uncouplers. The malfunctioning of the macromolecular site of action results in the binding of insecticide, that initiates a cascade of events

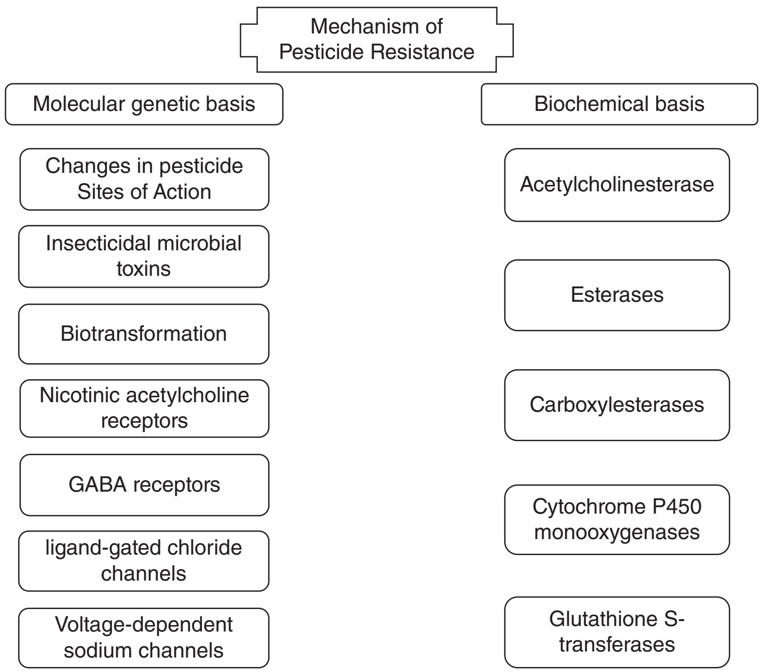

Figure 1.1 Schematic diagram for pesticide resistance mechanisms.

which leads to the death of the particular insect. Changes in insecticide binding to the site of action, or to disturb its functions after binding, must lead to major changes in the overall impact on the insect. There is plenty of evidence that changes are the initial cause of resistance to different types of pesticides. Mostly, the point mutations lead to critical changes in amino acid residues in the receptor molecule compared to changes in the expression level of existing receptors. However, in some cases it seems that a functional target site is not dangerous for the existence of the insect even though its interaction with the pesticide leads to death. Significant changes on sites, either through mutation or decreased expression, are not always disadvantageous, and sometimes the complete elimination of the gene product (null mutation) is a viable pathway to greater levels of resistance. There have been several researches which like, Gahan et al. (2001) confirmed the loss of a cadhedrin-binding protein for *B. thuringiensis* toxin in *Heliothis virescens*; the loss of the nicotinic acetylcholine receptor subunit that binds the spinosyns in resistant Drosophila (Orr et al. 2006); and the loss of a binding protein for juvenile hormone analogs in Drosophila (Wilson and Ashok 1998).

1.5 Nicotinic Acetylcholine Receptors

There are two different types of neurotransmitter ACh available in insects, including muscarinic receptors, which were linked to slower G-protein mediated postsynaptic actions, and a nicotinic receptor that open ion channels through the neuronal membrane prominent to a rapid but transient shift in membrane polarization. In resistant strains of *Drosophila melanogaster* formed by mutagenesis, resistance is attributable to numerous different mutations in a gene encoding the Dα6nAChR subunit, which in some cases leads to the complete loss of this site of action for the insecticide (Orr et al. 2006). Resistance to neonicotinoids is increased with their extensive use and has been reviewed by Nauen and Denholm (2005). In a few cases, increased degradative metabolism, mainly by monooxygenases, is involved. However, in other cases, evidence for an alteration in the sensitivity of the nervous system to the effects of the insecticide has been reported (Liu et al. 2005; Mota-Sanchez et al. 2006). In the brown plant hopper, resistance to imidacloprid has been attributed to a mutation in AChR subunits that decreases specific binding to nervous system membrane preparations (Liu et al. 2005). The occurrence of a single mutation (Tyr151Ser) in a conserved region believed to be involved in ACh binding was found in two subunits, Nlα1 and Nlα3, and associated well with the existence of imidacloprid resistance. When these insects α-subunits were co-expressed in rat β2-subunits to form chimeric receptors, a virtually complete loss of imidacloprid binding was observed compared with the same subunits from susceptible insects.

1.6 GABA Receptors and Other Ligand-gated Chloride Channels

γ-Aminobutyric acid (GABA) is the main inhibitory transmitter in the insect nervous system, with GABA-ergic transmission occurring both within the central nervous system and at the neuromuscular junction (Casida 1993; Hawkinson and Casida 1993; Casida and Pulman 1994; Ozoe and Akamatsu 2001; Buckingham et al. 2005; Ozoe et al. 2009; Ozoe 2013). These fast-acting GABA receptors (GABARs) are linked to the channels that gate chloride ions and they lead to hyperpolarization. Another group of GABARs in insects' gate cations is having an electrifying effect, but very less is known about their functions and their insecticide action. Their MOA is distinct from that of the GABARs gating chloride channels (Gisselmann et al. 2004). The structures, functions, molecular genetics, and interactions of inhibitory GABARs with insecticides have been reviewed by Buckingham and Sattelle (2005) and Buckingham et al. (2005). The structure of inhibitory GABARs is pentamers with the subunits organized to form a central ion-conducting pore. Each subunit has four transmembrane domains and the receptor has abundant distinct binding sites for xenobiotics. There are several forms of the subunits which exist *in vivo* even though somewhat little is known about the number, nature, specific functions, or localization of the native receptors. Ionotropic receptors that are gate chloride ions occur in the insect nervous system having either glutamate or histamine as their activator. Particularly, glutamate-gated channels (glutamate H receptor) (GluHR) have a vital role in insecticide action. For certain compounds, including the avermectins, which act on both GluHR and GABAR are possibly involved in their complete toxicity to insects.

1.7 Voltage-Dependent Sodium Channels

Sodium ions often move across the axonal membrane is an important factor in the enhancement of a nerve action potential. The opening of voltage-dependent sodium-specific channels produced as the wave of depolarization induced by an approaching action potential reaches a critical value. The sodium channels are quickly inactivated, after the opening, which switch over the inward flow of sodium current and consequently limits the depolarization. Three different groups of insecticides influence this process by their actions on voltage-dependent sodium channels (VDSCs). Even though having different structures, its relatives and the pyrethroids have their MOA as a common feature. They slow channel deactivation and trap sodium channels in the open configuration, to modify the sodium channels, resulting in elongated channel opening evidenced by a large tail current associated with repolarization (Vijverberg et al. 1982; Narahashi 1988). This may lead to the repetitive discharge of action potentials, or, if depolarization is in an elevation state, a complete block on axonal transmission will formed (Narahashi 2002). Both of the actions have greater effects on the nervous system. Indoxacarb is an oxadiazine insecticide that acts on VDSCs by a different mechanism of actions. Indoxacarb and its N-decarbomethoxylated metabolite block the sodium channels of various insects and mammalian neurons by maintaining VDSCs in an inactivated form (Wing et al. 2010). These studies proposed that the actions of indoxacarb and DCJW (N-decarbomethoxylated metabolite) share some similarities with that of local anesthetics, which impede sodium currents by binding selectively to the inactivated state of the sodium channel (Hille 1977). Changes in target site mediated resistance to indoxacarb have not yet been reported, however, mutations in the VDSC are a general cause of resistance to Dichloro diphenyl trichloroethanes (DDT) and the pyrethroids (Soderlund et al. 2002; Soderlund and Knipple 2003; Khambay and Jewess 2005; Soderlund 2005; Dong 2007).

1.8 Insecticidal Microbial Toxins

Currently different types of proteinaceous bacterial toxins were utilized for the insect control. Among which, *B. thuringiensis* (*Bt*) and *Bacillus sphaericus* (*Bs*) are the two major sources. *Bt* strains produce arrays of crystal protein δ-endotoxins which are availed either in prevalent spray-on applications or are genetically engineered into plants. More than 200 disparate toxin-producing *Cry* genes have been established. The collection, nomenclature, and uses of *Bt* toxins have been summarized by Bravo et al. (2005). Certain groups of endotoxins are produced which are intended to be profoundly specific for different orders of insects, including lepidopterans, coleopterans, and mosquitoes. Behind the solubilization and proteolytic activation into the insect gut, it is considered that *Bt* endotoxins apply their toxicity by binding to receptors on the mid gut epithelial cells of sensitive insects. The group of several toxin molecules then directs their insertion into the luminal membrane to form a pore. This comes up with the loss of ionic control which leads to the osmotic disruption of the mid gut cells causes swelling and lysis, which is lethal. Some types of receptor, including glycosyl phophatidylinositol-anchored aminopeptidase-*N* (APN), a digestive enzyme and cadherins for the *Bt* toxins, which are toxic to lepidopterans have been identified on the mid gut epithelial cell surface, known to act as intercellular adhesion molecules. Various receptor types are visibly involved in the actions of different *Bt* toxins, while binding site-based resistance to Cry1A toxins does not direct to cross-resistance to Cry1C toxins (Ferré and Van Rie 2002). The different mechanisms of resistance to *Bt* genes which have been discovered until now, have been reviewed by Ferré and Van Rie (2002), Bravo *et al.* (2005), and Griffitts and Aroian (2005). The diamond-back moth (*Plutella xylostella*), is the only one insect species known to have resistance to *Bt* toxins during field exposure and the complete mechanism for this resistance is not identified. Yet, it is monogenic and partially recessive and is characterized by a remarkable decrease in the specific binding of radiolabelled Cry1A toxins to encounter the border membrane vesicles isolated from the resistant mid guts (Ferré and Van Rie 2002; Sayyed et al. 2004, 2005). The exposure of a premature stop codon into the cadherin coding sequence has also been directly linked to Cry1Ac resistance in *Helicoverpa armigera* (Xu et al. 2005). In this perspective, it is fascinating that three different recessive cadherin mutations were recognized in the field-identified pink bollworms, *Pectinophera gossypiella* (Morin et al. 2003). Respective genetic changes lead to the deletion of at least eight amino acids upstream of the presumed binding zone. Even though, resistance only arose from a combination of any two of the deletion-bearing alleles were closely linked.

1.9 Biotransformation

The metabolic changes of an insecticide within the target organism are a frequent defensive mechanism that directed toward a decrease in the period and intensity of the exposure to the target site, which minimize the probability of lethal condition. Several insects have developed wide and rapidly inducible defenses against pivotal toxic xenobiotics that are initially taken in during the diet; therefore, these defenses may be modified to behave as the path of resistance. Three key mechanisms of metabolic transformation of insecticides cause the huge number of examples of biotransformation-based resistance: (i) oxidation; (ii) ester hydrolysis; and (iii) glutathione conjugation. Although, the products of these reactions are most frequently less toxic than the parent, there are numerous examples of an increase in toxicity as a result of a biotransformation reaction, in which the insecticide is applied as a pro-pesticide. Apparently, an increase in the rate of metabolic conversion in this case should result in being more toxic to the insect, and a decrease in the rate of activation is one of the routes to resistance. By comparing to the resistance evolving from site-of-action changes in which mutations in the structural genes are most predominate as a fundamental mechanism, biotransformation-based resistance frequently involves the overexpression of prevailing metabolic enzymes by modifications in their regulatory systems and by gene duplication. Esterases, cytochrome P450 monooxygenases, and glutathione transferases are the most significant factors in specific cases of resistance. Further conjugation reactions, including glucose and sulfate conjugations likewise appear to involve little role in well-known cases of resistance.

1.10 Acetylcholinesterase

Acetyl cholinesterase (AChE) play a primary role in the removal of excitatory neurotransmitters and the acetylcholine (ACh) from cholinergic synapses, which is the target region for carbamate and organophosphate inhibitors in both insects and vertebrates (Giacobini 2000). The inhibition of AChE through accumulation of ACh leads to the continuous stimulation and the desensitization of the ACh receptors (AChR), severe neurological disruption, and eventually to death. The molecular architecture of the homodimeric AChE enzyme and its catalytic site were initially described based on crystallographic analysis in Torpedo (Sussman et al. 1991) and the parallel majority of structural features in the Drosophila enzyme were described by Harel et al. (2000) and revised by Oakeshott et al. (2005). Resistance to the insecticides like organophosphorus and carbamate in most of the arthropod pests were conferred by a series of common/shared point mutations in acetylcholinesterase (AChE). However, the mutations linked with the insecticide sensitivity often results in reduced catalytic efficiency and leads to a fitness disadvantage (Lee et al. 2015).

AChE has different allosteric sites that modify the activity of the enzyme, but these are not usually considered as the primary site of action of pesticides. Diptera family insects have only one gene for AChE whereas other insects, including mites and mosquitoes, have two, while possibly only one (ACE-1) is expressed in, and is active in eradicating ACh from the central nervous system (Russell et al. 2004). Interestingly, this gene is not the orthologous or even the single enzyme in the higher Diptera, so it seems that two somewhat distinct genes encode for the neuronal AChE even in different members of the same order, Diptera (Weill et al. 2002). Recently the third ACE gene has identified by Fournier (2005) and the functions of the other ACE genes are unknown, but they do not appear to be related to resistance.

1.11 Esterases

The majority of Esters are the most widely used insecticides. Those are almost all carbamates and OPs, maximum of pyrethroids and other compounds such as indoxacarb, methoprene and similar juvenoids, fluacrypyrim, and bifenazate. Most of the cases, the hydrolysis of the ester group lead to a significant reduction in, or total removal of toxicity. Only in a few cases does ester or amide hydrolysis act as an activation reaction; for example, indoxacarb, acequinocyl, or dinitrophenol esters such as dinocap are all influenced by ester hydrolysis for their toxicity. Subsequently, esterase activity frequently plays an important role in determining the comparative responses and resistance to present insecticides. In insects, esterases hydrolyse the esters of carboxylic acids so therefore they are termed as carboxylesterases. The nature and consequence of esterases in insecticide toxicology and resistance have been reviewed by Oakeshott et al. (2005) and Wheelock et al. (2005). The different types of structural features of the substrate also changes the rates of ester hydrolysis.

1.12 Carboxylesterases (B-Esterases)

More than thirty genes of insects have been involved in the production of esterases that hydrolyse the carboxylic acid esters. They are members of the large and versatile family of enzymes that contain the α/β hydrolase fold with a nucleophile–acid–histidine catalytic triad (Oakeshott et al. 1999), and which was distributed into several subgroups (Oakeshott et al. 2005; Wheelock et al. 2005). One subgroup which is inhibited by OPs and most importantly, which hydrolyses aliphatic substrates is generally termed as carboxylesterases. These include phosphotriesterases. Calcium-dependent phosphotriesterases promptly hydrolyse many insecticidal Ops; to be precise the more labile phosphate esters, mostly cleaving the ester at the most anhydride bonds (Vilanova and Sogorb 1999). Mostly, in mammals, it presents predominantly but much less in fish, birds, and several insects OP metabolism (Dauterman 1983; Vilanova and Sogorb 1999).

1.13 Cytochrome P450 Monooxygenases

The cytochrome P450 catalyzes the multifunctional monoxygensases, which encompasses the highly versatile system for the metabolism of insecticides. These enzymes play a major role in the toxicity of many pesticides and are a key player in the development of resistance in insects. The cytochrome P450-dependent monooxygenases mediated resistance has been reviewed by Bergé et al. (1998), Scott (1999), and Feyereisen (1999, 2005). The monooxygenase was regulated by NADPH through a flavoprotein, NADPH: cytochrome P450 oxidoreductase. Cytochrome b5 will possibly involve electron transfers with some forms of cytochrome P450. A huge number of the cytochrome P450 (CYP) superfamily genes is present in insects, with nearly 100 so far characterized in some insect genomes. Some of the genes possess known physiological functions in intermediary metabolism, but others play a role in defenses against the many xenobiotic chemicals. Previously in several studies P450 inhibitors have been used, mainly piperonyl butoxide (PBO), that synergize compounds degraded by P450. The role of P450 in resistance was indicated by a decline in the resistance level in synergized insects. The PBO is not completely specific for oxygenase reactions; besides it may decrease the proportions of insecticide penetration (Sanchez-Arroyo et al. 2001) and may inhibit certain esterases by synergistic activities (Young et al. 2005). In order to conclude the resistant strains has greater level of P450 catalyzed reactions compared to susceptible strains; model P450 substrates are used widely. This has been produced useful information and caution is necessary, although meanwhile several P450 isoforms are available which varied extensively in substrate specifications.

1.14 Glutathione S-Transferases

The Glutathione S-transferases (GST) are a huge group of enzymes that enrich the reaction of the cysteine sulphydryl group of the tripeptide glutathione (GSH) with xenobiotics. In xenobiotics, the sulphydryl group of GSH a nucleophile which reacts with the electrophilic sites that leads to the GSH conjugate formation. The less toxic conjugates are most freely evacuated than the parental insecticide. The overall properties and toxicological significance of GSTs have been reviewed by Eaton and Bammler (1999), Sheehan et al. (2001), and Hayes et al. (2005). Even though a cluster of microsomal GSTs exists in insects, the transferases of high toxicological interest is soluble, which are relatively small (50–55 kDa) proteins with a dimeric structure. Certain GSTs are very quickly inducible by improved transcription after exposure of the insect to xenobiotics, such as pesticides and phytochemicals however GST activity in resistance systems is unknown (Yu 1996).

1.15 Other Resistance Mechanisms

In toting with changes in target sites and biotransformation mode of resistance systems, many other biochemical mechanisms may also involve in resistance to the insecticides. Even though individually, which has adequate influences on toxicity, and in the same insect has the ability to play as key roles in resistance while accommodating with the major mechanisms. They possess the wide range of activities, but moderately slight consideration only paid in these mechanisms and poorly understood in many cases. The evolution of resistance is a population genetics paradox, afflicted by manifold interactions amid pest biology and ecology, assets of the pesticide and patterns of pesticide use (Georghiou and Taylor 1977a,b; Carrière et al. 2015); in the spatially complex model, as suggested by Caprio (2001) that for source–sink dynamics, certain degree of isolation concerning a refuge and a toxic crop involved in delaying resistance is considerably less than having random mating among the different habitats. According to Alstad and Andow (1995), these dynamics will produce greater damage to the surrounding areas established to refuges. Using the spatially complex models, Peck et al. (1999) and Sisterson et al. (2005) established that resistance initially evolved from the places with a high density of transgenic fields and before it spread toward the outside. The selection process in these small areas emphases the high resistance allele frequencies in a greater frequency of resistant homozygotes and also the rapid rate of resistance evolution. When resistance developed in these regions, migration spread resistance alleles across all the areas.

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2

Fungicide Toxicity to Legumes and Its Microbial Remediation: A Current Perspective

Mohammad Shahid, Mohammad Saghir Khan and Almas Zaidi

Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, 202002, India

2.1 Introduction

Legumes are one of the most important dietary components of many countries including India. The high concentration of protein in legume grains is considered a valuable and cheap source of protein in numerous emerging nations. Some of important grain legumes grown worldwide include pigeon pea, pea, broad bean, chick pea, cowpea, and soybean; important pasture species include alfalfa, clovers, sweet clovers, trefoil, and vetches (Graham and Vance 2003; Kaur et al. 2014). These legume species together contribute around 33.0% of the nutritional human protein (Vance et al. 2000). Legume production is however, severely decreased by diseases caused by numerous soil borne phytopathogenic fungi (Katan 2017). So, to protect legumes from phytopathogens and consequently to maintain its nutritional value, various synthetic fungicides for example, carbendazim, mancozeb, kitazin, hexaconazole, thiram, etc. are applied indiscriminately in handling pulses. The majority of the applied fungicides, however, persist in the soil and are non-biodegradable. Such fungicides in turn destroy soil fertility (Anuradha et al. 2016) leading eventually to the loss of growth, symbiotic attributes, and yields of legumes (Garg et al. 2017). Some fungicides have been reported to even abolish nodule formation and nitrogen fixation of several grains and fodder legumes. Considering the nutritional importance of legumes on the one hand and the toxicity of fungicides to legumes and conflicting reports on the management of phytopathogens employing fungicides on the other hand, scientists are working hard to find a nontoxic and practicable option to facilitate the production of legumes in areas even contaminated with fungicides. In this perspective, the application of microbes especially plant beneficial soil microorganisms often termed as "PGPR," consisting of multiple growth enhancing properties have been found effective in solving such problems when used as microbial inoculants (Patil et al. 2017). Here, focus is given to better understand the mechanisms/process by which fungicides affect legumes and how the toxicity of fungicides to legumes could be reduced, if not completely eliminated.

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2.2 Nutritional Importance of Legumes

Legumes, a chief pulse crop are grown and consumed around the world. They essentially supply protein and carbohydrate besides acting as a good source of energy. In addition, they also provide significant amounts of vitamins, minerals, lipids, and some important micro and macro nutrients (Table 2.1). Some legumes (especially green gram) are well

Table 2.1 Nutritive importance of some commonly grown legumes

	Examples of some common legumes			
Nutritional value (per 100 g)	**Pea**	**Chickpea**	**Green gram**	**Lentil**
Energy (kJ)	339	686	1452	1477
Carbohydrates (g)	14.47	27.42	62.62	60
Sugars (g)	5.67	4.8	6.6	2
Dietary Fibers (g)	5.1	7.6	16.3	3.1
Fats (g)	0.4	2.59	1.15	1
Saturated (g)	—	0.269	—	—
Monounsaturated (g)	—	0.583	—	—
Polyunsaturated (g)	—	1.156	—	—
Proteins (g)	—	5.42	8.86	26
Vitamins				
Vitamin A eqiv (μg)	38	1	—	—
Thiamine(B1) (mg)	0.266	0.116	0.621	0.87
Riboflavin(B2) (mg)	0.132	0.063	0.233	0.211
Niacin(B3) (mg)	2.09	0.526	2.251	2.605
Pantothenic acid (B5) (g)	—	0.286	1.91	2.120
Vitamin B6 (mg)	0.169	0.139	0.382	0.54
Folate (B9) (μg)	65	172	625	79
Vitamin B12 (μg)	—	0	—	—
Vitamin C (mg)	40	1.3	4.8	4.4
Vitamin E (mg)	0.13	0.35	0.51	—
Vitamin K (μg)	24.8	4	9	—
Minerals				
Calcium (mg)	25	49	132	56
Iron (mg)	1.47	2.89	6.74	7.54
Magnesium (mg)	33	48	189	122
Phosphorous (mg)	108	168	367	451
Potassium (mg)	244	291	1246	955
Sodium (mg)	5	7	—	6
Zinc (mg)	1.24	1.53	2.68	4.56
Other constituents				
Water (g)	—	60.21	—	10.4

Source: Modified from Jukanti et al. (2012).

recognized for reclamation actions and are used to energize mentality, lessen heat hyperpyrexia, and decrease inflammation in summertime (Tang et al. 2014). Apart from these, legumes also contain high levels of antioxidants, anti-inflammatory, antimicrobial, and antitumor activities and may be involved in the regulatory metabolism of lipids (Anjum et al. 2011). Phytochemicals of legumes counteract oxidative damage, induce enzyme detoxification, stimulate the immune system, and lessen cancer hazards, etc. (Meher and Mishra 2017).

2.3 Fungal Diseases of Legumes: A General Perspective

It is estimated that 10–15% of legumes production in India is lost due only to fungal diseases. Some common symptoms produced due to fungal infection in legumes include chlorotic and necrotic spots, lesions, specks, defoliation, and stunning in plants (Kumar 2016). Most of the fungal pathogens belong to division Ascomycetes, Basidiomycetes, or Oomycota. Some of the diseases caused by phytopathogenic fungi are powdery mildews, rusts, wilts, root rots, damping off, gray mold, alternaria blight, ascochyta, and botrytis, etc. For example, in chickpea, the major diseases that typically cause yield losses include fusarium wilt (c/o *Fusarium oxysporum)*, dry root rot (c/o *Rhizoctonia bataticola*) and the damping off seed rot in pea (c/o *Pythium* sp.) (Table 2.2). These pathogens together cause 1–14% loss annually in important crops such as legumes, grains, and some other crops equivalent to 80 billion dollars per year (Shuping and Eloff 2017).

2.4 Types of Fungicides and Their Mode of Action

Fungicides can be categorized into two key groups- (i) contact (non-systemic); and (ii) systemic fungicides.

(i) *Contact Fungicides*. Kills or hinders the phytopathogenic fungi/mycological reproductive structures before the mycelial growth and development inside the tissues of plants (Landschoot et al. 2017). However, after the establishment of infection, this fungicide becomes ineffective. Therefore, such types of fungicides are applied only as a protectant. Compounds that contains the inorganic Cu such as copper carbonate, Bordeaux/bordo mix, inorganic sulfur (elementary S) and lime Sulfur are a few common and important examples that comes in the category of main non-systematic fungicides (Mabbett 2016). Within this category, dialkyl dithiocarbamate is an example of organic fungicides which include ziram, ferbam, thiram, etc. which regulate the pathogenic illness since they are more active, effective, and comparatively less toxic than inorganic complexes (Hunsche et al. 2007). These fungicides act as multiple site inhibitors and have various types of toxic action in fungal cells, for example chelation of metals, formation of mixed disulfide bonds and heavy metals transport across the membranes. Dialkyl dithiocarbamate inhibits a widespread fungal enzyme, but the scheme of pyruvic dehydrogenase is predominantly delicate to such types of fungicides (Sisler 2014). Ethylenebis dithiocarbamate, another class of contact and organic fungicide, extensively used and comprised of mancozeb, maneb, thiram, and zineb. In the manner of stroke, these types of fungicide are dissimilar from dialkyl dithiocarbamates in

Table 2.2 Some fungal diseases in legumes, their causal agents and symptoms

Fungal diseases	Phytopathogens involved	Disease symptoms	References
Chickpea			
Ascochyta blight	*Ascochyta rabiei*	Dark brown lesions at the basal part of stem, small necrotic specks in newly formed leaves	Tadesse et al. (2017)
Fusarium wilt	*Fusarium oxysporum*	Limp and drooping in young growing tips, dark browning fungus streak in pith region	Jendoubi et al. (2017)
Dry root rot	*Rhizoctonia bataticola*	Black sclerotic bodies of fungus on the main root below bark	Ravichandran and Hegde (2017)
Black root rot	*Fusarium solani*	Yellowing and wilting in plants, root system is rotten, finely root disappears, and remainder turns black. Affected plants dry permanently	Mitiku (2017)
Pea			
Alternaria blight	*Alternaria alternata*	Pale brown lesions on leaf, elongated lesions on stems, petioles, flowers, and pods, Slight stunting in plants	Sharma et al. (2013)
Ascochyta blight	*Ascochyta pisi*	Circular lesions, small light-colored specks on leaf, lesions occur on stems, petioles, and pods resulting in plant tissue coalesce and defoliation, breakage of stem lodging	Kumar and Banniza (2017)
Damping off seed rot	*Pythium* spp.	Failing of emergence of seedlings	Lamichhane et al. (2017)
Black root rot	*Thielaviopsis basicola*	Yellowing of plants, defoliation, stunting, red brown lenticels and swelling of crown. Entire root appears black	Helyer et al. (2014)
Fusarium Root Rot	*Fusarium solani* f. *pisi.*	Brown, spreading lesions on taproots, decay, and reddening of root vascular tissue, seedling collapse, stunted growth. Diseased plants appear unthrifty, variously dwarfed	Tönnberg (2016)

Mungbean			
Powdery mildew	*Erysiphe polygoni*	Small, irregular powdery spots on leaves, yellowing of foliage instigating untimely defoliation, whitish dry spots on the petioles, shoots, and grains, emergence of gray and white coloration on different plant organs	Yadav et al. (2017)
Wilt	*Fusarium oxysporum*	Occurrence of plant withering on flowering and seedling stages, discoloration of stem and vascular system. a few branches are affected by partial wilt	Bhupendra and Jyant (2016)
Cercospora leafspot	*Cercospora* spp.	Small violet red color rounded spots with gray colored center on leaves and pods	Sumrtini (2017)
Anthracnose	*Colletotrichum capsica*	Dark brown circular spots with concentric ridges on leaves	Kulkarni (2009)
Lentil			
Gray mold disease	*Botrytus cinereal*	Grayish colored soft, necrotic lesions on leaves, stems, and flowers	Zada et al. (2016)
Sclerotinia disease	*Sclerotinia sclerotiorum*	Cottony and white mycelium on leaves, shoots, and grains.	Ahmed and Akhond (2015)
Wilt disease	*Fusarium oxysporum*	Symptoms in the form of black patches appears both on seedlings and adult stages	Garkoti et al. 2014

the form that they endure their conversion to ethylene diisothiocyanate, which deactivates the enzymes of thiol groups and various metabolic activities of fungal cells. Contact/non-systemic group of fungicides are cheap/low-cost and resistance among fungal cells arises infrequently. Due to these properties, this group of chemicals are still extensively used for protecting the plants from pathogenic soil borne disease even though, in the modern era various novel, more powerful systemic fungicides have been established (Connolly et al. 2017).

(ii) *Systemic Fungicides.* These types of fungicides are taken up by plants system and are passed to the site of contagion/infection through translocation. Systemic fungicides act by killing fungal cells after the mycelium has entered in to the parenchymatous tissues, and thus, stop the dispersion or infection/contagion inside the plant organs (Smitamana and McGovern 2018). These fungicides can be used as eradicant, protectant, or both and are considered the very auspicious types of fungicide (Ellis et al. 2017). Though the systemic type of fungicides is very site specific against target pathogens, fungal pathogens may eagerly develop resistance/tolerance to them if they are not managed appropriately. Fungicides that falls in the category of systemic ones, include an extensive group of composites having inconstant modes of action. For instance, dicarboximide is the biggest and very imperative class of systemic fungicides that are applied in agricultural practices used to manage the diseases caused by numerous phytopathogenic fungus. Dicarboximide fungicides such as procymidone, chlozolinate, iprodione, vinclozolin, and metomeclan are predominantly important and used for the control of phytopathogenic diseases produced by the species of *Sclerotinia*, *Botrytis*, *Alternaria*, *Monilinia,* and *Sclerotium* (Dias 2012). The dicarboximide fungicide inhibits the triglyceride biosynthesis of fungi (Sisler et al. 2013). Benzimidazole having the systemic action, is an organic fungicide, applied in agronomic practices to control fungal diseases. These types of chemicals regulate a wide-ranging fungus at comparatively low rates of application (Di et al. 2016). For instance, benomyl is the benzimidazole group of fungicide that is the most operative/active chemical and widely used in the management of fungal diseases (Tashiro and Nita 2017). Some fungicides, for example, carbendazim, benzimidazoles benomyl, phenyl carbamate, diethofencarb, and thiabendazole unambiguously restrict the establishment of microtubule formation, which is considered as the principal component in variable cellular processes of fungi including mitosis and cell shape maintenance (Zhou et al. 2016). This type of agrochemicals (fungicides) precisely binds to the structural protein and their subunits called tubulin (Sáez-Calvo et al. 2017).

2.5 Fungicides Uptake, Metabolism and Their Persistence

Fungicide uptake by the plant system occurs mainly through the roots which are then trans located to aerial organs such as leaves and fruits. Once these fungicides are taken up by plants, they translocate to leaves or shoots either via phloem or xylem, or in the root systems through phloem alone. However, uptake and accumulation of these fungicides depends on various factors such as lipophilicity and dissociation constant, physical and chemical characteristics of the fungicides and soil, and environmental conditions such as precipitation, temperature, relative humidity, and sunlight (Wang and Liu 2007).

Also, the mobility of pesticides depends on their solubility and can be stored adjacent to the absorption site, in storage cells adjoining the translocation pathway or in the areas that are very actively metabolizing (Wu et al. 2016).

2.6 Phytotoxicity of Fungicides to Legumes: A General Perspective

Fungicides are known to have a deleterious impact on growth and development of many leguminous crops. The genotoxic, cytotoxic and morpho toxic effects of fungicides at various cellular stages on the components of some commonly grown legumes are summarized in Figure 2.1 and Table 2.3. The toxicity of fungicides to legumes are deliberated in following section.

2.7 Impact of Fungicides on Plant Growth

Fungicides are often applied in the form of seed protectant. But fungicides have conflicting impact on the plants. For example, Sharma (2012) observed that seed dressings of *Cicer aritinum* with synthetic fungicide (Captan) with the combination of *Mesorhizobium ciceri* strain had no any adversative impact on plant height. But it was also observed that higher than the recommended rates did cause deleterious effects. Some workers found the systemic fungicide triadimefon affected plant weight, plant length, and symbiotic nitrogen fixation only at levels in soil much greater than those applied in practice. The accumulation of fungicides in soil ecosystem has led to destruction in soil microbial compositions (Xu et al. 2017; Gomes et al. 2017a,b) and soil enzymatic activity (Malik et al. 2017). As a consequence, the high concentrations of fungicides/pesticides cause loss in soil fertility and agricultural output (Fox et al. 2007). For example, tebuconazole, a triazole group of fungicide has been found to reduce the growth of above

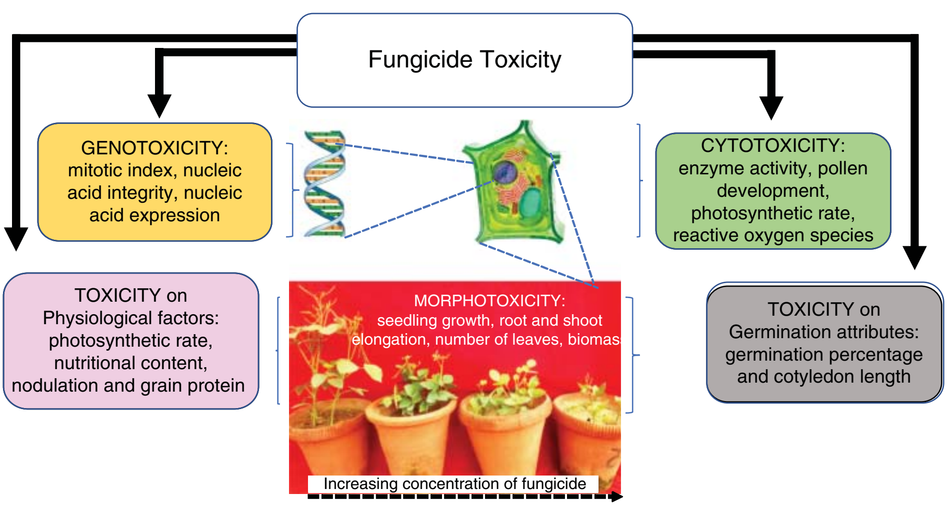

Figure 2.1 Various levels of phytotoxicity of fungicide to legumes.

Table 2.3 Phytotoxic impact of fungicides on some commonly grown legumes

Fungicides used	Concentration	Experimental conditions	Legumes	Effect	Reference
Propineb 70 WP	0.25%	*In vitro*	*Vigna radiata*	Chlorosis of young leaves followed by marginal scorching, reduction in the size of newly emerged leaves	Khalko and Pan (2009)
Prochloraz 45 EC	0.15%	*In vitro*	*Vigna radiata*	—	Khalko and Pan (2009)
Difenoconazole 25 EC	0.15%	*In vitro*	*Vigna radiata*	—	Khalko and Pan (2009)
Tebuconzole	100–300 $\mu g\,kg^{-1}$ soil	Pot trials.	*Cicer aritinum*	Decreased nodule number, biomass, dry biomass, N and Phosphorous content, grain yield and seed protein	Ahmad and Khan (2010)
Tebuconazole	100–300 $\mu g\,kg^{-1}$ soil	Pot trials.	*Pisum sativum*	Reduced dry biomass, nodule number, and nodulation	Ahmad and Khan (2011)
Tebuconazole	100–300 $\mu g\,kg^{-1}$ soil	Pot trials.	*Vigna radiata*	Decreased nodulation, leghemoglobin content, dry biomass, nutritional uptake grain yield and seed protein	Ahmad and Khan (2012)
Carbendazim	2 $\mu l\,kg^{-1}$ soil	Field trials	*Pisum sativum*	Reduced nodule number and pod yield	Tariq et al. (2016)
Thiophanate methyl	2 $g\,kg^{-1}$ seed	Field trials	*Pisum sativum*	Decreased nodule number and pod yield	Tariq et al. (2016)
Hexaconazole	0–20 $mg\,l^{-1}$	Field trials	Common bean		Chehelpar et al. (2016)
Mancozeb	10–150 ppm	*In vitro*	*Vigna mungo, Vigna radiata*	Decrease in rate of germination, survival, root, and shoot ratio, tolerance index and vigor index at higher concentration	Fatima et al. (2017)
Hexaconazole	0.4–1.6 $ml\,l^{-1}$	Pot trial	*Phaseolus vulgaris*	Negative impact on elongation, leaf area, photosynthetic pigments and grain protein	Mourad et al. (2017)

ground (shoot) and the root of green gram plants. Moreover, the fungicide disrupted the symbiotic interaction of *Rhizobium*-legume and henceforth, N_2-fixation resulting in the reduced growth of plants (Ahemad and Khan 2012). The deleterious impact of fungicides might be due to the deterioration of the growth regulatory enzymes involved in the progression and improvements of legumes or due to the distraction of signaling between phyto-chemicals, for instance luteolin, apigenin, and Nod Dreceptors of *Rhizobium*, that are the principal components for the initiation of nodule formation and nitrogen fixation (Fox et al. 2007). Likewise, Bashir et al. (2007) reported the influence of the non-systemic fungicide mancozeb on the morphology and biological attributes of *Lens culinaris* L. at several growing stages. From this study, they come to the conclusion that the lower concentrations of fungicide did not affect the morpho-biological attributes negatively; however, the higher concentrations of this fungicide caused a considerable decline in all the measured biological parameters. Apart from these, the application of pesticides also causes a drastic variation in photosynthetic machinery and reduces the rate of photorespiration. Concomitantly, the fungicides increase the probabilities of injury/impairment, which results in reduced growth and production of biomass in legumes. In another study, Schwarzbacherová et al. (2017) reported a genotoxic effect of some fungicides.

2.8 Effect on Symbiosis and Yield

Pesticides including fungicides interrupt the systems involved in the signaling of phytoestrogen between *Rhizobium* and legumes that controls their symbiotic association (Lira et al. 2015). They also disrupt the preliminary binding of *Rhizobia* to lectins that are contemporarily found on the root hairs (Fox et al. 2004). Consequently, pesticidal species severely affect the symbiotic association between the legume and *Rhizobium* due to antagonistic behavior of pesticides for the attachment on the binding sites of *Rhizobium* on root hair systems of leguminous plants. Fungicides obstruct the formation of nodules in the leguminous crops by dropping the number of existing sites for binding of rhizobial species by lessening the source and supply of simple sugars such as glucose, fructose, and sucrose to the nodules. Accordingly, fungicides reduced the viability and persistence of symbiotic rhizobia and also cause hindrance in the signaling system that is obligatory to initiate the formation of nodulation process of in the legumes. Several agricultural chemicals as well as fungicides induce the symbiotic proficiency by distracting the rhizobial attachment to the root system of leguminous crops, decreasing the nodules formation and activity of nitrogenase enzyme, which in turn decreases the nutritional uptake (N content) and diminishes the yield and growth of plants (Fox et al. 2007). The combined application of the fungicides carbendazim and thiram reflects the decrease ingrains of soybean (*Glycine max*) and produces fewer pods in number/plant and grains (Gomes et al. 2017a,b). Fungicides effectively manage/control the fungal diseases resulting in higher biomass yields. Application of fungicides on the contrary, contaminates the soil and influences oil microbial community, which further distresses the fecundity of soil (Malik et al. 2017). However, Dubey et al. (2012) in a study determined the influence of different fungicides on soil microbial diversity of legume plants and they observed that no substantial variations occurred in bacterial viability. In their conclusion, they further confirmed that bacteria inhabiting in the soil rhizosphere has the potential ability to degrade/detoxify the agrochemicals.

2.9 Effect on Chlorophyll Content and Photosynthetic Rates

Photoreceptor pigments (chlorophylls and carotenoids) play a significant role in the capture of light energy and its transformation into potential chemical energy for the reduction of atmospheric CO_2 into organic molecules such as carbohydrates and proteins (Guidi et al. 2017). Fungicides generally decrease the chlorophyll content and other photosynthetic pigments of plants (Sankar et al. 2016). In this regard, Mourad et al. (2017) reported that application of the systemic fungicide hexaconazole decreased the chlorophyll and carotenoid pigment in *Phaseolus vulgaris*. Fungicides also affect the thylakoidal membrane of photosystem I (Yoon et al. 2011) and also, modify the phytosterol profile and thylakoidal functions. In several reports, it was observed that Chlorophyll "a"fluorescence parameters of fungicide treated plants demonstrated that fungicide exposure affects the light reactions of photosynthesis (Xia et al. 2006). Fungicides unfavorably disturb the metabolic enzyme; therefore, it seems probable that the triazole fungicides inhibited metabolic activity of the enzymes involved in the photosynthetic Carbon Reduction (PCSR) cycle such as Rubisco, 3PGA Nicotinamide adenine dinucleotide phosphate, NAD-Glyceraldehyde-3-Phosphate dehydrogenase and aldolases and kinases. Any reduction in the area of leaf diminishes the parameters of photosynthetic pigments, which consequences the reduction in growth and yield. In a similar finding, it was observed that the chlorophyll content of *Vigna radiata* declined with increasing concentrations of fungicide used. Furthermore, it additionally results in the stimulation of oxidative progression (Kaushik 2006). In a finding, Parween et al. (2012) described that *V. radiata* L. under the influence of pesticide displayed a decline in the growth attributes with increasing concentration, showed a reduction in number and area of leaf, which might be due the hinderance in the translocation of photosynthetic rate and reduction in chlorophyll content. The investigation of numerous photosynthetic pigments and fluorescence parameters of plants raised in fungicides stressed environments (Junqueira et al. 2017) validated that light reactions of photosynthesis are also very sensitive to exposure of these chemicals (Dias 2012). Fungicide pyrimorph strappingly inhibits the reactions of electron transport occurring in chloroplasts, as reported first by Xiao et al. (2014). Likewise, application of some common systemic and synthetic fungicides such as triazole and benzimidazoles, and anon-systemic fungicide (dithiocarbamate) severely affected the effective quantum yield of photosystem-II (ΦPSII) as well as the maximal quantum efficiency of photosystem-II (Fv/Fm). The decline in the above attributes was recognized to the decrease in photochemical quenching (qP) (Xia et al. 2006; Burbulis et al. 2017). In *G. max*, the application of strobilurin reduced the ratio of Fv/Fm (Nason et al. 2007). Strobilurin binds to Qi site of the chloroplast cytochrome bf complex and blocks the electron transport system between PSI and PSII (Nason et al. 2007; Lamberth 2016). Since the growth and overall performance of plant vigor depend on photosynthesis to assimilate the carbon, the impairment of photosynthesis has destructive impacts on plant yield and the production of biomass. Application of flusilazol (systemic fungicide) and mancozeb (contact fungicide) modifies the dark reaction of respiration in some edible crops (Junqueira et al. 2017). The proliferation in the dark reaction of respiration can be elucidated by the requirement of superfluous energy, compounds obtained after the metabolic breakdown, and/or instigation of alternative pathways.

2.10 Fungicide Toxicity to Legume Rhizobium Symbiosis

2.10.1 Effect on Nodulation

Ever increasing application of fungicides in intensive cropping system decreases the nodule formation in leguminous crops by limiting the supply of carbohydrate to already created nodules (Stovold and Evans 2006). As a result, decreased nourishment of rhizobia, biochemical signaling and cell division, eventually leading to inhibition of nodule development was observed (Anderson et al. 2004; Mondal and Kaur 2017). However, there are contradictory reports on the effect of fungicides on legume-*Rhizobium* interactions. (Tariq et al. 2016). For example, Stovold and Evans (2006) observed that dressing of seeds with fungicides like thiram, captan and captafol had a steady protective impact on soybean against seedling diseases, but inhibited nodulation. On the contrary, fungicide (copper oxychloride) seed application in combination with rhizobial inoculation has been found beneficial and enhanced nodulation in food legumes (Muthomi et al. 2007). In other similar reports, when fungicides were applied on rhizobial primed seeds, this decreased the nodule number per plant in *P. vulgaris* but had no impact on Hyacinth bean and green gram plants. (Yang et al. 2012; Fox et al. 2007). Apart from nodulation, it has also been reported that the decrease/decline in nodule numbers in pea plants grown as benzimidazole treated plants might be due to cytokin in like growth regulatory activity of benzimidazole fungicide.

2.10.2 Effect of Fungicides on Nitrogenase and Leghaemoglobin

Nitrogenase enzyme found predominantly in nitrogen fixing organisms catalyzes the conversion of nitrogen gas to usable ammonia. In legumes, it occurs only within the bacteroides. The reaction requires hydrogen as well as energy from adenosine triphosphate production (ATP). However, the nitrogenase activity is also affected by fungicides. For instance, a reduction in the activity of nitrogenase enzyme leading to a reduction in yield was reported in legume *Medicago sativa* L. when grown under fungicide stressed condition (Fox et al. 2007). Similarly, reduction in nitrogenase enzyme activity was observed in some commonly grown legumes such as, *Lens esculentum*, *Cicer arietinum*, *Vigna radiate,* and *Pisum sativum* after application of the fungicide pyriproxyfen. The reduction in nitrogenase activity of legumes was owing to lessening in leghaemoglobin content within the nodules of leguminous crops (Ahemad 2014). Reduction in nitrogenase enzyme of soil bacteria for example, *Bradyrhizobium* and *Rhizobium* was seen in the leguminous plants such as *Trifolium, Medicago,* and *Ornithopus* after the use of the fungicides by several workers. The symbiotic interaction by rhizobia improved nodule formation and nitrogen fixation, and the accessibility of more nitrogen to plants augmented the yield and productivity (Koskey et al. 2017). As the number of nodules increase/decrease, there are induced differences in the fixed nitrogen which in turn disturb the plant development and yield of host. The existence and viability of *Bradyrhizobium* on cowpea, lablab, and soybean grains was inhibited by fungicides mefenoxam and fludioxonil. Considering this, the symbioses of *Bradyrhizobium* with tested legumes were inhibited, resulting in diminishing the nitrogen fixation (Bulyaba and Lenssen 2017). All these and other similar studies validate the fact that fungicide pressure declines the symbiotic association between leguminous plants and nitrogen-fixing bacteria, which in effect weakens the nodulation process and the activity of nitrogenase enzyme. Concomitantly, under such types of situation, the quantity of fixed nitrogen drops which diminishes the yield and growth of legumes.

2.10.3 Effect on Dry Biomass

Application of fungicides also decreases the dry biomass of leguminous crops. The drop in dry phyto mass of leguminous crops upon the application of fungicide may probably be due to modification/inhibition of several enzymes involved in growth, development, physiology, and metabolic activities of plants (Lindroth 2017). Besides these, synthetic fungicides applied to defend/safeguard the seed grains against phytopathogenic diseases, are noxious to growth and metabolic activity and cell viability of rhizobacterial cells; in maximum belongings, even if the rhizobacteria persist active and viable, their host–legume nodulating efficiency or nitrogen fixing ability is lessened (Guene et al. 2003). However, Muthomi et al. (2007) observed that a when rhizobia are applied with the mixture of fungicide (Copper oxychloride), it was observed that there were no any impacts on shoot dry biomass relative to the control in food grain legumes.

2.11 Microbial Remediation of Fungicide Toxicity

Rhizosphere microorganisms commonly known as plant growth promoting rhizobacteria (PGPR) augments the growth and efficiency of plants by various direct and indirect mechanisms (Shahid et al. 2017) under both stressed and conventional environment (Figure 2.2). Plant beneficial rhizobacteria have been reported to directly increase plant growth by a variety of mechanisms: (i) fixation of atmospheric nitrogen that is transferred to the plants; (ii) production of siderophores that chelate iron and make it available to the plant roots; (iii) solubilization of minerals such as phosphorus; and (iv) synthesis of phytohormones (auxins, cytokinins, gibberellins, etc.). Among the PGPR strains, the agronomically important PGPR belongs to the genera *Azotobacter, Azospirillum, Bacillus, Cellulomonas, Pseudomonas, Rhizobium,* and *Xanthomonas,* etc. The PGPR strains also affect the growth of plants indirectly through the production of siderophores and antifungal metabolites (e.g. HCN, antibiotics), which in turn suppress the growth of phytopathogen and hence, increase the growth of plant (Glick 1995).

Plant beneficial bacteria may also degrade/reduce or hydrolyze the toxic action of pesticides including fungicides by means of several enzymatic actions (Yang and Lee 2008). Recent research has revealed several microbial systems capable of biodegrading organic compounds including synthetic pesticides (Parte et al. 2017). In a study, Tariq et al. (2016) applied potent bioinoculant *Rhizobium leguminosarum* PS1 and PS2 strains which significantly increased the nodule number in *P. sativum* plants comparatively to those of fungicide supplemented and uninoculated plants. Furthermore, when *R. leguminosarum* PS2 applied together with thiophanate methyl (fungicide), expressively improves the nodule numbers (122) and nodule dry weight (34.5 mg). Also, when *R. leguminosarum* strain PS1 was applied as a potent biological inoculant (biofertilizer) in contrary to carbendazim treated pea plants, the bioinoculant PS1 augmented the pod

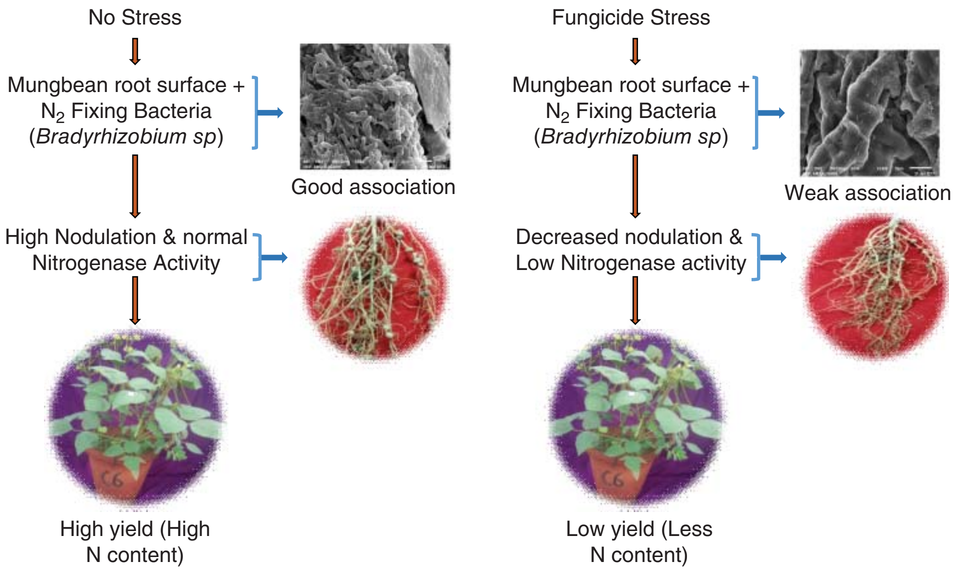

Figure 2.2 An example of effect of fungicide stress and bioinoculant *Bradyrhizobium* sp. on nodule formation, nitrogenase activity, association of nitrogen-fixing symbiotic rhizobacteria on root surface and growth and yield of *Vigna radiata* (L). Wilczek.

yield by 43% compared with the fungicide supplemented and non-inoculated/control plants. The enhancement in nodule formation by the bio-inoculants was due to nitrogen fixing symbiosis and growth stimulating action of rhizobial inoculants. Also, pea symbiotic bacteria *R. leguminosarum* was found resistant against benzimidazole fungicide and significantly improved pea nodulation. A significant increase of 34–43% in yield of pod was achieved in pea plants treated with bioinoculant *R. leguminosarum* compared with the plants developed in fungicide treated and uninoculated control. In a similar experiment Sharma and Singh (2014) observed that *Rhizobium* strain inoculated lentil plants raised in presence of fungicides like thiram, captan, dithan M-45, and dithane Z-78 had more grain and straw yields over corresponding un-inoculated control. The fungicide tolerant strain of *Rhizobium* increased the number of nodules significantly at 30, 60, and 90 days stage of crop growth over uninoculated control fungicides. Gaind et al. (2007) described that combined application of fungicides and bioinoculants *M. ciceri* strain SP4 and *Azotobacter chroococcum* strain CBD-15 improve shoot and root dry biomass and seed attributes in *C. aritinum*. It was concluded from this study that combined application of fungicides and bioinoculants could facilitate plant growth by controlling fungal diseases and supplying essential nutrients to plants. Also, the nodule microorganisms especially rhizobia synthesize plant growth substances like indole acetic acid (IAA) (Bose et al. 2016) and iron chelating compounds siderophores (Nimnoi et al. 2014), which together promote the growth of leguminous crop seven in pesticide-polluted soil (Hassen et al. 2016). Gurikar et al. (2016) stated that pesticide tolerant strains *A. chroococcum* and *Azotobacter vinelandii* tolerated some commonly used pesticides including fungicides at the rate of 5% and proved their biodegradation efficiency. These strains synthesized IAA, gibberellic acid, arginine, etc. and other plant growth bioactive molecules and directly stimulate the length of root and shoot as well as seed development of numerous legumes under stressed environment. From these findings, it is evident that bacterial inoculants not only produce/secretes the bioactive molecules including growth regulatory enzymes but are also capable of tolerating pesticides and could be used as bio inoculant. Plant growth promoting rhizobia as well assymbiotic nitrogen fixing bacteria have been reported to enhance/expand the growth and metabolic activities of plants by several mechanisms, like, by defending the developing plants from the toxic action of pesticides through catabolic activity (Yang and Lee 2008). Various fungicide resistant/tolerant PGPR strains have been isolated from different rhizospheres having multiple PGP activities (Bose et al. 2016).

2.12 Concluding Remarks

Fungicides after uptake by plant from different environments move to several plant organs through vascular systems. Fungicides can also persist in plants for a long-time due to their long half-lives. However, following entry inside plant tissues, pesticides adversely affect various plant organs, root-shoot morphology, nodulation, nitrogenase enzymes, chlorophyll content, photosynthetic and transpiration rate, dry biomass, macronutrients and also the action, effectiveness, and existence of leguminous symbiotic microorganisms. Additionally, N_2 fixed by the symbiotic Rhizobia and other growth promoting substances like HCN, siderophores, and phytohormones produced by them can be negatively affected by fungicides. Soil bacteria on the contrary have the ability to detoxify/degrade the synthetic fungicides and hence can facilitate growth of plants even under fungicide stress. Considering these, the use of potent and efficient PGPR strains as bioinoculants in pulse cultivation practices appears to be exciting that could be applied even under polluted environment.

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3

Pesticide Metabolism in Plants, Insects, Soil Microbes and Fishes: An Overview

Anket Sharma[1,2,3], Vinod Kumar[1,2], Sukhmeen Kaur Kohli[1], Ravdeep Kaur[1], Tajinder Kaur[1], Saroj Arora[1], Ashwani Kumar Thukral[1] and Renu Bhardwaj[1]

[1] *Plant Stress Physiology Laboratory, Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar 143005, India*
[2] *Department of Botany, DAV University, Sarmastpur, Jalandhar 144012, India*
[3] *State Key Laboratory of Subtropical Silviculture, Zhejiang A&F University, Hangzhou 311300, China*

3.1 Introduction

Pesticides are utilized throughout the globe for the protection of crops. These substances repel, destroy, or prevent pests, and are generally grouped as fungicides, herbicides, and insecticides. In plants, pesticides are applied via spray, soil, or seed priming before sowing and these directly affect the plants, insect pests, soil microbes, and aquatic organisms. Extensive agriculture has been successful for the last few decades because of the use of pesticides along with high yielding varieties. Due to the excessive application of pesticides there is a need to check their contamination which is of great significance. Their degradation depends upon biotic and abiotic factors which are particular for a specific pesticide.

Plants uptake pesticides by the process of absorption through roots or leaf surface. However, their uptake as well as metabolism in plants is affected by various factors like humidity, rain, temperature, mode of application, plant developmental stage, and physiochemical characteristics of soil as well as pesticides (Finlayson and MacCarthy 1973; Führ 1991). In plants, pesticides after their absorption undergo the process of degradation by the plant's xenobiotic detoxification system or they may accumulate in plant parts which ultimately results in their bio-magnification (Mwevura et al. 2002). Additionally, the usage of pesticides also causes toxicity to plants which results in their retarded growth and development (Xia et al. 2009; Sharma et al. 2016a,b, 2017a). However, plants also degrade these harmful pesticides through the pesticide detoxification mechanisms which consists of enzymatic and non-enzymatic antioxidative systems (Xia et al. 2009; Zhou et al. 2015; Sharma et al. 2016c,d, 2017b,c).

Insecticides enter into insects when they feed an insecticide-treated plant. However, insecticides also have negative effect for non-target insects like honey bees (James and Xu 2012). Moreover, to avoid insecticide toxicity, insects have their own enzymatic driven detoxification mechanisms to metabolize the insecticides into less toxic compounds (Wu et al. 2009).

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Pesticides also get accumulated in soil and persist for longer times (Navarro et al. 2007). Only about 0.1% of the applied pesticide reached the target organism and the remaining part is responsible for the contamination of soil (Carriger et al. 2006). With the increasing usage of pesticides in modern agriculture, the problem of the negative effects of these agro-chemicals on soil microbes has got more attention (Andrea et al. 2000; Baxter and Cummings 2008). The applied pesticides may cause a harmful effect on the local microorganisms, disrupt the soil ecosystem and finally affect human health by gaining entry into the food chain. Their persistence in soil may also affect fertility of soil (Chowdhury et al. 2008). Bioremediation is an efficient technique for the removal of contaminants from the polluted ecosystem and various bacterial and fungal species have been isolated and identified that cause the breakdown of pesticides (Singh and Walker 2006). Pesticide biodegradation by bacteria and fungi is ecofriendly and, is the most effective and economic method for their detoxification (Rani and Dhania 2014).

Chemicals such as pesticides used in agriculture can influence aquatic animals specially fishes, directly or indirectly by negatively affecting their food requirements. These chemicals also have the ability to cause death of aquatic organisms within a short period of exposure time. These pesticides also cause a reduction in fish reproduction efficiency by damaging fish testis, causing late development of oocytes and hindering the biosynthesis of hormones (Kim 1998).

Since pesticides enter plants, insects, soil microbes, and aquatic organisms like fishes after application, these organisms have their own mechanisms of pesticide metabolism to cope with the harmful effects of pesticides. The present review gives a detailed account on the pesticide metabolism mechanism in these organisms.

3.2 Metabolism of Pesticides in Plants

Absorption or uptake of pesticides by plants depends upon its mode of application. Pesticides are usually applied as a soil treatment or sprayed over foliage thus roots and leaves are major sites pesticides absorption by plants (Shimabukuro et al. 1982). Pesticide uptake is influenced by multiple factors like physicochemical properties of pesticide, nature of plant, soil, and other abiotic conditions (Gao et al. 2000; Bouldin et al. 2006; Hwang et al. 2015). Atrazine and cycloxidim were absorbed more by roots and shoots of *Myriophyllum aquaticum* than terbutryn and trifluralin (Turgut 2005). The potential of two aquatic plants, *Juncus effusus* and *Ludwigia peploides* for atrazine and lambda-cyhalothrin uptake was assessed by Bouldin et al. (2006). Uptake of atrazine was more in *J. effusus* although lambda-cyhalothrin was absorbed more by *L. peploides*. Uptake and translocation of dieldrin, an organochlorine pesticide (OCP) was better in cucurbits than in non-cucurbits as they only translocated a considerable amount of dieldrin to their shoots from roots while non-cucurbits accumulated only in their roots (Murano et al. 2010). Pesticide uptake also varies with the concentration of pesticide in soil and plant growth. Exposure to $20\,mg\,kg^{-1}$ endosulfan to cucumber resulted in 7.8 and $3.8\,mg\,kg^{-1}$ uptake after 15 and 30 days respectively, while exposure to $40\,mg\,kg^{-1}$ resulted in 14.5 and $7.9\,mg\,kg^{-1}$ uptake after 15 and 30 days. Indicating that uptake of pesticide enhances with an increase in the concentration of pesticide and growth of the plant dilutes the concentration of absorbed pesticides (Hwang et al. 2015). In addition pesticides may undergo degradation in water as well as soil and form simple metabolites which could be easily up-taken by plants (Trapp 2000). Difference in uptake of a different pesticide by a same species due to difference in their physico-chemical properties such as molecular weight and lipophilicity. A compound's lipophilicity is generally expressed by octanol-water coefficient (K_{ow}), the root uptake is generally more for a lipophilic compound and translocation being a passive process favors compounds with intermediate polarity (Briggs et al. 1982). Highly hydrophobic compounds having $K_{ow} > 3$ bind strongly to roots minimizing their translocation to shoots while hydrophilic compounds having $K_{ow} < 1$, can't bind sufficiently with roots nor can be translocated (Dietz and Schnoor 2001). Organic compounds with K_{ow} ranging 1–3 have highest uptake and translocation potential (Chang et al. 2005). Translocation of pesticides inside plants is not uniform and depends upon type of pesticide and site of absorption. It occurs by vascular tissues (xylem and phloem). Usually, pesticides are translocated from roots to transpiring leaves passively, i.e. apoplastically by xylem. In phloem they are translocated actively via symplast from mature leaves to the growing points of shoots and roots (Shimabukuro et al. 1982). Foliar uptake takes place by diffusion of pesticides through epicuticular wax, cuticle, and plasmalemma of epidermal cells of leaves. Therefore, it depends upon plant species and the physico-chemical properties of a pesticide like molecular weight and lipophilicity. However, these factors do not have a linear relationship with uptake of pesticide and uptake can't be predicted by them (Wang and Liu 2007). Uptake and translocation of polar and non-polar pesticides was observed in leaves of maize, sugar beet, strawberry and rape after 24, 48, and 72 hours of their foliar spray by Baker et al. (1992). They observed that pesticide uptake and translocation to adjacent site was better in plants with more waxy leaves (rape and strawberry) than less-waxy (maize and sugar beet). Methyl ester herbicide derivatives increase total absorption of herbicide through lipophilic leaf cuticle by assisting their penetration (Bell et al. 2011).

Plant can not only uptake pesticides but also metabolize them. Several studies have shown the potential of plants for the degradation of pesticides in them making useful for phytoremediation of xenobiotics like pesticides. For example, *Eichhornia crassipes* could metabolize ethion (organophosphate insecticide) and its concentration decreases in shoots by 55–91% and by 74–81% in roots after one week (Xia and Ma 2006). *Lemna minor* removed herbicides namely glyphosate and isoproturon from hydroponic medium by 8% and 25%, respectively (Dosnon-Olette et al. 2011). Similarly, four wetland plants namely *Typha latifolia*, *Phragmites australis*, *Iris pseudacorus*, and *J. effusus* were able to metabolize and remove absorbed fungicides, tebuconazole by 25–42% and imazalil by 46–96% (Lv et al. 2016).

Plants metabolize pesticides by three phased detoxification system (Xia et al. 2009). Phase I and II are also known as chemical transformation or activation and conjugation, respectively and Phase III as compartmentalization and storage (Coleman et al. 1997; Sandermann 1992). Generally, the pesticide molecules are highly lipophilic in nature, chemical transformation targets conversion of the parent compound to more reactive and polar substrate (Cole 1994). Phase I is activation of pesticide by addition of –OH, $-NH_2$, or –SH groups through oxidization, reduction, or hydrolysis reactions. These reactions transforms the parent pesticide molecule to more hydrophilic and less toxic product (Dietz and Schnoor 2001; Van Eerd et al. 2003). Cytochrome P450 monooxygenases are crucial enzymes of phase I metabolism of herbicides in plants (Siminszky 2006). Transgenic plants with human cytochrome P450 monooxygenases were more

Table 3.1 Enzymes involved in phase I and phase II chemical transformation of pesticide (Van Eerd et al. 2003)

Process	Reaction	Enzymes
Phase I (Activation)	Oxidation	Oxidases (cytochrome P450s, peroxidases, phenol oxidases, oxidoreductases, etc.)
	Reduction	Reductases (nitroreductase, reductive dehalogenases, etc.)
	Hydrolysis	Esterases, amidases, nitrilases, etc.
Phase II (Conjugation)	Conjugation to glutathione	Glutathione-S-transferases
	Conjugation to sugar	Uridine diphosphatase-glucosyl (UDPG) transferase

resistant to herbicides like atrazine and metolachlor and were more useful in degrading them from soil (Kawahigashi et al. 2008). Metabolism of metamitron in *Chenopodium album* involved cytochrome P450 monooxygenases and its primary degradation products were deamino-metamitron and metamitron-N-glucoside (Aper et al. 2012). In rice Cytochrome P450s (Rong Tan et al. 2015) and laccases (Huang et al. 2016) in involved in detoxification of atrazine and its degradation products.

During phase II, pesticide or phase I metabolized product is conjugated with glutathione (GSH), sugar or amino acid and leads to formation of more water soluble product with little or no phytotoxicity (Van Eerd et al. 2003). Various enzymes catalyzing phase I and II reactions are enlisted in Table 3.1.

Phase III involves secondary conjugation, compartmentalization or internal storage of soluble pesticide metabolized products inside vacuoles and insoluble in apoplast (Van Eerd et al. 2003; Xia et al. 2009). In higher plants pesticides–sugar conjugates may undergo secondary conjugation with malonate by malonyl CoA transferase forming N-malonyl-glucose conjugate (Van Eerd et al. 2003). Metabolism of several pesticides by *L. minor* in hoagland's medium was analyzed by Fujisawa et al. (2006). *L. minor* metabolized 3,5-dichloroaniline by phase II conjugation with glucose, 3-phenoxybenzoic acid with glucose and glutamic acid (R,S)-2-(4-chlorophenyl)-3-methylbutanoic acid with malic acid and malonyl glucose.

Many pesticides induce expression glutathione-S-transferases (GST) encoding genes indicating the crucial role of GSH conjugation in their detoxification. GSH conjugation with pesticides or its primary metabolized derivatives occur by nucleophilic addition reactions which are catalyzed by GST and these glutathione-pesticide conjugates can be stored in vacuoles (Peuke and Rennenberg 2005). To protect the crop species from herbicide attack, herbicide safeners are used alongside or before herbicide application. These substances increase herbicide detoxification in crop species by enhancing herbicide-GSH conjugation by stimulating GSH content or GST activity (Dietz and Schnoor 2001). The adenosine triphosphate production (ATP)-dependent tonoplast transporters export glutathione S-conjugates from cytosol to the vacuoles (Coleman et al. 1997).

Chlorpyrifos, an organophosphorus insecticide was absorbed in the roots and shoots of several *Populus* sp. and *Salix* sp., but did not persist in their tissues with time indicating further metabolism of chlorpyrifos in them (Lee et al. 2012).

3.3 Metabolism of Pesticides in Insects

Insects present the gravest threat to agriculture production and pest management. The application of insecticides plays an imperative role in restraining the population of insect pests (Panini et al. 2016). Also, the entomologist are facing wide array of challenges to aid the humans and animals for protection from the insect pests. Insecticides are the most significant constituents of insect-pest control concerns world-wide (Bulter 2011). The interaction between pesticides and insects have been previously studied, keeping in view two criteria: (i) various pest management strategies have been examined for their ability to elevate by application of certain microbial pesticides; or (ii) are their sub-lethal doses of these pesticides which have negative impact on the non-target insects such as bees (James and Xu 2012)? An insecticide to reach a target site must first penetrate the cuticle of the insects, which is considered are primary line of defense of insects. The resistance to entry of insecticides in insects is via physiological and biochemical changes in the structure of the cuticle (Panini et al. 2016). The alteration in the cuticle structure results in lowered absorption of the insecticides. The lowered and slow entry of insecticides increases the time for detoxification of chemical entities via phase I enzymes (Strycharz et al. 2013; Kasai et al. 2014). Various cuticular proteins were reported to be over-expressed in green peach aphid which was found resistant to neonicotinoid (Puinean et al. 2010). Various enzyme families including esterases, GSTs, and mixed oxidases have been noticed to be involved in pesticide absorption, detoxification, and its excretion.

A wide array of insecticides have been reported to inhibit the activities of antioxidative enzymes including GSTs (Wu et al. 2009) and superoxide dismutase (SOD) (Buyukguzel 2009), cytochrome C (cyt. C) and catalase (CAT) (Turrens 2003). Most of the insects detoxify the toxins via oxidation which is a feedback mechanism in response to enhanced production of reactive oxygen species (ROS). ROS synthesis results in cellular damage and enhanced in-activation of scavenging species and reduction reactions. Enhancement in the GSTs enzyme activities was recorded in midgut and fat body of the honeybee in response to *Nosema ceranae* infestation (Vidau et al. 2011). Stimulation of CAT activity was observed in response to varied infestation by entomopathogenic fungi and toxins like destruxin (Sowjanya Sree et al. 2010). All these enzymes detoxify the insecticides into a non-toxin form which is rapidly excreted from the body. The detoxification of insecticides is divided into two phases: (i) phase I or primary phase involving hydrolysis and oxidation of toxin; and (ii) phase II or secondary phase involving the by-products of phase I to conjugate with various endogenous constituents including antioxidants and antioxidative enzymes (Yu 2008; Berenbaum and Johnson 2015). The enzymes primarily involved in the detoxification of pesticides in insects include esterases, mono-oxygenases (mixed oxidases), and GSTs. A constant homeostasis is to be maintained between the production and scavenging of ROS, which is imperative for bacterial defense as they have anti-bacterial bioactivity and are also required for proper functioning of hydrolytic enzymes. ROS also play a crucial role in the modulation of apoptosis as well as the detoxification of pesticides (Turrens 2003).

A huge variety of exogenous and endogenous substrates are reported to have altered metabolism by a large category of phase I enzyme, i.e. esterase. A wide array of reports suggests the involvement of esterase enzyme in detoxification of insecticides, such as carbamates, pyrethroids, and organophosphates (Hollingworth and Dong 2008). The detoxification of insecticides occurs via enzymatic cleavage or absorption of these insecticides. Esterase enzyme triggers the hydrolysis process of ester-insecticides to their corresponding acidic as well as alcoholic compounds. This ultimately increases the polarity of insecticide-derived metabolites which are easily excreted from the plant cell. Esterase is also reported to elevate sequestration of insecticides, as a result is not available to interact with specific proteins (Wheelock et al. 2005). The increased expression of the enzyme esterase might be due to up-regulation of gene expression or amplification, for example a report of increased levels of carboxylesterase activity in *Myrus persicae* (green peach aphid) in response to gene amplification (Bizzaro et al. 2005; Bass et al. 2014). Another report by Cao et al. (2008) suggested increased activity of esterase in response to elevation in transcriptional levels due to corresponding enhancement in gene expression of encoding genes in *Bemisia tabaci* and *Aphis gossypii*. Demythylation and chromosomal re-arrangement also results in enhancement in activity of esterases (Bizzaro et al. 2005; Rivi et al. 2012, 2013).

Mono-oxygenases or mixed oxidases are also categorized as phase I enzymes. They are involved in the detoxification of insecticides and also alteration of the metabolism of other hormones, pheromones, and fatty acids. The mixed oxidases convert lipophilic entities to polar metabolites which are easily excreted from the insect's digestive track (Feyereisen 2015; Liu et al. 2015). Cytochrome P450 (Cyt. P450s) is another imperative mono-oxygenases which are heme-thiolate proteins, these enzymes catalyze the transfer of O_2 to substrates as well as reduction of second O_2 atom to form water. The reaction requires presence of enzyme NADPH cyt. P450s reductase (Guengerich 2008). Cyt. P450s catalyze various other metabolic mechanisms such as epoxidation, N-dealkylation, O-dealkylation, hydroxylation, and desulfurization. This also affects the metabolism of several insecticides including organophosphates, neonicotinoids, pyrethroids, etc. (Yu 2008; Puinean et al. 2010; Alptekin et al. 2016).

GSTs play a crucial role in the detoxification of insecticides and other xenobiotic compounds via modulation of transportation, biosynthesis of hormones and proteins (Ketterman et al. 2011). GSTs help in the conjugation of electrophilic substrates with reduced glutathione, converting these electrophilic entities into water-soluble forms which are readily excreted from the insect body (Konanz and Nauen 2004). The GSTs in insect system are categorized into two classifications, i.e. microsomal and cytosolic, dependent upon their location in cells (Enayati et al. 2005). Another insecticide, i.e. acetylcholine has been reported to be an excitatory of CNS neurotransmitter at synapses (Pichon 1974). Few insecticides have been observed to be inhibitors of acetylcholinesterase which has been reported to hydrolyze acetylcholine resulting in termination of its synaptic actions (Corbett 1974). It was reported by Gepner et al. (1978) that *Periplaneta Americana* when exposed to nicotine and isothiocyanate (having insecticidal activity) are agonist of acetylcholine receptor in the CNS. A recent report of Meng et al. (2015) suggested enhanced expression of varied subclassifications of P450s and GSTs, sequence diversity in nicotinic acetylcholinesterase (nAchRs) and 17-gama-amino butyric acid (GABA) was observed.

3.4 Metabolism of Pesticides in Soil Microbes

Pesticides have an adverse effect on the soil microbial diversity (Ingram et al. 2005; Littlefield-Wyer et al. 2008; Niewiadomska 2004; Wang et al. 2006). They have great influence on the microbial mineralization of organic compounds and associated biotransformations, i.e. nutrient kinetics (Demanou et al. 2004; Kinney et al. 2005; Mahía et al. 2008; Niewiadomska 2004). Pesticides also alter the behavior of soil enzymes which are the main component of soil micro-flora (Antonious 2003; Monkiedje and Spiteller 2002).

The effect of pesticides on the diversity of microorganisms in soil is controlled by various ecological factors in addition to the amount, persistency, bioavailability, and its toxicity (Abdel-Mallek et al. 1994). Bioavailability is the main factor in preventing the effect of pesticides on the soil microbes in the ecosystem. The processes such as adsorption and desorption control the content of pollutant in the soils and therefore its bioactivity, degradability, and bioavailability in the soil environment (Bonczek and Nkedi-Kizza 2007; Katagi 2008). The impact of three insecticides such as cypermethrin, monochrotophos, and quinalphos on microbial populations in black clay soil was analyzed by Gundi et al. (2005). Synergistic effects were reported at low content, and harmful effects were found at the high content of the insecticides. Organic matter presence and vegetation have great influence on the toxicity of pesticides to the microbes in soil environment. Pesticide toxicity against fungal species was enhanced by the addition of carbon sources such as glucose, acetate and glutamine, serine, tryptophan, and arginine (Mishra and Pandey 1989). Rapid industrialization has resulted in large production of xenobiotic chemicals in the ecosystem. The OCPs pose great danger to the worldwide ecosystem and human health because of their affinity for dispersal, transportation over long distances, and bioaccumulation in the food chain.

Pesticide metabolism in microorganisms is important for their safe and good use as well as for making pesticide bioremediation approaches for polluted soil (Van Eerd et al. 2003). More usage of pesticides is a key issue for the scientists to develop some techniques for the transformations of these pesticides. Physiochemical characteristics of soil have great impact on the transformation of these pesticides to some extent. Soil microbial mechanism largely influences the breakdown of pesticides (Pal et al. 2006). Olchanheski et al. (2014) studied the strategies that are used by bacteria (*Escherichia coli*) to conquer the oxidative stress caused by herbicide mesotrione which inhibits 4-hydroxyphenylpyruvate dioxygenase. They also studied the antioxidative stress systems, variations in lipid membrane and the ability of bacteria to break down mesotrione. The results indicated that *E. coli* were able to endure elevated amount of the herbicide and almost breakdown the mesotrione following three hours of treatment. This bacterium system response is probably a general adaptive process by which bacterial strains protect themselves from the presence of herbicides in agricultural soils. Microbial degradation has a great impact on the persistence of herbicides in the soil (Araújo et al. 2003). For degradation of pesticides, it is essential to identify the processes that are responsible for enzymatic catalysis, that are possibly to develop new efficient techniques for the management of pesticides (Ortiz-Hernández et al. 2013). Degradation of pesticides with the help of enzymes is a novel technique for elimination of such chemicals from the contaminated environment. Pesticide breakdown with the help of enzymes is more efficient as compared to chemical methods. Enzymes are essential to the approach for the action of various pesticides. Activation of pesticides is done by *in situ* enzymatic method, whereas several pesticides play the role by targeting specific enzymes with significant biological function (Scott et al. 2008). Major enzyme systems that are concerned with degradation of pesticides are: esterases, hydrolases, mixed function oxidases (MFO) and GST (Li et al. 2007). Hydrolase enzyme is involved in the biodegradation of pesticides. It catalyzes the hydrolysis of many biochemical types of pesticide, i.e. peptide bonds, esters, ureas, etc. and normally functions in the lack of redox cofactors making them suitable for the bioremediation strategies (Scott et al. 2008). Phosphotriesterases (PTEs) are the most important group of enzymes that are involved in pesticide degradation and are concerned with hydrolysis and detoxification of organophosphate pesticides (Chino-Flores et al. 2012). Esterases enzymes are also involved in the hydrolysis of organophosphate pesticides (Rosario-Cruz et al. 2000; Galego et al. 2006; Baffi et al. 2008). Persistent organic pollutants (POPs) are stable and harmful compounds with the capability to oppose environmental degradation are the reasons of increasing distress worldwide even at lower concentrations. Because of environmental toxicity and lipophilic characteristics, OCPs are regarded as POPs (UNEP 2003; Arslan et al. 2015). Aislabie et al. (1997) studied the microbial breakdown of 1,1,1-trichloro-2,2-bis (p-chlorophenyl)ethane (DDT). DDT biodegradation takes place in the soil at a slow rate. *In situ* a number of mechanisms are proposed to enhance their degradation. They comprise the accumulation of DDT-metabolizing microbes to the contaminated soil. Chlorpyrifos is the most commonly used OCPs in agriculture. Microbial degradation is regarded as efficient and cost efficient approach for remedial action of OCPs from the environment (Bhagobaty et al. 2010). Many reports indicate that a broad variety of ecosystems may be contaminated with OCPs. These compounds contain more mammalian toxicity and as a result it is important to remediate them from the environment. Microbial bioremediation is regarded as one of the most important process for the elimination of coat protein (CP) from the environment (Rayu et al. 2017).

Degradation of pesticides by microbes is important method. Some researchers identified that genetically modified microorganisms (GMMOs) have the capability to breakdown specific pesticides but the issue is that they are not used in the fields, due to the reason that it creates other environmental issues. Microbes that are present in nature and, when native, are isolated from the specific polluted environment have the capability to breakdown the pesticides at a high rate. The bio-augmentation mechanisms such as accumulation of important nutrients or organic matter are needed to increase the degradation rate of pollutants by native microbes (Verma et al. 2014).

The native microbial strains are more efficient in breakdown of pesticides than the exogenous microbial strains because they survived and breed well in a specific soil environment. Pesticide degradation by microbial consortium is an effective and ecofriendly technique for sustainable agriculture formation (Jaiswal et al. 2017). Abraham and Silambarasan (2016) studied the chlorpyrifos biodegradation by JAS2 bacterial strain isolated from paddy rhizosphere soil. There is a need to develop techniques that reduces

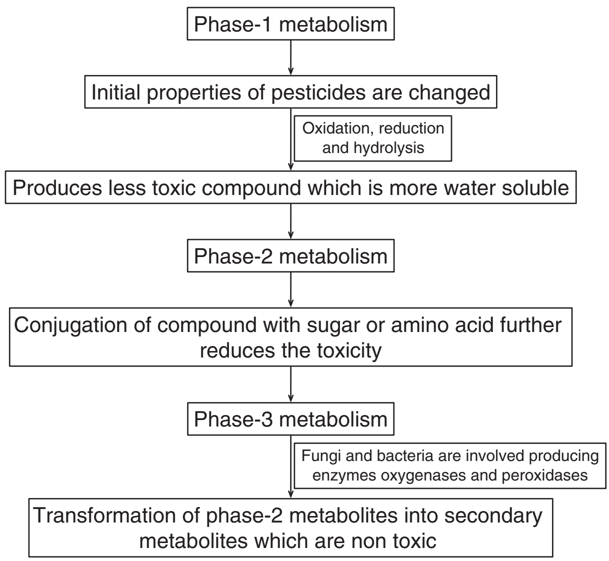

Figure 3.1 Process of pesticide metabolism by microbes. Source: Van Eerd et al. 2003; Ortiz-Hernández et al. 2011.

the soil pesticide residues and their toxicity (Singh and Jauhari 2017). Figure 3.1 shows the general process of the metabolism of pesticides by microbes.

3.5 Metabolism of Pesticides in Fishes

Ecological pollution of water systems is an extremely severe problem all over the world. Discharge of various chemicals from commercial, agricultural, or industrial sources into the aquatic environment have certain harmful effects on aquatic organisms. These pollutants were also identified to build up in fish bodies either directly from the contaminated water or indirectly from the food chain (Mohamed 2009; Chaudhry and Jabeen 2011). The major cause of concern in surface water is the presence of certain chemical fertilizers and pesticides. The most common use of pesticides in agriculture is to check the vector-borne diseases in order to increase production by reducing the chances of crop diseases. But their improper use due to the lack of proper knowledge results in detrimental effects on the environment and ultimately affects the living organisms. The excessive use of pesticides is unsafe to the ecosystem, as this results in contamination of soil, surface, and underground water resources. While pesticides effectively control pests and weeds in agricultural farming, their residue can travel from fields to water and air affecting the relevant organisms negatively (Arias-Estévez et al. 2008). The absorption of pesticides by the aquatic organisms such as fish can cause harm to the fish health as well as eating quality of its meat for human beings (M'Anampiu 2011). These pesticides are not easily biodegradable and can survive for long periods of time in the environment after their application (Richterova and Svobodova 2012).

Fish species are sensitive to enzymatic and hormone disruptors. The species exposed to low concentrations of pesticides for a longer period may be subjected to major effects than acute poisoning. Lower concentrations of pesticides are found to be related with minor changes in behavior and physiology that may affect both survival and reproduction in certain species (Kegley et al. 1999). The pesticides that have been shown to cause oxidative stress include OCPs, organofluorine pesticides, organophosphates, carbamates, pyrethroids, bipyridyl herbicides, triazine herbicides, chloroacetanilide herbicides, and other pesticides (Slaninova et al. 2009). Biochemical changes stimulated by pesticide stress often leads to the inhibition of important enzymes, metabolic disturbances, retardation of growth, and reduction in the fecundity and longevity of the organism (Murty 1986). The most susceptible organs of a fish exposed to any type of toxicants are gills, liver, brain, and kidneys (Jana and Bandyopadhyaya 1987). Biochemical changes occurring in body tissues can be employed as an important indicator of physiological stress and health of fish (Ali Muhammad Yousafzai 2004).

The most important indicators to assess the level of pesticides pollution in fresh water systems are fish samples (Schantz et al. 2001). The variability of pesticide accumulation within fish tissues depends on the route of its uptake. Their possible use as biomonitoring agents is therefore an important factor in the evaluation of bioaccumulation and biomagnification of contaminants within the ecosystem (Haider and Inbaraj 1986). Many hazardous chemical elements, if released into the environment have the ability to accumulate in the soil and sediments of water bodies. These chemicals are then absorbed by lower aquatic organisms and transferred to higher trophic levels via the food chain. The free divalent ions of many pollutants may be directly absorbed by fish gills from the water under acidic conditions (Haider and Inbaraj 1986). The protein levels in brain, gills, muscle, kidney, and liver were found to be reduced when fish were exposed to pesticides under experimental conditions. Tilak and Rao 1991 showed significant decrease in the protein content in the kidney and the liver due to oxidative stress resulting from their elimination and also in metabolism. The normal development of fish is interrupted resulting in male fish having female characteristics on interference of pesticides with endocrine hormones. These external symptoms of developmental disruption are associated with reduced fertility and even sterility in adults, as well as lesser hatching rates and viability of offspring. Certain defects of the skeletal system, resulting in deformities and stunted growth were also observed during development of young fish on disruption of the balance of endocrine hormones (Ewing 1999; Goodbred et al. 1997).

The chlorinated organic pesticides are resistant to photo degradation due to their stability in both fresh and salt water (Kegley et al. 1999). The absorption of these pesticides occurs via secondary mechanisms such as, biological breakdown by microflora and fauna, absorption on sediment, and absorption by fish through gills, skin, and feeding which will lead to their disappearance from water systems. They cannot be hydrolyzed easily leading to their accumulation in animal tissues. The absorption of these pesticides in fish bodies can be directly from water or by ingesting contaminated food. The gills are in direct contact with water. Therefore, the level of pesticides in gills indicates their concentration in water where the fish live (Haider and Inbaraj 1986).

Among carbamates, Carbofuran is a systemic insecticide, acaricide, and nematicide for its use worldwide. Due to its extensive use, it has been found to be present in surface, ground, and rain waters. Carbofuran is very harmful to aquatic organisms such as fish (Ensibi et al. 2012). Fish contaminated with carbofuran may cause health problems for the people consuming contaminated fish. At lower temperature the pyrethroids are more toxic to mammals and birds than at higher temperatures and these are found to be toxic more than 100 times more for fish due to insufficient hydrolytic enzymes for pyrethroids in fish (Aydın et al. 2005).

They are metabolized to sulphates and glucuronides after distribution to the kidney, bile, liver, and blood cells where significant adverse effects can cause multiple damage to fish meat quality and even the survival of these fish (Richterova and Svobodova 2012; Gautam and Gupta 2008; Yang et al. 2014). Pyrethroids acts by interfering with various ion channels in the nerve axon. The disturbance of concentration gradients across membranes can cause osmotic stress in aquatic organisms (Murthy et al. 2013). At very low concentrations in the water, they have a high rate of gill absorption due to their lipophilicity. This is also one of the contributing factors affecting fish sensitivity to pyrethroids as they are unable to metabolize these pesticides efficiently (Viran et al. 2003). One of the widely used pyrethroid synthetic insecticides is cypermethrin, but it is highly toxic to aquatic invertebrates and fish populations (Gautam and Gupta 2008).

The hydrophobic chemicals which are persistent in nature may accumulate in aquatic organisms through different pathways such as the direct uptake from water by gills or skin (bio-concentration), uptake of suspended particles (ingestion), and the consumption of contaminated food (biomagnification). The rate of uptake depends on the concentrations of pesticides in water, which will usually be higher for less hydrophobic compounds (Gobas 1993). The rate of uptake of hydrophobic chemicals in fish generally increases with higher lipid content of the biological membranes (Spacie and Hamelink 1982). The most common reason for high concentration of pesticides in fish samples may be their mobility resulting in exposure to compounds in other parts of the hydrologic system and the presence of fat content in their tissues (Upadhi and Wokoma 2012). However, these pesticides can build up in fish tissues due to their lipophilic nature when they are released into water bodies.

3.6 Conclusion

It has been concluded that plants, insects, soil microbes, and fishes try to attenuate the negative effects of pesticide toxicity by an enzyme mediated pesticide detoxification system.

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4

Bioaccumulation of Pesticides and Its Impact on Biological Systems

Shubhra Gupta and Kapil Gupta

Department of Biotechnology, School of Life Sciences, Central University of Rajasthan, Ajmer, 305817 Rajasthan, India

4.1 Introduction

Pests are one of the important parts of the ecosystem, which are a big threat to human derived crop production and beneficial plants. Pest management programs generally aim to maximize the farmer's profits while minimizing the input costs. A number of insect practices have been developed to control pests and can be broadly classified into cultural methods viz. crop rotation, trap and catch crop, fallowing, hand-pulling, fertilization, and time of planting; physical methods like solarization; biological methods includes use of fungi and insects; chemical methods like insecticide and host plant resistance, which is obtained by raising resistant or tolerant crop varieties. Chemical approaches are more prominently effective so are continuously being used. Among the chemical methods fumigation and pesticides are the main approaches being used. Fumigation is tedious and more hazardous to the system and is used for the complete elimination of pests. All known fumigants are toxic to man and proper aeration is required for the process. Due to the toxicity to man and animals it should be applied only by trained, experienced operators. Pesticides are naturally or artificially derived chemicals that destroy or suppress the life cycle of pests. Under Pesticides Act (1999), pesticides cover herbicides, insecticides, fungicides, rodenticides, and many other types of substances. The herbicides may be selective like 2,4-D, mecoprop, dicamba which work only on broadleaf or may be non-selective like Paraquat, glufosinate, glyphosate which work on all type of herbs. Insecticides are used to control insects and pests by killing the insects, their eggs, and larvae. It include Organochlorides (Aldrin, deldrin, DDT-Dichlorodiphenyltrichloroethane-it attack on nervous system), Organophosphates (OPs) (Malathion -disrupting nerve impulses), carbamates (Aldicarb, Bendiocarb), Neonicotinoids (works on nervous system), Pyrethrins, Cyfluthrin, Aluminum phosphide (produces phosphine), etc.

Pesticides are intentionally applied to the environment to kill targeted pest and so its management is problematic because it is found everywhere where is human life. Pesticides act by disturbing the biological process of the pests after absorbing through body and kills the targeted organism. Pesticides are sprayed on the ground or foliage and reach inside through leaching with water and target the organism along with other non-target

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organisms. These pesticide residues are left on agricultural products and enter the food chain; from there it gradually moves to a higher level of food web; which is dangerous to human health and other animals too. These practices should be limited to avoid the accumulation of these pesticides in the ecosystem and harm organisms. The presence of pesticides in food causes medical problems, from headaches to cancer, failure in reproductive and endocrine system. Due to the routine use over some time the insect and pests develop resistance against these pesticides and thus create crop loss. The use of pesticides began in the 1940s, which facilitated large benefits in food production, but this use has decreased since 1990 although increase in productivity of some important crop was achieved. The adverse effect of pesticides on the human and environment was first reported during the 1960s (Carson 1962). The impact of pesticides on the environment depends on the quantity used and its chemical characteristics. (Severn and Ballard 1990; Emans et al. 1992). Environmental risk assessments of pesticides carried out by regulatory bodies involves the assessment of pesticide exposure and its effects (Klein et al. 1993).

Farmers and workers suffered from pesticide intoxication because of exposure during pesticide application in farms. According to an estimate of WHO 355 000 people were killed globally per year due to exposure to harmful chemicals. (WHO 1990, 2012; Alavanja 2009; Alavanja and Bonner 2012). On a local scale, pesticides sprayed on crops, are carried by surface runoff to contaminate nearby lagoons and enter in the aquatic food chain; for example toxaphene that is applied onto cotton crops in Nicaragua, retained in soils and reached to nearby watershed (Carvalho et al. 1992, 2003). DDT applied to fields was transported to the lagoons where it was converted to dichloro diphenyl trichloroethylene (DDE) and entered in aquatic food chains which were finally consumed by humans (Kale et al. 1999). Sometime pesticides are transported to faraway areas, where they were not supposed to be present. Hexachlorocyclohexanes (HCH), chlordane, and toxaphene are some compounds that were used in south USA and reached to the Great Lakes at Canada after being volatilized and transported through the atmosphere (Li and Jin 2013). A similar case is HCH applied on rice fields in South Asia, which was transported to higher latitudes (Iwata et al. 1993; Simonich and Hites 1995). Compounds that are highly volatile were reaching far away from their application areas. This process of evaporation and condensation was first reported for organochlorine compounds (OCs), but later was also reported for organophosphates (OPs). The OCs pesticides were reported as environmentally persistent, accumulating in animals and humans with shocking toxic effects (Köhler and Triebskorn 2013; *Discussed in Bioaccumulation of pesticides in Animals*).

4.2 Dispersion of Pesticides into the Environment

Agricultural pesticides are applied on the crop or to the field, injected into the field or used in seed treatment. An application of pesticide depends on crop stage, intended target, application technique, weather conditions, and distribution between soils. When pesticides are applied from an aircraft, half of it drifts out of the target area and 10–30% loss occurs when pesticides are sprayed on crops (Pimentel and Levitan 1986). The presence of pesticides in surface water came to knowledge in 1960s, when residues of chlorinated hydrocarbon insecticides accumulating into water bodies were shown to be toxic to aquatic organisms (Carson 1962; Cope 1965). During the 1970s and 1980s, the number of pesticides found in groundwater increased (US Environmental Protection Agency 1977; Cohen et al. 1984; Leistra and Boesten 1989; Schiavon et al. 1995), which raised great concern, because groundwater is the major source of drinking water. The presence of pesticides in the atmosphere came to knowledge in 1970s and 1980s. Even122 very long transport and deposition of pesticides may occur through long distances, even in ocean fog (Schomburg and Glotfelty 1991) and arctic snow (Gregor and Gummer 1989). This tells us about reaching of pesticides to areas where there is no human activity found but through dispersal and other ways. The presence of pesticides shows the reach and degree of pollution of pesticides which increases risk to unfarmed areas too.

4.3 Behavior of Pesticides in Soil

Pesticide behavior in soils can be understood by following processes: Degradation by soil microorganisms; chemical degradation (hydrolysis); photo-degradation sorption and binding by organic and soil components; uptake by plant; and volatilizations. Microbes metabolize pesticides and are responsible for its degradation. Microbes like bacteria and fungi utilize pesticides as their carbon and energy source. Also, pH, temperature, and organic content affect its degradation. Sometimes microbes may take up DNA molecules and acquire pesticide degradation biochemical machinery. Chemical degradation may occur through hydrolysis, oxidation-reduction, and ionization reactions; all of which are dependent on the pH of the soil. In photo-degradation, pesticides are degraded by the light energy coming on soil or plant surfaces. Photochemical energy by photons breaks the chemical bonds of pesticides.

Sorption is the binding of pesticides to particulates and dissolved organic matter (DOM); and adsorption to humic acids, sediments, and other suspended macromolecules. This leads to reduction in bioavailability of hydrophobic pesticides in soil. Uptake of pesticides from soil occurs in plants through root or foliage. The accumulation volatilization is conversion of solid or liquid to gaseous form and release into air and is lost from the site of application. Rate of volatilization depends on temperature, humidity, air property of pesticide and this cause transfer of pesticide to distant areas.

Pesticides in the soil are degraded via microbiological and chemical means which increases with rise in temperature and soil water content (Walker 1976). In water limiting or dry conditions half-life of pesticide is extended. Degradation rate is presented by a half-life time; higher half-life means longer retention time in soil which means there is more chance of the pesticide entering biological systems. Degradation and fate of pesticide degradation products is of great concern and should also be taken into consideration because the degradation product may have similar characteristics and persistence in soil as well. Fenamiphos is degraded to its sulfoxide and sulfone forms though its pesticidal property remained unchanged (Kookana and Aylmore 1994). Degradation of fenamiphos and its two metabolites has to be taken as total and account for half-life of 70 days. Sometime the bioaccumulation properties of degradation product of pesticide may be observed higher than the parent molecule. For degradation product of Fipronil, fipronil sulfone was found to be more recalcitrant ($t_{1/2}$ three times higher) in fish, and had greater bioaccumulation potential than its parent compound, fipronil. Therefore risk assessment of fipronil in aquatic systems must be considered along with fipronil sulfone.

4.4 Bioaccumulation and Biomagnifications of Pesticide

Dietary uptake of pesticide via contaminated food is termed biomagnification while ecological magnification is known for accumulation of pesticides in the food chain. The term bioaccumulation is used for pesticide uptake from food and water. Pesticides enter in organisms via direct uptake from water or through food chain along different trophic levels. The bioaccumulation not always exhibit direct effects but develop complex symptoms like reduced fertility, health risks for humans, animals, and also to environment. Some effects are only recognized in late phases of life; sometime it may takes many generations to develop any symptoms. The maximum bioaccumulation of pesticides and their effect has been observed in the members present at higher trophic level predators and humans (Travis et al. 1988; Czub and McLachlan 2004) and is a highly undesired. DDE affects thickness of bird eggshell (Henny and Bennet 1990; Mullie et al. 1992), and Polychlorinated Biphenyls (PCBs) affects hatching of eggs. These things didn't appear earlier but after the passage of pesticide for a few generations it became prominent (Tillit et al. 1992).

The bioaccumulation property of a chemical is represented in terms of bioaccumulation factor (BAF). Higher BAF shows the high tendency of a chemical to accumulate in a biosystem while chemicals with smaller BAF value is les accumulative in biological system. In the European regulatory framework chemicals with a BAF > 2000 are treated as bioaccumulative and those with BAF > 5000 to be highly bioaccumulative (EC 1996/2003). There is a clear relationship between the bioaccumulation factor on a wet weight basis (BCFW) of pesticides, like trichlorobenzene, lindane (*g*-HCH), pentachlorophenol, chlorinated benzenes, etc. and lipid content of organism (Geyer et al. 1985, 1994, 1997).

The bioaccumulation of OCs in fish was higher than plants and shrimps in aquatic system. Heptachlor compounds were the predominant OCs contaminants in Nansi Lake, China other than hexachlorocyclohexanes (HCHs) and DDTs. DDTs has highest accumulation property in plants, fish, and shrimp followed by HCHs, and drins (Zhang et al. 2014). Organism size influences the bioaccumulation, but the trophic position of the organism and lipid content plays a major factor in it. Level of PCB in bio samples were observed highest in European eel (*Anguilla anguilla*), (846–2190 ng/g wet weight (ng/g ww) while the lowest in common periwinkle (*Littorina littorea*), a mollusk (17.6–28.0 ng/g w/w). The highest concentration in European eel correlates with accumulation of lipophilic substances compared to other predators because of their high lipid content upto 18.6% of body (Van Ael et al. 2013).

The bioavailability depends on the environmental factors like DOM; humic acids, sediments, and suspended macromolecules. Formation of pesticides colloidal suspensions of with hydrophobic substances, reduce the bioavailability. Effects of DOM in Canadian lakes on 1, 3, 6, 8-tetrachloro dibenzo-*p* dioxin bioavailability in *Crangonyx laurentianus* was studied by Servos and Muir (1989) and positive relationship between presence of organic material and pesticides uptake was observed. The role of organic matter in reduction of chlorinated dioxins bioavailability and bioaccumulation in aquatic organisms (Servos and Muir 1989). DOM makes aggregates with the pesticides and thus reduces their uptake by organisms. Bioconcentration factors are related to the bioavailability of chemical in dissolved fraction are only taken up (Franke 1996). BMFs/BCFs are expressed on the basis of weight of fresh, dry, and lipid weight. Pesticides persistence is higher in low organic matter content soil than with high organic content soil.

A high bioaccumulation/bioconcentration potential (BAF/BCF) pesticide is much toxic to organisms including humans. Regulatory classification, guidelines, and risk assessments use bioaccumulation property to categorize it as hazardous if the BCF is found to be above threshold values (Franke et al. 1994; Zeeman 1997; Franke 1996). In European Union (EU), chemicals with BCF on a fresh weight basis (BCFW) > 100 are categorized as potential bioaccumulative chemical and dangerous to environment and European Commission recommended BCFW > 100 as hazardous (EC 1996). The U.S. Environment Protection Agency (EPA) categorized chemicals of BCFW > 1000 as high potential bioaccumulation property (Zeeman 1995).

4.4.1 Bioaccumulation of Pesticides in Plants

Uptake of pesticides by plants is a major entry route to the food chain which results in bioaccumulation (biomagnification) in humans and animals (Paterson et al. 1990). Absorption of pesticides takes place either by foliar uptake of volatile components or through root. Foliar uptake of pesticides volatilized from the soil surface contribute more than root uptake (Topp et al. 1986).

Predominance of DDT and HCH in food produced was reported in 1980–1990, and due to the ban for its use in agriculture, the concentrations present were reduced in level up to twofold in cereals and pulses (Kannan et al. 1992). Patel et al. (1996) studied DDT and HCH residues in rice grains of four districts of Gujarat, India, and found that both average values and ranges were within the permissible value. In other cereals the DDT and HCH residues were found to be lower than the rice values (Kashyap et al. 1994).

Uptake of pesticide in vapor form occurs in plant foliage (Whitacre and Ware 1967; Nash and Beal 1970). Level of PCBs found in the foliage of beans, broad beans, tomatoes, and cucumbers when grown in PCB supplemented or normal sand up to 28 days did not correlate to the level present in the soil grown. Accumulation of PCB in foliage occurred due to the PCB-vapor in green-house. PCBs mobility through the root is very less and thus their accumulation in bean foliage is mainly due to the vapor uptake Bacci and Gaggi (1985).

Pesticides uptake and accumulation in plants may be highly toxic and can be hazardous to human health and ecosystems (Trapp 2004). Entry of pesticides into plants occurs via (i) root uptake from soil; and (ii) transfer through deposition of particles on plant surfaces, and enters into the plant (Collins et al. 2006), where the transfer depends on specific transfer and permeability properties of chemicals to cross the plant cuticle. Concentration of Imidacloprid in tomato fruits due to foliar spray was found to be higher than the fruits treated with soil application. Application of pesticides by drip-irrigation systems is advantageous over spray applications as it reduces the amount applied, and pesticide exposure, to prevent its entry into the environment (Juraske et al. 2009).

Studies have shown that the application of pesticides affects the symbiotic relation of rhizobia and plants which ultimately affect SNF (symbiotic Nitrogen fixation). Plant growth of Alfalfa by *Rhizoctonia soloni* was found to be reduced due to disruption of signaling. Use of Organochlorine pesticides at high rates onto agricultural land enters the soil (Fox et al. 2007; Sharma 2012). Due to pesticides, a decline in plant growth promotion by *Mesorhizobium* sp. strain MRC4 was observed (Ahemad and Khan 2012). Further pesticide-rhizobacteria interaction should be studied at molecular level to identify genes up or down regulated due to pesticide-stress in Rhizobacteria.

4.4.2 Bioaccumulation of Pesticides in Animals

Animals may take up pesticides through food and water, respiration or contact with skin or exoskeleton. Pesticides cross various barriers of the body after entering the body to reach body tissue. Pesticides cause unintended environmental effects in animals, since they are not selective to the target organism and affect other animals too.

Pesticide which move from soil along with rain water or runoff water is the main source for contaminating water reservoirs, while pesticide leaching contaminates groundwater. Toxaphene applied in cotton crops in Nicaragua, persisted in soils year after year and is carried to watersheds and coastal lagoons by surface runoff where residues contaminated aquatic biota (Carvalho et al. 2003). DDT applied to crops is transported to the aquatic environment where it is metabolized to DDE and bio-accumulated in aquatic food chains (Kale et al. 1999). Toxicity of pesticide is measured on basis of number of indices (oral and dermal LD50) based on tests carried out on laboratory animals. If the rate of excretion or metabolism of pesticide is slow, or they get absorbed or fat-soluble chemicals, the final concentration of chemical in the organism will become higher than its concentration in surroundings of the organism (Madhun and Freed 1990).

Pesticides accumulation damage the immune system (Culliney et al. 1992), sometime it may mimic the hormones of the organism system thus disrupt the endocrine system in both humans and animals (LeBlanc 1995). By the early 1950s, it was well established that dead birds were killed by spraying with DDT or other insecticides in the fields (Madhun and Freed 1990). Birds eating on insects which can't escape due to the effects of the insecticide, develop insecticide accumulation from such insects. When less than a lethal dose of pesticide is ingested or accumulated it cause sub-lethal effects. DDT accumulation disturbs reproductive behavior in birds (LeBlanc 1995) and causes eggshell thinning (Hall 1987). The predatory mammals accumulate higher residues than herbivores as they are on top level of food chain. Widespread death of wild mammals has occurred due to major pest control programs where organochlorine pesticides were used (Madhun and Freed 1990). Aquatic toxicity of pesticides is often assessed by determining toxicity to algae, crustaceans and fish, representing three major trophic levels.

Even in the Arctic Ocean; pesticide accumulation was found in marine mammals and fishes. In ringed seals and polar bears the concentration of β-endosulfan was found to be highest among pesticides currently being used. Capelin of Arctic Ocean among other fishes had higher concentrations of chlorothalonil, chlorpyrifos, dacthal, endosulfan sulfate, and endosulfan (Morris et al. 2016). Organophosphates were reported to be highly toxic to arthropods, which includes insects, but it also affected shrimp, crabs, and other crustaceans, and also to vertebrates as well. Pyrethroids also have an effect on insects and vertebrates; other compounds like herbicides affect the central nervous system and excretory system of mammals as well (Casida 2009; Singh et al. 2016). The use of imidacloprid in seed treatments poses risks to small birds, and ingestion of even a few treated seeds could cause toxicity to smaller-bodied species such as house sparrows (*Passer domesticus*), *Serinus canaria* and gray partridge (*Perdix perdix*) (Gibbons et al. 2016)."

4.4.3 Bioaccumulation of Pesticides in Human and Toxicity

Each year one million cases of human poisoning occur due to pesticide poisonings in world, with 20 000 deaths (WHO-UNEP 1989). Approximately 1.8 billion people engaged in agriculture use pesticides against pests to protect the crop production. People are also exposed during the application of pesticides in the lawn and garden (Alavanja 2009). Humans are also exposed to pesticides by consumption of food or air inhalation, etc. Pesticides persist in the environments for years, and cause health threat through bioaccumulation (Domingo and Bocio 2007; Van Ael et al. 2012). Toxaphene after its application to cotton in Nicaragua, persisted in soils and carried away to coastal lagoons and watersheds by surface runoff from where it entered in aquatic biota (Carvalho et al. 1992, 2003) which was further returned back to human by means of fishes and other animals. DDT use on crops is transported to the water bodies and is bio-accumulated in aquatic animals, finally return back to humans (Kale et al. 1999).

OCs residues are transferred to the food chains where they impact human health adversely. Its impact was observed heavily in animals of the top order in terrestrial food chain, OCs accumulate in adipose tissues of animals and are finally returned to humans as endocrine disruptor (EEA 2013), which is transferred to newborns with the milk fat. There is concern about herbicides, which are routinely used in fields effects on human health, Glyphosate is the most widely used herbicide to kill weeds, is reported to be carcinogenic (Araujo et al. 2016; Benbrook 2016).

Without pesticides or agricultural chemicals crop yield could drop by one third and this will result in a hike in food price. The mixing–loading step before applying the pesticide is the most dangerous step and accounts for major pesticide exposure. Unskilled handling or spraying of these chemicals causes high health hazards (Gupta 2004). According to the Centre for Science and Environment (CSE) pesticide exposure causes poisoning, cancer, neuroproblems, and infertility (Takagi et al. 1997; Arora 2007).

Epidemic studies and toxicology data regarding the health risks assessment against pesticide exposure need to be more accurately estimated. There are many pesticides with proven health hazards to humans, e.g. Lindane, which has been reported to be a human carcinogen causing immunosuppressive effects in humans and DDT, which stimulates colon and liver cancer cell proliferation *in vitro* (International Agency for Research on Cancer 2016). Pesticides like glyphosate, malathion, and diazinon are carcinogenic to humans (International Agency for Research on Cancer 2017). Damage to the chromosomal DNA has also been reported by Malathion and Diazinon. After absorption Malathion is metabolized to the bioactive form malaoxon which damages DNA and chromosome (International Agency for Research on Cancer 2017), while chromosomal damage is done by Diazinon (Hatjian et al. 2000).

4.5 Regulatory Activity

An increasing number of pesticide effects have raised the eyes for requirement of regulatory bodies which deal with restrictions on the excessive use of pesticides or to ban a particular pesticide having a very adverse environment and biological effect. Bioaccumulation is a complex process and assessment of bioaccumulation of a substance enables further use in agriculture. Bioaccumulation of a pesticide constitutes a potential risk to the environment by long term persistence and adverse effects on ecosystems which are not visualized even in laboratory testing.

Indian farmers use a wide range of pesticides to minimize the crop loss from pests. Among pesticides usage, insecticides account for 73%, herbicides 14%, and fungicides 11% (Grace et al. 2007). In pesticide registration a wide variety of aspects are associated with the use of a pesticide, and its effect on human health and the environment is assessed (Monaco et al. 2002; Environmental Protection Agency, EPA 2009). Pesticide registration is an important step and helps in the selection of a suitable pesticide to be used; for purposes, usage rates, claims, labeling, and packaging (WHO 2010). The WHO (World Health Organization) and IARC (International Agency for Research on Cancer) keep close regulation and revision of the toxicity of new and old chemicals being used in agriculture. Many agrochemicals are reported to cause prostate and other types of cancers and are therefore regulated for their use (Singh et al. 2016; ECA 2017).

The fate of pesticides in soils is of more importance due to their impact on the ecosystem and on drinking water sources. Although the production and usage of many types of OC pesticides were limited in many developing countries, they were highly used due to their low cost and effect (Postel 1988; Goldberg 1991; Tanabe et al. 1991).

4.6 Conclusion and Future Perspectives

Pesticides use has helped to double food production in the last century, and currently there is need to increase food production to fulfill the food requirement of the rapidly growing population. This condition forces the farmer into intensive use of fertilizers and pesticides to increase crop production. The toxic effects of pesticide residues on humans and nonhuman biota in terrestrial and aquatic ecosystems are increasing. The increase in food production must be with better quality and less contaminants.

There should be programs to make all farmers aware of reasonable pesticide use in order to minimize the harmful effect of the pesticides. Use needs to be made of biotechnology and bio-pesticides (natural products like *Azadirachta indica* (neem)) and use of integrated pest management (IPM) to manage agriculture, biodiversity, ecosystem, and human health. In an alternate way, to minimize the pesticide input in agriculture, drip based pesticide application can be used. Using drip based application of pesticides, only the required amount will be released into the environment and this little amount can be easily degraded by the abiotic and biotic factors of soil or degraded itself. Organic farming is also a very good alternative of farming in which use of natural pesticides and fertilizers will reduce the synthetic pesticide usage and ultimately lower the toxic effects in the environment. This option is getting more known and its products in consumers because of good quality of crops and fruits and it is devoid of toxic pesticides and other chemicals.

Agro industries need to develop practices which require the regulated use of pesticides through testing, risk assessment, and licensing. Farming practices through education of farmers and public will help in better protection of ecosystems, and sustainable development of agriculture and fisheries. Bioremediation of pesticides can also be attempted through regulating pH of soil, using DOM, or enrichment of microbial population, etc. However, use of herbal plant materials are less studied. Plants can be utilized for bioremediation of pesticides from the soil. Use of herbs as well as weeds like Parthenium and Lantana which grow luxuriantly everywhere or such plants which are not consumed by animal or human are an option to be used for bioremediation of the pesticide accumulated site. Thereafter they can be uprooted and destroyed and eventually reduce pesticide concentrations in soil.

Scientific research for developments in techniques for food production, food safety, and environmental protection, is necessary. Further use of genetically engineered crops (GM Crops) is the demand for future and success of safe food production. Use of genetically engineered plants for resistance against pests can avoid use of these harmful chemicals.

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5

Impact of Pesticide Exposure and Associated Health Effects

Jyoti Upadhayay[1,2], Mahendra Rana[1], Vijay Juyal[1], Satpal Singh Bisht[3] and Rohit Joshi[4]

[1] *Department of Pharmaceutical Sciences, Kumaun University Campus, Bhimtal, Uttarakhand, 293136, India*
[2] *School of Health Sciences, University of Petroleum & Energy Studies, Dehradun, Uttarakhand, 248007, India*
[3] *Department of Zoology, D.S.B. Campus, Kumaun University, Nainital, Uttarakhand, 263001, India*
[4] *Division of Biotechnology, CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, 176061, India*

5.1 Introduction

Pesticides are ubiquitous chemicals that persist in the environment for longer duration. Because of their long half-lives, they persist for decades in the human body and shows various health effects like skin rashes, developmental delays, and cancer, depending upon their exposure level. The term "pesticides" covers a broad range of compounds which includes insecticides, fungicides, herbicides, rodenticides, plant growth regulators, and others. Globally, these chemicals are controlled by Stockholm Convention, a treaty approved by the global convention. United Nation Environment Program (UNEP) organized this Stockholm Convention, which was created initially in 2001 and signed in 2004. The purpose of this convention is to eliminate or reduce the unintended production of the pesticides called "dirty dozen" recognized in 2001. Pesticides like aldrin, chlordane, dieldrin, dichlorodiphenyltrichloroethane (DDT), heptachlor, hexachlorobenzene, enderin, mirex, polychlorinated biphenyls (PCB) and dibenzofurans (Haffner and Schecter 2014). The primary goal of mankind is to obtain food for the survival and to improve the quality of life. More than sixty percent of the population in some countries are involved in agriculture for producing food not only for their population and but for other countries. For increasing productivity there is a need to control pests, weeds, insects, and pathogens using crop protection products. According to World Health Organization, pesticide poisoning cases reported approximately 3 million each year and 220 000 deaths occur in developing countries. Harmful effects of pesticide exposure generally occur in children and particularly in the young and the developing organism like fetus during gestation period. Also, very low level of pesticide exposure may have serious adverse health effects during the developmental process. Neurological symptoms like memory loss, cognitive dysfunction, reduced visual ability, alteration in mood and behavior, reduced response to stimuli, and reduced motor skills

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are caused by pesticide exposure. It is difficult to recognize these symptoms by the medical community as a clinical effect. Other probable manifestations include allergies, asthma, endocrine disruption, cancer, reproductive problems, and fetal development disorder. Generally, the formula of pesticide contains both “active” as well as “inert” ingredients. An active ingredient kills pests, whereas an inert material aids active ingredients to work more efficiently (Lah 2011).

In developing countries, the largest consumer of pesticides is agriculture and they are also used in controlling vector-borne diseases like malaria and dengue (insecticides), preventing growth of unwanted plants, avoiding the proliferation of pests, insects, bacteria, in various household equipment and food packaging materials (Gliden et al. 2010). They are designed to be poisonous and their unintended exposure can be extremely hazardous. Some detectable levels of pesticide metabolites in urine samples of the general population have been reported, which indicates potential exposure from both dietary (food and water) and non-dietary (air) exposures. Long term use of pesticides in farmland near residential proximity is an important pathway of pesticide exposure that ultimately affects human health.

5.2 History of Evolution of Pesticides

History of pesticide production begins with the evolution of pest controlling agents from naturally occurring non-selective agents to highly specific biological and synthetic materials that control pests. Earlier naturally occurring and basic poisons were used as pest control products. They were highly persistent, non-selective, and toxic to many live forms. They were arsenic, fluoride, lead as insecticides, ashes, salts, smelter sludges as herbicides, chalk, woodash, and sulfur as fungicides. In the 1800s insecticides included botanicals, rotenone, pyrethrums, and nicotine. During this period sulfur and copper compounds were used as disease controlling agents on fruits, vegetables, and ornamental plants. During 1930s the modern era of synthetic organic pesticides began. Introduction of 2, 4-D in 1940 was a real breakthrough in weed control in cereal crops. Organomercurials and organochlorines (DDT) were introduced in early twentieth century. New classes of insecticides called organophosphates were introduced with lower risks for both user and environment. The evolution of pesticides continued as new chemical agents were discovered with decreased persistence in the environment and which possess attractive and valued benefits to both producers and end users (Bhattacharyya et al. 2009).

5.3 Pesticides Regulations

Pesticides regulations and their use have always been controversial. Rachel Carson, a biologist published “Silent Spring,” which prominently describes the risks associated with the use of DDT (Carson 1962). After this, the US authority canceled the use of DDT for agricultural purpose. Pesticide residues persist for longer duration and are toxic to human health. They also kill bees, domestic animals and fishes. Also, their long-term use causes development of pesticides resistance in insects, weeds, and pathogens. The distribution of hazardous pesticides and their use has been a major issue of concern. In 1982

Pesticides Action Network (PAN) an international organization calling for successful action on the elimination of toxic pesticides globally. PAN has been one of the driving forces among various non-governmental organizations (NGO) which helps in framing policies toward safer, effective, economically viable and environmentally sustainable pest management systems. The first international "Code of Conduct" on pesticide distribution and use was adopted by United Nation Food and Agriculture Organization (FAO) which respond to the associated risk and harm with the pesticides use. The code article in the first version of "Code of Conduct" stated that "industry should halt sale and recall products when handling or use pose an unacceptable risk under any use directions or restrictions"(PAN 2015). In 2009, new pesticide authorization regulation was made by the European Union which emphasizes the necessity of taking the substance of hazardous pesticides into account. Pesticides that are carcinogenic, mutagenic, toxic, cause infertility and endocrine disruption shall not be considered acceptable by the EU (EC 2009). The FAO in November 2006 endorsed the Strategic Approach to International Chemical Management (SAICM) that includes a broad range of activities like ban on highly hazardous pesticides, promotion of good agricultural practices, and environment friendly disposal of pesticides etc. In 2007, a Joint Meeting of (FAO/WHO) on Pesticide Management (JMPM) outlined the criteria which identifies Highly Hazardous Pesticides (HHP). In 2009 PAN International publishes its first PAN list of HHPs (PAN and IPEN 2013). The criteria for identifying hazardous pesticides are acute toxicity, chronic effects, environmental toxicity and global pesticide-related convention. The definition of HHP according to the FAO includes pesticides linked with a high incidence of severe or irreversible adverse effects on human health or the environment (PAN 2015).

5.4 Impact on Environment

Pesticides are designed to be toxic and have considerable adverse effects on living creatures as well as in various environmental media like soil, air, and water (Aktar et al. 2009). Pesticides like aldrin, dialdrin, dichloro DDT, heptachlor, and hexachlorobenzene contain persistent organic pollutants (POPs) which remain in the environment for a longer period of time (Yadav et al. 2015). Repeated use of pesticides caused biodiversity losses and increased incidences of pest resistance and pest resurgence (Damalas and Eleftherohorinos 2011). Airborne pollution caused by pesticides mainly occurs through pesticide drift like aerial spray drift and post-application volatilization (Rull and Ritz 2003). Indoor pesticide exposure is created by mechanisms like heating, cooling, and ventilation system. Use of pesticides accounts for about 6% of the total ozone level in the tropospheric region (Coxall 2014). A study from White et al. 2006 shows the measured concentration of pesticides in ambient air from three potato farm sites in Prince Edward Island, Canada. The fungicide Chlorothalonil, was found at relatively high concentration showing the effect of its repeated application on potato farms. Another study showing a ground level concentration of carbofuran and methamidophos in air was found to be 219 and 637 ng m^{-3}. The concentration of these pesticides was a matter of high concern in case of possible exposure to wildlife. Pesticide residues were also found in rain as well as ground water. The most common pesticides found in the water were diazinon (1%), propachlor (1.5%), metolachlor (1.5%), acetochlor (4%), and atrazine (6%) (Szekacs et al.

2015). Bulut et al. (2010) determined the pesticides concentration in drinking water sample and few major pesticides as beta-hexachlorocyclohexane (β-HCH) 0.281 $\mu g\,l^{-1}$, 4,4^{1}–dichloro diphenyl trichloroethane (4-4^{1}-DDT) 0.138 $\mu g\,l^{-1}$, endrin 0.120 $\mu g\,l^{-1}$, ketone, and methoxychlor.

5.5 Impact on Human Health

5.5.1 Pesticide Exposure

Pesticide exposure is a hazard or risk with a degree of danger under certain conditions. Hazard generally depends upon the toxicity of the pesticides and amount of pesticide exposure expressed with the given equation: **Hazard = Toxicity × Exposure.**

Pesticide toxicity is a measure of the capacity or ability of the pesticide to cause illness or injury (Lorenz 2009). The exposure of pesticides occurs through both the routes direct and indirect. In direct exposure a person individually applies pesticides in their residential, occupational, or agricultural settings which results in highest level of exposure, whereas in the case of indirect route, exposure occurs through air, dust, drinking water, and food and presents long-term low level exposure. It may occur more frequently than direct exposure of pesticides (Fenske 1997; Gladen et al. 1998; Semchuk and McDuffie 2003). Methods for assessment of pesticide exposure are more refined for evaluating occupational exposures, pesticides use near residual proximity; information regarding pesticide application are important tool for estimating exposure to others in the family members, rural residents, and population. Uses of geographic information, mapping, and remote sensing data provides information for estimation of pesticides and other environmental exposures. All these evaluation parameters enhance the ability for assessing the effects of pesticides on health in agricultural as well as other settings. Epidemiological studies show recent improvements in assessment of pesticides exposure (Alavanja et al. 2004b).

5.5.1.1 Pesticide Exposure Routes in Humans

Pesticide exposure can occur from both direct route and indirect route. The direct exposure occurs from agricultural, household, and occupational use whereas pesticides indirectly transferred through food. Air, water, soil, flora, fauna, and food chain are the main routes of exposure to pesticides in humans (Anderson and Meade 2014). Table 5.1 shows the route of pesticide exposure in humans.

5.5.1.2 Acute Toxicity of Pesticides

It is the chemical ability of the pesticides to cause injury from a single exposure for short duration of time. “Acute effects” are harmful effects that occur after single exposure to pesticides from any route of entry. The routes of exposure are oral, inhalation, eyes, and dermal. The acute effects of organophosphate and carbamate pesticides occur on the sympathetic, parasympathetic, and central nervous system. They act by interfering with the acetylcholine (ACh) metabolism by inhibiting acetylcholinesterase (AChE) (Ecobichon 1994). Acetylcholine is a neurotransmitter present at the neuronal junctions responsible for continued stimulation and then neurotransmission suppression to organs. It is the transmitter of somatic motor neurons to skeletal muscles, preganglionic

Table 5.1 Routes of pesticide exposure in humans

S.N	Routes	Mechanism of exposure	References
1	Dermal exposure	Absorption occurs as a result of a splash, spill, or spray drift, during mixing, loading, disposing, cleaning of pesticides.	Salvatore et al. (2008)
2	Oral exposure	Accidental cases due to carelessness or intentional purposes.	Damalas and Eleftherohorinos (2011)
		During transfer from original labeled container to an unlabeled container.	Gliden et al. (2010)
		Pesticides in soft drink bottles or after drinking water stored in pesticides contaminated bottles.	U. S. Environmental Protection Agency USEPA (2007).
3	Respiratory exposure	Spraying of pesticides causing production of smaller droplets with conventional application equipment.	Amaral (2014)
4	Eye exposure	Some pesticides reported being absorbed by the eyes causing serious and fatal illness.	Gliden et al. (2010)

fibers of both sympathetic and parasympathetic nerves, postganglionic parasympathetic nerve fibers and central nervous system. When acetyl choline gets accumulated in the motor nerves it results in fatigue, muscle cramps, weakness, fasciculations, and muscular weakness of respiratory system. Accumulation of acetyl choline at autonomic ganglia causes increased heartbeat and blood pressure, hypoglycemia and pallor. At muscarinic receptors, its accumulation results in visual disturbances, wheezing caused by bronchoconstriction, chest tightness, increased bronchial secretions, salivation, sweating, lacrimation, peristalsis, and urination. An effect in the CNS includes headache, convulsions, ataxia, anxiety, respiratory depression, circulation, tremor, slurred speech, and generalized weakness (Ecobichon 1994; Sherman 1995). Like other organophosphates, carbamates do not inhibit acetylcholinesterase irreversibly. During pregnancy the toxicity of carbamates poses an increased risk with time as there is a reduced activity of acetylcholinesterase in the first trimester (Howard et al. 1978; Evans et al. 1988). Herbicide paraquat causes progressive and severe damage to lungs causing anoxia and finally death. During the first phase, alveolitis along with neutrophil infiltration occurs which leads to progressive pulmonary edema. Rapid and progressive intense interalveolar and intraalveolar fibrosis damages alveolar structure in the second phase. In paraquat toxicity, tubular necrosis occurs causing renal failure and liver dysfunction also occurs (Blain 1990).

Anhydrous ammonia gas used as fertilizer handled as a pressurized liquid during transportation. Due to its high pressure and a temperature of −28 °F, this form of anhydrous ammonia penetrates in any tissue and causes burns of the part of that tissue it strikes like skin and eyes. Also, inhalation of this gas causes laryngospasm, bronchitis, tracheitis with oedema in lungs. Injury to lungs is usually reversible but eye injury is an irreversible damage (Helmers et al. 1971).

5.5.1.3 Neurobehavioral Effects After Acute Toxicity

WHO protocol core test battery demonstrates neurobehavioral effects like lack of verbal attention, reduction in visual memory, affectivity, and motricity (Maroni et al. 1986; WHO 1986). A study of Organophosphate Poisoning cases registered in California State was carried out consists of 128 subjects showed lack of sustained visual attention, symbol digit tests, vibrotactile sensitivity. Excessive poisoned subjects (hospitalized) showed an increased level of neurobehavioral impairment (Steenland et al. 1994). An abnormal vibrotactile threshold was observed in one fourth of patients previously poisoned with methamidophos, out of 36 male workers hospitalized due to acute OP poisoning (McConnell et al. 1994).

5.5.1.4 Chronic Toxicity of Pesticides

Chronic effects are harmful effects that are caused when small doses of pesticides are repeated over a period of time. These effects from exposure to pesticides include birth defects, fetal toxicity, malignant or benign tumors, blood disorders, genetic disorders, nerve disorders, reproductive effects, and endocrine disruption. The chronic toxicity of pesticides on the nervous system is less well understood but evidence of neuro-developmental toxicity arise from low-level exposure during the gestational period or early postnatal life is accumulating. Health assessment parameters indicate that persons working on agricultural lands face chronic health related problems when exposed to pesticides for a longer duration. Organs which are significantly affected due to long-term exposure to pesticides are eyes, skin, lungs, kidney, and neurons. The following are the pesticides related health abnormalities:

5.5.1.5 Disruption of Endocrine System

The endocrine system comprises of various hormone secreting glands which control growth and development, metabolism in tissue, reproductive functions, and other physiological changes. Use of synthetic chemicals like pesticides has been increased extensively. Clinical evidence and epidemiological studies suggest that these endocrine disrupting chemicals possess a major risk for human health by affecting different organs and systems in the body. The target organs involve estrogen receptors, nuclear receptors, and steroidal receptors. Mechanisms involving xenobiotics induced stimulation of signaling pathways, genetic mutation, or DNA methylation help us to understand the results of these xenobiotics action on the endocrine system. Any disturbances in the endocrine system result in various problems like breast cancer, ovarian cancer, testicular carcinoma, thyroid eruptions, schizophrenia, Alzheimer disease, nerve damage, and obesity (Maqbool et al. 2016). Endocrine disruptors defined by U.S. Environmental Protection Agency (USEPA), as an agent that interferes with the synthesis, secretion, transport, binding, or elimination of hormones in the body. These hormones are responsible for maintaining homeostasis, growth and development, behavior, and reproduction (Kavlock et al. 1996).

Human physiology affected by endocrine disrupting chemicals may be genomic and non-genomic through various receptors linked or non-receptor linked pathway. Figure 5.1 shows the action of endocrine disrupting molecules, i.e. pesticides at both receptor and hormonal level. In steroid biosynthesis various enzymes are involved which are ideal targets for these pesticides. Cytochrome P-450 (CYP-450) has a major role in biosynthesis of steroid hormones, regulated by various organs like adrenal

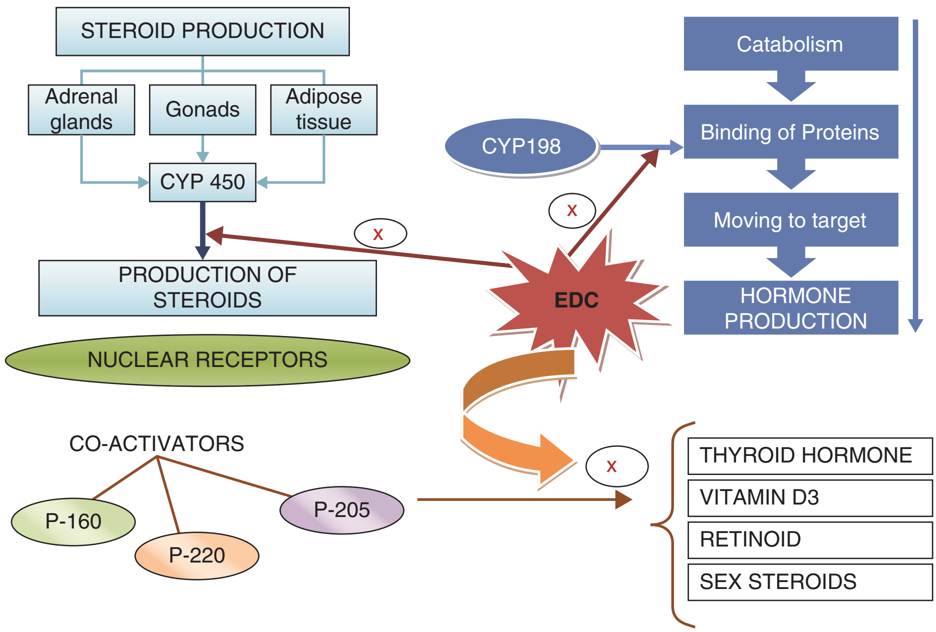

Figure 5.1 Action of endocrine disrupting chemicals at receptor and hormonal level.

glands, testes, ovary, adipose tissue, brain, and placenta. Organotin compounds used especially in paints, agriculture, and industries were found to inhibit the activity of Cytochrome P450 (CYP450), Cytochrome P1A1 (CYP1A1), aromatase in fish (Fent and Stegeman 1991). Nuclear receptors are transcription factors (ligand inducible) which modulate specific gene expressions which are involved in differentiation, metabolism, and sexual functions. They regulate the response in target cells to hormones like thyroid, vitamin D_3, sex steroids, and adrenal steroids. Co-activators are natural or synthetic ligands that have been known to stimulate actions of nuclear receptors and they are P-160 family with intrinsic histone acetyl transferase activity (Freedman 1999). P-220 is a thyroid hormone receptor activator protein and P-205 is vitamin D receptor interacting protein and binding protein lacking intrinsic histone acetyl transferase activity (Rachez et al. 2000). Blockage of steroid receptor co-activators has also been evidenced in protein degradation mediated by proteasome, caused by alteration of gene expressions and inhibition of activity of co-activators. A study shows activation of nuclear receptors by organotins under the influence of high fat and high calorie diet, stimulates adipocyte differentiation and predisposition progresses toward obesity (Grun and Blumberg 2006). Apart from organotins, Bisphenol A (BPA), and phthalates have also been evidenced as stimulator of nuclear hormones and nuclear receptor linked pathways causing metabolic syndrome (Grun and Blumberg 2007).

Hormones stimulate and catalyze a number of biological reactions in the body. Endocrine disrupting chemicals are reported to affect production cycle of hormones (You et al. 2001). Chemicals like PCB, dioxins, phthalates, and furans induce the stimulatory actions of thyroid hormones in animal study whereas flame retardants reduce the thyroid hormones level in treated rodents (Moriyama et al. 2002).

5.5.2 Carcinogenicity

Pesticides including organochlorine (like DDT, chlordane, aldrin, etc.), lead arsenate, sulfate, and creosote are found carcinogenic, reported in some animal studies but many of them continued to be used in developing countries (Repetto and Baliga 1996). Some carcinogenic solvents are also present in pesticides formulations (Petrelli et al. 1993). A study on children and infants living near agricultural areas and using household pesticides has shown an increased prevalence of leukemia and lymphoma (Alexander et al. 2001). In a population-based case control study, Parron et al. 2014, shows higher prevalence rates and cancer risks in districts with greater pesticide use as compared to those with lower pesticides use. This study was conducted in ten health districts from Andalusia (South Spain) which were categorized into low and high levels of environmental pesticide exposure dependent upon two quantitative categories, i.e. the number of hectares devoted to intensive agriculture and pesticides sales per capita. The study population comprises of 34 205 cancer cases and 1 832 969 health districts match control. Data were collected between 1998 and 2005, from computerized hospital records. Prevalence rates of stomach cancer, colorectal cancer, liver cancer, skin cancer, bladder cancer, and brain cancer were significantly greater in areas having higher environmental pesticides exposure in comparison with lower exposure. Hodgkin and non-Hodgkin lymphoma prevalence rates were significantly reduced. Leukemia and multiple myeloma prevalence were found greater in highly exposed areas, but the difference was not statistically significant. In male's greater prevalence of prostate cancer, testicular cancer, and lung cancer were observed whereas in case of females, breast cancer, cervical cancer, and ovarian cancer were highly prevalent.

Non-Hodgkin's Lymphoma (NHL): It is the most widely observed cancer in relation to pesticide use. Blair and Zahm (1991, 1995) observed NHL linked with herbicides, i.e. phenoxyacetic acid, organochlorine, and organophosphate pesticides in epidemiological studies. A case control population based study shows the association of NHL risk with specific pesticides (organochlorine) through agricultural exposures (Cantor et al. 1992; McDuffie et al. 2001). There was a statistically significant increase in the risk of NHL with increased exposure to insecticides like carbamates and phosphates, herbicides like phenoxy and benzoic acid, amide fungicides and fumigant like carbon tetrachloride (McDuffie et al. 2001). A case control hospital-based study shows association of NHL risk with serum chlordane and related compounds which were collected post diagnostically (Hardell et al. 1996). Another population- based case control study uses prediagnostic serum levels of various organochlorine compounds does not confirm this study (Cantor et al. 2003). The inconsistency of these two studies shows the importance of incorporating prediagnostic biological samples for determining the etiological associations if possible.

Leukemia: Some epidemiological studies shows an association between agricultural exposure and leukemia, but no clear pattern of risk has been established. In leukemia, hematopoietic malignancies occur in both acute and chronic forms affecting both adults and children. In northeastern Italy, population-based case control study in animal breeding and farming areas shows an association between pesticide use and chronic lymphocytic leukemia (CLL). In the pesticides organophosphates, DDT, and carbamates show CLLs association with persons working in these areas (Nanni et al. 1996). In France a hospital-based study shows interrelation between organophosphate insecticide and hairy-cell leukemia (Cavel et al. 1996). Exposure to herbicides increases the risk of both hairy cell leukemia and non-Hodgkin lymphoma, but other categories of pesticides do not show any effect (Hardell et al. 2002).

Multiple Myeloma: Plasma cell hematopoietic malignancy occurs in most parts of the world. A 32 peer reviewed study of multiple myeloma and agricultural exposure was analyzed and published between the years 1981 and 1996. The study indicates an increased risk of multiple myeloma among male farmers, i.e.1.23 and also the same risk was analyzed in female farmers (Khuder and Mutgi 1997; Kruder et al. 1999). Possible exposure agents for this increased risk of multiple myeloma includes pesticides, infectious agents, and organic solvents, but supporting evidence related to this etiological factors is not strong.

Soft-Tissue Sarcoma: There are some studies showing the association between pesticides exposure and soft tissue sarcoma (Hoar et al. 1986; Woods et al. 1987; Petrovitch et al. 2002). A population-based case control study shows associated risk of soft tissue sarcoma among farmers of Kansas with increasing time (Zahm et al. 1988). A study determined the use of herbicides associated with fibriohistiocytic sarcoma but not with liposarcoma (Hoppin et al. 1999).

Prostate Cancer: A cohort study among registered pesticides users in the United States shows a significant association of chlorinated pesticides use with the risk of prostate cancer. Also, a fumigant, methyl bromide was also related with prostate cancer risk when lifetime exposure was given. Pesticides like organophosphates insecticides, pyrethroids, herbicides like thiocarbamates showed an increased risk of prostate cancer with the family history of this type of cancer. This family history pesticide exposure and prostate cancer interaction suggests gene-environment interactions (Alavanja et al. 2003).

Pancreatic Cancer: A number of studies related with occupational exposures and pesticides users like farmers shows elevated pancreatic cancer risk (Falk et al. 1990; Forastiere et al. 1993). Cases of pancreatic cancer were observed among DDT manufacturing workers (Garabrant et al. 1992). Fivefold increase in the risk of pancreatic cancer among outdoor workers was observed in Australia with the DDT application (Beard et al. 2003).

Lung Cancer: The risk of lung cancer was associated with arsenical compounds (IARC 1986), and was observed in arsenical pesticides manufacturer (Mabuchi et al. 1979 and Mabuchi et al. 1980) and vineyard workers (Luchtrath 1983). In Germany a cohort study shows exposure to herbicide containing phenoxy group or contaminants like dioxins and furans was observed for lung cancer mortality in workers from manufacturing units (Becher et al. 1996).

Ovarian Cancer: A hospital-based case control study in Italy suggested role of triazine herbicides in etiology of ovarian cancer. The relative risk for ovarian cancer was observed 4.4 in women with either "probable" or "definite" exposure to these herbicides (Donna et al. 1984). A prospective cohort study among pesticides users shows significant ovarian cancer risk among females (Alavanja et al. 2004a). Herbicide atrazine induces tumors in rats (Pinter et al. 1990; Wetzel et al. 1994) and mice both male and female (Donna et al. 1981 and Donna et al. 1986). Both atrazine and cyanazine detected in surface water in the corn belt of the United States (EPA 1990).

5.5.2.1 Neurological and Neuro-developmental Effects

Some pesticides are highly toxic to the central nervous system of pests like insects and mammals such as rodents. This neurotoxicity acts as a useful tool for the development of pesticides. High level of pesticide exposure like organophosphates, carbamates, fungicides can result in neurotoxicity (Keifer and Mahurin 1997). Organophosphate exposure response occurs in a minute and causes manifestations like headache, dizziness, papillary constriction, vomiting, excessive sweating, and salivation. The most severe symptoms include muscle weakness, abnormal heart rate, bronchospasm which further progresses to convulsions and finally coma and death. The cause behind these symptoms is overstimulation of postsynaptic cholinergic receptors and organophosphates induced acetylcholinesterase inhibition. After four days of exposure an intermediate syndrome occurs characterized by muscle weakness and become fatal when respiratory muscles become affected. Organophosphate-induced delayed polyneuropathy (OPIDP), a syndrome occurs after two to five weeks involve symptoms like sensory abnormalities, muscle weakness, cramps, and paralysis especially in legs, caused by axonal death of neurons by inhibiting neural enzyme neuropathy target esterase. This inhibition is irreversible. Several studies show an increased prevalence of symptoms like deficits in cognitive and psychomotor function, impaired nerve conduction, and decreased vibration sensitivity, among farm workers or the general population having a history of pesticide poisoning (Savage et al. 1988; Steenland et al. 1994). Some studies show that both acute and chronic occupational exposure result in minimum to maximum deterioration in neurological functions which may be irreversible (Ecobichon et al. 1990). Organochlorine, carbamates, organophosphates, fungicides (diphenyl, hexachlorobenzene, hexachlorophene, mercurials), and fumigants (methyl bromide, sulfuryl fluoride) are pesticides that cause chronic neurologic effects (Ecobichon et al. 1990; Dennis and Weisenburger 1993).

5.5.2.2 Parkinson's Disease (PD)

Many studies and literature suggest that people, having farming as occupation, living in rural areas, drinking well water are at greater risk of Parkinson's disease. Also, numerous studies have determined that PD risk was associated with pesticide exposure (Priyadarshi et al. 2001).

5.5.2.3 Immunologic Effects

Animal study reports that pesticide toxicants can alter or modulate immune response. Contact dermatitis and asthma type reactions in humans are triggered by pesticide exposures. Also, alterations in complement and immunoglobulin level and changes in T-cell population were reported in humans exposed to pesticides. Occupational exposure to OP causes impaired neutrophil chemotaxis followed by an increased level of respiratory tract interferons was analyzed in one study. There is a need for further research in the studies of the immune effects of pesticide exposure in humans because low immunity predisposes humans to a variety of cancers like non-Hodgkin lymphoma (Dennis and Weisenburger 1993; Thomas et al. 1990).

5.5.2.4 Reproductive Effects

These pesticides damage the normal physiological functions related to the reproductive system. A number of *in vivo* and *in vitro* evidences are available showing ovarian and testicular abnormalities. Reduction in quality as well as number of sperms, as well as increased incidences of testicular, ovarian, and breast cancer have been reported in some studies (Toppari et al. 1996).

Several animal studies evaluated potential reproductive toxicity for many associated pesticide exposures. Pesticides including organochlorine (dibromochloropropane DBCP, Chlordecone) act as reproductive toxic agents in the human male. DBCP reports cases of reduced sperm motility, oligospermia, and azoospermia whereas chlordecone also reports cases of oligospermia and reduced sperm motility. Higher prevalence of abortion in females has been reported in those whose husbands were exposed to dibromochloropropane pesticide (Mattiscm et al. 1990). Reduced fertility, sperm counts, sperm motility, viability, and abnormal morphology were observed with exposure of ethylene dibromide. There is an increased level of abnormal sperm in males caused by exposure to insecticides, carbaryl, and carbamates (Mattiscm et al. 1990). Occupational exposure of 2, 4 Dichlorophenoxyacetic acid in males revealed reduction in sperm counts, sperm motility, viability, and morphological changes (Lerda and Rizzi 1991).

5.5.2.5 Estrogenic Effects of Pesticides on Human Estrogen-Sensitive Cells

DDT and Chlordecone are estrogenic pesticides have a deleterious effect on the reproductive system. The estrogenic activity of several pesticides was assayed by an "in culture" technique. In this method the E-screen test uses human breast estrogen-sensitive cells MCF7. The cell yield was compared after six days of culture in a medium containing 5% charcoal–dextran stripped human serum in the presence of estradiol, i.e. positive control, and its absence, i.e. negative control, and with several concentrations of pesticides suspected of being estrogenic. Organochlorine pesticides: endosulfan, dieldrin, and toxaphene possess estrogenic properties as compared to DDT and Chlordecone (Soto et al. 1994).

5.5.2.6 Diethyl Stilbestrol (DES) Syndrome (Model for Estrogenic Chemicals Exposure in the Environment)

A synthetic estrogen diethyl stilbestrol (DES) was used by doctors for preventing frequent abortions in women from the years 1948 until 1971. The use of DES was banned for this purpose but humans exposed to DES serve as a model for exposure to estrogenic chemicals during early life, involving pollutants which are estrogen agonists. Estrogenic activity of a pesticide was determined by the activation of mitotic activity in the cells or tissues of the female genital tract during puberty, early ontogeny, and in the adult (Hertz 1981). Estrogenic chemical exposure also affects other tissues in both male and female (vom Saal et al. 1992 and vom Saal et al. 1993). In-utero exposure of estrogenic chemicals (DES, Estrogenic agonist) in females especially at an age at which incidences of reproductive organ cancer occurs are at much higher risk than unexposed females. Exposure of these estrogenic chemicals in adult men has been found responsible in the etiology of prostate hyperplasia (vom Saal et al. 1993; Ghanadian 1983). Prostate cancer and benign prostate hyperplasia in men and cancer of estrogen-responsive tissues in women are a major health issue faced by elderly people.

5.5.2.7 Developmental Effects

The percentage of incidences of pesticide related developmental toxicity in humans is not known. In animal studies approximately 50% of pesticides tested are teratogenic

(Johnson et al. 1990). Developmental disorders finally lead to death, functional disorders, growth retardation and malformations. Many studies of pesticide exposure have failed in finding fetal malformations. Data from some epidemiological studies shows congenital malformations in rural areas and pesticide use. Malformations include limb reduction defects, congenital heart disease, facial clefts, musculoskeletal defects, and urogenital defects. (Dennis and Weisenburger 1993). During pregnancy prenatal exposure to pesticides has been associated with cognitive development defects, behavior and mental problems in childhood. Some studies have determined associated abnormal reflexes during newborn period. There is a need of further research to examine the early neurobehavioral effects of pesticide exposure. Some epidemiological and experimental research suggests that both prenatal and early postnatal pesticide exposure adversely affects neurodevelopment (Bjorling- Poulsen et al. 2008). The presence of organochlorine pesticides, DDT (dichlorodiphenyl trichloroethane), and HCH (hexachlorocyclohexane) have been determined in human breast milk. This study was conducted in the Dibrugarh and Nagaon districts of Assam. The results show mean levels of total DDT, $3210\,ng\,g^{-1}$ and HCH $2720\,ng\,g^{-1}$ lipid weight in Nagaon district and $2870\,ng\,g^{-1}$ of DDT and $2330\,ng\,g^{-1}$ of HCH in Dibrugarh district respectively. No significant difference was observed in investigated pollutants levels between the two districts. After investigating the organochlorine pesticides levels in human breast milk ADI (Average daily intake) by the infants has been determined. It was observed that high daily intake of DDT and HCH in infants exceeds the TDI (Tolerable daily intake) level, indicating that infants of these two districts are at greater risk from these pesticides which is a matter of concern for health related issues in infants (Mishra and Sharma 2011).

5.6 Other Health Problems

5.6.1 Eye Problems

Pesticides like acetamide and 2, 4 D are called as eye irritants. Chronic irritation of the eye due to exposure of these pesticides leads to the pterygium, i.e. formation of vascular membrane on the cornea, which leads to diminished vision (Morgan 1977; Antle and Pingali 2015).

5.6.2 Respiratory Problems

Lungs are the target site where binding and metabolism of most organophosphate compounds occurs. The most common lung disorder caused by long term exposure of pesticides is Bronchial asthma. Chronic exposure to pesticide like organochlorine and organophosphates among agricultural farmers shows respiratory impairment (Dennis and Weisenburger 1993). Cases of persistent pulmonary fibrosis were reported in paraquat poisoning survivors, and broncholitis, bronchiectasis, and chronic cough have been observed in anhydrous ammonia exposure (Kass et al. 1972).

5.6.3 Determination of Pollution Potential of Pesticides

It is essential for us to determine the extent of the real health problem associated with the use of pesticides. Various methods have been proposed for estimating the impact

Table 5.2 Methods determining pollution potential of pesticides

S.N	Method	Procedure	References
1	Transfer model	Target the fate of the substance Knowledge about physical, chemical, and microbial processes controlling their persistence in the environment. Main focus of this method is on the pesticides behavior in the environment. Neglect the effect of these pesticides on the receptor population.	Jury et al. (1987) Leonard (1990)
2	Ranking method	Incorporate different effects of pesticides Risk assessment method includes both fate and exposure.	Kovach et al. (1992) Jouany (1995) Newman (1995)
3	Environmental Life Cycle Assessment (LCA)	It captures the environmental impact of products over their complete life cycle. Methods: CML 92, Ecoindicator 99 USES LCA, evaluate pesticides combining fate and exposure analysis with toxic assessment for humans and ecosystems	Heijungs et al. (1992) Goedkoop (1995) Goedkoop et al. (1999) Huijbregts et al. (2000)

of pesticide exposure in the environment, but some of them have limitations (Margni et al. 2002). Six methods have been proposed to assess the environmental impacts of pesticides and associated problems (Van der Werf 1996).

Methods include a lack in the explicit definition of the impact of pesticide use in the environment, ignoring important fate processes, the amount of pesticide applied, or toxicity related information. Table 5.2 shows the already existing methods for identifying the pollution potential of pesticides.

In order to reduce the risks and impacts of pesticide exposure on the environment and human health, the European Commission Directive 2009/128/EC (2009) established a framework, whose main aim is to achieve the sustainable use of pesticides by promoting integrated pest management system (IPM) and other approaches of pest control and techniques (alternative to pesticides, i.e. non-chemical). A specific program, Ecophyto plan in France has led to the development of a new cropping system that contributes to the reduction of reliance on pesticides, built on biological, agronomic, mechanical, and physical principles (Barzman et al. 2015; Ecophyto 2015).

5.7 Conclusion

In the field of agricultural the use of pesticides and fertilizers has grown significantly over the past few years. Recently, approximately 600 active pesticides are used but the availability of their adequate toxicological data is only for 100 of these. Environmental exposure of these pesticides to humans is very common and can result in both acute and chronic health effects like neurotoxicity both acute and chronic caused by insecticides, fumigants, and fungicides. Various types of cancers are also linked with these pesticide exposures. Reproductive effects and developmental abnormalities have also been reported. The health effects linked with pesticides use do not appear to be restricted to only a few categories of these pesticide classes. Therefore, additional efforts are required in order to control or eliminate exposure of human to these pesticides wherever possible. Future research is also needed in order to better characterize the adverse effects of these agrichemicals on human. The use of pesticides increases cancer risk through various mechanisms like tumor promotion, genotoxicity, hormonal actions, epigenetic effects, and immunotoxicity. Most of the pesticides are not mutagenic in genotoxicity assays. Some epidemiological studies related to pesticides exposure indicates that these pesticides might contribute to cancer by possible mechanisms other than DNA damage and mutagenicity (Alavanja et al. 2013). Oxidative stress and receptor mediated signaling are important determinants in pesticide mediated carcinogenesis, further experimental research is needed in order to identify the relationship between pesticide exposure and cancer at molecular level. There is a need for detailed investigation and monitoring of pesticides level in dietary (food and water) and non-dietary sources (air).

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6

Microbiome as Sensitive Markers for Risk Assessment of Pesticides

Upma Singh, Varsha Ashok Walvekar and Shilpi Sharma

Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, New Delhi 110016, India

6.1 Introduction

Ecosystem is sustained by intricately entwined inter-dependencies between organisms and the environment in which the organisms thrive. These dependencies also involve relationships between plant, soil, and microbial communities, all of which are inter-dependent. The soil microbial communities form a diverse network of inter-connected and inter-dependent group of communities from bacteria to fungi, which altogether play a vital role in deciding the fate of soil fertility. Soil microbial communities improve soil richness by forming a major and mobile pool in life-essential functions including nutrient cycling, biochemical processes, and organic material decomposition (Sheeba et al. 2011). Microorganisms' small size and high surface area to volume ratio provide them with a large contact interface to their contiguous environment (Liu et al. 2006; Kirk et al. 2004). Hence, soil microorganisms are very sensitive to environmental amendments, and work as powerful bioindicators for pesticide contamination and other ecosystem disturbances (Xiaoqiang et al. 2008).

In the modern scenario, agricultural practices completely rely on the benefits of fertilizers and pesticides to fulfill the needs of global food demands. Pesticides are xenobiotic compounds, majorly categorized based on their targets, and encompasses herbicides, fungicides, insecticides, defoliants, nematicides, avicides, and rodenticides (Imfeld and Vuilleumier 2012). The most widely used chemical pesticides in the world are insecticides (45%), herbicides (30%), and fungicides (22%) (Yadav et al. 2015) that are further classified based on their active compound. Worldwide, around 3^*10^9 kg of pesticides are applied annually to enhance crop production (Hussain et al. 2009). Research facts also articulate that only 0.1% of applied pesticides target pests, rest 99.9% accumulate and percolate in the environment (Bhardwaj and Sharma 2013). Hydrophobic pesticides are known to have longer persistence as they adhere to soil particles. Hydrophilic pesticides have proved to be equally toxic as their hydrophilic nature enables them to leach and contaminate groundwater. The complex interplay

★Upma Singh and Varsha Ashok Walvekar contributed equally.

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between pesticides and soil microorganisms makes it interesting, and of utmost importance to study the impact of these chemical compounds on soil health.

Apart from the advantages that we appreciate from pesticide amendments, it is crucial to also note the adverse effects manifested on soil microbial community, which forms an integral part in preserving soil fertility for the next cropping season. The interplay between pesticides and soil microbiome is a complicated series of events that influences major biogeochemical cycles driven by microorganisms. There have been many contradictory reports on the effects of pesticide application on soil microbial community, be attributed to the complexity of soil microbial environment (Das and Debnath 2006; López et al. 2006; Jeenie et al. 2011; Ahemad and Khan 2012a,b). While some microbial communities are extremely sensitive toward pesticide application, others play key role in deciding the fate of pesticide metabolism in the soil (Singh and Walker 2006; Yang et al. 2006; Zhang et al. 2006; Wang et al. 2009a,b; Supreeth et al. 2016). Understanding and analyzing the toxicity of pesticides on soil microbiome is an utmost necessity as it aids in decision making for farmers, agriculturists, and environmentalists toward other safer alternatives, without compromising with soil fertility. Such toxicological analysis not only serves to distinguish most harmful pesticides, but also seems to be critical in deciding recommended field dosages for the application. Although some transient perturbations to soil microbiome can be manifested by application of pesticides at recommended level, it is quite evident from several reports that repeated pesticide application can cause loss of fertility and productivity (Bromilow et al. 1996; Kaur et al. 2017). Repeated application of pesticides leads to build-up of toxic metabolites (that cannot be metabolized further) that pose a serious threat to soil fertility, which is reflected over crop yield as plant probiotic soil microorganisms are affected (Stratton and Corke 1982; Osano et al. 2002).

6.2 The Rhizosphere

In general terms, the rhizosphere includes the soil surrounding the plant root system. The rhizosphere is a home to several key and intensive interactions between the soil, plant, and the soil microbiome. The terminology was first coined by a pioneer in rhizosphere microbial ecology, Lorenz Hiltner in 1904. His work yielded two important concepts in soil microbiology, first that the region surrounding the plant root system is rich in diversity of microbial communities, and the soil microbial communities that thrive near the root region are different from those that are present in the bulk soil (without plant); second, root exudates act as substrates upon which the rhizospheric community survives and provides various benefits to the plant itself. This symbiotic relationship between plant and the rhizospheric community depends on several factors that include plant species, age of the plant, and soil type. Age of the plant is a crucial factor in determining what kind of community thrives in the rhizosphere and this phenomenon of community selection is driven by the type of root exudates (Micallef et al. 2009). The process of root exudation is termed as rhizodeposition and is one of the sources for carbon input to the soil (Kuzyakov and Schneckenberger 2006). The process attracts several microorganisms as such exudates are rich sources of carbon and energy. A cocktail of root exudate varies greatly from mycorrhizal and non-mycorrhizal plant species, thus stating that the type of plant species is a major driving factor for attracting colonization of a particular group of soil microorganisms (Schwab et al. 1984). The microorganisms adapt to the root environment and bestow upon the plant with nitrogen and phosphorus sources, protection from plant pathogens, and plant growth promoting hormones (Pohlman and McColl 1982; Arshad and Frankenberger 1991; Kannan and Sureendar 2009). This intricate series of inter-dependencies between the plant and the rhizospheric community confers ecological fitness to the plant (Berendsen et al. 2012). A thorough understanding of various aspects of the rhizospheric community, including an analysis of its diverse nature of functions, is a key to understanding plant and soil health.

Amongst several bacterial and fungal species that colonize a plant rhizosphere, special importance is being given to the *Bacillus* genera (Wang et al. 2009a,b). The bacteria belonging to this genus are not only known for production of indole acetic acid (IAA) and gibberellins that induce plant growth but are also actively involved in the secretion of unique compounds like lipopeptides that act by creating pores in the cell walls and cell membranes of pathogenic fungi (Shafi et al. 2017). The plethora of advantages from this genus is being harnessed by artificially introducing consortium of *Bacillus* sp. (combined with other genera) as bioinoculants for achieving enhanced plant growth. The rhizospheric community is also known to accentuate the plant defence system by a mechanism termed as rhizospheric induced systemic resistance (ISR). Reports have confirmed that the mechanism of ISR is completely independent and distinct of salicylic and jasmonic acid pathways, which are majorly activated upon pathogen's attack (Zhang et al. 2002). Although the exact mechanism by which ISR is elicited in plants by rhizo-microorganisms is unknown, secretion of few volatile organic compounds have been suspected to be involved in rendering resistance against plant pathogens (Naznin et al. 2014). Protection against plant pathogens is also exhibited by depriving pathogenic organisms of the sources of iron, which is achieved by producing several iron chelating compounds known as siderophores (Kloepper et al. 1980).

6.3 Effect of Chemical Pesticides on Soil Microbial Communities

Soil microbiome is an important part of the environment but extensive agrochemical applications like chemical pesticides, affect the total soil microflora as well as individual species as shown in Table 6.1. Primarily, pesticide application decreases the abundance of microbes and their activities, but the microbes have been observed to persist and develop resistance against pesticide and recolonize (DeLorenzo et al. 2001; Moorman 1989; Kalia and Gosal 2017). Jena et al. (1987) demonstrated the influence of butachlor herbicide alone; butachlor + carbaryl on nitrogenase activity, and butachlor + carbofuran on nitrogen fixing bacteria *Azotobacter*, *Azospirillum*, and anaerobic nitrogen fixers in two different tropical paddy soil (loam alluvial soil and acid sulfate saline Pokkali soil). Butachlor + carbofuran ($2\ \mu g\ g^{-1}$) application in non-flooded sandy loam alluvial soil reduced *Azospirillum* and anaerobic N-fixers populations. In comparison, butachlor alone enhanced anaerobic N-fixers' population in non-flooded acid sulfate saline Pokkali soil. However, soil stressed with a combination of pesticides repressed the *Azospirillum* population.

Commercial pesticides are labeled with recommended doses for field applications, however, the criteria for deciding recommended field dosages completely ignore toxic

Table 6.1 Effect of chemical pesticides on soil microbiome

S. No.	Pesticide	Chemical group	Type	Application method	Effects	Method	Reference
1.	Aldicarb Chlorpyrifos Deltamethrin Tebuconazole Metalaxyl+ mancozeb	Carbamate Organophosphate Pyrethroid Triazole Phenylamide + Ethylenebis (dithiocarbamate)	Insecticide Insecticide Insecticide Fungicide Fungicide	Soil mixing and spray	Significant shift observed in culturable soil bacterial communities (CSBC) in first two harvesting points.	PCR-DGGE fingerprint-ing of CSBC	Ferreira et al. (2009)
2.	Pendimethalin Difenzoquat Thiobencarb Folpet Captafol	Dinitroaniline Pyrazoles Thiocarbamate Dicarboximides Sulfanilamide	Herbicide Herbicide Herbicide Fungicide Fungicide	Spray	No significant effects observed with herbicides treatments. Fungicide temporary inhibited some C cycling activities and soil fungi.		Atlas et al. (1978)
3.	Acetochlor Fenvalerate Thiophanate-Methyl	Acetamide Pyrethroid Thiophanates	Herbicide Insecticide fungicides	Soil mixing	Microbial community structure showed variation after pesticide and fertilizer treatments.	BIOLOG GN2 microplates	Chen et al. (2007)
4.	Azoxystrobin	Strobilurins	fungicide	Soil mixing	Soil bacterial diversity was unaffected. Fungal diversity increased by light incubation and decreased by dark incubations.	PCR DGGE analysis of 16S and 18S rRNA genes	Adetutu et al. (2008)
5.	Methamidophos	Organophosphate	Insecticide	Soil mixing	Soil organic C value increased with high level of pesticide treatment. C_{mic}/C_{org}, $C_{org}/_{tol}$, and C_{org}/P_{tol} values decreased. Respiratory activity, N, P increased in pesticide treated soils.	soil microbial biomass analysis, CLPP	Wang et al. (2006)
6.	Napropramide	Amide	Herbicide	Soil mixing	Fungal and bacterial population decreased on day 1, but at end of experiment total biomass, fungi, and gram negative bacteria significantly increased with field rate dosage of pesticide.	PLFA analysis	Cycoń et al. (2013)

7.	Parathionmethyl Mancozeb Carbaryl Atrazin Prometryne 2,4-D Glyphosate Diuron Linuron	Organophosphate Carbamate Carbamate Triazine Triazine Phenoxy acid Organophosphate Urea Urea	Insecticide Fungicide Herbicide Herbicide Herbicide Herbicide Herbicide Herbicide Herbicide	Soil mixing	Phenol oxidase activity used as an early indicator of pesticide contamination; and arylamidase and β-glucosidase used for evaluation of soil resilience after pesticide disturbance. Analysis of functional diversity of bacterial communities using CLPP less efficient for determining pesticide contamination compared to soil enzyme activities.	Biolog Ecoplates	Floch et al. (2011)
8.	2,4-D (2,4-dichloro-phenoxyacetic acid) MCPA (4-chloro-2-methylphenoxy-acetic acid)	phenoxyacetic acid phenoxyacetic acid	Herbicide Herbicide	Soil mixing	Expression of tfdA functional gene (involved in phenoxyacetic acid degradation) increased at time of MCPA and 2,4-D degradation.	mRNA quan-tification by RT-Q-PCR	Bælum et al. (2008)
9.	Penconazol-ecypermethrin	triazoles	Fungicide Insecticide	Pesticide containing sterile water mixed with soil	Increased number of total viable soil microbiota in presence of insecticide or fungicide with plant growth hormone treated soil. Adverse effect on nitrogen fixing bacteria. Morphological deformations found in *Aspergillus flavus*.	Colony forming unit (CFU) count	Al Abboud (2014)
10.	Butachlor fluchloralin Oxadiazono xyfluorfen	Acetanilide Chloroaniline Oxidiazole diphenyl-ether	Herbicide Herbicide Herbicide Herbicide	Spraying	Increased phosphorus availability in soil by means of enhanced microbial activity leading to mineralization	CFU count	Das and Debnath (2006)

effects manifested on non-target soil microorganisms. Perucci and Scarponi (1994) investigated post-application effects of imazethapyr and imidazolinone applications using three application dosages: recommended field level as stated by the manufacturer, 10 and 100 times the recommended rate. In both type of trials with the recommended field rate, the herbicide posed no negative effect on microbiological processes. At 10 and 100-fold higher field application rate, imazethapyr caused reduction in biomass and dehydrogenase activity, in addition to enhancing protease, catalase, and hydrolytic capacity. In a similar study to examine over application of metalaxyl, a pesticide used to control fungal pathogens *Peronosporales* and *Pythiales,* observed inhibition toward bacterial and fungal population until 28 days post application (Wang et al. 2015). Effects of diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea), tebuthiuron, linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea), chlorotoluron (3-(3-chloro-p-tolyl)-1,1-dimethylurea), and bromacil (urea herbicides) were studied by El Fantroussi et al. (1999) on soil microbial communities of 10-year long treated soil. While drastic shifts in bacterial diversity was observed using denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene, community level physiological profiling (CLPP) exposed alterations caused to functional properties of soil microbial population. These data clearly suggest that over application of chemical pesticides is not the only factor responsible for loss in soil fertility, but extensive and repetitive applications also cause imbalances in soil microbiome. Pesticides' exploiting effects were also studied by Yen et al. (2009), who investigated the impact of two fungicides, triadimefon and propiconazole at 10 mg kg^{-1} and 100 mg kg^{-1}on rhizobacterial communities of strawberry plants. Although enhancement in microbial population was seen at 1st time point (10 days post application), higher concentrations of propiconazole proved to be severely toxic toward microbial communities. In addition, disturbances in microbial community composition could be observed until 75 days post-pesticide treatment, supporting the notion that perturbations caused by agrochemical usages could not recovered by then.

Effects of pesticide application on soil microflora fluctuate not only with different soil types but also with different environmental conditions. Adetutu et al. (2008) studied the effect of azoxystrobin on non-target soil bacteria and fungi, and reported that bacterial diversity of soil was unchanged but the fungal soil diversity increased with azoxystrobin treatment under light conditions. Three insecticides (aldicarb, chlorpyrifos, deltamethrin), and two fungicides (tebuconazole and metalaxylþmancozeb) when applied on soil of the region of Seropédica, Rio de Janeiro, Brazil were found to cause significant shifts in microbial bacterial community structure during first two annual harvests compared to control (Ferreira et al. 2009).

Pesticides of hydrophobic nature cause surface sedimentation and eventual accumulation in lakes and estuaries, which lead to their entry into food chains. Megharaj et al. (2000) observed significant decline in bacterial and algal populations in soil with long term Dichlorodiphenyltrichloroethane (DDT) exposure. Additionally, Baoguo et al. (2008) demonstrated the effects of cypermethrin application on phyllospheric microbial community in cucumber plants. Phospholipid fatty acid profiles depicted significant shifts in the ratio of gram-positive to gram-negative bacteria in cypermethrin treated soil compared to untreated.

Chemical pesticides affect individual bacterial species by inhibiting enzymes and genes involved in vital processes. This functional alteration directly affects the survivability of microbes and also changes necessary biochemical processes, which directly relate to maintenance of soil ecosystem. Boldt and Jacobsen (1998) revealed the effects of various concentrations of metsulfuron methyl, chlorsulfuron, and thifensulfuron methyl on 77 genetically different strains of fluorescent *Pseudomonas*. The sulfonylurea herbicides caused growth reduction in fluorescent *Pseudomonas* but the sensitivity fluctuated within strains, which was suspected to be a result of differences in acetolactate synthase (ALS) enzyme, a key participant in the synthesis of valine, leucine, and isoleucine.

Although there are several reports supporting the notion that pesticides alter rhizosphere community (Gupta et al. 2013; Nettles et al. 2016; Newman et al. 2016; Walvekar et al. 2017), thereby affecting physiological aspects of plant growth and development, fewer reports state the damaging effects exhibited by pesticide application at cellular level. Studying the aspect of pesticide toxicity at single cell level rather than considering the whole microbial community is of utmost importance when one wants to discern the exact mechanism by which pesticides alter microorganisms. Manikar et al. (2013) studied the effects of an organophosphorus pesticide, malathion on cyanobacteria. The study reported activation of several antioxidants by cyanobacteria in order to counter the oxidative stress caused during growth in malathion containing medium. Another study that aimed to characterize inhibition of different respiratory enzymes in cyanobacteria upon addition of endosulfan and tebuconazole, suggested significant reduction in succinate dehydrogenase activity, impairing respiratory pathway (Kumar et al. 2012). In addition, a dose-dependent inhibition was also observed for the two pesticides on glutamine synthetase activity. Direct inhibition to photosynthetic machinery was encountered in *Tolypothrix scytonemoides* with monocrotophos and nimbicidin treatments, which resulted in reduced oxygen evolution as a consequence of damage caused to thylakoid membrane (Rajendran et al. 2007). Upon pesticide treatment, the bacterial cells were able to produce more ammonia and secrete carbohydrates as a result of membrane permeabilization. Transmission electron microscopy images depicted changes in cell volume for *Ankistrodesmus gracilis* under chlorpyriphos stressed conditions in addition to impedement in cell division (Asselborn et al. 2015).

6.4 Effect of Pesticides on Plant Growth Parameters as a Result of Impact on Microbiome

Pesticides are included in agricultural practices to kill or control menace caused by target plants and animals. However, these chemical compounds are not specific to their respective targets, and produce several toxic effects on non-target organisms. Pesticides affect plant growth parameters by direct and indirect mechanisms. In the direct inhibition mechanism, pesticides directly affect plant processes like photosynthesis, cell division, and also interfere with the synthesis of enzymes, proteins, and certain pigments (Parween et al. 2014). Pesticides hinder plant growth by indirect mechanisms that involve posing toxicity toward plant growth-promoting rhizobacteria (PGPR), which will be the focus of this section.

Pesticides differ enormously in their mode of toxicology and there are several factors, such as the presence of functional groups, biodegradability, and dosage at which the pesticides are applied, that influence their toxic nature (Divito et al. 2007). The toxicity of most pesticides is dose-dependent with several reports showing that higher concentrations are potentially more harmful to the plants than the lower doses (Tiyagi et al. 2004; Baćmaga et al. 2015; Borowik et al. 2017; Juan et al. 2017). While pesticides serve as poisonous chemicals against the colonization of the indigenous rhizosphere community, at non-toxic levels the same serve as carbon and energy sources for the growth of soil microorganisms that ultimately reflects upon plant growth.

Under pesticide stress conditions, a significant reduction in plant growth was observed for chickpea plants as a result of direct inhibition toward PGPR (Ahmed et al. 2012). The study which evaluated different types of agrochemicals: herbicides, insecticides, and fungicides on plant growth promoting (PGP) activities in the rhizosphere of chickpea plants, demonstrated prominent negative effects of chemical amendments on key substances like growth hormones and secretion of siderophores. However, toxicological assessment of pesticides carried out in liquid medium revealed no inhibitory effect of pesticides on PGPR consortium, instead the degradation of pesticides significantly enhanced the growth of PGPR (Myresiotis et al. 2012). Chauhan et al. (1999) observed toxicity of cypermethrin against meristematic growth in root cells of *Allium cepa* which attributed to a drastic reduction in root length. Decrease in root length of tomato plants caused by oxidative stress was also reported upon application of cypermethrin by Chahid et al. (2013). Additionally, a significant reduction in shoot and root length of snake cucumber plants was also manifested upon treatment with pesticides from the carbamate group (Gafar et al. 2013). Decrease in plant growth parameters upon pesticide amendment is attributed to the toxicity of pesticides toward stomatal opening and inhibition toward photosynthetic machinery (Kilic et al. 2015). Fungal and bacterial strains from the rhizosphere of pesticide stressed plants have been isolated and characterized for pesticide degradation potential (Wang et al. 2011; Dubey and Fulekar 2013; Kryuchkovaa et al. 2014). In addition, few bacterial and fungal strains are also known to possess metabolic pathways for complete degradation of pesticides such as chlorpyriphos and its toxic metabolite 3,5,6-trichloro-2-pyridinol (TCP) (Chen et al. 2012; Jabeen et al. 2015; Abraham and Silambarasan 2016). TCP is the most toxic product obtained from chlorpyriphos degradation, exhibiting potent antimicrobial activity and longer persistence in the environment.

6.5 Impact of Safer Alternatives, Biological Pesticides

Of the total chemical pesticides in application, only a small proportion target their pests, while the rest accumulate by adhering to soil organic matter. Vaporization, solubilization, mineralization, and degradation are various ways by which pesticides enter into the environment. After entering into the food web, these pesticides are biomagnified in lipid bodies, cells, and tissues of higher organisms (Hafez and Thiemann 2003). Knowledge regarding the negative impacts of chemical pesticides on soil microbiota has led to a shift toward safer alternatives with the introduction of biopesticides.

Biopesticides are naturally occurring substances that are believed to control pests without damaging the non-target organisms. Biopesticides includes living organisms such as bacteria, fungi, virus, or their products (microbial products, phytochemicals) or byproducts (semi-chemical substances), and living organisms termed as biological control agents (BCA) (Ombudsman, Biopesticides and Pollution Prevention Division

2012; Panda 2012). The biological pesticides came into the picture because of their eco-friendly nature by being less destructive to the environment as well as to human health, and have also been confirmed as powerful plant disease management tools. According to U.S. Environmental Protection Agency (EPA) definition, biopesticides are designed to enhance crop production with repeated application. In the modern agricultural era, biopesticides are much more popular because of their natural origin in comparison to the chemical pesticides.

Many studies have mentioned the effects of biopesticide application on soil microbiome and some of these studies have been summarized in Table 6.2. Johansen et al. (2005) studied the effects of *Pseudomonas fluorescens* DR54 and *Clonostachys rosea* IK726 (bacterial and fungal BCA) on microflora of bulk soil, and rhizospheres of barley and sugar beet in a greenhouse experiment. They demonstrated that after 193 days, DR54 and IK726 had declined by 10^6 and 20 factor, respectively. DR54 temporary increased the sugar beet rhizosphere and displaced the indigenous pseudomonads, while IK726 had general stimulating effects on soil microbial enzyme activity and soil microflora. Phospholipid fatty acid (PLFA) analysis detected perturbations in rhizospheric community structure in BCA stressed treatment, and at the end of the experiment (193 days), it showed a decline in rhizospheric microbial population. BCA are effective in controlling plant diseases but may also pose a threat to non-target organisms including saprophytic fungi, mycorrhizal fungi, soil bacteria, etc. (Brimner and Boland 2003). On the contrary Winding et al. (2004) studied the effects of application of *Pseudomonas* (F113, CHA0, DR54, SBW25, Q2-87, and WCS358r) and *Bacillus cereus* BCA in different test systems, and observed no major fluctuations in the microbial community structure.

Fungal BCA, that include species of *Trichoderma, Gliocladium, Talaromyces flavus, Ampelomyces quisqualis, Pythium oligandrum,* and *Coniothyrium minitans*, control pathogens by different mechanisms that include mycoparasitism, competition for nutrients, production of antibiotics, enzymes, and also through the induction of the plant host defence system (Brimner and Boland 2003). *Trichoderma* spp. is used in fields to control numerous soilborne diseases caused by *Pythium ultimum, Fusarium oxysporum,* and *Sclerotinia sclerotiorum* and also positively influence growth and propagation of mycorrhizal fungi. Vázquez et al. (2000) reported the stimulatory effect of *Trichoderma harzianum* on corn root colonization by *Gliocladium mosseae,* an arbuscular mycorrhizal (AM) species. On the contrary, Gerlagh et al. (1999) reported the unwanted effects of pathogen controlling strategy, mycoparasitism (one fungal species directly attacks and feeds on another), of *Trichoderma* sp. to resist *S. sclerotiorum* infection, and observed an increase in the number of pathogenic reproductive structures compared to untreated plants. Thus, in some cases applications of BCA might result in enhancement of disease and spread of the pathogen. Some BCA have also been reported to hinder the symbiotic associations between *Rhizobium* and legumes. When Anusuya and Sullia (1985) noted the effects of *T. viride* on the association of *Rhizobium* spp. with peanut plants, a significant reduction in weight and number of root nodules was examined. *T. viride*'s fast growing and plant surface colonizing abilities prevented successive invasion of roots by the bacteria.

Bacillus thuringiensis (Bt) is another bacterial species commonly used as biological pesticide. It produces host specific protein called Bt d-endotoxin, harmful for host insects. Many research studies have reported its non-toxic nature toward vertebrates

Table 6.2 Effect of biological pesticides on soil microbiome

S.No.	Biological Pesticide	Type	Type	Effects	Methods	Reference
1	Azadirachtin Pyrethrum Spinosad Terpene	Biochemical produced from *Azadirachtaindica* Biochemical produced from *Chrysanthemum cinerariaefolium* Bacterial derived component Plant derived components	Insecticide Insecticide Insecticide Insecticide and nematicides	Azadirachtin application led to reduction in shoot and root biomass. Significant and persistent shifts in AM fungal community (only in the case of azadirachtin).	Plant growth parameters. Arbuscular mycorrhizal (AM) fungi community analysis.	Ipsilantis et al. (2012)
2.	Folicon *Paeciliomyces lilacinus* *Bacillus subtilis* *Pseudomonas fluorescens* *Beauveria bassiana*	Neem based biochemical Fungal BCA Bacterial BCA Bacterial BCA Fungal BCA	Insecticide Nematicides Fungicide Fungicide Insecticide	Soil microbial biomass C content increased with time in biopesticide treatment. Maximum increase found with *Paeciliomyces lilacinus*	Chloroform fumigation incubation method for C content analysis.	Sethi and Gupta (2013)
3.	*Glomus mosseae* *Glomus deserticola* *Azospirillum brasilense* Sp245 *P. fluorescens* WCS 365 *Trichoderma harzianum* T12	Arbuscular mycorrhizal fungi (AMF) AMF Bacterial BCA Bacterial BCA Fungal BCA	Fungicide Fungicide Fungicide Fungicide Fungicide	Mycorrhizal colonization prompted qualitative changes in bacterial population. Both mycorrhizal colonization and microbial inoculation modified the microbial community structure and ecology.	CFU count, Enzyme activities assays(esterase, phosphatase, trehalase, and chitinase)	Vázquez et al. (2000)

4.	*Pseudomonas fluorescens* DR54 *Clonostachys rosea* IK726	Bacterial BCA Fungal BCA	Fungicide Fungicide	*P. fluorescens* DR54 displaced indigenous pseudomonads. PLFA profile showed perturbations of the soil microbial population and structure.	PLFA analysis	Johansen et al. (2005)
5.	Bt corn	Transgenic crop with cry endotoxin of *Bacillus thuringiensis*	Insecticide	Effect of Cry protein on soil microbial community was limited in lab experiment. No difference in PLFA biomarker of fungal, AM fungal.	PLFA analysis and CLPP	Blackwood and Buyer (2004)
6.	*Bacillus thuringiensis* and its crystal protein	Bacterial BCA	Insecticide	Heterotrophic bacteria populations not affected by *B. thuringiensis*. Increased number of nodules in treatment with Cry+ strain and insecticidal crystal protein. Inhibited mycorrhizal colonization in treatment of Cry+ and Cry− strains.	CFU count	Ferreira et al. (2003)
7.	*Bacillus subtilis* HJ5, and DF14	Bacterial BCA	Fungicide	Strengthened enzyme activities and antagonistic bacterial abundance and suppressed pathogens.	CFU counts Soil enzymatic assays qPCR analysis of some functional genes (related to *Trichoderma, Bacillus, Pseudomonas, Fusarium,* and *Verticillium dahliae*)	Li et al. (2017)

(Continued)

Table 6.2 (Continued)

S.No.	Biological Pesticide	Type	Type	Effects	Methods	Reference
8.	*Paecilomyces lilacinus* strain 251	Fungal BCA	Nematicides	Stimulated effect on the growth of fungi and copiotrophic gram negative bacteria. Terminal restriction fragment length polymorphism (TRFLP) and qPCR analysis with *amoA* gene indicated time-dependent inhibitory effect on Ammonia-oxidizing bacteria/ Ammonia-oxidizing archaea AOB/AOA abundance upto 20 d post application.	Terminal restriction fragments length polymorphism (T-RFLP) and qPCR analysis of amoA gene. PLFA analysis	Rousidou et al. (2013)
9.	*Trichoderma harzianum* (CCTCC-RW0024)	Fungal BCA	Fungicide	Strain showed high antagonistic activity, disease reduction against *Fusarium graminearium* and biocontrol enzymes and gene expression. Exogenous inoculation in maize rhizosphere increased acidobacteria.	eGFP tagging qRT-PCR Pyrosequencing	Saravanakumar et al. (2017)

and the environment. Ferreira et al. (2003) demonstrated the effect of *B. thuringiensis* and Bt endotoxin on soybean var. Br 322. Br322 was grown in non-sterile soil with three *B. thuringiensis* inocula; a mutant non-producer (Cry−), insecticidal crystal protein producer (Cry+), or insecticidal crystal protein (ICP) and measurement of the effects on rhizospheric soil samples were evaluated at five time points. From the study it was observed that heterotrophic bacterial populations inoculated with *Bt* were not affected, while some differences were detected in functional groups of C-cycling microbes. Cry+ and ICP increased the nodule formation and plant growth in comparison to control. Deleterious effects with Cry+ and Cry− strains were also observed on AM fungi colonization compared to uninoculated control plants. Blackwood and Buyer (2004) demonstrated the effect of Bt corn's Cry endotoxin on soil microflora by comparing two transgenic Bt corn line expressing different Cry endotoxin and non-transgenic isolines in three soil types. There was no significant effect on the soil microbial diversity because of the expression of Cry endotoxin and corn lines, except for high clay soil where both dramatically affected bacterial CLPP profiles. Thus, they concluded that the effects on the soil microbial community because of Bt corn was small and temporary.

Azadirachtin, spinosad, and terpenes are the most used botanical pesticides, with a non-toxic mode of action. They act by disturbing the growth, development, reproduction, and ecology of the pests. Azadirachtin is extracted from *Azadirachta indica* seed kernels. It is reported to exert several positive and negative impacts on non-target soil microbial community (Singh et al. 2015a,b). Kızılkaya et al. (2012) studied the effects of azadirachtin on dehydrogenase (DHA) and catalase activity (CA) in loamy field soil of Perm, Russia. They added azadirachtin in concentrations of 0, 15, 30 and 60 ml da^{-1} and analyzed DHA and CA at three time points post application. Results showed a positive influence of azadirachtin on DHA and CA at different sampling times, and the effect was dose dependent. On the contrary, Singh et al. (2015b) carried out a comparative study of two chemical pesticides (cypermethrin and chlorpyrifos), and one biological pesticide (azadirachtin) on *Vigna radiata* rhizospheric microbial community by cultivation-dependent and -independent techniques. These pesticides were applied to the soil at two dosages (recommended and 5x of recommended dose). Employing a cultivation-dependent approach, they unraveled the adverse effects of both chemical and biological pesticides on rhizospheric bacteria, and fungal diversity at different plant growth stages. Cultivation-independent technique showed an adverse effect on nitrogen cycle genes and transcripts, stating that both biological and chemical pesticide had an equally negative impact on rhizospheric microbial population. Ipsilantis et al. (2012) demonstrated the outcomes of biological pesticides (azadirachtin, spinosad, pyrethrum, and terpenes) and synthetic fungicide carbendazim on mycorrhizal fungi in pot and field experiments. DGGE analysis showed that biopesticides spinosad, pyrethrum, and terpenes did not exert any effect on the AM fungal community structure. On the other hand, azadirachin in pot experiment selectively inhibited *Glomus etunicatum* strain and showed significant enhancement and persistent change in AM fungal colonization at field conditions. Synthetic pesticide carbendazim increased AM colonization in pot application while at field level the same pesticide caused transitory effect on community structure.

Biological pesticides can activate the plant defense response or can interfere with the functioning of pathogen infection ability. Thus, timing and frequency of application should be a critical dimension. Biopesticides should be applied before infection or immediately after appearances of first symptoms of a disease. They work as a protective fence on plant surface that restricts invasion by a pathogen. But in foliar conditions, biopesticides are not so effective because of their faster degradation, and because they are washed out by rain or irrigation of foliage and fruits, hence the need for repeated applications (McGrath et al. 2010).

In the United States, the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) requires that EPA always evaluate the proposed pesticide before launching into the market, and ensures that the pesticide is devoid of potential risks to the environment and to human health. FIFRA has also defined some biopesticides under minimal-risk pesticide because of their recognized active and inert elements. The registration process for biological pesticides is quicker than the conventional pesticides and also encourages the use of biopesticides (Pesticide Registration Manual 2017).

6.6 Conclusion and Future Perspectives

The agricultural practices today only focus on the benefits related to crop production and economic strength. To achieve this goal, pesticides of both chemical and biological nature are used indiscriminately. Pertaining to a natural origin, biological pesticides have been considered relatively safe for use in agriculture. However, recent reports contradict this widely accepted belief. The most elementary and important part of agricultural ecosystem is the soil microbiome. Unselective, repeated long-term and over usage of pesticides alter soil microbial diversity, which indirectly alters soil aggregation, richness, and fertility. To minimize such impacts on soil health, we need to critically perform risk assessment of any agricultural amendment before its widespread application, including its impact on resident microbiome. For this, newer protocols with precise application need to be developed, together with social awareness about pesticide use and the effects on the environment and human health.

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7

Arms Race between Insecticide and Insecticide Resistance and Evolution of Insect Management Strategies

Pritam Chattopadhyay[1] *and Goutam Banerjee*[2]

[1] *Department of Botany, M.U.C. Women's College, University of Bardwan, Bardhaman, West Bengal, 713104, India*
[2] *Department of Biochemistry, University of Calcutta, Kolkata, West Bengal, 700019, India*

7.1 Introduction

Pesticide resistance in agriculture and health sector is one of the major Global problems. Due to the natural genetic difference within a pest insect population, some members of the population may survive against a wide range of insecticidal agents. The surviving members of that insect population are called resistant to the insecticide. According to the Insecticide Resistance Action Committee (IRAC) insecticide resistance can be defined as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species" (IRAC 2010).

The first paper concerned about insecticide resistance was published in 1914, more than 100 years ago (Melander 1914). In agriculture and forestry the most challenging problem of insecticide resistance ever faced was from the members of Lepidoptera (*Helicoverpa armigera*, *Spodoptera exempta*, *Cydia pomonella*, etc.) along with Coleoptera (*Leptinotarsa decemlineata*, *Popillia japonica*, *Tribolium confusum*, etc.), and Hymenoptera (*Megastigmus spermotrophus*, *Megachile frontalis*, *Xylocopa aruana*, etc.) (Chattopadhyay et al. 2017). In the year 1949, Cagle reported the spider mite (*Tetranychus urticae*) as the first registrant pest in the horticulture field (Cagle 1949). Thereafter, leafminers, aphids, whiteflies, and thrips also have been recorded to develop resistance against a wide spectrum of insecticides (Chattopadhyay et al. 2017). Whereas, in public health sectors Global challenges of insecticide resistance is mainly from the members of Diptera (*Aedes* spp., *Anopheles* spp., *Culex* spp., etc.). Several species of mosquito such as *Aedes aegypti* and *Aedes albopictus* are considered as carrying vectors for different life-threatening virus like chikungunya virus, dengue virus, Rift Valley fever virus, yellow fever virus, and Zika virus (Moyes et al. 2017). *Anopheles* is a genus of mosquitoes having about 460 recognized species, and among these species, *Anopheles gambiae* is well known for acting as a vector of deadliest human malaria parasite – *Plasmodium falciparum* (Gnankiné and Bassolé 2017). Some *Anopheles* spp. also can serve as the vectors for filarial causative agent (*Wuchereria bancrofti* and *Brugia malayi*), canine heartworm (*Dirofilaria immitis*), and viruses

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(e.g. Onyongnyong fever virus). Similarly, Culex is another mosquito genus and several species of which serve as vectors of different diseases in humans, birds, and other animals (Benelli et al. 2017). Therefore, insecticide resistance is an important factor that converted mosquitoes into the worldwide burden of infectious disease. Bed bugs (*Cimex lectularius*) are parasitic insects (they belong to the cimicid family) prefer to feed on human blood, and cause different types of skin infections and rashes (Doggett and Russell 2009). Bed bugs are another example of insect pest which have developed a resistant mechanism against a wide range of insecticides. In the present chapter, we have summarized the insecticide resistance mechanism in all three sectors, namely agriculture, horticulture, and public health. Insecticides damage or prevent specific important biological processes in an insect pest. Mechanisms of resistance to insecticides in insect pests can include alterations in insecticides, alteration of insecticide-receptor interactions, alterations in insecticide detoxification metabolism, and alterations in insecticide solubilization. However, mutation in the target site and metabolic resistance are considered as major resistance mechanisms in most of the cases (Moyes et al. 2017). Here we have categorically discussed the basic resistance development mechanism in all the insecticide classes presently in use in agriculture, horticulture, and public health sectors.

Integrated pest management (IPM) has addressed a broad range of strategies such as biological control, cultural control, autocidal techniques, crop rotation, chemical control, semiochemicals, host plant resistance, and genetically modified (GMO) plants to combat the resistance issues (National Research Council 2000). Except for host plant resistance and GMO plants, all of the above approaches reduce the pest pressure below the economic damage thresholds on a crop. Effective insecticide resistance management (IRM) as the Specialist Technical Group within Crop Life International focused on the development of the Mode of Action (MoA) classification scheme to combat the resistance problem in insects (Sparks and Nauen 2015). In light of the importance of insecticide resistance, approaches to studying IRM have been widely discussed (e.g. Onstad et al. 2014; Sparks and Nauen 2015). In the present chapter, we have provided a guideline to combat the insecticide resistance problem in real time. Regarding the choice of terminology, we have followed the suggestions of Chattopadhyay et al. (2017).

7.2 Different Types of Insecticide

According to origin and mode of synthesis insecticides may be broadly classified into chemical insecticides and biological insecticides (Table 7.1). Chemical insecticides are broadly classified into seven classes: (i) neonicotinoid (e.g. acetamiprid, clothianidin, imidacloprid, nitenpyram, nithiazine, thiacloprid, and thiamethoxam), (ii) organophosphates (e.g. azamethiphos, azinphos-methyl, chlorpyrifos, diazinon, dichlorvos, fenitrothion, malathion, methyl parathion, parathion, phosmet, and tetrachlorvinphos), (iii) carbamates (e.g. aldicarb, carbofuran, carbaryl, ethienocarb, fenobucarb, oxamyl, and methomyl), (iv) organochlorine (e.g. aldrin, chlordane, chlordecone, Dichloro dipheynl trichloroethane (DDT), dieldrin, endosulfan, endrin, heptachlor, hexachlorobenzene, lindane, methoxychlor, mirex, pentachlorophenol, *T*etrachloro*d*iphenyl*e*thane (TDE)), (v) pyrethroids (e.g. allethrin, bifenthrin,

Table 7.1 Different insecticide type, their examples, mode of action and resistance mechanism

Sr. no.	Insecticide type		Example	Mode of action	Resistance mechanism	References
1		Neonicotinoid	acetamiprid, clothianidin, imidacloprid, nitenpyram, nithiazine, thiacloprid, thiamethoxam	nAChR agonists	Reduced penetration Mutation of nAChR Over-expression of cytochrome P450	Bass et al. (2014) Bass et al. (2014) Bass et al. (2014)
2	**Chemical**	Organophosphates	azamethiphos, azinphos-methyl, chlorpyrifos, dichlorvos, fenitrothion, malathion, parathion, phosmet	Disrupt AChE and other cholinesterases activity	Mutation of AChE gene Carboxylesterases overproduction	Bass et al. (2014)
3		Carbamates	aldicarb, carbofuran, carbaryl, ethienocarb, fenobucarb, oxamyl, methomyl	Disrupt AChE and other cholinesterases activity	Carboxylesterases overproduction Mutation of AChE gene	Bass et al. (2014)
4		Organochlorine	aldrin, chlordane, chlordecone, DDT, endosulfan, endrin, hexachlorobenzene, lindane, methoxychlor, TDE	Targeting the GABA receptor Open sodium channels in the insect's nerve cells	*Rdl* mutation Mutations in VGSC (also known as *kdr*) Epsilon GSTs through direct dechlorination	Bass et al. (2014) Bass et al. (2014) Bass et al. (2014)

(Continued)

Table 7.1 (Continued)

Sr. no.	Insecticide type		Example	Mode of action	Resistance mechanism	References
5		Pyrethroids	allethrin, cyfluthrin, cypermethrin, flumethrin, imiprothrin, λ cyhalothrin, prallethrin, τ fluvalinate	Nonpersistent sodium channel modulators	Mutations in VGSC (also known as *kdr*)	Bass et al. (2014)
					Overexpression of cytochrome P450	Bass et al. (2014)
6		Ryanoids	chlorantraniliprole, cyantraniliprole, flubendiamide	Bind to calcium channels in cardiac and skeletal muscle blocking nerve transmission	Amino acid polymorphisms in the ryanodine receptor	Troczka et al. (2017)
7	Biological	IGR analogs	Diflubenzuron	Inhibit chitin (exoskeleton) biosynthesis	Virtually no reports of resistance have been found	El-Sheikh et al. (2016)
			Hydroprene, kinoprene, methoprene, ecdysone	Agonist (mimic) tebufenozide		
8		Viral	nuclear polyhedrosis virus, granulovirus	Infect secretory cells of midgut and cells of other tissues	Virtually no reports of resistance have been found	Cory (2000)
9		Bacterial	*Bacillus popilliae*, *B. sphaericus*, *B. thuringiensis*	*Cry*, *Cyt*, and *VIP* toxins	Reduced binding to midgut proteins like cadherin and aminopeptidase N	Pardo-Lopez et al. (2013)
					Mutations in ATP-binding cassette of ABC transporter	Atsumi et al. (2012)
					Missplicing of the ABCC2 gene	Xiao et al. (2014)

		Clostridium bifermentans, Pseudomonas alcaligenes, P. aureofaciens,	Different toxins	Virtually no reports of resistance have been found	Bensidhoum et al. (2016)
		Saccharopolyspora spinosa, Streptomyces avermitilis,	Bind with postsynaptic nicotinic acetylcholine and GABA receptors	Virtually no reports of resistance have been found	Samri et al. (2017)
		Serratia entomophila	Antifeeding activity and physical pressure	Virtually no reports of resistance have been found	Chattopadhyay et al. (2017)
10	Fungal	*Beauveria bassiana*, *Lecanicillium lecanii*, *Metarhizium anisopliae*	Competitive exclusion, mycoparasitism and production of toxic metabolites	Virtually no reports of resistance have been found	Roberts and Leger (2004)
11	Protozoa	Microsporidia, such as *Nosema locustae*	Debilitating effect on reproduction and overall fitness	Virtually no reports of resistance have been found	Cai et al. (2012)

(Continued)

Table 7.1 (Continued)

Sr. no.	Insecticide type		Example	Mode of action	Resistance mechanism	References
12		Botanical	anabasine, azadirachtin, cinnamaldehyde, cinnamyl acetate, citral, spinosyn A, spinosyn D, thymol	Repel insects Antifeedants	Virtually no reports of resistance have been found	Nisha et al. (2012), Senthil-Nathan (2013)
13		Nematode	*Steinernema feltiae*	Their symbiotic the bacteria cause septicemia	Virtually no reports of resistance have been found	Loya and Hower (2002)
14		Pheromones	Mating-disruption pheromone	Incorporated into the insects' olfactory systems and react behaviorally	Virtually no reports of resistance have been found	Baker and Heath (2004)
15		Plant-incorporated protectants (PIPs)	Bt cotton, Bt corn	Cry toxins	Same as *B. thuringiensis*	Ferré and Van Rie (2002)
			RNAi	Disrupt hormonal control, chi	Virtually no reports of resistance have been found	Mamta and Rajam (2017)

cypermethrin, esfenvalerate, flucythrinate, prallethrin, resmethrin, silafluofen, tefluthrin, transfluthrin), (vi) ryanoids (e.g. chlorantraniliprole, cyantraniliprole, and flubendiamide), and (vii) insect growth regulator (IGR) analogs (e.g. diflubenzuron, flufenoxuron, cyromazine, methoprene, hydroprene, tebufenozide) (Table 7.1). Many of these chemical pesticides are broad-spectrum in nature and kill the agro-economically beneficial species community and thereby disrupt the ecological balance. As for example, many neonicotinoids and organochlorine are reported to be linked with the honeybee colony collapse disorder (CCD) and loss in bird populations (Gill et al. 2012; Jensen 2015). Thus, in the year 2013, the European Union and other countries have banned the use of such neonicotinoids in the agricultural fields (Alemanno 2013). Some of the chemical pesticides are toxic to human beings also. Toxic effects of organophosphates on humans were discovered in 1932 (Lange and Krueger 1932). Irrespective of environmental and health-related issues, insect resistance has been recorded against all the chemical insecticide classes available in the market (Sparks and Nauen 2015).

Apart from chemical insecticides, biological insecticides may be classified broadly into eight categories: (i) viral (e.g. nuclear polyhedrosis virus, granulovirus etc.), (ii) bacterial (e.g. *Bacillus popilliae*, *Bacillus sphaericus*, *Bacillus thuringiensis*, *Clostridium bifermentans*, *Pseudomonas alcaligenes, Pseudomonas aureofaciens, Saccharopolyspora spinosa, Streptomyces avermitilis, Serratia entomophila*, (iii) fungal (e.g. *Beauveria bassiana*, *Lecanicillium lecanii*, *Metarhizium anisopliae* etc.), (iv) protozoan (e.g. *Nosema locustae* etc.), (v) botanical (e.g. anabasine, anethole, annonin, asimina, azadirachtin, caffeine, carapa, cinnamaldehyde, cinnamon, cinnamyl acetate, citral, deguelin, derris or rotenone, eugenol, linalool, myristicin, nicotine, polyketide, pyrethrum, ryanodine, spinosad, spinosyn A, spinosyn D, tetranortriterpenoid, thymol etc.), (vi) Nematode (e.g. *Steinernema feltiae*), (vii) pheromones (e.g. mating-disruption pheromone), and (viii) plant incorporated protectant or PIP (e.g. GMO plant with cry toxin, RNA interference or RNAi) (Table 7.1). Pheromones are communicating chemicals produced by insects and are considered to be highly species specific. Copping and Menn (2000) have reported 30 products capable of disruption to pheromone based mating phenomenon in lepidopteran pests, and these products have been registered by the US EPA as biocontrol agents. The controlling of the insect pest by using pheromone lures is a useful strategy and is economically profitable (Leskey et al. 2012; Peng et al. 2012). Insect control by pheromones can be applied in integrated crop management or ICM (Reddy and Guerrero 2004). Toxins produced by different bacterial strains of *Bt* are widely applied for insect control as PIP in transgenic plants; however, their efficiency reduced when pests evolve resistance (Banerjee et al. 2017; Yang et al. 2017; Chattopadhyay and Banerjee 2018). In RNAi technology, Double-stranded RNA may be utilized as a pesticide that is expressed as a PIP (though it may have multiple application methods including foliar spray, post-directed spray or drench, seed treatment, or granule/powder). RNAi-mediated silencing of different insect genes involved in various physiological processes was found to be detrimental to insect growth, development, and survival (Mamta and Rajam 2017). The sequence-specific gene silencing via RNAi holds a great promise for effective management of agricultural pests.

7.3 Different Types of Insecticide Resistance

In most of the recorded cases (>50%) insecticide resistance is being found between two-five different classes of insecticides (Couso-Ferrer et al. 2011). It is quite alarming as multi-resistance or cross-resistance is very difficult to deal with. Interestingly, in most of the cases, insecticide resistance is unstable in the field. Based on the nature of the insecticide resistance, it can be categorically divided into following subheadings.

7.3.1 Cross Insecticide Resistance

Sometimes changes in a single major genetic factor of the insect pest confer resistance to more than one pesticide (Couso-Ferrer et al. 2011). This phenomenon is known as cross- resistance. Malathion (organophosphate insecticide) is a good example of cross-resistance, which confers moderate levels of cross-resistance (3–16-fold) compared to other organophosphates (such as phosmet and methyl-chlorpyrifos), the carbamate carbaryl, the pyrethroid lambda-cyhalothrin, and the benzoylphenylurea derivative lufenuron. However, spinosad exhibited cross-resistance below twofold in field populations of the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae) (Couso-Ferrer et al. 2011). Among Bt toxins, Cry2Ab2-corn selected *Spodoptera frugiperda* was found to be strong cross-resistant to Cry2Ae (Yang et al. 2017).

7.3.2 Multiple Insecticide Resistance

Changes in more than one genetic factor of the insect pest confer resistance to more than one pesticides, it is known as multiple insecticide resistance (Menze et al. 2016). Differentiation between cross-resistance and multiple-resistance is a difficult job and only possible to check in a laboratory condition by performing genetic studies. An example of multiple-insecticide resistance includes spider mites to cyhexatin and dicofol (Dennehy et al. 1988), and malarial vector *Anopheles funestus* to organochlorines and pyrethroids (Djouaka et al. 2016). Factors responsible for multiple-insecticide resistance in *A. funestus* from Northern Cameroon were identified as metabolic resistance due to mutations in target sites (Menze et al. 2016).

7.3.3 Stable Insecticide Resistance

A stable insecticide resistance means increases in resistance frequency with the continuous application of a pesticide that does not decline appreciably even after withdrawal of that pesticide. However, insecticides that frequently confer the chance of developing stable resistance were not favored in pest management programme. A classic example of stable resistance is the Western flower thrips (*Frankliniella occidentalis*). In this direction, Contreras et al. (2008) have reported the selective insecticides (acrinathrin, formetanate, and methiocarb) resistance of *F. occidentalis* in laboratory condition over a significant number of generations and concluded it as a stable resistance.

7.3.4 Unstable Insecticide Resistance

An unstable insecticide resistance means increase in resistance frequency with the continuous application of a pesticide that does decline appreciably after withdrawal of that pesticide. Insecticides that lead toward unstable resistance due to higher reversion rate are favored in pest management. For example, resistance in the leafminer *Liriomyza trifolii* (Diptera: Agromyzidae) collected from commercial ornamental production greenhouses in the United States to three insecticides (viz. abamectin, cyromazine, and spinosad) was reported to be unstable as the resistant population turns to be susceptible within five to six generations (Ferguson 2004). In another report, unstable resistance of *Spodoptera litura* (Lepidoptera: Noctuidae) in the absence of the selection pressure, i.e. insecticide spinosad. Spinosad might be included in the control program of *S. litura*, due to its lower stability and higher reversion rate (Rehan et al. 2011).

7.4 Reasons for Insecticide Resistance

Insecticide resistance has led to compromise the enormous effort put into the control of insect pest populations (Barbosa et al. 2011). Selection pressure plays a critical role in developing resistance in insect pests (Figure 7.1). Hidayati et al. (2011) have proved that 45 consecutive generations of *A. aegypti* under the selective pressure of malathion exhibited high, moderate, and low resistance to DDT, propoxur, and cyfluthrin, respectively. The working efficacy of an insecticide directly depends on the resistance intensity of the insect pest in field conditions (Ffrench-Constant 2013). Selection pressure may be quantified from the frequency of resistance alleles before and after administration of an insecticide (Barbosa et al. 2011). As for example, the organophosphorus resistance ability in *Culex pipiens quinquefasciatus* (Diptera, Culicidae) from Martinique has been identified as a selection pressure resulting in two definite changes in allele frequency: first, a decrease of susceptible genotype at two loci (Ester and ace-1), and second, allele replacement at the Ester locus (Yebakima et al. 2004).

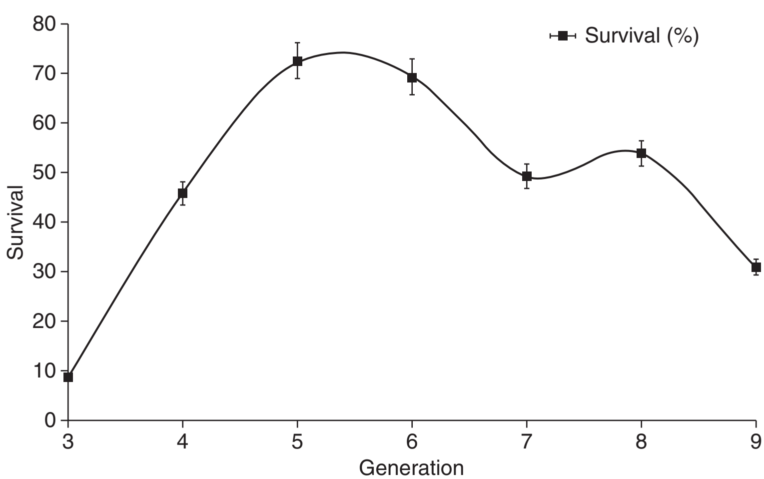

Figure 7.1 Generalized insecticide resistance process in the laboratory. Survival dynamics of a resistant insect pest population submitted to controlled selection in the laboratory. Survival rate from F3 to F9 under continuous selection pressure of insecticide would typically give a sigmoid curve like this.

7.5 Mechanisms of Insecticide Resistance

There are different mechanisms of insecticide resistance. A single resistance mechanism can convey cross-resistance and/or multiple-resistance to other insecticides. Different mechanisms employed by the insects to combat with insecticide are listed below.

7.5.1 Alterations in Insecticide Detoxification Capacity

Insect pests having enhanced metabolic detoxification mechanism are able to cope with insecticides (Table 7.1). Therefore, a much lower dose of the active pesticide sprayed on the field reaches the target site in the pest. For example, overproduction of carboxyl esterases that breaks carboxyl ester bond of pesticide confers resistance to organophosphate and carbamate insecticides in peach potato aphid, *Myzus persicae* (Bass et al. 2014).

7.5.2 Alteration of Toxin-Receptor Interactions

With the second common mechanism, the proteins targeted by insecticides play a crucial role in the functioning of the biologically important event of an insect pest (Table 7.1).

Case I. Mutation in acetylcholinesterase (AChE) enzyme confers resistance to organophosphates, carbamates, and neonicotinoid: Different classes of insecticides that share a common target like AChE can be tackled through mutation. For example, insect pests like *M. persicae* and *Aedes* undergo mutation in AChE, which confers resistance against organophosphates and carbamates (Moyes et al. 2017). On the other hand, mutation of the nicotinic acetylcholine receptor (nAChR) confers resistance against neonicotinoid insecticides (Bass et al. 2014).

Case II. Mutations in the voltage-gated sodium channel (VGSC) confers resistance to organochlorine and pyrethroids: Different classes of insecticide that share a common target like VGSC can be managed through mutation. For example, knockdown resistance (*kdr*) mutations in *A. aegypti* help to develop resistance against pyrethroids and DDT (Kushwah et al. 2015).

Case III. Mutations in the gamma aminobutyric acid (GABA) receptor enhance resistance to organochlorines. Duplication and mutation of the GABA receptor subunit gene was related with resistance to cyclodiene insecticides in *M. persicae* (Bass et al. 2014).

Case IV. Mutations in the ryanodine receptor may also confer resistance to ryanoids: For example, rapid development for resistance to diamide insecticides (such as flubendiamide and chlorantraniliprole) in *Plutella xylostella* was reported to be correlated with polymorphisms of amino acids in the ryanodine receptor (Troczka et al. 2017).

Case V. Mutation in multiple target sites are responsible for developing resistance to Cry toxins. As for example, resistance to Cry toxins in Lepidoptera is being related with reduced binding of the toxins to receptors like cadherin and aminopeptidase N (Pardo-Lopez et al. 2013).

7.5.3 Alterations in Detoxification Metabolism

Alterations in detoxification mechanism through over expression or conformational change of enzymes that are involved in the processes of insecticide metabolism, sequestration, and excretion are a common mechanism of pesticide resistance (Table 7.1). Examples of such enzymes include cytochrome P450 monooxygenases or P450s, glutathione S-transferases or GSTs, and carboxy/cholinesterases or CCEs (Liu et al. 2016).

Case I. Over expression of cytochrome P450: Resistance against nicotine and neonicotinoid insecticides in *M. persicae* has been found to be related with over expression of cytochrome P450 (Bass et al. 2014). Furthermore, comparative transcriptomic studies have revealed that multiple P450 genes (including CYP6 and CYP9 subfamilies) are responsible in conferring resistance via over expression.

Case II. Disruption of GSTs: RNAi mediated disruption of detoxification genes like GSTs are reported to be associated with insecticide resistance. For example, RNAi knockdown of *GSTE2* and *GSTE7* in *A. aegypti* contributes to deltamethrin resistance (Lumjuan et al. 2011).

Case III. Over-expression of Uridine 5′-diphospho (UDP)-glycosyltransferases (UGTs): Over-expression of UGTs was recorded to confer resistance against pyrethroids and temephos in *Aedes* strains (Faucon et al. 2015).

7.5.4 Alterations in Insecticide Penetration

The third and relatively uncommon mechanism of insecticide resistance is to reduce cuticular penetration (Table 7.1). Reduced penetration results in poor availability to its target site. As for example, reduced penetration of insecticides through the cuticle in *M. persicae* confers resistance to neonicotinoid insecticides (Bass et al. 2014).

7.5.5 Other Potential Mechanisms of Resistance

7.5.5.1 Induced Resistance

All of the resistance mechanisms described above is constitutive in nature. Induced resistance includes plant immunity.

7.5.5.2 Behavioral Resistance

It is likely that some behavioral aspect of pest insects enable them to reduce contact with pesticides on treated areas. As for example, there are reports for both “modified” behavior (Nauen and Elbert 1997) and “avoiding” behavior (Fray et al. 2014) of aphids on insecticides that confers insecticide resistance.

7.6 Factors Influencing Insecticide Resistance

Insecticide resistance development in insect pest populations is influenced by biological, ecological, genetic, and operational factors (Figure 7.2).

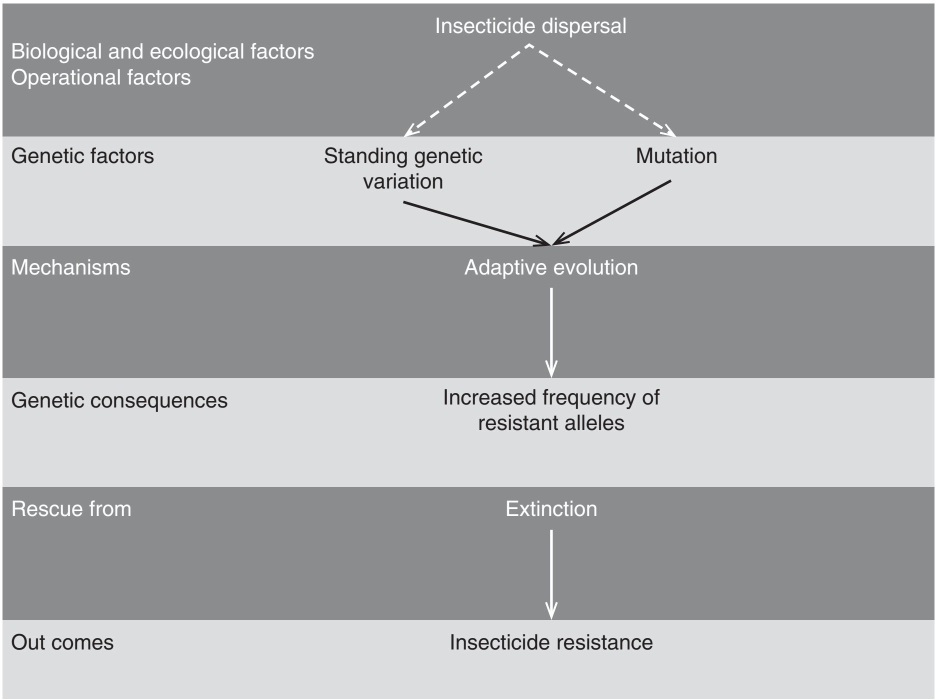

Figure 7.2 Development of insecticidal resistance in terms of biological, ecological, and genetic factors, mechanisms, genetic consequences, and populations are being rescued.

7.6.1 Biological and Ecological Factors

It was recorded that mutations related to insecticide resistance may occur in the nature biologically even without any stress of insecticide. For example, Hartley et al. (2006) reported that the mutations conferring resistance to malathion were present even before the introduction of the organophosphorus insecticides. Some resistance alleles (e.g. Cyp6g1) were found to be associated with a fitness benefit, even in the absence of pesticide (McCart and Buckling 2005). Biological factors like mobility, reproduction rate, and number of progeny per year have tremendous influences on the evolution of insecticide resistance. For example, rapid reproductive rate is an important contributing factor for rapid resistance evolution in spider mites and predatory mites (Croft and Van de Baan 1988).

Geographical isolation is the most important contributing factors influencing the evolution of insecticide resistance in pest insects. As for example, insecticide resistance *kdr* mutation allele frequencies are distributed heterogeneously from southern to central China and was found as a result of geographic isolation in the mosquito populations (Chang et al. 2016).

Food-limitation and immigration are two other important ecological factors that limit resistance evolution under field conditions. Thus for example, pesticide treatment causes shortage of food to the predatory mites that resulted in reduced reproduction, starvation, or migration (Croft and Van de Baan 1988), whereas the level of immigration of susceptible individuals into treated habitats will again influence insecticide resistance evolution. Other ecological factors, like presence of alternative hosts, proximity to untreated areas, and efficacy of biological control are among the important contributing factors. For example, significant correlations among the distribution of *A. gambiae* sub-species, resistant genotype (pyrethroid target-site mutations, *kdr*) and agricultural activity were reported by Nkya et al. (2014).

7.6.2 Genetic Factors

Environmental changes, such as administration of insecticide, population persistence hinges on phenotypic plasticity, dispersal, or adaptive evolution are identified as influencing genetic factors in developing resistance in insect pest (Merila and Hendry 2014). Understanding genetics in insecticide resistant insect pest can assist in the development of management programs to control the development and spread of resistant insect populations (Carlson et al. 2014). Mutation is an important factor responsible for development of resistance alleles (Feyereisen et al. 2015). Resistance development can also be addressed by investigating the frequency of dominant, recessive, and co-dominant forms of genes encoding (Naqqash et al. 2016).

Case I. Gene duplication: In resistant strains of *Myzus*, the numbers of copies of the E4 gene was found proportional with the levels of resistance (Devonshire and Sawicki 1979). The similar event was also recorded with cytochrome P450 (Ffrench-Constant 2013).

Case II. Single gene mutation with single origin: Cyclodienes and endosulfan resistant *M. persicae* at peach orchards in Washington State of USA, was found to carry point mutations (replacements of alanine 301) in their *Rdl*-encoded GABA-gated chloride channels (Anthony et al. 1998). Neonicotinoid resistance of the *Myzus* was also reported to be a result of a point mutation in the β1-subunit of nicotinic acetylcholine receptor (Bass et al. 2011).

Case III. Single gene mutation with multiple origins: The prospect of multiple different mutational events (e.g. point mutation, insertions, and duplications) giving rise to a range of complex alleles at a single locus (either structural or regulatory). Different alleles at the same locus used to increase pesticide. For example, three non-synonymous mutations (L1014F, L1014C, and L1014S) were detected at the knockdown resistance (*kdr)* codon L1014 of *para-type* sodium channel gene in adults or larvae of *Anopheles sinensis* (Chang et al. 2016). A classic example of a single regulatory gene mutation through transposonal insertions followed by duplication conferring different level of resistance (against DDT) is identified as Cyp6g1 in *Drosophila melanogaster* (Schmidt et al. 2010).

Case IV. Gene conversion: Chimeric P450 (e.g. CYP337B3 in *H. armigera*) can metabolize pesticide like fenvalerate to the nontoxic 49-hydroxyfenvalerate. Therefore, gene conversion via unequal crossing over between two parental P450 genes was responsible for resistance to the pyrethroid fenvalerate (Jousen et al. 2012).

Case V. Multiple mechanisms: Multiple mechanisms of insecticide resistance are well documented in different mosquito species and aphid (Edi et al. 2012; Djouaka et al. 2016; Menze et al. 2016).

7.6.3 Operational Factors

Operational factors are another important concern that influences the process of insecticide resistance. Among the important operational factors that directly indulge in the process are chemical nature of the insecticide, a persistence of residues, and the number of applications (Rust 2016). The rate of resistance development increased as the number of applications and concentration increased. For example, the R allele frequency in *L. trifolii* exceeded 0.50 in 1.3 crop cycles at 6 sprays per crop cycle versus 1.0 crop cycles at 12 sprays per crop cycle (Mason et al. 1989). But, consecutive application of higher concentration may accelerate the resistance process. For example, a high concentration with 6–12 applications resulted in resistance before two crop cycles was reported in *L. trifolii* (Mason et al. 1989). An operational procedure like refuge strategy may effectively reduce the rate of insecticide resistance. For example, a refuge for 10% of the adults in *Liriomyza* leafminers exhibited slow resistance development in all cases (Mason et al. 1989).

7.7 Managing Pesticide Resistance

Based on the circumstances, it is important to develop a resistance management depending upon the crop, season, and geographical locations.

7.7.1 Insecticide Resistance Database

To manage overwhelming cases of insecticide resistance (which varies in time and space) databases need to be developed. One such important database is the Arthropod Pesticide Resistance Database (APRD) that reports resistance cases from 1914 to the present was developed by Michigan State University, USA. The website corresponding to this database is www.pesticideresistance.org. Another important database is the global insecticide resistance database established in 2014 by WHO reporting the status of insecticide susceptibility of *Anopheles* mosquitoes in malaria-endemic countries (http://www.who.intmalaria/areas/vector_control/insecticide_resistance_database/en/).

7.7.2 Chemical Use Strategies for Resistance Management

Since the selection and use of insecticides are variables, they can be effectively altered to manage resistance problems. Chemical use strategies are formulated based on one of the following principals:

7.7.2.1 Management by Moderation

Management by moderation is probably the most widely used principle for successfully managing resistance. It involves:

(i) optimal dosages of insecticides;
(ii) higher treatment thresholds;
(iii) chemicals with shorter residual activity;
(iv) treatment of only limited areas where the attack is maximum;

(v) maintaining refuge areas for susceptible individuals; and
(vi) application of insecticides at the specific stage of insect development.

Many of these points are already common components of IPM programs.

7.7.2.2 Management by Multiple Attacks

Management by multiple attacks means the simultaneous application of multiple insecticides or rotations of insecticides over the course of time.

7.7.2.3 Management by Saturation

Management by saturation means the application of insecticide in such a dose that kills all the target insects including the resistant individuals. Alternatively, insecticide synergists may be used.

7.7.3 Reactive Resistance Management

Reactive resistance management is an approach to combat the resistance problem only after occurence of the resistance issue. This is an older approach of insecticide resistant management programme.

7.7.4 Proactive Resistance Management

Protective resistance management is a solution to overcome the resistance problem even before it has arrived. This approach reflects an overall sophistication of the IRM programme.

7.7.5 Resistance Management as a Component of IPM

IRM will extend the importance of IPM-compatible practices including:

(i) pest monitor;
(ii) crop rotation;
(iii) use reasonable treatment thresholds; and
(iv) full use of non-chemical insecticides (such as biological and cultural control, sanitation, and host plant resistance).

7.8 Technical Strategies to Combat Insecticide Resistance

7.8.1 Searching and Characterizing New and Novel Insecticide

At present, most of the agricultural pests are resistant to the insecticides available in the market, and thus screening and characterization of novel insecticides are essential. However, the replacement of old insecticides by a new one is time consuming and expensive. Sparks (2013) has characterized a novel insecticide named sulfoxaflor, which acts on different nicotinic acetylcholine receptors (nAChRs) of a wide variety of pests, specially sap-feeding resistant insect pests. Due to unique the characteristic, sulfoxaflor is considered as an alternative option in IPM program. Another such example is anatoxin-a, which is also known as Very Fast Death Factor (VFDF). In general, it is a secondary metabolite produced by cyanobacteria and act as a neurotoxin. Like sulfoxaflor, anatoxin-a binds to nicotinic acetylcholine receptors and lead to respiratory paralysis and un-synchronized muscular contraction (Aráoz et al. 2010). Similarly, Cui et al. (2007) have also reported a novel insecticide; esterases against insecticides resistant mosquito.

7.8.2 Amending Biocontrol

Nowadays, farmers are paying more attention to biopesticides compared to chemical pesticides, as they have several advantages like being environment friendly, target specificity, less shelf life, and easy degradability (Kumar and Singh 2015). Furthermore, the use of chemical pesticides is restricted in different countries, as they enters the body through the food chain. Several researchers have reported and identified endo- and exo-pathogens of insects; however, few are available commercially on the market (Chattopadhyay et al. 2017). In general, three biocontrol procedures have been applied in the field; (i) classical biological: Pathogens of insect pest has been introduced, (ii) conservation biological control: Provide suitable environment and additional food for insect pest, and (iii) augmentation biological control: Increase the number of natural plants and animals through inoculation that has biocontrol efficacy. However, the increasing number of predators in a new area is not always good, because due to the lack of natural prey, it might become the pest itself.

7.8.3 Exploring Novel Insect Pest Resistant Varieties

7.8.3.1 Plant Immunity and Insect Resistance

Allelochemicals are secondary metabolites produced by plants, which play an important defensive role against herbivore attack, other plants, disease, and pests. To date, researchers have isolated and characterized different types of allelochemicals and explored their MOA. For example, pyrethroid (an organic compound) obtained from the flowers of pyrethrums (*Chrysanthemum cinerariaefolium* and *Chrysanthemum coccineum*) has a toxic effect against insect pest, and thus it is randomly used as an ingredient of common insecticides (Vijverberg and vanden Bercken 1990). In detail, pyrethrums have binding efficiency with piperonyl butoxide, which is recognized as an inhibitor of microsomal cytochrome P450 enzymes. Other examples included; *Peganum harmala* (seeds and roots), *Desmodium caudatum* (leaves and roots), and *Quassia* (South American plant genus). However, like herbivores, few insect pests also have developed resistant against these defensive chemicals.

7.8.4 Combining Known Insecticides in Appropriate Proportion

In recent years, several strategies have been developed to encounter the insect pest. Among these strategies, the combination of two or more insecticides in appropriate proportion gain huge popularity. Fabrick et al. (2015) have reported the efficacy of the combination of different Bt proteins produced by transgenic plant “pyramids” to combat with the resistant pest. Combination and expression of several bacterial insecticides producing genes in crop plant might be effective against insect pests. Production of novel insecticides using genetic combination will definitely solve the problem. For example, plant-colonizing beneficial bacteria can be used for the successful delivery of Bt genes to enhance the efficiency of insecticides. In this regard, Manker et al. (2002) have stated that the combinational effect of a Bt enhancer and Bt protein is very effective to control the attack Lepidopteran caterpillar. Until now, the research in this direction is not well explored and thus needs much more attention. The combinational use of biotechnological, genetic and protein engineering will be helpful to prepare novel insecticides against agricultural pests.

7.8.5 Modifying Known Insecticidal Toxins

Protein fusion is a part of genetic engineering to develop chimeric protein (joining of several proteins produced by different gene). Development of chimeric insecticides (known as next-generation biopesticides) is very important for effective pest control management. In recent years, Yang et al. (2015) have reported a fusion protein (Pl1a/GNA and Hv1a/GNA attached to snowdrop lectin), which kills the insecticide registrant strain of peach-potato aphid (*M. persicae*) by targeting the VGSCs. Similarly, Ahmad et al. (2015) have reported the *in-silico* insecticidal effect of Vip3Aa-Cry1Ac against Lepidopteran pest. Compared to single insecticide, fusion proteins are more effective against a wide variety of insect pests.

7.9 Future Perspective

The war between the resistant pest and insecticides is a never-ending process. Regular application of insecticides in agriculture and health sectors creates pressure for evolving resistant pest and insects. Till now, different types of chemical and biological insecticides are available, however, these will not continue to work for a long time, and thus, searching of the new insecticidal agent is mandatory. Bioinformatics analysis together with wet laboratory application might be helpful for discovering novel toxins from unexplored microbiome. Furthermore, TILLING and Eco-TILLING are also very helpful to determine the resistant varieties in insects, which will open a new window to combat the pest resistance problem in agriculture and horticulture sectors.

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Conflict of Interest

None of the authors have any conflict of interest.

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8

Agricultural Herbicides and Fungi in Soil Exposed to Herbicides

Barberis Carla, Magnoli Carina, Carranza Cecilia, Benito Nicolás and Aluffi Melisa

Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas Físico Químicas y Naturales, Universidad Nacional de Río Cuarto, Río Cuarto, 5800 Argentina

8.1 Introduction

Over the past century, the increased demand of food grains and vegetables has led to an extensive use of chemical pesticides in agriculture (Narra 2016). Their use allowed more and better products to be obtained, therefore many countries could increase their agricultural production and exportation. Despite these positive aspects of the use of pesticides, the negative aspects of their intensive and repeated application should be considered. Some researches establish that about 5% of the used pesticides are absorbed by the target organisms, while more than 50% remains in the soil and water bodies (Nawaz et al. 2011). Thus, the environment pesticide contamination is a great concern.

Herbicides are one of the most applied, comprising about 40% of the world market, with some variations according to the countries considered. Although herbicides have certain beneficial effects for agricultural production, they or their degradation products can be bioavailable for aquatic organisms and, due to lipid affinity, they can be incorporate in fatty tissues. Nevertheless, the biota is exposed to several pesticides that result from mixed applications in the crops. Therefore their concomitant presence, even at low concentrations, needs to be evaluated because it may lead to an increased toxicity or higher effects than were expected (Mhadhbi and Beiras 2012).

Considering the negative effects of pesticides, several researchers worldwide have focused their attention on removal strategies of pesticides from the environment using different physical, chemical, and biological approaches (Diez 2010; Kulshrestha and Kumari 2010; Hussain et al. 2015). From these, the microbial biodegradation of pesticides is one of the most studied methods for removal of pesticides of soil and aqueous media (Diez 2010; Yin and Lian 2012; Hussain et al. 2015). The use of microbial biotechnology for the elimination of pesticides from the environment has several advantages such a slow cost and environmental friendly. In this regard, several microorganisms as bacteria (Yang et al. 2005; Li et al. 2007; Hussain et al. 2009, 2011; Zhang et al. 2017; Háhn et al. 2017), filamentous fungi (Badawi et al. 2009; Sene et al. 2010; Mohamed et al. 2011; Peng et al. 2012; Peter et al. 2015) and

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yeast (Salam et al. 2013) have been isolated from natural sources and characterized for biodegradation of pesticides. Several studies reported pesticides biodegradation ability by bacteria, only a few fungi of the genera *Aspergillus* spp. (Sene et al. 2010; Mohamed et al. 2011; Fu et al. 2017), *Trichoderma* sp. (Sene et al. 2010; Perissini-Lopes et al. 2016), *Penicillium* spp. (Peng et al. 2012), *Fusarium* spp. (Sene et al. 2010), *Phanerochaete* sp. (Reddy and Mathew 2007; Chrinside et al. 2011), *Rhizopus* sp. (Sene et al. 2010), *Trametes* sp. and *Paecilomyces* sp. (Bastos and Magan 2009; Chan-Cupul et al. 2016), *Lentinus* sp. (Nwachukwu and Osuji 2007) and *Mortierella* sp. (Badawi et al. 2009; Ellegaard-Jensen et al. 2013) have shown the ability to biodegradate different pesticides.

8.2 General Aspects of Main Herbicides

8.2.1 Clodinafop Propargyl

The clodinafop propargyl (CF) (prop-2-ynyl (R)-2-(5-chloro-3-fluoro-2-pyridyloxy) phenoxy]propanoate) is a aryloxyphenoxypropionate herbicide (fops), applied to eliminate weeds after emergence in cereal crops. Their mechanism is explained as a herbicide that interferes the enzyme acetyl coenzyme-A-carboxylase, essential for lipid biosynthesis (Singh 2013).

8.2.2 Toxicity of CF

World Health Organization classified CF as a metabolite class III, whereas for the U.S. Environmental Protection Agency consider it as "Likely to be Carcinogenic to Humans" (U.S. EPA 2004).

The persistence of CF is low. The literature reported a half-life for this herbicide in soil of 3–5 d, dependent of the chemical properties and the microbiota (Roy and Singh 2006; Singh 2013). In the environment CF is incorporate as an ester derivative, then undergoes fast hydrolysis to an acid, which increase their mobility and persistence in the soil. In this condition their half-life is increased at 33.6 d (U.S. EPA 2000). However, some studies have established that CF is highly toxic to aquatic species, but the underlying mechanism of its biocide activities still remains unknown. Fops are particularly sensitive at CF. Potential genotoxicity at low concentrations has been informed *in vitro* studies (Kashanian et al. 2008). CF at low concentration interacts with calf thymus DNA and disrupts the posterior and ventral development of zebrafish embryos (Gui et al. 2011; Jaquet et al. 2014).

One of the most promising alternative methods to replace animal testing concerning aquatic toxicology is the zebrafish embryo toxicity test (OECD 2013). With recent developments, zebrafish early life stage testing has been suggested as a tool to bridge the gap between *in vitro* cell-based models and *in vivo* mammalian models (van Woudenberg et al. 2013). In this way, in the research done by Gui et al. (2011) evaluated the development of zebrafish embryos. These authors showed that CF exposure impair the normal embryonic development. Similar effects were also observed for embryos exposed to clodinafop (the metabolite of CF degradation). In addition, these compounds resulted toxic and carcinogenic to humans and several animal species (Kashanian et al. 2008; Gui et al. 2011).

8.2.3 2,4-Dichlorophenoxyacetic Acid

The dichlorophenoxyacetic acid (2,4-D), the first commercial herbicide, is the most thoroughly researched and world widely used. This chlorinated compound is formulated as amine salts (mainly dimethyl-amine salt), which are more soluble in water than acid, and ester derivatives (2-ethyhexyl ester), which are less water soluble. The 2,4-D amine salts and esters rapidly turn to the 2,4-D acid when is applied in the environment (U.S. EPA 1997). They are frequently applied for the selective activity and highly effective for control of weeds (broad-leaved) resistant to glyphosate (GP) in agriculture crops, pastures, and forests. The product is used both agriculturally and domestically for post-emergent control of weeds.

With respect to the mode of action, some works establish that 2,4-D produces an increase of levels of the auxin in the plant, leading to a stimulation of plant growth and death. In addition, an induction of ethylene production also been attributed to 2,4-D, and it produces defoliation in the plant. Despite this, some authors establish that the exact mechanism by which this herbicide affects the weed cells is not understood (Gonzalez et al. 2005; Marrón-Montiel et al. 2006).

8.2.3.1 Toxicity of 2,4-D

The herbicide 2,4-D is classified by WHO as a hormonal herbicide of level II by their potential toxicity. The carcinogenic, teratogenic, neurotoxic, immunosuppressive, cytotoxic, and hepatoxic effects have been attributed to this herbicide on both animals and humans. In recent decades the toxicity of 2,4-D has been widely studied (Bortolozzi et al. 2001; Garabrant and Philbert 2002; Bukowska 2006; Mikov et al. 2010; Burns and Swaen 2012; Atamaniuk et al. 2013; Coady et al. 2013; Neal et al. 2017). In mammals, 2,4-D disrupts the adenosine triphosphate production (ATP) (Palmeira et al. 1994) and affects the DNA, inhibiting both cell growth and protein synthesis (Gonzalez et al. 2005). In addition, the carcinogenic effects are related to the development of lymphomas and a type of tissue sarcoma (Garabrant and Philbert 2002; Holland et al. 2002).

8.2.4 Glyphosate

Glyphosate (GP)(N-[phosphonomethyl] glycine) is the main ingredient of several commercial herbicides used to control annual and perennial weeds. It is a broad-spectrum herbicide that is applied to control weeds mainly in agriculture no-till cropping systems. Its agricultural uses increased considerably after the development of glyphosate-resistant genetically modified (GM) varieties. It acts by inhibition of the enzyme 3-enol-pyruvylshikimate-5-phosphate synthase (EPSP synthase), located in the chloroplast, interfering in the biosynthesis of aromatic amino acids to then interfere in the synthesis of proteins (Roberts et al. 1998). The EPSP synthase is an enzyme that forms part of the metabolic pathway of the shikimic acid. This is a process that only occurs in plants, bacteria, and fungi and it does not exist in animals; due to this fact the acute toxicity in animals is low.

8.2.4.1 Toxicity of GP

Glyphosate was considered an advantageous herbicide until its use led to the evolution of GP-resistant weeds (Duke and Powles 2008). GP interest has increased exponentially among scientists, due to concern about the toxicity in humans and all types of organisms. The majority of the work is conducted with commercial GP, formulated with GP as an active ingredient and other ingredients (adjuvants). Some ingredients may be more toxic than GP for no target organisms (Nobels et al. 2011; Kim et al. 2013; Mesnage et al. 2013).

Although GP is considered to be a relatively safe compound, today, several reports informed that GP has negative effects on human health. In addition, GP and aminomethylphosphonic acid (AMPA one of its main degradation metabolites) have been frequently detected in fumigates areas, mainly surface waters and soil next to the cultivated fields. The concentrations detected are determined by contamination source, hydrology, and water movement pathways (Coupe et al. 2012; Aparicio et al. 2013). The concern of the adverse effects of GP on aquatic and soil species has increased due to their extensive use and the large amount that is annually applied (Schuette 1998; Contardo-Jara et al. 2009; Paganelli et al. 2010).

GP has been the subject of regular assessments by different regulatory agencies (Williams et al. 2000; JMPR 2006). All of them had established that GP has a relatively low toxicity in mammals. However, a recent report from the International Agency for Research on Cancer (IARC) concluded that the herbicide and its formulated products are probably carcinogenic in humans (Guyton et al. 2015; IARC 2015). Currently, the carcinogenic potential of GP is a controversial issue (Tarazona et al. 2017a,2017b)

8.2.5 Atrazine

Atrazine is technically described as [2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine]. It is a selective herbicide belonging to the family of the s-triazines, which is widely used. It has been banned in some regions such as the European Union. However, it is one of the most used herbicides in North America and in several other countries for pre- and post-emergence control of broadleaf weeds in several crops (Sass and Colangelo 2006; Ghosh and Philip 2006).

Residues of atrazine and its metabolites have often been detected in surface and ground waters at concentrations well above the permissible limits. This situation is propitiated for massive application, their high mobility in soil, long half-life, and high persistence under reductive conditions (Solomon et al. 2013; WHO 2011). The inhibition of weeds produced by atrazine is attributed to the interference on photosynthesis and its associated noncyclic photophosphorylation (Shelton et al. 1996).

8.2.5.1 Toxicity of Atrazine

Atrazine is a common herbicide used worldwide. In Europe, the use of this herbicide decreased because it caused undesirables effects on wildlife and humans (Zeljezic et al. 2006; Frank 2007).

The endocrine system in humans and animals is documented as the main target of atrazine. Jablonowski et al. (2008) showed that 14C-marked atrazine remained in soil even 22 years later. These results revealed a potential risk of atrazine chronic exposure. Atrazine is considered as a carcinogen that affects nervous, reproductive system, immune, and cardiovascular system. Atrazine effects in animals are related to non-Hodgkin's lymphoma in humans. Atrazine is a possible disruptor of sexual differentiation in male frogs (Oka et al. 2008; Hayes et al. 2010) and it also alters the immune response (Forson and Storfer 2006). Some studies have associated this herbicide with alterations in the locomotion activity in rodents (Bardullas et al. 2011). It was also related to decreases in catecholamine content of the striatum and decreases in the amount of a specific type of neurons (Hayes et al. 2002). Due to the high toxicity of this herbicide at low levels, the development of bioremediation strategies is very necessary.

8.2.6 Metolachlor

The transformation of metolachlor in soil by microorganisms has as the main degradation products metolachlor ethane sulfonic acid (ESA) and metolachlor oxanilic acid (OA) (Barbash et al. 1999). In soil the degradation of this pesticide take place mostly by decomposition of microorganisms and photo-degradation (Senseman 2007; Xu et al. 2008). The temperature, volume of microbial community, soil depth, amount of dissolved oxygen and organic carbon were influenced by the incidence of microbial degradation. In case of sandy soils, the half-life of this herbicide is ranged between 67 and 81 days. Just the presence of metolachlor on soil cover promotes photo-degradation. This occurs only when metolachlor is present on the soil surface (Long et al. 2014). McGahen and Tiedje (1978) were the first to describe the *Chaetomium globosum* metabolism of metolachlor. These authors reported that 45% of the metolachlor was removed after six days. The 18.4% of ^{14}C-metolachlor applied in soil was mineralized by inhabitants of the same like *Rhizopus*, *Actinomyces,* and *Streptomyces* (Liu et al. 1988). Other fungi (*Fusarium* sp., *Mucor racemosus*, *Phanerochaete chrysosporium*, *Rhizoctonia praticola* and *Syncephalastrum racemosum)* were described as metolachlor degrading (Saxena et al. 1987; Liu et al. 1991, 1995; Libra et al. 1996). Metolachlor hydrolysis by a crude extract of *Aspergillus flavus* was investigated by Sanyal and Kulshrestha (2004). They found that there is a parallel between the increase of amounts of crude extract of metolachlor (20 and 100 μg ml^{-1}) in samples with the degradation rate detected in them. These authors found that the crude extract contained enzymes responsible for dechlorination, hydroxylation, and N-dealkylation reactions. Nwachukwu and Osuji (2007) studied the efficacy of *Lentinus subnudus* to degrade three herbicides, being metolachlor one of them. About 94% of metolachlor degradation was observed after 25 days in liquid media. More recently, Słaba et al. (2015) reported that alachlor (another chloroacetinilide herbicide) degradation by *Paecilomyces marquandii* was increased on liquid batches when the environment conditions were optimal and controlled (by 20% to compared with flask cultures).

8.2.6.1 Toxicity of Metolachlor

Metolachlor was classified by the US Environmental Protection Agency (US EPA) as a "not likely to be carcinogenic to humans". This classification was done based on studies in rodent, where a significant increase in liver neoplasms was seen in female rats at high levels (U.S.EPA 2018).

Laville et al. (2006) observed an increase in the aromatase enzyme activity in human cell culture. This enzyme converts testosterone into estradiol. Alterations on the activity of the aromatase enzyme might cause deficiencies in male reproduction. Mai et al. (2013) detected low success in fertilization and high defects in oyster embryo development with low doses of metolachlor. Furthermore, Zeilinger et al. (2009) found that acetochlor (acetamide-type herbicide) was able to alter the thyroid system of fish species at high concentrations.

Recently, Quintaneiro et al. (2017) informed the adverse effects of linuron and S-metolachloron zebrafish (*Danio rerio*) embryos. These authors showed that both herbicides affect neurotransmission and energy production, induce steroidogenesis and interfere with hypothalamus-pituitary-thyroid and adrenal axis, which are potential targets for endocrine disruption compounds.

In humans, Silver et al. (2015) evaluated cancer incidence through 2010/2011 for 49 616 workers, 53% of them reported having ever used metolachlor. These authors observed no relationship between the use of metolachlor and the prevalence of all type of cancers ($n = 5701$) or site-specific cancers. In addition, they suggested that the relationship between this herbicide and liver cancer among rural workers has never been informed and they remarked the importance of their finding about the higher amount of liver neoplasms in animal assays. However, the observations of both liver cancer and follicular cell lymphoma justify follow-up to assess the effects of the intensive use of metolachlor.

8.2.7 Diuron

Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is a systemic herbicide belonging to the phenylurea family. It is used to control weeds and mosses in agricultural and non-agricultural areas (Castillo et al. 2006; Stasinakis et al. 2009). Diuron is a wide-spectrum herbicide, it can be applied alone or in combination with other herbicides (bromacil, hexazinone, paraquat, thiadiazuron, imazapyr, monosodium, sodium chlorate, sodium metaborate and copper sulfate) (USEPA 2004).

The main effect of Diuron in the plants is blocking the Hill reaction in the photosynthesis process. This imitates the production energy compounds such as ATP. Diuron blocks the electron transport between QA and QB by binding to the QB-binding site on D1. The D1 protein belongs to the photosystem-II complex inside the chloroplast. This process avoids the fixation of CO_2 and the assembly of high energy compounds. The interruption in electron transport from QA caused the creation of triplet-state chlorophyll, which reacts with ground-state oxygen producing singlet oxygen. Both triplet chlorophyll and singlet oxygen produce lipid radicals by extracting hydrogen from unsaturated lipids, therefore they initiate a chain reaction for lipid per-oxidation. These reactions cause oxidation of lipids and proteins that result in chlorophyll and carotenoids losses, therefore the organelles and cells dry and disintegrate (Hess and Warren 2002).

8.2.7.1 Toxicity of Diuron

The US Environmental Protection Agency classified diuron as: likely human carcinogen (USEPA 1997). Diuron has been restricted or even banned in Denmark, Finland, Germany, UK, and Sweden due to its potential risks to humans and environment. Despite this, diuron is widely used around the world and accepted for protection of European plant products (Finnish Safety and Chemicals Agency 2013).

The ban of diuron is based on studies in rats and mice where carcinomas in bladder, kidney, and mammary gland were found after diuron exposition. In addition, diuron exposition caused several changes in the rat urothelium such as increase cell proliferation and hyperplasia (Nascimento et al. 2006; Cardoso et al. 2013), and it produced necrosis on bladder mucosa (Da Rocha et al. 2010; Cardoso et al. 2013). This herbicide is metabolized and then excreted by urine. It has been demonstrated that mechanisms of genotoxicity may be involved in the cytotoxicity of urothelial cells (Da Rocha et al. 2010, 2014). Besides, Comet assay revealed diuron genotoxicity in oysters (Akcha et al. 2012; Barranger et al. 2014).

Diuron toxicity was evaluated testing two human cell lines (breast adenocarcinoma–MCF-7 and placental choriocarcinoma–BeWo) (Huovinen et al. 2015). In both cell lines, the highest diuron concentration (200 μM) increased the reactive oxygen species (ROS) production. This herbicide significantly reduced BeWo viability, but not for MCF-7. While diuron inhibits cell proliferation in both cell lines due to the relative cell number was reduced. DNA damage was tested by the Comet assay showing that diuron caused an increase in DNA fragmentation only in MCF-7. Both p53 (marker of cell stress) and p21 protein (target of p53) expressions increased in the presence of diuron only in MCF-7 line. In conclusion, these authors suggested that diuron has cytotoxic and potentially genotoxic effect in specific tissues and the toxicity is due to the production of ROS. Thus, diuron exposition may have negative effects on fetal development and therefore affect human health.

Diuron photodegradation occurs by hydrolysis and leads to 3,4-dichloraniline (3,4-DCA) formation, which is a highly toxic compound (Giacomazzi and Cochet 2004) also after its biotransformation (Xiao et al. 2016). Thus, both parent and degradation compounds can be found in soil, sediment, surface, and ground water (Giacomazzi and Cochet 2004). Diuron is quite recalcitrant in the environment due to its slow degradation rate in water. Diuron half-life is around 100 days in soil and around two weeks in water (Guardiola et al. 2012).

8.2.8 Imazapyr

Imazapyr, technically 2-(4,5-dihydro-4-methyl-4-[1-methylethyl]-5-oxo-1H-imidazol-2-yl)-3-pyridinecarboxylic acid, is a non-selective herbicide that belongs to the imidazolin one family and to the group 2 of broad spectrum herbicides. It has action on annual and perennial grasses, broad leaf branch, sedge, and a variety of shrubs and deciduous trees. Imazapyr enters the plant by leaves and roots, then it rapidly passes through the xylem and phloem and then enters into the meristematic regions. In these regions, the herbicide accumulates and disrupts protein synthesis and interferes with cell growth and DNA synthesis. As a result of this, imazapyr causes the death of growing leaves. The herbicide effect of this compound is to stop the biosynthesis of essential amino acids in plants by binding to the acetolactate synthase enzyme. Animals take these amino acids of the plants; therefore, the specific toxic effect can only happen in plants (Singh and Singh 2016).

8.2.8.1 Toxicity of Imazapyr

Imazapyr is very recalcitrant on soil, its half-life being around 17 months (Gianelli et al. 2014). Imazapyr is considered as non-toxic to animals; however, it irritates eyes, and it can produce rashes, redness, and swelling in the site exposed. The toxic effects in animals evaluated occur at a much higher dose (Yahnke et al. 2013).

8.2.9 Pendimethalin

Pendimethalin (PND) (N-[1-ethylpropyl]-3, 4-dimethyl-2, 6-dinitrobenzenenamine), is a dinitroaniline-type herbicide used at both pre-emergence and early post-emergence stage. It has action on most annual grasses and broad-leaved weeds in agricultural practices. PND acts by inhibiting mitotic cell division in developing root systems (Singh and Singh 2016).

8.2.9.1 Toxicity of Pendimethalin

The US EPA has classified PND as bioaccumulative toxic due to its persistence in the environment. Although PND has almost no acute toxicity, it has been linked to thyroid follicular cell adenoma. It is very toxic to aquatic animals (fish and invertebrates). Due to PND being moderately persistent on aerobic soil environments, this herbicide has been defined as not persistent in the EC regulation 1107/2009 Annex II (Vighi et al. 2017).

8.2.10 Paraquat

Paraquat (PQ), technically1,1-dimethyl-4,4-bipyridinium, is a herbicide classified into the quaternary-nitrogen group. It has action on several broad leaf weeds. PQ is an on selective herbicide that disrupts photosynthesis and ruptures cell membranes. These processes allow water to get out the cell and lead rapid drying of foliage. PQ has strong affinity to clay minerals and organic matter in the soil (Singh and Singh 2016).

8.2.10.1 Toxicity of PQ

PQ is widely used in emergent countries, so their use can cause severe damage in animals and human. The deaths caused by PQ poisoning reached up to a third of all suicides worldwide (Sabzghabaee et al. 2010).

PQ can be absorbed by several ways, i.e. mucous membranes, skin, lungs; therefore many living organisms can be damaged. The acute toxicity of PQ has been well studied in lung, liver, and kidney of animals (Huang et al. 2012; Wua et al. 2013). With experimental animals, PQ showed a high toxicity in the inhalation route and it has been placed in the highest levels of toxicity (Category I) for acute inhalation effects. Pulmonary fibrosis is the typical disease caused after the ingestion of PQ, which lasted even weeks after the poisoning (Suntres 2002). Although this disease has high mortality, the molecular mechanism and the treatment are not known yet.

8.3 Biodegradation of Most-Used Herbicides by Fungi

Nowadays, different kinds of chemical herbicides are applied in rural systems for weed control. The most common herbicides used are clondinafop propargyl (CF), 2,4-D, atrazine, metolachlor, diuron, GP, imazapyr, pendimethalin, and PQ. Their degradation processes occurs at different modes and with variable efficiency. The rate of degradation of the different herbicides is influenced by several factors which include pollutants chemical structure, soil pH, hydrogen peroxide concentration and iron concentration. Acceleration of degradation processes results in decontamination in short span of time. Thus, photocatalytic degradation, biodegradation, ozonation, and photo-Fenton reactions are commonly evaluated for herbicides removal studies (Wyss et al. 2006). The continuous use of these chemicals caused an extensive environmental pollution; hence new eco-friendly strategies are being designed to reduce herbicides residues (Singh and Singh 2016).

Soil microorganisms are essential for the bioremediation of herbicides. The phenomenon of biotransformation is very common and sometimes very important for the survival of microorganisms, responsible for biodegradation of applied herbicides. There is a natural balance between microbial evolution and bioremediation (Häggblom 1992). Biodegradation can be approached via microbes and also this process can be enhanced by artificial means. This approach possesses a number of benefits, such as minimal environmental disruption, low cost, and low risk of secondary exposure (Hinchee et al. 1994; Chaudhry 1994). Therefore, the search of microbial species capable to degrade herbicides and their residues are the main topics under investigation in the last two decades. Fungi are a key part in the biogeochemical cycles of the environment. In addition, they are capable to degrade a large number of xenobiotics including herbicides. In most cases, fungi can do slight structural changes on herbicides and another xenobiotics. This results in the release of degradation products which are metabolized by another soil microorganisms (Gianfreda and Rao 2004). Biodegradation studies with fungi increased in the recent years due to these microorganisms having certain advantages with respect to other ones. Mycelial growth, unspecific catabolic enzymes, and broad-spectrum of growth substrates make fungi an interesting tool for bioremediation (Harms et al. 2011; Chen et al. 2012). Several studies have been carried out, aimed to isolate and characterize herbicide-degrading strains from contaminated sites (Badawi et al. 2009; Sene et al. 2010; Caihong et al. 2011; Arfarita et al. 2011; Munoz et al. 2011). In the same way, the more adapted degrading microorganism is those that are isolated from contaminated sites.

8.3.1 2,4-D Degradation

The herbicide 2,4-D is a chlorinated acidic phenoxy herbicide widely used. It is an analogue of auxin, the growth hormone. 2,4-D is moderately recalcitrant, and its half-life is between 20 and 200 days. This persistence in soil is in regard to the type of soil. It solubility in water is high and it has not high affinity to organic matter (El-Bestawy and Hans-Jorgen 2007); therefore 2,4-D reaches streams, rivers, or lakes almost directly from the agricultural fields (Lagana et al. 2002; Muller and Babel 2004; Mikov et al. 2010). Microbial degradation of phenoxyacetic acids can be achieved by two metabolic routes. While bacteria seem to have the ability to degrade these compounds cleaving the side of the chain, fungi can hydroxylate the ring structure (Häggblom 1992). *Aspergillus niger*, *Fusarium oxysporum, Penicillium rugulosum, P. chrysosporium* and *Dichomitus squalens* were the first 2,4-D-degrading fungi (Fournier and Catroux 1980; Shailubhai et al. 1983; Valli and Gold 1991; Donnelly et al. 1993; Yadav and Reddy 1993). Vroumsia et al. (2005) evaluated the *in vitro* 2,4-D and 2,4-dicholorophenol (2,4-DCP) degradation ability of 90 fungal strains isolated from soil, decomposed wood and walnuts. From all strains tested, 20 and 54 of them degraded more than 20% of applied 2,4-D and 2,4-DCP at 100 ppm, respectively. *Aspergillus penicilloides* and *Mortierella isabelina* were the most effective 2,4-D-degrading strains, while *Chrysosporium pannorum* and *Mucor genevensis* degrade 2,4-DCP more effectively. In another *in vitro* study, Silva et al. (2007) found that *Penicillium* sp. isolated from Brazil soils was degraded 30% of 2,4-D. These results suggest that, several soil fungi are potentially able to degrade the herbicide and the degrading residues, and they represent a great potential for bioremediation.

8.3.2 Atrazine Degradation

Atrazine's soil adsorption is low and it has moderate solubility in water. Nevertheless, this herbicide has a long half-life and the risk of contamination of surface and ground water is high. Atrazine use was banned several years ago in some countries; however, this compound and its metabolites are still found in surface and groundwater (Chan and Chu 2003). As for many herbicides, the key mechanism to degrade atrazine in soil is microbial metabolism. The degradation of atrazine includes enzymes that N-dealkylate, dechlorinate and cleave the ring. The biodegradation process indicates by an N-dealkylation reaction that affects the ethyl or isopropyl side chains and produce deethylatrazine (DEA) or deisopropylatrazine (DIA). Dechlorination is another reaction that occurs early and two s-triazine hydrolase enzymes were identified. The first data of atrazine degradation by fungi were informed in the 1970s (Kaufman and Blake 1970).

These authors reported that several fungi species such as *Aspergillus fumigatus*, *Aspergillus ustus*, *Aspergillus flavipes*, *Rhizopus stolonifer*, *Fusarium moniliforme*, *Fusarium roseum*, *F. oxysporum*, *Penicillium decumbens*, *Penicillium janthinellum*, *P. rugulosum*, *Penicillium luteum* and *Trichoderma viride* degrade atrazine N-dealkylating alkylamino groups. The degradation rates were varied as well as the different degradation products.

Filamentous fungi may be having certain advantages when translocation of essential factors such as nutrients, water or the pollutant itself is needed. The main atrazine degrading fungi are wood-degrading basidiomycetes. This ability can be attributed to the lignin-degrading system. These fungi are promising bioremediation tools due to their tolerance to varied environmental conditions, such as temperature, nutrients, and moisture contents (Devers et al. 2007). *Trametes versicolor* grows and effectively degrade atrazine in natural soil under water stress conditions (Bastos and Magan 2009). Other white-rot fungi, *P. chrysosporium* was able to degrade several environmental pollutants. This fungus removed 48% of 2 μM of atrazine in growth medium within the first four days of incubation and the enzymes involved were lignin peroxidases and manganese peroxidases (Mougin et al. 1994). Elgueta et al. (2016) recently studied the *Anthracophyllum discolor* immobilization to improve the atrazine degradation by a biopurification system. The half-life of atrazine was shorter in the inoculated assays, it decays up to six days. Brown-rot fungi were also able to degrade xenobiotics by the Fenton mechanism with and without enzymes involved. A nonsporulating mycelia fungi strain was able to produce cellobiose dehydrogenase and grew on agarized medium with high atrazine concentration (500 $mg\,l^{-1}$). This fungus removed by 50 times 20 $mg\,l^{-1}$ of atrazine in 40 days. While atrazine decreased, extracellular cellobiose dehydrogenase accumulated in the culture medium. This enzyme was also identified in *P. chrysosporium*, *T. versicolor*, *Schizophyllum commune*, *Pycnoporus cinnabarinus*, *Myceliophthora thermophila*, *Humicola insolens* and *Sclerotium rolfsii* (Khromonygina et al. 2004). A consortium between bacteria and white-rot fungi enhanced the remotion of atrazine from contaminated waste water samples. Hai et al. (2012) reported that the mixed fungal consortium removed 98% of 10 $mg\,l^{-1}$ of atrazine after 14 days.

8.3.3 Metolachlor Degradation

The transformation of metolachlor in soil by microorganisms has as the main degradation products ESA and metolachlor OA (Barbash et al. 1999). In soil the degradation of this pesticide takes place mostly by decomposition of microorganisms and photo-degradation (Senseman 2007; Xu et al. 2008). The temperature, volume of microbial community, soil depth, amount of dissolved oxygen and organic carbon were influenced by incidence of microbial degradation. In the case of sandy soils, the half-life of this herbicide ranged between 67 and 81 days. Just the presence of metolachlor on soil cover promotes the photo-degradation (Long et al. 2014). McGahen and Tiedje (1978) were the first on described the *C. globosum* metabolism of metolachlor. These authors reported that 45% of the metolachlor was removed after six days. The 18.4% of $_{14}$C-metolachlor applied in soil was mineralized by inhabitants of the same like *Rhizopus*, *Actinomyces,* and *Streptomyces* (Liu et al. 1988). Other fungi (*Fusarium* sp., *M. racemosus*, *P. chrysosporium*, *R. praticola* and *Syncephalastrum racemosus)* were described as metolachlor degrading (Saxena et al. 1987; Liu et al. 1991, 1995; Libra et al. 1996). Metolachlor hydrolysis by a crude extract of *A. flavus* was investigated by Sanyal and Kulshrestha (2004). They found that there is a parallelism between the increase of amounts of crude extract of metolachlor (20 and 100 $\mu g\, ml^{-1}$) in samples with the degradation rate detected on them. These authors found that the crude extract contained enzymes responsible of dechlorination, hydroxylation, and N-dealkylation reactions. Nwachukwu and Osuji (2007) studied the efficacy of *L. subnudus* to degrade three herbicides, being metolachlor one of them. About 94% of metolachlor degradation was observed after 25 days in liquid media. More recently, Słaba et al. (2015) reported that alachlor (another chloroacetinilide herbicide) degradation by *P. marquandii* was increased on liquid batches when the environment conditions were optimal and controlled (by 20% to compare with flasks cultures).

8.4 Effect of Herbicides on Fungi

Many of the studies done about the interaction of herbicides and soil microorganisms have been focused on herbicide persistence. In addition, several studies were done to determinate the effect of herbicides on the mycobiota of the rhizosphere taking into account each particular herbicide. Temperate ecosystems are influenced (overall nutrient flowing and general development) by arbuscular mycorrhizal fungi (AMF) (Gianinazzi et al. 2010). More than 80% of vascular plants optimize the supply of nutrients (Cameron 2010) and soil aggregation (Siddiky et al. 2012) through the formation of mycorrhizae with different fungal species.

8.4.1 Glyphosate

Glyphosate is a systemic herbicide; in most plants it is not metabolized. The GP was easily translated in the metabolic ducts, as the roots (Duke 1988) to be finally removed into the rhizosphere (Coupland and Caseley 1979). In a greenhouse experiment with white clover (*Trifolium repens*) Zaller et al. (2014) investigated how the globally used GP herbicide influences the relationship between earthworms and AMF, both essential organisms on soil. They determined that glyphosate reduced the development of mycorrhizae in roots, the volume of conidia, vesicles, and inoculum in the soil. The amount of herbicides and the increase of earthworms increased the fungal mass to and decrease the infiltration of water into the soil after a simulated heavy rain. The use of herbicides in relation to AMF led to slightly fatter and passive worms.

They detected a 40% decrease on mycorrhization after GP addition in soils, in which the fungi *Glomus mosseae* were present. The GP could affect directly active metabolite production in the plant with adverse effects on root AMF establishment (Savin et al. 2009). The intraradical stage of AMF it is sensible to different metabolites produced by the plants; these, together with secondary effects of GP could regulate AMF population (Zaller et al. 2014).

In other work, Malty et al. (2006) analyzed the effect of different concentrations of GP on AMF in soybean roots. These authors found a great inhibition due to GP in the growth of *Gigaspora margarita, Glomus etunicatum,* and *Scutellospora heterogama*. Particularly, *G. margarita* showed a slight stimulation on its development that began to decrease as the GP concentration increases. This herbicide also influenced the growth pattern of germinated spores. For *G. margarita*, high incidence of high germination growth of the spores was observed at GP concentrations up to 120 μM. The germinated spores of *S. heterogama* and *G. etunicatum* practically did not grow in a medium with GP. This fact evidence a greater inhibitory effect of the herbicide on these species than the effect observed for *G. margarita*. As germination growth is related to mycorrhizal formation, an inhibition of this parameter implies low root colonization capacity.

Previously, Morandi (1989) found that soybean colonization by *G. mosseae* was favored by addition of GP (0.5 $\mu g\, ml^{-1}$ equivalent to 2.9 mM) after plant emergence. This researcher suggest that this promotive influence of GP may be a result of the higher production of isoflavonoids in the roots, which may promote mycorrhizal colonization, as it was also reported by Siqueira et al. (1991).

8.4.2 2,4-Dichlorophenoxy Acetic Acid and Others Herbicides

The first reference to 2,4-D appeared in an article by Pokorny (1941). However the effects on rhizosphere microorganisms were reported many years later. Mahakhode (2016) used six concentrations of 2,4-D to analyze the influence of the herbicide on the rhizosphere mycobiota of *Psoralea corylifolia* (Asian medicinal plant). They informed that the herbicide had fungicidal effect. Fungal species belonging to Deuteromycetes were dominant as compared to Chytridiomycetes, Zygomycetes, and Ascomycetes. Among all the concentrations tested, the lethal dose was found to be 1000 ppm. The total number of colonies g^{-1} continuously diminished with incrementing 2,4-D amounts. The highest number of colonies was observed with 600 and 800 ppm.

Adhikary et al. (2014) evaluated the impact of three herbicides used in chilli-producing fields (pendimethalin, oxyfluorfen, and propaquizafop) on microbial soil populations. They informed that inhibition of fungal growth occurs according to the chemical structure of the herbicides. At the recommended rate of application in the field, these substances would be moderately toxic for the development of fungal colonies, producing a reduction of 54%.

In 2013, Majid and Mazharuddin Khan, performed container assays on tomato plants to analyze the influence of carbendazim (fungicide), 2,4-D and metribuzin (herbicide) on some propitious microorganisms of the radical environment. The total number of bacteria, actinomycetes, and fungi were more reduced in the handled soil than the unhandled soil. This adverse influence of the herbicides and fungicide on the fungi population of, *Azotobacter* and *Azospirillum* were described (Majid and Mazharuddin Khan 2013).

Recently, Nongmaithem and Pal (2016) reported the effects of different plant extracts and chemical herbicides in different crops with conventional practice of hand-weeding on the soil microbiota. They analyzed the effect of the herbicides quizalofop-p-ethyl and fenoxaprop-p-ethyl. Regarding the actinomycetes, quizalofop-p-ethyl and fenoxaprop-pethyl produced a population decrease of 25% and 21% at 30 days, respectively; while 41.5% and 40% of reduction was recorded in the case of fungi for each compound. This effect might be assigned to the toxic influence of the applied chemical herbicides. Also, it has been cited that the influence of herbicides on the soil microbiota is usually more evident immediately after herbicide application when the concentration in soil is the highest.

Recently many multinational companies have released new herbicide molecules which again open a huge scope for studying the influence of these molecules on soil microorganisms and soil characteristics. Trimurtulu et al. (2015) analyzed the impact of selected new herbicide molecules on soil microbiota and also on soil characteristics. In this study the authors selected a short duration crop like Blackgram (*Vigna mungo L*) in the post-rainy season where herbicide application is mandatory to control weed incidence and to reduce the loss of soil moisture and nutrients. They used pendimethalin, oxyflourfen, pursuit and pertialachlor and analyzed the effect of these on rhizosphere soil microbiota of Blackgram field. A gradual raise was observed in the population of some microbial groups (bacteria, fungi, Actinomycetes, and Rhizobia) with the application of the herbicides. Among all the herbicides, oxyflourfen caused an increase in microbial population with respect to the controls. This increase could be attributed to the direct action of herbicide particle as a substrate for microbial growth or due to the indirect effect of enhanced release of root exudates in the rhizosphere soil.

All herbicides caused an increase in nodule number. Oxyflourfen provokes the highest increase, these authors observed a microbial population 17% higher than the microbial population in the control. They obtained the evidence that pre-emergence herbicide molecules applied during the crop season in vertisols with recommended dosages does not alter the soil microbial communities tested in the study. In fact, the applied herbicide molecules had a synergistic effect on soil microbiota and also led to stimulation of the growth of microbial communities in vertisols.

Otherwise, different works analyze the impact of herbicides on beneficial fungi such as *Trichoderma*, used commonly as an antifungal and it has been implicated in antibiosis, parasitism, and competition. It is applicated on leaves, grains, and soil for the inhibition of several fungal diseases (Elad et al. 1980; Papavizas 1985). Commercial bioproducts such as 3Tac has been effective against *Botrytis, Fusarium,* and *Penicillium* species. Besides is useful on enzyme production (Singh et al. 2014). The use of incompatible pesticides with this fungus may inhibit their development and reproduction. Abbas and Amini (2015) studied the effects of several pesticides, between them the herbicides haloxyfop-r-methyl and ethalfluralin, on growth rate and spore germination of *Trichoderma harzianum*.

Ethalfluralin effectively controls several weeds (foxtail, barn yard grass, fall panicum, crabgrass, pigweed, kochia, and black nightshade) when applied prior to planting or to the plant seedlings (Thriveni et al. 2009). In the presence of ethalfluralin, spore germination is inhibited and the growth rate of the *Trichoderma* was less than 1 cm per week. It was found that the ethalfluralin not only controlled the weeds, it also suppressed the *Trichoderma* growth in the fields.

Haloxyfop is a selective pre- and post-emergent herbicide and belongs to the pyridine chemical group. It is applied to control the annual and perennial grasses in several crops as onions, sunflowers, and strawberries (Zhang et al. 2004). In a laboratory work, this herbicide completely restricted the spore germination of *T. harzianum* at 2000 ppm and significantly controlled the spore germination and colony growth at lower concentrations.

8.5 Effect of Herbicides on Toxicogenic Fungi and Mycotoxins Production

In soils destined for maize crop and in near vegetation, the prevalent mycobiota was composed by *Aspergillus*, *Fusarium*, *Penicillum,* and *Alternaria* genera (Gonzalez et al. 1995; Magnoli et al. 2006). Among these, *Aspergillus* section *Flavi* species are important colonizers in crop cereals both in warm and moist areas. The extensive frequency of *A. flavus* in soil has been related to its capacity to invade vegetable debris (stubble) (Abbas et al. 2004, 2009). In several countries toxigenic species of *Flavi* section have been isolated from maize seeds. Aflatoxins (AFs) are extensively known to be a strong carcinogenic, teratogenic, and they repress to some metabolic systems (Minto and Townsend 1997; Zain 2011). From mycotoxins, aflatoxin B_1 (AFB_1) is one of the main causes of risk in animal and human health (carcinogen group A) and is generally the most important AFs produced by aflatoxigenic strains (IARC 1993). To prevent the effects associated with the ingestion of contaminated agricultural products, the FDA has determined AFs limits of 20 ng g^{-1}, for food and 20 to 200 ng g^{-1} for animal feed in US, some regions of the European Community are more restricted (Commission of the European Communities 2006).

Some studies have shown that the addition of GP can inhibit the development of mycorrhizal microorganism and consequently could increase the development of phytopathogen and toxigenic fungi as *Fusarium* (Krzysko-Lupicka and Sudol 2008; Fernandez et al. 2009). While in some bacteria and fungi sensitive to GP this herbicide can inhibit protein synthesis via the shikimic acid pathway (Zablotowicz and Reddy 2004)

Few data have been informed on the influence of pesticide compounds on the agricultural ecosystem on opportunistic phytopathogen such as *Aspergillus* section *Flavi*, and its effect on the subsequent AFs accumulation (Hasan 1999a,1999b; Reddy et al. 2007). Barberis et al. (2013), evaluated in the laboratory the influence of six GP amounts on growth parameters and AFB_1accumulation by strains of *Aspergillus* section *Flavi* isolated by agricultural soils under different environmental conditions. All the isolated samples tested behaved in the same way at different conditions assayed, the time prior to growth decreased as GP amounts incremented to. The *A. flavus* and *Aspergillus parasiticus* isolated presented a significant decrease in their lag phase with 2 mM of pesticide at 0.980 of water activity (a_W), while with 1.5 mM the same behavior was observed now at 0.950 and 0.930 a_W depending of strains tested. At 5 and 10 mM, the minors lag phases occurred in all strains. The different GP concentrations used increase hugely the mycelial development in all *Aspergillus* section *Flavi* isolated in dissimilar proportions respect to control according to herbicide amount and a_W condition. When the authors analyzed the effect of GP treatments on AFB_1 production showed that, mostly, AFB_1 accumulation exhibited a different behavior than the observed with growth rate. AFB_1

accumulation presented small differences between the several pesticide amounts tested at all a_W levels. A significant stimulation in AFB_1 production compared with control was exhibited in *A. parasiticus* with concentrations from 1.5 to 5.0 mM of GP at 0.95 $a_{W.}$ In *A. flavus* strain this fact was more noticeable at the same a_W condition at concentrations above 1.5 mM at 7 and 14 days of incubation.

8.6 Effect of Herbicides on Phytopathogen Fungi

Weeds decreased the effectiveness and quality of crops, spoil the culture and harvest techniques, the use of pesticides worldwide allows to infer that weeds are the pest that causes the greatest economic losses around the world. In addition to the target organisms, as a collateral action, the biological activity of the herbicides affects the plant–pathogen relationship, through a direct influence on the pathogen, the plant and the rhizosphere microorganisms, also influencing the symbiotic relationships between them (Kortekamp 2011).

Many works reported that the development of *S. rolfsii* and other pathogenic fungal species has been inhibited by GP, this fungi commonly survives on vegetal debris until the next seed time. Banana field workers detected that the debris that was sprayed early with the GP showed a few fungal mycelia and a poor sclerotium production that has not been sprayed with the herbicide. *In vitro* cultures added with glyphosate at recommended doses, diminished the *S. rolfsii* development, respect to the control treatments assayed; therefore, GP could be a greatest inhibitor (Westerhuis et al. 2007). Some fungi that cause diseases on plants, like *Pythium ultimum* and *Fusarium solani* shows a reduction in their growth, sporulation, and germination when the amounts of pesticides on the environments increased (Kawate et al. 1992; Sanogo et al. 2000).

Many cases have been reported about the restricted influence of GP on some leaf diseases in several crops. Feng et al. (2005) determined that diseases caused by *Puccinia striiformis* f.sp. tritici, *Puccinia triticina*, and *Phakopsora pachyrhizi*, can be reduced when GP-resistant crops are fumigated with commercial GP. These researchers suggested that when fungal conidia where on direct contact whit GP, the enzyme EPSPS from these fungi was inhibit, therefore, they described that can be occur the same mechanism that on weeds. Other authors has been proposed that glyphosate can decrease the fungal and disease development of *Septoria nodorum* on wheat (Harris and Grossbard 1979), *Rhizoctonia* root rot (Wong et al. 1993), these effects has been shows on *Rhynchosporium secalis* and *Drechslera teres* on barley (Toubia-Rahme et al. 1995; Turkington et al. 2001). There are also intra-specific variations in *Rhizoctonia solani* as shown by Verma and McKenzie (1985).

Wyss et al. (2004) described that some pesticides and their adjuvants influenced conidial germination and development of *Phomopsis amaranthicola*, a useful bioherbicide against *Amaranthus* species. Some herbicides such as GP present negative effects on conidial germination of *P. setariae* (Peng and Byer 2005).

Krzysko-Lupicka and Sudol (2008) investigated the permanence of indigenous fungal strains on soils with liquid GP. They found that the prevalent fungi detected was associated with the genus *Fusarium*: *F. solani*, and *F. oxysporum* and defined the relationship between the fungal strains and different amounts of GP. This work shows that the isolated examples have a minimal sensitivity to the doses of GP (0.5–2.0 mM) tested and when GP acting as the unique source of phosphorus (1.0–1.5 mM) the increment on fungal biomass was highly significant.

The effects of diquat, paraquat, GP, dicamba, trifluralin, chlorsulfuron, and chlorthal dimethyl at amounts of 0–500 ppm on the hifal development, vigor, and virulence of *Gaeumannomyces graminis* var. tritici (Ggt) on wheat were analyzed by Mekwatanakarn and Sivasithamparam (1987). These authors observed that, the total of pesticides tested, except for dicamba and chlorsulfuron, completely reduce the mycelial development on Potato Dextrose Agar medium (PDA) at 10–500 times over the recommended doses that applies to crops. The mycelial development of the pathogen fungi that grows out on PDA added with 100 ppm diquat + paraquat or GP was decreased by 47.4% and 42.4%, respectively. Straw colonized by Ggt on agar amended with amounts of diquat + paraquat at all of concentrations of GP tested produced a minimal root disease in wheat seedlings. They proposed that the decreased virulence of conidia on agar added with these two herbicides, should be by the minimal colonization of the fungi on the culture medium, and in this way, this pattern of behavior could be extrapolated to the field.

Vargas de Álvarez et al. (2002) studied the relationship between the intensive use of GP and sheath blight of rice *(Oryza sativa* L.) incidence caused by *R. solani* Kuhn. The authors found that increasing concentrations of the herbicide caused fungal growth reduction, and the detrimental effect reached a plateau at 2500 mg l^{-1}. The expected amount of GP on soil after spraying at commercial dosages would be 0.75 mg kg^{-1}. These results do not support the view that GP, as a herbicide treatment in rice fields, may affect in any way the population densities of *R. sotaní*.

Zain et al. (2013) analyzed the soil mycobiota exposed to paraquat, GP, glufosinate-ammonium and metsulfuron-methyl at 0.5, 1, and 2 times over the recommended applies doses, and this experience was made on laboratory and on field soil. The authors show that in laboratory tests as well as in soil, the herbicides decreased significantly the mycelial development, and that exist a parallelism between this decrease with the increment of herbicides. The decrease percentage analyzed in the laboratory was in order of paraquat and glufosinate-ammonium >GP > metsulfuron-methyl. The strains decreased growth and effect of exposure time were assessment and they determined that a different behavior for fungal strains, pesticides, and their doses.

8.7 Conclusions

In view of the reported toxicological aspects of the most used herbicides, it is necessary to incorporate innovative agricultural systems to protect natural resources and also to increase productivity. This transformative process toward more ecological approaches needs the commitment of the different areas involved in the conservation of agriculture, which are also built upon traditional knowledge.

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9

Pesticides Usage, Uptake and Mode of Action in Plants with Special Emphasis on Photosynthetic Characteristics

Nivedita Chaudhary[1], *Krishna Kumar Choudhary*[2], *S.B. Agrawal*[3] *and Madhoolika Agrawal*[3]

[1] *Department of Vegetable and Field Crop Research, Agricultural Research Organization, Gilat Research Center, Negev, 85280, Israel*
[2] *Department of Botany, School of Basic and Applied Sciences, Central University of Punjab, Bathinda 151001, India*
[3] *Department of Botany, Institute of Science, Banaras Hindu University, Varanasi 221 005, India*

9.1 Introduction

Pesticides are an essential part of modern life because of their utilization to check the development of undesirable living organisms. Pesticides might be natural, synthetic, or organic materials which are used for prevention of cultivated crops, animals, shrubs, trees, timbers, home, or things that are having economic importance to human beings. Over past few decades, application of pesticides has increased tremendously, not only confined to agricultural sector but also widely utilized at homes and public places for prevention of various pests. Globally, pesticides consumption in various sectors is about 5200 million pounds per year (Mahmood et al. 2016). Usage of pesticides could be traced from Roman times, where sulfur, arsenic, salts, and ashes were used against different pests. Progressive development has been observed in this field from the utilization of honey and arsenic mixture for controlling ants in the sixteenth century to use of chemicals, i.e. nicotine sulfate and calcium arsenate during the eighteenth century (Mahmood et al. 2016). A major development in the pesticides industry has been observed after the discovery of DDT (dichloro diphenyl trichloroethane) in 1939, and later BHC (β-Benzene Hexachloride) and 2,4-D (2,4-Dichlorophenoxyacetic acid). Further, development of insecticides like organophosphate (the 1960s), carbamates (1970s), and pyrethroids (1980s) along with the introduction of herbicides and fungicides introduced in 1970s–1980s led to significant control of pests worldwide along with the increments in agricultural productions (Aktar et al. 2009; Mahmood et al. 2016). In India, pesticides production started in 1952 after the establishment of BHC plant in Kolkata, India is the second largest manufacturer of pesticides after China in Asia and holds a twelfth position at World level. In India, about 45% of total pesticides consumption is only for cotton followed by paddy and wheat crops (Aktar et al. 2009).

Pesticides provide protection to plants mainly by killing or suppressing the growth of pests, and also act as a repellent to certain pests, therefore "Pesticide" is a broad term as elaborated in Figure 9.1.

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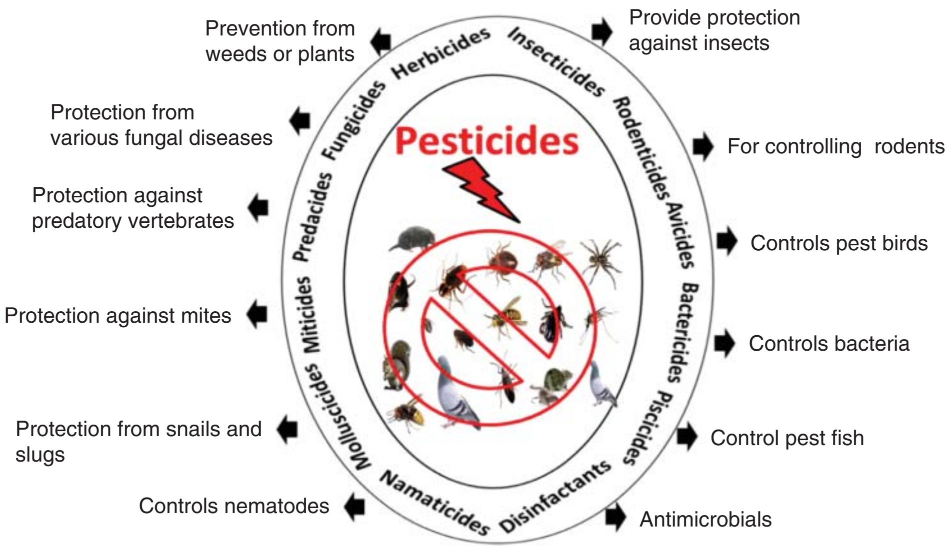

Figure 9.1 Various types of pesticides and their role.

Appropriate pesticide usage can protect our plants, animals, or houses from destruction. However, improper pesticides usage might lead to damaging effects on plants, human health can be impaired, and expected pest control could not be achieved. Besides this, pesticides also contaminate soil, air, and water. Before using any pesticides, everyone should be aware of their user manual and proper management.

9.1.1 Usage and Requirement of Pesticides on Plants

For the regulation of pest growth, pesticides can be used without causing harm to humans and plants. The term pesticide covers a wide range of compounds including insecticides, fungicides, herbicides, rodenticides, molluscicides, and nematicides (Perry et al. 1998). Among the vast range of pesticides, insecticides, herbicides, and fungicides, these perform on biochemical targets in weeds, insects, and harmful fungi (Casida 2009). Agriculture is one of the most important sectors of pesticides utilization. Before applying pesticides in agricultural fields, farmers are required to first explore whether the pest is really causing any harm to their crops or not, because in certain cases farmers unnecessarily use lots of pesticide products due to misidentification of problems on the plants, although the damaging effects on plants might be due to poor irrigation and drainage system. Furthermore, farmers should detect how many or what kind of pests are attacking plants, and further selection of which pesticide would be best suitable for the particular situation. After the identification of pest and selection of pesticides for agricultural fields, application techniques and its concentration also play an important factor for controlling the pest. Presently, most of the pesticides commercially available in the market and clearly labeled regarding its use and management, which should be strictly followed for better performance and safety.

"Prevention is better than cure," so regular inspection of agricultural fields is furthermost good practice. Occasionally, some pests present on the plants might not be the problem as there are several natural enemies also present for their eradication. Else, eradication could also be managed manually when there will be a low population of pest or at the onset of the problem to avoid the unneeded or excess use of pesticides. Although pesticides have beneficial protective roles, meanwhile they have side-effects also as they are toxic to other animals, birds, and humans. Therefore, pesticides usage must be reduced possibly, which will help to reduce the burden of additional expenditure on the agricultural sector along with reducing the risk of contamination to food, soil, water, and the environment (Maksymiv 2015). Also, farmers should be informed about the conditions, weather, or environmental factors that play a major role in affecting the life cycle of pests. Farmers should also be trained about pest management through changes or variations in agricultural practices.

9.1.1.1 Integrated Pest Management (IPM)

Presently integrated pest management (IPM) is becoming very popular among farmers, which is an effective plan developed for protection against the pest and also to avoid reliance on pesticides. A good IPM program must be focused on following aspects:

i) Identification of pest and monitoring of problems
ii) Selection of best management strategy
iii) Maintenance of records for evaluation program.

IPM practices should be broadened as they are very beneficial for the farmers as well as for the environment also. Some of the beneficial roles of IPM compared to pesticides are as follows:

i) IPM is very much needed for the balance of the ecosystem, as pesticides can alter the balance of ecosystem by destroying certain beneficial species.
ii) Pesticides might be ineffective as the pest became resistant, and also they may survive where the proper application is not done but IPM will be always beneficial.
iii) Pesticides are a major source of contamination of groundwater level along with soil and air. Moreover, these chemicals persist in the environment and pose harmful impacts on living creatures.
iv) Overall, IPM can prevent unnecessary expenses of pesticide and reduce the burden on farmers.

During implementation, one should keep in mind that the selected methods are effective as well as not harmful to people or environment. Natural control of the pest is the best way for sustainable development of agricultural sector. Several ways of pest management adopted by the agricultural sector are discussed below.

9.1.1.2 Cultural Control

Cultural control deals with the adjustment of environmental conditions for the host or pest, to avoid or suppress the infestation. Cultural control is done with the help of "cultural practices" and "sanitation." Cultural practices include selection of pest-resistant varieties, crop rotation, the suitable timing for sowing and harvesting, good irrigation management (Maksymiv 2015; Saeedi Saravi and Shokrzadeh 2011). Trap crops are also very useful in the prevention or reduction of the population of weeds, insects, mites, microorganisms, etc. Mulching is another satisfactory way for weed management. On the other hand, sanitation is done by eliminating the basic requirements of pests that are necessary for their survival, like shelter, food, water, etc. Removal of weeds that harbor pests is also a good practice along with their elimination before seed production. Infected plant stuff should be destroyed, also agricultural fields and surrounding areas should be checked for pest breeding sites.

9.1.1.3 Mechanical Control

This includes the use of machines, various devices, and a physical approach for supervising pests like traps, barriers, fences, nets, etc. (Maksymiv 2015). During cultivation, the use of tillage devices like plows, disc blades, rollers, cultivators, etc. leads to the destruction of weeds, and disturbs the soil environment beneficial for some harmful microorganism and insects. Exclusion techniques are also very useful for prevention against pests which includes fences and ditches for vertebrate pests and, wire or cloth mesh to protect fruit trees from birds. Further, traps are also mechanical devices that are frequently used for the relocation or destruction of pests. Sometimes sticky surfaces are maintained for trapping crawling insects (Maksymiv 2015; Saeedi Saravi and Shokrzadeh 2011).

9.1.1.4 Biological Control

Under this method, natural enemies are used for the destruction or regulation of the pest's population efficiently in several conditions (Maksymiv 2015). Natural enemies can be pathogens or insects that can manage certain weeds, fungus, insects, mites, etc. For example, predatory mites are useful for controlling spider mites that feed on plants. As they do not have long-lasting effects, so they must be released periodically. Also, rearing and culturing of various natural enemies are done commercially.

9.1.1.5 Genetic Control

This includes the conventional and molecular breeding programs for plants and animals to avoid/resist particular pest problems. Plant resistance to pests could be achieved by the transfer of genetic material from certain pest destroying organisms to hybrid seeds (Maksymiv 2015). These gene manipulation techniques are widely used and can be a promising tool for future pest management programs.

9.1.1.6 Chemical Control

Chemical controls involve the use of pesticides which are naturally derived or synthesized. Pesticides play very important role in pest management, and they are widely and frequently utilized by farmers. Pesticides are widely utilized because of their better effectiveness, high speed, and easy control of pests (Saeedi Saravi and Shokrzadeh 2011).

9.1.2 Generalized Mode of Action and Uptake of Pesticides in Plants

Functioning of pesticides in the plants is required to be operative, selective, and harmless; however, the beneficial effects of management of pest growth have to outweigh the human health, economic, and environmental costs. Selection of insecticides depends on the toxicity to insects only which causes damage to the insects, and are comparatively harmless to plants; meanwhile, herbicides intended to execute weeds essentially required not to harm closely related crops. Fungicides should control the fungus but should not inhibit the useful properties of plants. Therefore, in the existing scenario of pesticides usage on plants are intended to demonstrate a higher degree of organismal specificity thus, typical physiological and biochemical process of pesticides are required to sufficiently elucidate to understand usage of pesticides, its approach and function at the target along with the uptake, distribution, and degradation in the environment (Aktar et al. 2009).

Herbicides generally constrain the plant definite traits, obstructing the amino acid or fatty acid biosynthesis and photosynthesis to affect the particular plant considered as weed, therefore herbicides own the plant-specific pathways affecting photosystem II (PSII), acetyl CoA, acetolacetate synthase, acetylcholine receptor, Na-channel, therefore, it may also affect the growth and development of the plants rather than weed (Aktar et al. 2009).

Insecticides function depends mainly on the insect's neurotransmission and based on the insect's behavior or survival it is essential to immediately induce the action because of this the insects are attacked quickly within few hours and days, which comes under the category of limited biological range such as aphids or caterpillars. Therefore, insecticides need to show responses quickly which may affect major characteristics of plant physiology and biochemistry of the plants. Its performance is regulated basically through the cellular functions which are vital to the emergence of the hyphal tip and it similarly disrupts the oomycetes owning motile phases and operated by oomyceticides. Sometimes fungicides influence the plant resistance due to defense mechanisms and are able to persist conditions of energy depletion with the fungicide pretending more as a fungistat (Igbedioh 1991).

Now the use of pesticides on plants is significantly a matter of action to the species characteristics, amount of the applied pesticide, application timing relative to the age and growth stage, environmental conditions at the time of application and later the plant morphological characteristics also act as a major characteristic action during application of pesticides (Zacharia 2011).

Besides the plants growing point, its structure and form, pesticide uptake varies therefore, plants situated underneath the surface of the soil are generally protected from the contact with the pesticides. Treatment of plants with pesticides by spraying may also trouble to reach toward the plants as the droplets of pesticides are inclined to flow or recoil off and lose the track off slender, erect leaves as opposed to wide and flat leaves which easily uptake pesticides. Plant leaf morphology such as cuticles and thick leaf hairs or waxy surface may also decrease the pesticides absorption, undertaking, and movement into the leaves (Wang and Liu 2007). Therefore, according to the action of herbicides, the classification is done whether it kills the plant material non-selectively or selectively. In the case of the systemic herbicides the difference in translocation is shown and the desirable plant provides the selectivity. However, non-selective herbicides basically disinfect the soil and their action is possibly temporary, for example methyl bromide and more insistent high dose of rates of simazine or atrazine. Herbicides which are applied in soil are classified depending on the application timings such as pre-plant, pre-, and post-emergent treatments. Example of a pre-plant herbicide like glyphosate applied to plants may be able to execute all vegetation, and also considered to be extremely volatile, therefore it has to be integrated into the soil before planting the plants. Another example atrazine and diuron showed no effect on developed weeds but execute germinating seeds and their application before the emergence of seedlings, however, certain herbicides that have no effect on weed seeds and only selectively kill emerged weeds when applied post-emergence such as paraquat, 2,4-D, glyphosate (Zacharia 2011). Therefore, depending on these phases the intake of the pesticides occurs over the soil and foliage, most frequent mode of pesticide application is the foliar spray and also considered to be non-selective e.g. glyphosate, paraquat, diquat diesel oil (known as herbicide).

The consequences of the pesticide application on plants also depends on the manner of application such as band application used in the crops in between the plants; basal application at the basal part of the plants, broad application on the entire area, foliar application is the most common application on the plants; soil application includes insertion of pesticides in the soil. Soil incorporated with a pesticide can be a function of tillage, rainfall, and irrigation to proceed the movement of pesticides into the soil also include soil injection beneath the soil according to the space, spot, and tree basal place injection of the soil (Aktar et al. 2009).

Most frequently the mode of pesticide application used is foliar spray and its uptake depends on the type of pesticide in accordance to the plant age when treated to the leaf exterior surface where it affects mainly the process of translocation and the activity in the plant, susceptibility occurs frequently in younger ones than the older plants (Baker et al. 1992). Therefore, the absorption of pesticides in the leaves is mostly affected by the surface hairiness, leaf position, and existence or non-existence of a superficial waxy surface.

Environmental factors also influence the functioning of pesticides such as soil moisture, rainfall, wind, relative humidity temperature, and light (Waltz et al. 2004; Abhilash and Singh 2007). Additionally, due to imposition of water stress plants do not willingly absorb pesticides and due to very low relative humidity, increment in the evaporation from the surface of the leaf also reduces moisture content and create the conditions which are unfavorable to the foliar uptake. Besides plant growth in less water condition, soil developed thick cuticle to prevent moisture loss further affects the plants uptake of pesticides. Environmental factors also induce the significant influence on the pesticide translocation to the shoot via xylem because the water movement in plants from the exterior of mesophyll cells, into the intercellular space and finally through the stomata. Drought conditions or water stress conditions enhanced stomatal closing, further led to the limit the water movement to the shoot and reduce pesticide translocation within the plants. High light intensity also reduces pesticide efficiency to affect the plants as the photodecomposition of the pesticide, therefore for more diffusion to the leaf surfaces, it is more favorable to these certain pesticides in the dark, i.e. late spray in the day. High temperature usually favors absorption, even though volatilization of pesticide may increase. Besides this, the strong wind increases the drying of the surface of the leaves and reduces pesticide uptake (Caseley 1987; Datta et al. 2009) (Figure 9.2).

The herbicides are applied either to the soil or plant foliage, the absorption in plants are categorized from the movement to the surface into the plant and the process of penetration up to the plant tissues (Haage et al. 2007). Therefore, absorption of pesticides depends upon the technique of application and the type and part of the plant with which the chemical gets in the contact. Absorption through the soil and foliar application translocation varied in plants, former generally carried by the root or shoot of the emerging seedlings of the plants where mainly water, salts, and water-soluble pesticides are receives by root hairs and cortex, and the molecules of pesticides moved through xylem to foliage by the transpiration stream.

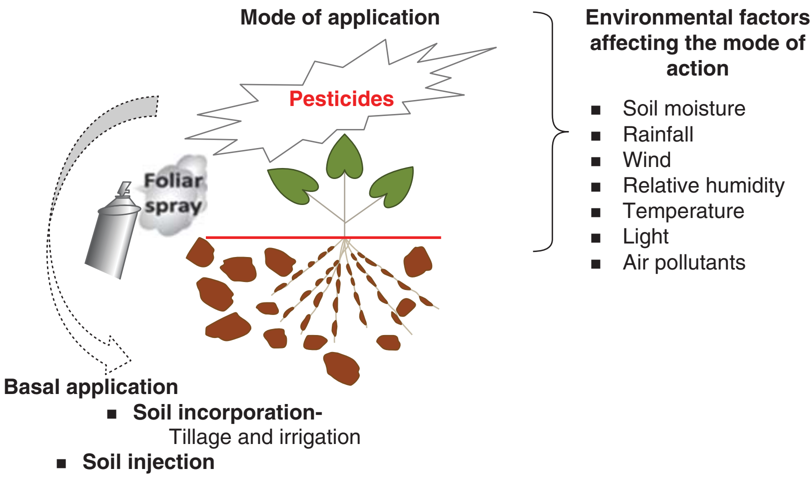

Figure 9.2 Mode of pesticide application in plants and environmental factors affecting uptake.

Absorption is also divided into passive and active types as like inorganic ions are primarily dependent upon the physical and chemical properties of the molecules. Many pesticides such as herbicides, insecticides, and fungicides applied to soil to control the weed. The typical application procedure involves treating the soil pre-or post-emergence of the plants or may be applied during planting or direct spray to the soil (Vryzas 2016). In plants, the main route of herbicide movement is xylem from the root toward shoot; besides if it is also absorbed through the shoot and do not need to move significant distances in the xylem to reach the specific site of action (Fedtke 1982). The root translocation of pesticides occurs mainly through xylem since it is considered to be the major water carrying tissue in the plants and correspondingly not comprises cytoplasm or plasma membrane and thus, makes the pesticide movement easier to cross over the Casparian strip, without any movement from the cell membranes. Therefore, mature leaves tend to receive more pesticides as it transpires more water and pesticides translocating toward shoot using the xylem tends to accumulate in mature leaves. For the proper movement of pesticide in xylem it is essential to not be extremely lipophilic or hydrophilic, former causes division into cell membranes and lipid bodies of the root, although later causes a problem in navigating around the Casparian strip. The passive entry of pesticide in plants is mainly through inter-cellular spaces and inter-connecting cell walls, together with the water or air-filled xylem elements regarded as a non-living system that can absorb water and the pesticides move with the water throughout the plant (Russell 1978). Pesticides correspondingly absorbed by developing shoots, seeds, tubers, rhizomes, and further vegetative parts of the particular plants like perennial weeds. Besides roots absorption and soil supplied herbicides such as atrazine and urea are also absorbed through shoots and kill the weeds.

Superficial or narrow application of pesticide application on plants will ensure greater selectivity for controlling shallow-rooted weeds then profoundly rooted plants (Hwang et al. 2017). The effectiveness of pesticide also depends on the its concentration, particularly in the zone usually considered to the depth of 2–8 cm of the soil surface. After absorption, the herbicides translocation is vital, as it is transferring from one part to

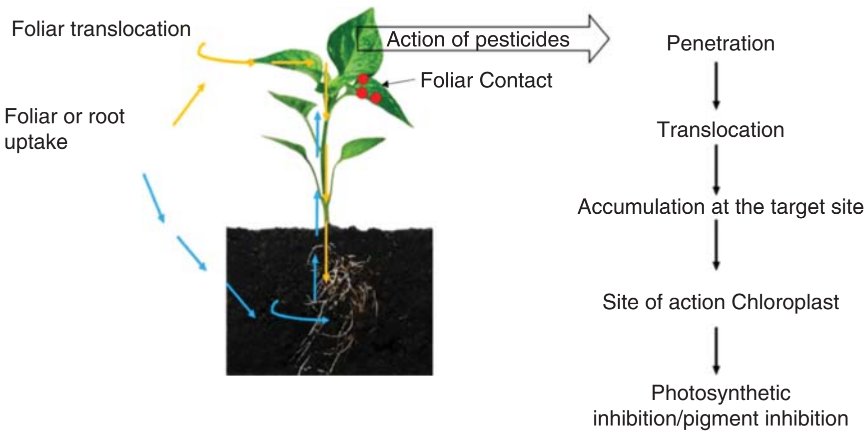

Figure 9.3 Uptake of pesticides and mode of actions in plants.

another within the plants. Therefore, pesticide translocation within the plant depends on the structure of plant and environmental conditions are essential to lead to the alterations for the physiological characteristics in the plants (Figure 9.3).

9.2 Effects of Pesticides on the Physiological Characteristics of the Plants

Pesticides are utilized to control pests affecting the vegetative growth and reproductive development of plants, however; they may induce impairment to humans or crops depending on their usage, dose, and their manner of action (Stevens et al. 2012). Pesticides may apply straight to the to the soil or grown plants, however if it is beyond the recommended doses and beyond normal rates, leads to significant effects on the physiology of the plants (Aktar et al. 2009). Pesticides are toxic materials at the target site, and the target proposed to be physiologically active only when it reaches inside the target organism. Pesticides performance also depends on the dose causing relative toxicity to pests and non-toxicity to the treated plants. Therefore, numerous pesticides affect the plant physiological processes, such as leaf photosynthesis, stomatal conductance, transpiration, respiration which may lead to affect plant's growth and development leading finally to the reproductivity of the plants (Dias 2012). Pesticides cause detrimental consequences on plant physiology dependent on the doses of application and uncertainty of a high dose has significant effects on plant even at a single application being able to reduce the photosynthetic rates; therefore, the potential effects of an insecticide on crop physiological process should be evaluated or documented prior to being used to modify pest populations and damage in yield loss (Parween et al. 2016).

The photosynthetic process in the plants is the ability of plants to utilize light energy from the photon to extract the electron from water to create highly reducing compounds NADPH and adenosine triphosphate production (ATP) which are utilized for fixation of CO_2 (Singh et al. 2014). Therefore, considered as the light-dependent reaction of photosynthesis comprises the alteration of light energy into chemical energy to induce the production of the molecular oxygen (Lodish et al., 2000). The photosynthetic process occurs in the chloroplast where two photosystems (PSII and photosystem I PSI) work for the procedure of extracting the light energy and the consumption of the energy in the fixation of CO_2. The former process of conversion of light energy in the photosystems I and II is connected by the intermediate membrane complex called cytochrome *b6f*. Absorption of light is by photosynthetic pigments chlorophyll a and b for sufficient light harvesting. Later, Nicotinamide Adenine Dinucleotide Phosphate (NADPH) and ATP utilized for the CO_2 fixation by the enzymes ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) situated in the stroma of the chloroplast and the reaction is known as the carboxylation. Therefore, the energy produced in the form of ATP and NADPH during the procedure of light dependent reaction utilized for the carboxylation occurs in the chloroplast stroma and transformed to starch and sucrose occurs in the cytosol. Meanwhile, during the photosynthetic process photorespiration also takes place simultaneously, which consumes O_2 and converts RuBisCO into CO_2. These competing reactions are both catalyzed by RuBisCO, and both utilize ribulose 1,5-bisphosphate (RuBP). Therefore, CO_2 fixation reaction is favored by a high concentration of CO_2 and low O_2 pressures; photorespiration reaction while respiration occurs at low CO_2 and high O_2 pressures. This former physiological trait is fundamental for plants and is reflected by both photosynthetic rate and mobilization of carbohydrate reserves (Taiz and Zeiger 2002). Indeed, as plants depend on their capacity of carbon assimilation through photosynthesis for the growth, development, and entire plant vigor, however, the photosynthetic interruptions may reduce yield or development of the plant. Physiology of plants is affected by pesticides and designates that these pesticides may cause harmful effects on the plants particularly on the plant physiological characteristics (Varshney et al. 2012) (Figure 9.4). Alternation includes the decrease in photo-assimilate production which results in a diminution of both vegetative and reproductive growth characteristics of the crop plants.

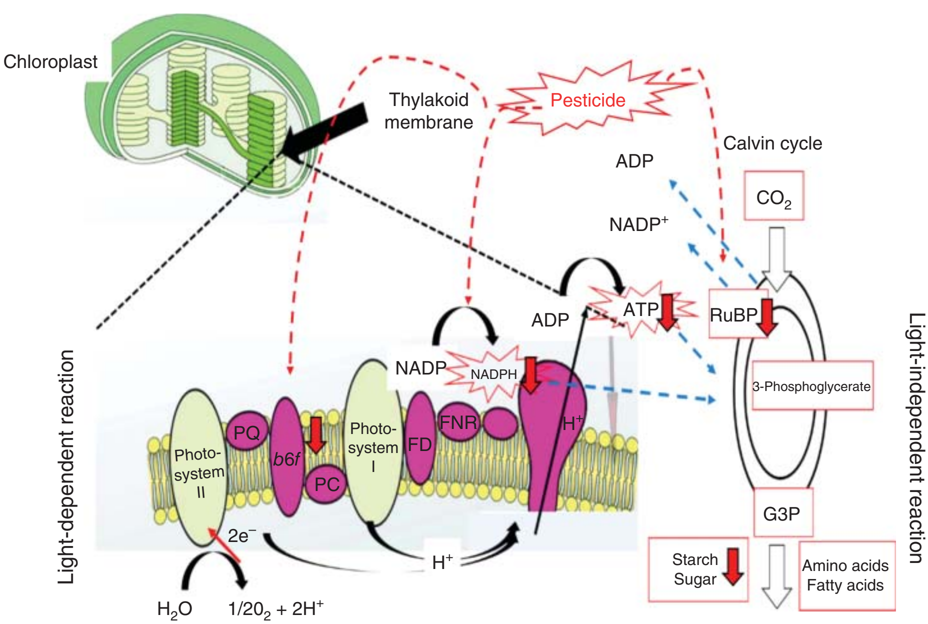

Figure 9.4 Target site of pesticides on dependent and independent reactions of the photosynthesis.

Commonly used fungicides are systemic fungicides also considered as remedial or eradication fungicides, function to destroy the fungus by subsequent penetration of mycelia into the parenchyma and discontinuing the dispersal or contamination within the plant, therefore, fungicides reaches to the active site of the plant meanwhile, fungicides application also contributes to nitrogen, and/or carbon metabolism later changes in the plant physiology lead to damage in the plant vigor (Yuste and Gostinear 1999).

The herbicidal action on plants is subject to the connections with a multitude of major biochemical reactions in the plants and is extensively held to possess the capability to affects the photosynthetic apparatus of the plant also respiration, phosphorylating process, and pigments (Ghanizadeh and Harrington 2017). Ghanizadeh and Harrington (2017) reported that herbicides affecting the physiology of the plant considered as the central series of metabolic reactions also offers as a specific target sites for an eminent number of commercially available herbicides. The initial effects of herbicides are reported by Moreland (1972) to define the constraining detrimental effects of pesticides ureas, triazines, uracils, and some amides and benzonitriles led to obstruction of the "Hill-reaction" of the plant physiology able even at low concentrations to disturb the characteristics of the chloroplast, particularly by the positive imino hydrogen and a negative carbonyl oxygen group of the herbicides. Studies also described that the plant canopy photosynthesis affected by pesticides particularly to the "Hill-reaction" (Kristevea and Kristev 1971).

Further, in the chloroplast the CO_2-independent reduction or else water splitting up process of photosynthesis, and separation the photosynthetic electron flow from phosphorylation, affected by the pesticides extended to inhibit ATP formation which later causes the suppression of energy transfer, interpreting disassociation of ATP into ADP and Pi (Younis and Mohanty 1980). Therefore, the prominent sites of action of pesticides are designated as PSII, therefore, Casida (2009) described that the PSI electron pathway is diverted by herbicides and perform as the reducing sites of PSI, considered the site I also affects the CO_2 fixation in the plants, therefore the action of pesticide localized at the initiation site of the electron transport chain between PSII and PSI. As the primary target, the consequence of herbicide on PSII causes inhibited and disrupts processes in electron transport and considered as the target site in the plants particularly denoted as the triazine, nitrile, and urea sites.

Also the early target pesticides also affects PSI electron transport pathway which contributed to the formation of singlet oxygen and causes lipid peroxidation, and disturbance of cell membrane (Murthy 1983). Therefore, pesticide particularly affects the protein D1 of PSII and able to block the electron transportation from PSII to plastoquinone which further prevents the conversion of absorbed light energy into electrochemical energy and extends the formation of triplet chlorophyll and singlet oxygen, which influence the peroxidation of membrane lipids. Therefore, photosynthesis inhibits by pesticides leads to affects the growth, physiological, and biochemical processes (Ghanizadeh and Harrington 2017; Petit et al. 2012).

9.2.1 Chlorophyll Fluorescence Affected by the Pesticides

Plant chlorophyll fluorescence characteristics is a vital physiological functioning and alternation in the chlorophyll fluorescence consider as a significant indicator of alterations in the photosynthetic activity (Maxwell and Johnson 2000; Srivastava et al. 2014). The condition of PS II provided by the fluorescence measurements, the electron movement through with PSII is the indicative under many conditions of the overall rate photosynthesis (Baker et al. 2007). Therefore, the light harvesting by the PSII antenna complex plant started the photophosphorylation starts by both the light collecting antenna complexes such as light harvesting complex (LHC) II and I related to PSII and PSI respectively, situated in the chloroplast thylakoid membrane of the leaves. The process induces transport of electron from water through a sequence of electron carriers to nicotinamide adenine dinucleotide phosphate (NADP), creating reducing power as NADPH and a H^+ electrochemical potential alteration within the thylakoid membrane and this proton motive force by the transition of H^+ back throughout the membrane over the ATPase initiates ATP production (Hillier and Babcock 2001).

The electrons route along a complex transfer chain finish with the formation of another active molecule, NADP, at that point ferredoxin is decreased by a photochemical reaction at PSI, and the ferredoxin–NADP+ reductase enzyme facilitates transfer of the electron to NADP with the formation of NADPH essential for CO_2 fixation, ATP, and NADPH further required for the next step, for instance, the energy taking reductive conversion of CO_2 into carbohydrates, and these reactions in a cyclic sequence are known as "Calvin-cycle." The important enzyme of the cycle, RuBisCO, is a prerequisite for CO_2 fixation and catalyzes the carboxylation of RuBP. It is substantially manifested that photosynthesis, which might be restricted by other biochemical processes occurs in the mesophyll, in accordance with the RuBisCO activity and RuBP regeneration (Krause and Weis 1991). Oxborough et al. (2000) reported that herbicide Diuron inhibits the efficacy as a result of which light absorbed by PSII antennae and used for photochemistry led to affect photosynthetic process across a leaf can be responsively demonstrated by images of PSII operating competence from a leaf (Figure 9.5).

Herbicides are also considered as the potent of PSII electron transport, preceding to the acceptance into the leaf lamina there was diminutive heterogeneity of the PSII functioning expeditiously across the leaf. PSII functioning efficiency in cells near to the vascular tissues, where the herbicide will be primarily transported to the leaf lamina and able to reduce to almost zero. Another herbicide Imazapyr designated to affect the efficiency of the fluorescence of the Arabidopsis and *Agrostis tenuis* seedlings efficacy of imaging fluorescence revealed that PSII efficiency factor altered similarly, the maximum quantum efficiency of PSII photochemistry also decreased by the treatment of herbicide imazapyr subsequently after the application of 24 hours (Barbagallo et al. 2003).

The photochemical reaction occurs at the PSII majorly showed that the transportation of electron and herbicides affects the secondary acceptor quinonen by shifting the redox potential toward negative values which leads to the inactivation of the acceptor (Idedan et al. 2011). Herbicides also displaced molecules present in the PSII and the quinone from the membrane regarded to be a protein which bound the plastoquinone causing obstruction of the photosynthetic flow of electrons. Herbicides are known to obstruct photosynthesis by adhering to the plastoquinone B (QB) binding site on the D1 protein of the PSII core complex, consequently preventing QB from binding at this position. The exclusion of QB from the binding site which blocks electron transfer from QA to QB, consequences in the limitation of electron flow in PSII. The block at PSII causes an increment in the fluorescence emissions from the chlorophyll proteins accompanying PSII (Mackay and O'Malley 2014). Various studies reported photosynthesis variations after

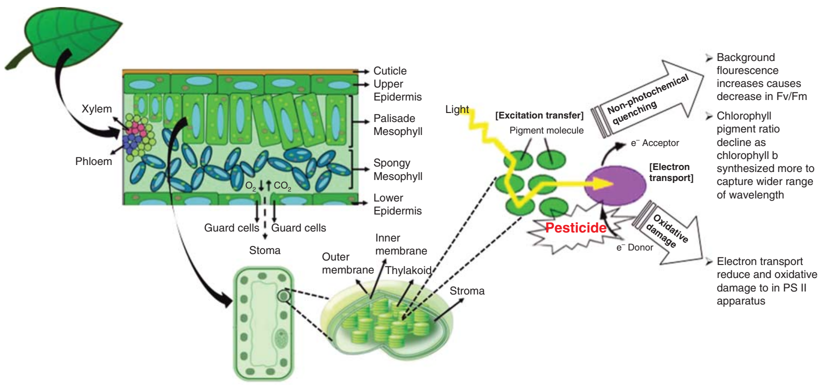

Figure 9.5 Effect of pesticides on chlorophyll fluorescence initiating oxidative stress.

the fungicide application on numerous crop plants which modify both photosynthetic activities and chlorophyll fluorescence (Petit et al. 2012; Xia et al. 2006).

9.2.2 Pesticides Affect Chlorophyll Content in the Plants

Pesticides affect the chlorophyll synthesis causing chlorosis of leaves because of ineffective biological synthesis of chlorophyll which ultimately affects photosynthetic mechanism (Su et al. 2017). Pesticide-induced chlorosis was reported by Arunachalam and Pandiaraj (1994) which correlates with the substantial reduction in the primary productivity and total chlorophyll of *Hydrilla verticillata* Royle with DDT. Similarly, loss of chlorophyll was observed in oilseed crops like soybean and mustard due to the treatment of DDT (Mitra and Raghu 1998). Pesticides presumed to be the chemical resulting in the disintegration of chloroplast structure (Singh and West 1967) might have inhibited the synthesis of protein in the chloroplast. Chlorophyll is structurally initiated from methyl phytolesters of dicarboxylic acid that comprise of porphyrin head with four rings linked centrally to Mg atom and a phytol tail ($C_{20}H_{39}OH$) along with an aliphatic chain of alcohol. Chlorophyll-a ($C_{55}H_{72}MgN_4O_5$) and chlorophyll-b ($C_{55}H_{70}MgN_4O_6$) perform solar energy absorption for the concern of photochemical reactions of the photosynthesis (Murray et al. 1986). Turkyilmaz and Esiz (2015) suggested the detrimental effects of an insecticide on two cultivars of maize. Harmful effects of pesticides were also observed on the chlorophyll contents of tomato and maize plants (Salem 2016). Another example of pesticide is use of carbendazim which results in the alteration to the foliar pigment concentrations further trigger to the photoprotection mechanism in plants through carotenoids (Garcia et al. 2002). Casida (2009) reported that carotenoids also get affected by the application of the pesticide on the plants. Carotenoids help in overactivation and destruction of light which helps in the protection of the photosynthetic pigments and the application of herbicides targeted to bleach the carotenoid, for instance, phytoene desaturase deactivated by the herbicides generally and Lycopene cyclase was inhibited by amitrole. Rózsavölgyi and Horváth (2008) described the impairment in the photosynthetic system contributes to the reduction of almost half of the chlorophyll content due to the application of pesticide on plant pea.

9.2.3 Effect of Pesticides on Photosynthesis

Application of pesticides on plants alters the physiology, in the form of growth and development of plants along with the metabolism of nitrogen, and carbon in the plants (Saladin and Clément 2005). Besides, certain pesticides lead to the alteration in the photosynthesis via stomatal closure, whereas some pesticides may affect the functioning and structure of chloroplast and able to manipulate the carbohydrate metabolism (Gomathinayagam et al. 2007).

Pesticides induced inhibition of photosynthesis in the plants also caused oxidative stress and consequently altered the source-sink association. Reduction in photosynthesis later causes the inhibition of growth of the plant further decreases carbohydrate in the plants and finally leads to a reaction showing the inhibition of photosynthesis in the plant as the feedback (Caspi et al. 1999; Vinit-Dunant et al. 2002).

Pesticide induced inhibition of the photosynthetic process during light-independent reaction particularly causing the disturbance in the processes of transmission of electrons to a low molecular quinone called plastoquinone. The inhibition occurs by the binding of the inhibitor to a specific protein D1 that regulates electron transfer.

Most of the destruction caused by the copper-containing pesticides which is a major component used together as the pesticide preventing of pest growth in plants and in developing various diseases (Petit et al. 2012). However, naturally present copper in the plants considered as an important microelement used in the photosynthesis in the form of protein, plastocyanin, and respiratory electron transport chain (Barón et al. 1995). Inhibition of photosynthesis may occur after use of pesticides containing copper and its application causes the disturbance in the photosynthetic process particularly by the alteration in the ultrastructure of the chloroplast (Baszynski et al. 1988) and chloroplast membrane (Szalontai et al. 1999), therefore, PSII gets affected during the light-dependent reaction of the photosynthetic process (Barón et al. 1995) Pesticide causes sensitivity of PSII by increasing inactivation due to the generation of photo-inhibitory effect leads to the action which gets localized within thylakoid membrane particularly PSI and the ferredoxin-dependent reactions and finally, a direct suppression of the enzyme RuBisCO (Petit et al. 2012).

Alongside copper, sulfur is commonly used as the element of fungicides for the supply of nutrient and protection of plants against fungal infections. Besides these, sulfur may probably be able to cause phytotoxicity in various plants. Studies conducted on the apple trees revealed that the photosynthesis is reduced due to the application of sulfur treatment. The pesticide, cuproxa which includes the copper sulfate exhibited the phytotoxic effects on the cucumber plants by the reduction in photosynthesis accompanied by the stomatal conductance and intercellular CO_2 (Xia et al. 2006). Fludioxonil (fdx) is a fungicide applied in the vineyards and disturbs the photosynthetic pigments (chlorophyll contents and carotenoids) after the application of fungicide which appeared to be effective according to applied dose (Saladin et al. 2003). Many other fungicides which are commonly used may show phytotoxicity, for instance, Benomyl which is a broad-spectrum fungicide commonly use in agriculture, benomyl breaks down to n-butyl isocyanate which may afterward respond to produce n-butylamine or N-N0-dibutylurea (DBU) later the by-products of benomyl undergo degradation also contributed to the phytotoxicity of the fungicide (van Iersel and Bugbee 1997). Furthermore, DBU is considered to be phytotoxic and its application in the plants through soil drench. Shilling et al. (1994) reported that cucumber photosynthesis affected by the application of DBU which causes negative effect on the PSII; although it increases the chlorophyll fluorescence, and, reduces oxygen production results into the photo-induction causing the reduction of the ferricyanide and NADP. ATP synthesis also gets affected by the fungicides comprising beta-methoxyacrylate compounds and leads to inhibit the respiration in fungi by blocking the electron transport. Coincidently, plants supplied with these fungicides resulted to lower the rate of transpiration, lower intercellular CO_2 causes lowering of photosynthesis compared with control plants.

Photosynthetic measurements in plants revealed the suppression of photosynthesis induced by the pesticide cyazofamid which gets associated with an increase of intercellular CO_2, resulting in the change due to non-stomatal factors (Xia et al. 2006). Besides this, another example of pesticides affecting photosynthesis is Triazole compounds, which have a phytotoxic effect on crops (Turkyilmaz and Esiz 2015) as well as stimulating an increase in the level of abscisic acid content resulting in inducing stomatal closure (Gopi et al. 1999).

9.2.4 Effects of Pesticides on Stomatal Conductance, Transpiration and Dark Respiration

Plant photosynthetic pigments absorb light, and along with the united systems of chloroplasts change light energy to chemical energy in the form of ATP. Pigments are considered as an important target site for pesticides as reported by Petit et al. (2008) where the fungicide fludioxonil (Fdx) applied on *Vitis vinifera* L. affects the reduction of the process of the fixation of CO_2, after alterations in function of stomata, interruption in carboxylation of rubisco and/or RuBP regeneration, and activity of PSII. Fungicide also reduced the photosynthetic rate very rapidly according to the dose of the application as it disrupts the rate of non-cyclic electron transport which is essential to maintain photosynthesis and the disruption in the stomatal closure which correlates to the reduction in the CO_2 fixation (Petit et al. 2008). Inhibition of the photosynthesis due to decrease in the RuBisCO caused by the application of fungicide (Tort and Türkyilmaz 2003).

In *Cucumis sativus*, pesticides caused reduction in net CO_2 assimilation as reported by Xia et al. (2006) due to alternation in the stomatal conductance and intercellular CO_2 concentration. The stomatal responses to pesticides may also induce changes in mesophyll, therefore, photosynthesis changes moreover by perceiving fluctuations in the concentration of intercellular CO_2 or C-fixing substrate (Nason et al. 2007). In addition, it has been reported by Haile et al. (2000) that spray of insecticides on lettuce plants showed the reduction in the rate photosynthesis and transpiration. Transpiration is directly connected to the stomatal opening, therefore, the reduction at the opening of the stomata leads to affect the intake of carbon dioxide for photosynthesis lead to the less fixation and sugar/carbohydrate production, the interrelated process cumulatively affects the plant's growth and development. Formation of ATP is a complex process occurs by the mitochondrial respiratory electron transport chain which involves a sequence of five membrane-bound and pesticides interrupt numerous sites by binding and inhibition or prevent oxidative phosphorylation and formation of the proton gradient (Casida 2009). Therefore, alterations like increment and later reduction in dark respiration were reported by successive application of fungicides mancozeb and flusilazol on *Malus domestica* (Untiedt and Blanke 2004). The increment in dark respiration can be elucidated by extra energy requirement, metabolic breakdown of the compound, and/or initiation of the substitute, cyanide-insensitive, respiration. However, some reports suggested inhibition of respiration in *Triticum aestivum* and in *Spinacia oleracea* (Nason et al. 2007). Besides, after their application on crops, residual of pesticides remains in the soil and may affect the root physiology such as root respiration.

9.3 Beneficial and Detrimental Effects of Pesticides

Globally, there is extensive pressure on farmers to satisfy the high demand of food. To fulfill this demand the use of pesticides is very common in agribusiness and it is anticipated to be increased in the near future. Due to several technological advancements in agricultural sector and developments of new pesticides for various problems this has facilitated farmers for better management of large farming areas with reduced labor cost (Bolognesi 2003; Saeedi Saravi and Shokrzadeh 2011).

9.3.1 Beneficial Effects

Remarkable benefits of various pesticides have been realized in public health, domestic sphere, forestry, and also in the agriculture sector globally. A significant example of benefits is related to malaria control which was accountable for 5000 deaths per day (Ross 2005). In India, food production tremendously increased by 50–198 million tons between 1948–1949 to 1996–1997 through 169 million hectares of cultivated land (Aktar et al. 2009). These results were obtained due to release of high-yield varieties, advancement in irrigation and agricultural technologies along with the development of agricultural chemicals (Aktar et al. 2009). Pesticides play a vital role toward the agricultural sector by protecting harvestable produce from weeds, disease, insect pests, etc. Pesticides provide direct benefits to the agricultural sector in various forms including immediate safety to crops, animals, and people. Good pesticide management had contributed to better quality of crops or livestock as well as controlled invasive species, low disturbances to the soil along with reduced inputs of fuels and labor cost. Pesticides benefits are not immediate but have long-term effects including global benefits dealing with food security. These effects include a boost in the global agricultural economy along with improved health and nutrition, also a significant contribution to improved life expectancy worldwide. Over the past 60 years, farmers achieved tremendous growth in food production and its quality through the use of pesticides, such as the production of maize in the USA (Kucharik and Ramankutty 2005), wheat in the United Kingdom (Austin 1999), as well as total yields of various crops in Russia (Cooper and Dobson 2007; Maksymiv 2015). Behera and Singh (1999) reported yield reduction in crops by 37–79% due to infestations of weeds. A significant decline was observed in rice by 28–48% (Behera and Singh 1999). Therefore, herbicides proved beneficial for farmers economically by reducing labor cost. Warren (1998) and Webster et al. (1999) also affirmed that without pesticides the world will face extensive economic losses.

9.3.2 Detrimental Effects

Pesticide use is also of serious concern to the environment, human, and animal health. As food, air, water, and soil get contaminated with these toxic chemicals. Several reports revealed that 98% of insecticides and about 95% of herbicides reach to non-targeted species along with water, air, and soil (Maksymiv 2015). In addition to protection against targeted insects/weeds, pesticides could be toxic to other organisms like birds, insects, fish, plants, and microorganisms. The toxic effects of pesticide on living being are through ingestion, breathing, or absorption by the skin. Continuous exposure to these pesticides may cause neurological disorders, abnormalities in physiology and behavior, genotoxicity leading to cancer, hormonal imbalances, defects in immune and reproductive system, etc. (Abarikwu et al. 2009; Bolognesi and Merlo 2011; Kubrak et al. 2012; Lushchak et al. 2009; Maksymiv et al. 2015). DNA damage has been seen due to exposure of 2,4-D in ovary cells of Hamster (Gonzalez et al. 2005). Evidence suggested that pesticides imposed harmful impacts on biochemical parameters including protein metabolism. Contamination of ground and surface water are of serious concern to aquatic flora and fauna, along with the human health. Heavy application of pesticides leads to contamination of soil, causing a decline in the population of beneficial soil microbes (Aktar et al. 2009). Overuse of these chemicals affected both bacterial and fungal population of the soil led to a severe loss of soil fertility (Aktar et al. 2009). Contrary to this, about 80–90% of applied pesticide gets volatilized in few days and is mainly accountable for the pollution of air and atmosphere (Majewski and Capel 1995; Aktar et al. 2009). Therefore, ideal pesticides should be developed that do not have any adverse effects on non-targeted organisms. There are certain pesticides which are safe and effective in use, but improper use can be lethal for living beings. As per WHO (2001), about 3 million cases per year reported for pesticide poisoning worldwide and about 2.2 million people of developing countries are at high risk of pesticides exposure (Aktar et al. 2009).

9.4 Conclusions

Pesticides are beneficial for the farmers and also to other peoples of society worldwide, as they contributed significantly by increasing agricultural yields and safety against pests through several ways. Besides being beneficial to crops against pests, pesticides possess harmful effects also, as they have a serious concern to human health as well as being accountable for environmental pollution. Therefore, IPM techniques should be promoted for pest control to minimize the dependency on these chemicals. Further, development of biopesticides should be encouraged that do not have harmful effects on non-targeted organisms, which will be beneficial for the plants along with the human health and environment. Peoples should be educated regarding the benefits, harmful effects, and proper use and disposal methods for pesticides to avoid their misuse. Moreover, regulatory authorities of government should ensure that pesticide products available in the markets are well labeled, containing all necessary information for safer use of pesticides in order to protect human health and environmental contamination. Researchers should be more concerned for formulations of eco-friendly pesticide which is the demand of the whole world in the present and future scenarios.

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10

Botanical Pesticides for Eco-Friendly Pest Management: Drawbacks and Limitations

Christos A. Damalas and Spyridon D. Koutroubas

Department of Agricultural Development, Democritus University of Thrace, 68200 Orestiada, Greece

10.1 Introduction

Ensuring sufficient food security to feed the ever-growing world population, while the production environment and the natural resources are continuously shrinking, is a great challenge of humankind today. The food situation is of major importance especially in developing countries, facing low productivity and huge food losses each year by pests and diseases (Parmer and Walia 2000). Over the centuries, crop protection by various pests has been a constant concern. Many of the earliest tools of crop protection were extracts of plants used for protecting crops during production and storage. Several plants were utilized as a source of commercial pesticides, but from 1940s, synthetic chemicals took over from botanicals in the market. Therefore, research on natural products for use in agriculture has been neglected for many years, but is now increasing, as natural products have great potential to play an important role in future crop protection for eco-friendly pest management.

Chemical pesticides continue to play a dominant role in world agriculture, but various new strategies for pest control are emerging. While modern agriculture produces high yields, more often than not, it is not sustainable. Over-reliance on pesticides, for example, had several unforeseen side effects, including contamination of surface- and ground-waters, destruction of the equilibrium between common crop pests and natural enemies, rapid development of resistance in pests, increased insect outbreaks, environmental and food chain contamination (Damalas and Eleftherohorinos 2011). Moreover, acute pesticide poisoning has emerged as a public health concern globally, with thousands of deaths every year (Goel and Aggarwal 2007). Adverse effects on non-target organisms, such as domestic animals, wildlife, and aquatic systems also occur. In recent decades, there is a distinct propensity toward a "trek back to nature" especially in the fields of pesticides and pharmaceuticals. The need for safer and more natural pesticides than the widely used chemical pesticides is now accepted without any serious contention. Therefore, a great interest in natural products, especially in plant extracts and oils, is becoming notable (Dimetry 2012; Kumar et al. 2013; Kedia et al. 2015; Xiong et al. 2016; Chaubey et al. 2017). Thus, botanical pesticides, namely, plant extracts or purified substances from plants attract great attention.

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Nevertheless, a lot of work is still required before large scale use of botanical pesticides could be enacted. Several plants exhibit some antifeedant activity or repellency to crop pests, regulation of insect growth, toxicity to other arthropods and invertebrate pests of agricultural importance, including antifungal, antibacterial, or antiviral properties against various plant pathogens (Dimetry and Abd-El Salam 2005; Dimetry 2014). Despite their advantages for eco-friendly pest management, the development and use of botanical pesticides on a large scale remains low (El-Wakeil 2013), indicating problems in their acceptance. The purpose of this chapter is to give an overview of botanical pesticides for pest control especially for agricultural pests and underline drawbacks and limitations in their use.

10.2 Overview of Botanical Pesticides

Botanical pesticides or biopesticides are substances derived from animals, plants, bacteria, or specific minerals used for the management of crop pests (EPA 2017). Currently, the biopesticide group comprises a small part of the market of crop protection products globally, reaching a value of about $3 billion and holding merely 5% of the relevant global market (Marrone 2014; Olson 2015). Over 200 products are available in the US market compared with 60 similar products in the EU market (Kumar and Singh 2015). Although the use of biopesticides is increasing at a global scale by almost 10% every year (Kumar and Singh 2015), it appears that the market of biopesticides globally should further increase, if this pesticide group is going to perform a visible role in replacing currently used conventional pesticides or reducing over-reliance on these chemicals. The use of biopesticides is estimated to outpace that of synthetic chemical pesticides, with compounded annual growth rates over 15% (Marrone 2014). It is projected that the use of biopesticides will equal the market size of synthetic chemical pesticides between the late 2040s and early 2050s, but variable rates of uptake, especially in Africa and Southeast Asia, may account for significant flexibility in those projections (Olson 2015).

Although slight differences in the classification of biopesticides may occur, according to Chandler et al. (2011), biopesticides are grouped into three major classes: microbial pesticides, biochemicals, and semiochemicals. Microbial pesticides are products that are made from beneficial microorganisms as the active ingredient. These types of pesticides can be effective on different pests, but each active ingredient is effective on specific target pest(s). For example, some fungi can control certain weeds, whereas other fungi can kill specific insects. The largest part of the microbial biopesticides now available in the global market is developed from only one entomopathogenic bacterium, *Bacillus thuringiensis* or Bt (Kumar and Singh 2015). Strains of Bt can produce a mixture of proteins with a capacity of killing one or a few larvae of different insect species. Thus, some Bt proteins are effective on some moth larvae commonly found on plants, whereas other Bt proteins are effective for larvae of flies and mosquitoes. Biochemicals include secondary metabolites that can impede herbivores from feeding on plants. Some of these secondary metabolites can be used as biopesticides (Figure 10.1).

This kind of pesticides includes pyrethrins, compounds produced by *Chrysanthemum cinerariaefolium* with insecticidal activities, as well as other compounds (Silverio et al. 2009). Biochemicals have low toxicity on mammals, but they decompose relatively shortly after application. Thus, the short residual action (persistence) of these

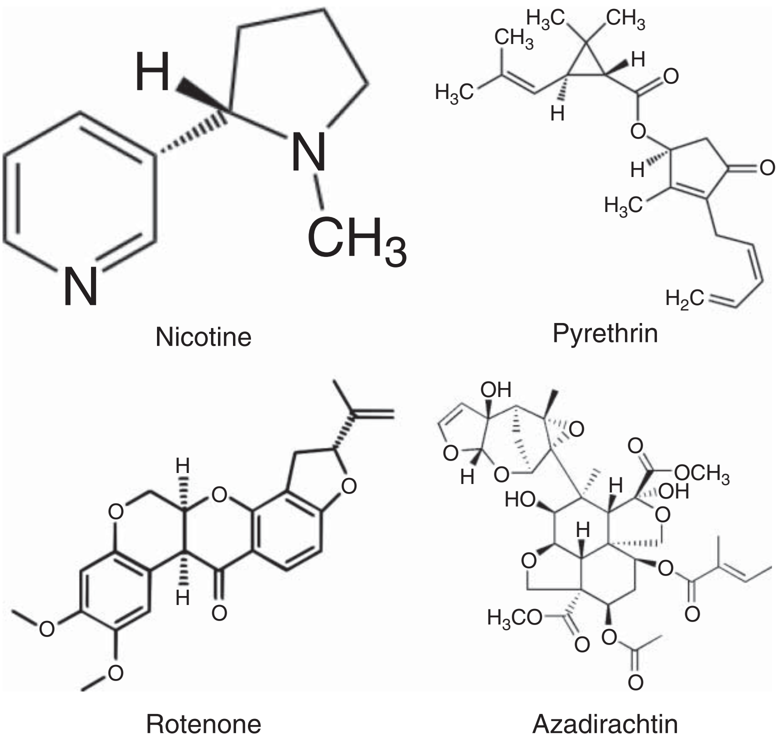

Figure 10.1 Common compounds used as botanical pesticides.

compounds led to the development of synthetic pyrethrins (pyrethroid insecticides). The most well-known botanical compound is neem oil, a substance with insecticidal properties that is produced in the seeds of the tree species *Azadirachta indica* (Campos et al. 2016). Semiochemicals are chemical signals by an organism that cause a behavioral change in an individual of the same or a different species. The most common semiochemicals used for crop protection are sex pheromones of insects. Some pheromones can be synthesized now and are routinely used for monitoring or pest control by mass trapping (Reddy et al. 2009), lure-and-kill systems (El-Sayed et al. 2009), and mating disruption. The latter (mating disruption) is used in large areas worldwide and has been useful in orchard crops (Witzgall et al. 2008).

Biopesticides possess several attractive properties that render these substances desirable components of an Integrated Pest Management (IPM) program. Most botanicals are selective, they produce little or no toxic residues, and their development costs lower than those of conventional chemical pesticides (Hajek 2004). Therefore, biopesticides are attracting great interest on the basis of their target-specificity, efficacy, biodegradability, environmental safety, and suitability in IPM programs. In this regard, biopesticides are promising alternatives to managing environmental pollution by synthetic chemical pesticides (Figure 10.2).

Though potential contribution of biopesticide use in environmental safety of pest control is well known, new interest in view of the growing demands for organic food has emerged. As the use of agrochemicals in modern crop protection seems

Figure 10.2 Biopesticides as alternatives for eco-friendly pest control.

indispensable to satisfy the increasing demands of food, there are now opportunities in selected crops where biopesticides can be used as a component of an IPM program. In this regard, the environmental safety of crop production can be largely enhanced through the wide application of biopesticides for crop protection. Biopesticides can be applied with existing spraying equipment of farmers and many of them can be produced locally. Nevertheless, the commercialization and adoption of new biopesticides in the market place has seriously lagged behind (El-Wakeil 2013; Isman 2015). Lately, strict regulatory regimes are relaxing some requirements for "low-risk" biopesticide products, thus facilitating the availability of more botanicals into the market.

10.3 Drawbacks and Limitations

Research has demonstrated a high potential of numerous plant products as botanical pesticides. In reality, botanicals may have advantages, but they also show some drawbacks in practical use. Therefore, despite many desirable properties, there are obstacles that hinder the effective large-scale use of botanical pesticides (Pavela 2014). These impediments must be overcome so that the high potential of these substances can be utilized in practice. In this context, focused efforts are hoped to remove limitations in biopesticide production, approval, and use.

10.4 Quality of Raw Material

Numerous plants from different botanical families have been assessed for pesticidal potential so far against various pests (Parmer and Walia 2000). The production of the active ingredients in plants comes to a maximum concentration at a certain growth stage. Environmental conditions highly affect plant growth and secondary metabolite synthesis, but research on the optimization of production areas are limited (Dong et al.

2011). For example, the substance azadirachtin-A found in neem fruits is formed at fruit ripening. Hence, it is fundamental to collect plant material at proper periods for reaching optimum results. Moreover, fruiting time may vary considerably in different climatic regions. For example, neem seeds normally mature in May to August in the northern part of India, whereas in March to May in the south part of the country (Parmer and Walia 2000). The accumulation of active ingredients in plants reflects to a great extent the influence of the interaction of multiple ecological factors on plant growth during the growing season, apart from genetic factors. Therefore, certain secondary metabolites are composed, or their contents largely increase under specific growth environments. Evidently, several factors must be taken into account for securing the supply of raw material with high quality.

10.5 Product Standardization

The standardization of natural products into commercial products has been the biggest constraint in biopesticide production that largely hindered the potential marketability of botanical pesticides compared with conventional pesticides. To increase acceptance, biopesticides should be capable of providing a reliable level of efficacy on target pest(s) to the end-user and, therefore, adequate standardization on the basis of the quality and quantity of the active ingredient(s) is required. In refined products based on rotenone, pyrethrum, and neem this standardization has been achieved to a great extent, but crude preparations may have low contents of active ingredients, which results in variable efficacy on the target pest(s). Moreover, production of botanicals with consistent purity is difficult due to variability in the content of active ingredients of the plant parts produced in different geographical areas. Thus, the mixture of botanicals with various contaminants is critical and requires attention. For instance, neem seeds can be contaminated with aflatoxins owing to poor processing and storage conditions (Dimetry 2012). Moreover, crude plant extracts may contain a mixture of chemical substances from different chemical groups, some of which may not exhibit biological activity. Therefore, chemical standardization is essential for a botanical pesticide to be effective. An appropriate analysis to ensure the desired level of biological activity and the use of standard procedures for each particular class of chemical molecules is important. Usually, mixtures of closely related compounds are involved in natural defenses of plants against herbivory rather than a single toxicant alone. This is a well-known phenomenon among botanical insecticides. Technical-grade products of natural compounds (e.g. pyrethrum, rotenone, azadirachtin) usually contain several active constituents with pesticidal properties. In neem, two compounds (azadirachtin A and B) account for most bioactivity. Mixtures of active constituents, as commonly found in several botanical pesticides, may also be beneficial as regards pest resistance and behavioral desensitization, e.g. multiple compounds found in the neem extract may weaken the selection pressure on pests, preventing the development of resistance (Feng and Isman 1995). Over the past decades, a large increase in the number of publications concerning the use of neem oil to control agricultural pests is observed (Montes-Molina et al. 2008; Da Costa et al. 2014; Gahukar 2014). However, most studies have only focused on testing at the laboratory level (*in vitro*), probably due to degradation of this substance under field conditions and therefore it is impossible to draw conclusions on the *in vivo* biological efficacy of the formulations due to unpredictable effect of several environmental variables. Therefore, standardized procedures are required for the identification and purification of proper active ingredients, e.g. as regards different morphogenetic, physiological and behavioral effects.

10.6 Rapid Degradation

Most botanical pesticides are usually less effective than synthetic chemical insecticides and, therefore, require high application rates in the field to achieve equal efficacy. Moreover, most botanical pesticides can decompose within a few days and often even within a few hours, which means that these pesticides have to be applied more frequently (Guleria and Tiku 2009). The relatively short time of persistence due to rapid biodegradation or rapid release in the surrounding environment can limit an effective pest control level (Pavela 2014). Non-persistence can be a two-edged sword, i.e. environmental contamination is limited, but repeated application may be necessary to achieve effective crop protection. On the other hand, the rapid degradation of the active product may be seen as an advantage as it reduces the risk of residues on food, since it is rapidly decomposed and thus it is less aggressive on natural enemies. Some natural compounds are rapidly degraded by UV light so that their residual action is low. This fact calls for precise timing of application or more frequent applications. Little degradation of pyrethrin occurred over time under dark conditions, whereas in the light, rapid degradation from 100% to less than 1% of pyrethrin within five hours has been observed (Grdiša and Gršić 2013). The pyrethrins are slowly degraded in water and, therefore, can show toxicity on some fish and aquatic invertebrates. However, in the presence of microbial communities, the degradation is expected to be faster through oxidative metabolism (Gunasekara 2004). Thus, botanical pesticides are considered to have a desirable environmentally friendly profile compared to conventional pesticides and, therefore, they present a perfect substitute for synthetic pesticides due to their high biodegradability; however, their use is often limited due to their instability. Emerging technologies such as nanoformulations and microencapsulation could increase residual action of botanical insecticides (De Oliveira et al. 2014) and this is expected to increase their field use.

10.7 Short Shelf-Life

The limited shelf-life of current formulations and the short persistence times of the active ingredients once applied to crops restrict cost-effectiveness of botanical pesticides and discourage wide use, even if suitable alternative products to chemical pesticides exist. Efficient storage, particularly of microbial biopesticides, is necessary on the basis of inconsistent and seasonal nature of the existing demand (Mishra et al. 2015). Therefore, special facilities and skills are needed for efficient storage of certain biopesticides, which most producers, shopkeepers, and farmers do not possess. New formulations, which can prolong the shelf-life of biopesticide products, could increase their use in practice. Combining specific strain and plant species, soil type, and environmental conditions is of major importance. Formulation technologies have been used for improving the delivery, the shelf-life, and the field efficacy of biopesticides, thereby increasing the number of commercial biological control products (Leggett et al. 2011; Ravensberg 2011). Despite limited information in this area of research, several studies have reported the emergence of new formulations with precise time or location delivery and formulations promoting activity persistence (Kohl et al. 1998; Townsend et al. 2004; Lacey 2007; Nuyttens et al. 2009; Hunter 2010).

10.8 Raw Material Availability

Steady supply of raw material used for the production of biopesticides is essential for commercial scale production, which means that the source plant should be responsive to cultivation (Guleria and Tiku 2009). Some plant species providing the raw material for the production of botanical pesticides may require a proper propagation strategy, because these plants grow in limited populations in the natural environment and thus uncontrolled harvesting can be destructive. In light of the above, development in production of such plants might reduce the amounts of raw material harvested from the natural environment. This approach would be beneficial, especially for large companies that are often skeptical about the return of an investment on a product with unknown markets and unreliable raw material supply, uncertain patent issues, and often questionable efficacy. In this context, large agrochemical companies view various phytochemicals positively, for instance, for the production of new classes of insecticides, but eventually might not be interested in developing botanical insecticides because of the dependency on natural resources often in a foreign country. However, completely controlled production of certain phytochemicals through plant cell culture or callus culture may provoke major companies to increase interest in the direct development of natural product-based pesticides than in the past.

10.9 Safety of Botanical Pesticides

Safety of botanical pesticides should not be always considered self-evident. Issues of phytotoxicity with the application of some botanicals raise concerns. Some formulations of neem oil can be phytotoxic, e.g. to tomato at a level above 1% (w/w), even causing yield losses (Sharma et al. 2012). Some common botanical pesticides, such as rotenone and nicotine, are more toxic to some non-target species (e.g. humans and fish) compared with a number of synthetically derived insecticides. Based on the LD_{50}, an indicator describing the lethal dose required to kill 50% of the test animals, botanical pesticides are generally perceived to be safer than synthetically derived insecticides, despite the fact that some registered botanicals are toxic to non-target organisms, such as fish, beneficial insects, and mammals. The LD_{50} is expressed as milligrams (mg) of toxicant per kilogram (kg) of body weight and the lower the LD_{50}, the more toxic the compound is to humans. Some botanical insecticides have lower values of LD_{50} than some synthetically derived insecticides, e.g. carbaryl and malathion. For the production of botanical pesticides, research normally focus on natural toxins extracted from plants, but it should be kept in mind that "natural" does not necessarily imply "safe" or "non-toxic". Some plants, such as *Ricinus communis* as well as species of the genus *Taxus* and *Aconitum*, are highly toxic (Fu et al. 2006; Bonnici et al. 2010; Hernandez et al. 2010). Some substances used as raw material for botanical pesticides are toxics to certain pests, so safe use is of major importance, as these materials are used in stored foods for the control of storage pests. Despite this fact there is little published work on the toxicity of plants species with pesticidal properties on vertebrates. Possibly, the cost of commercializing those plant species are prohibitive and so official tests are not required (Pavela 2014). Some plants can affect growth and development of mammals (Belmain et al. 2001). Moreover, several botanical insecticides might be toxic on honeybees (Xavier et al. 2015). More data on botanical pesticides, both in terms of effectiveness and chronic (long-term) toxicity are required. Modern society shows a shift toward "green consumerism" with consumers' tendency to buy products that have been made in a way that protects the natural environment. In food production, this is translated into the desire for fewer synthetic ingredients in food, which may favor plant-based products in eco-friendly management of agricultural pests as botanical pesticides (Dimetry 2012).

10.10 Regulatory Approval

Regulatory approval remains a major obstacle to the commercial availability of new botanical pesticides. This is because synthetic pesticides and biopesticides are treated the same in many assessments. Therefore, the development of biopesticides so far followed a chemical pesticide model that inadvertently devalues the beneficial biological properties of biopesticides. In developed countries, the market for botanical pesticides is mainly based on uses in greenhouse production, which are considered limited to offer high profits to offset the regulatory costs of the manufacturers. This situation prevents many botanical pesticides from reaching the market where there is great demand. It is not surprising that some natural products may pose some risk and, therefore, an absolute safety level cannot be taken for granted (Trumble 2002). Moreover, the regulatory approval process in many developed countries has become time-consuming and often costly, so that few companies (e.g. multinational agrochemical companies) have the necessary resources to meet the requirements of the regulatory approval process for their pesticides (Thacker 2002). Consequently, botanicals will probably be used in niche markets, such as production in controlled environments or certified organic production. In developing counties, however, regulation (if exists) is generally looser, particularly for domestic food production. Advocating the use of unregistered crop protection products for which health hazards have not been established is certainly not justified, but there is reasonable evidence to suggest that, with few exceptions, botanical preparations are not hazardous to human health compared with currently used conventional pesticides and are of substantially lower risk.

10.11 Future Perspectives

Biopesticides are attracting global attention as a safe strategy for the control of pests, such as insects, plant pathogens, and weeds, while posing less risk to human beings and the environment. Given that several pests of agricultural importance have already developed extensive resistance to many conventional insecticides, it seems that the pesticide industry is lacking resources to provide new products to the market. Evidently, botanical pesticides with new modes of action that are not susceptible to the development of resistance are required. Thus, development of already known botanicals, but also screening more plants and isolation of new and novel bioactive molecules with pest control properties should be targeted in future research.

While several biopesticides are developed, more work is required to confirm efficacy and safety, addressing limitations such as minimal residual activity with improvements in formulation technology. Bioactivity of plant derivatives on various pests is continuously documented in the literature, but only few botanicals are used in developed countries and also few prospects for market expansion with new products exist (El-Wakeil 2013; Isman 2015). With reference to research efforts, an increasing body of literature on botanical pesticides is noted (Isman 2014), but much of this literature is highly limited regarding reproducibility and often cannot be compared with existing or future studies so that this knowledge can be translated to practice (Isman and Grieneisen 2014; Isman 2017). Having this in mind, greater efforts should be put to study the utility of the available plant extracts for crop protection under field conditions, in close collaboration with local farmers, because such studies can more useful than only laboratory tests.

Biopesticide development in developed countries followed a chemical pesticide model that inadvertently devalued the beneficial biological properties of biopesticides (Waage 1997). This regulation model can offer improvements in the formulation, packaging, and application of biopesticides, but does not facilitate efficient registration and, therefore, it needs to be altered, focusing on the study of biopesticides more from a biological perspective. It should be kept in mind that biopesticides come from nature and these objections do not apply in the same way; however, this issue needs to be fully recognized by the regulatory legislation, while specific benefits of biopesticides have been overlooked. In the EU, there is a major challenge for the authorities of the various member states to synchronize regulations, handle submission files for biopesticides, and better evaluate risks (Balog et al. 2017).

Botanical pesticides that will emerge based on new scientific evidence may enhance the adoption of different policy implementation in different countries. These policies may be different, but to avoid repeating the mistakes of the era of chemical pesticides, a primary target would be not to consider biopesticides as another set of "silver bullet" solutions for pest control.

10.12 Conclusions

Botanical pesticides can offer an effective and economically viable alternative to common conventional pesticides for the management of major agricultural pests. Much research in the recent decades provided high knowledge about new plant materials. It is time now that this work focuses on the most effective plant species to develop new improved products for wide use. Concurrently, weaknesses of the biopesticide sector also need to be addressed to promote wide adoption in practice. Multi-stakeholder networks should assess both science and technology policies toward botanical pesticides. These networks should provide appropriate guidelines on the advantages and drawbacks of botanical pesticides, which will promote the use of optimized technologies. Thus, apart from continuous scientific support, continuous production of botanical pesticides following commercialized propagation and cultivation with selection of high-quality propagating material needs proper promotion. Moreover, harvesting protocols, optimized product preparations, and clear information about the efficacy of certain plant species on specific insects are required. Assumptions about efficacy, safety, and proper handing could be linked with failure of this technology, unless strong guidance is provided.

Innovations in science and technology allowing more effective and safer products of botanical pesticides should be developed and then efficiently promoted to farmers. Emerging technologies, such as nanoformulations and microencapsulation could raise residual action of current or future botanical insecticides, and this could promote their use under field conditions. Scientists from multiple disciplines need to co-operate closely to offer a broad skill base that will drive the research forward. Addressing several drawbacks of botanical pesticides is required by all stakeholders (the scientific community, institutions, policy makers, and farmers) to ensure a better future for these pesticides. Creating awareness about how botanical pesticides work better, target pests, and how variability may be overcome as well as good practices for harvesting and application is needed. It is more than evident that scientists and policy makers must closely co-operate for developing safe products and reasonably priced approval regulations. Advancing our knowledge on the efficacy, conservation, and regulation of botanical pesticides remains a big challenge, but meeting this challenge will promote acceptance of botanical pesticides, improve safety in food production, and ultimately improve livelihoods in the long run.

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11

Pesticide Interactions with Foodstuffs: Case Study of Apple

Géraldine Giacinti[1,2], *Christine Raynaud*[1,2] *and Valérie Simon*[1]

[1] *Laboratoire de Chimie Agro-Industrielle (LCA), Université de Toulouse, INRA, INPT, Toulouse, France*
[2] *Centre d'Application et de Traitement des AgroRessources (CATAR), INPT, Toulouse, France*

11.1 Introduction

Economic and productivity issues, together with the continual growth of the world population led to the exceptional development of pesticide chemistry in the last half of the twentieth century. The excessive use of plant health products has recently been shown to have had many negative effects (Bonnefoy 2012; ORP, consulted on January 2016):

- Environmental problems, due to the lipophilicity of the molecules used, which persist in the soil, water, air, and biological tissues.
- The development of resistance in some organisms, such as insects (14 types of resistance in 1948, 224 in 1969 and more than 500 in 1990).
- Sanitary problems in animals and humans, such as reproductive problems in some species, colony collapse disorder in bees, the decline of predatory species (e.g. bald eagles, peregrine falcons) and health problems in agricultural workers and local residents exposed to pesticides and in the general population due to exposure via foodstuffs.

Nowadays, the amounts of pesticides used tend to decrease worldwide, but particularly in Europe, with the regular banning of molecules, the development of more efficient products and increases in awareness of the negative impacts of pesticides.

Treatments are applied such that the active molecules reach the surface of plants: (i) as vapor, (ii) dissolved in droplets (aerosols), or (iii) as solid particles (Riederer 1990). Plants accumulate abiotic molecules by absorption via the roots of treatments applied to the soil or in water, or by absorption via leaves, flowers, fruits, and stems (Sabljic et al. 1990) of aerial treatments applied as sprays.

The level of ecosystem contamination varies and is correlated with many different factors: the numerous complex factors governing membrane penetration (e.g. plant species, the physicochemical properties of abiotic molecules and of their formulation), volatilization – which is greater from the plant than from the soil in which humic substances act as sequestering agents – photodegradation, the drying of deposits on

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the plant surface due to climatic conditions and to the properties of the formulation used, and leaching in rainwater (Lichiheb et al. 2015).

Many studies have investigated the contamination levels of fruits, including apples in particular. Such contamination may have major implications for health, because apples are the third most frequently consumed fruit worldwide, after citrus fruits and bananas (France Agroalimentaire 2015). There are more than 10 000 varieties of apple worldwide today, with annual global production reaching about 71 million tonnes. China is responsible for 53% of total production, and Europe produces 11.9 million tonnes, with 62% of apple production concentrated in Poland, Italy, and France. In 2014/2015, Europe was the main source of apple exports, supplying 66% all apples exported. France exports 40% of its production (Guiavarch 2015a,b).

Apples are among the most heavily treated crops in France. The French Ministry of Agriculture uses the treatment frequency index (TFI) as an indicator of the intensity of pesticide use (Brunet et al. 2008). Mean TFI is about 40 for dessert apples, versus only 17 for other fruit crops, and less than 4 for field crops (Butault et al. 2010, 2011). This value highlights the particular importance of apple crops in France, with apples the most widely consumed fruit in France, ahead of bananas and citrus fruits.

Plants are matrices in which multiple chemical interactions with anthropic molecules occur. It is essential to understand the nature and structure of the plant matrix and the physicochemical characteristics of the molecules with which it interacts, to comprehend the processes involved in these interactions and exchanges.

11.2 Apple Biology

11.2.1 General Botanical Presentation

The apple belongs to the Rosaceae family and the genus *Malus* (Ziadi 2001). It is a deciduous tree that may grow to 6–10 m in height. Its flowers have five petals, five sepals, multiple stamens (the male reproductive system) and a *gynoecium* of five carpels (the female reproductive system) (Bowes and Mauseth 2012; Prat 2010).

The fruit of *M. domestica* Borkh. (Figure 11.1a) is a complex fruit, the flesh of which is formed by hypertrophic development of the receptacle fused to the ovary (Bowes and Mauseth 2012; Prat 2010). The walls of the ovary expand to form the mesocarp, at the center of the fruit. The epicarp, the fine visible line on the cross section of the fruit in Figure 11.1a, defines the limit between the mesocarp and the fleshy receptacle. The internal epidermis of the lodges becomes lignified and forms the endocarp, generally referred to as the “core.” Together, the epicarp, mesocarp, and endocarp form the pericarp. The outer surface of the apple is protected by a membrane called the epidermis, generally referred to as the “peel” or “skin.” In apples, the epidermis is covered by a protective extracellular layer of lipids called the cuticle (Figure 11.1b).

11.2.2 Plant Structural Biochemistry

The plant cell wall consists mostly of polysaccharides, such as cellulose, hemicelluloses, and pectins, but it also contains structural proteins, enzymes, and phenolic polymers, such as lignin and suberin. These compounds can be classified as proposed below (Colin-Henrion 2008; Simon 2009):

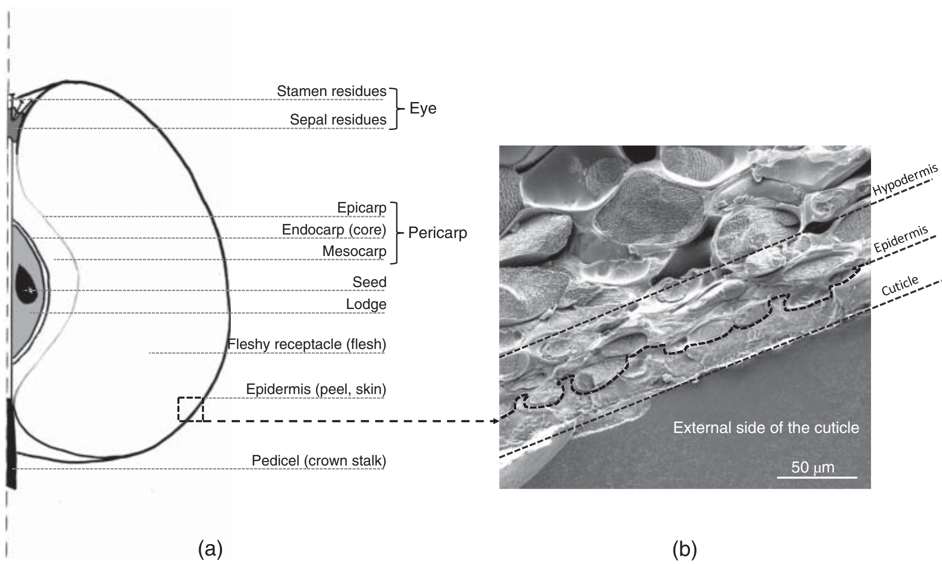

Figure 11.1 Anatomy of an apple fruit, in longitudinal section (a), based on (Bowes and Mauseth 2012; Prat 2010), and a cryo-SEM* micrograph of a cross-section of fresh epidermis from a Royal Gala apple obtained on a FEI Quanta 250 FEG electron microscope (FEI Company, Eindhoven, the Netherlands) operating at 5 kV (b) (*Scanning Electron Microscopy).

- *Permanent compounds*. Polysaccharides, such as cellulose, hemicelluloses, and pectins, which are synthesized in the cytoplasm of the cell and then secreted to form the cell wall,
- *Embedded compounds*. Lignins, minerals, polysaccharides, such as gums and mucilages, which accumulate among the cellulose microfibrils of the primary and secondary cell walls in place of the usual matrix compounds,
- *Deposited compounds*. Lipids, such as cutin, waxes, and suberin, forming an outer layer over the membrane that may disappear. This layer is reputed to be waterproof, limiting exchanges of water and gases.

The principal parietal polysaccharides of apple are pectins, galacturonic acid, galactose, arabinose, rhamnose, xylose, glucose, and mannose (Massiot et al. 1994), cellulose and hemicelluloses. The lipids form the cuticle, the protective outer film covering the plant epicarp or the epidermis of complex fruits like apple.

11.2.3 Chemical Composition of the Tissues of the Fruit of *Malus domestica* Borkh

Apple fruits consist of three principal tissues (Figure 11.1a):

- the epidermis (peel or skin),
- the receptacle (flesh or pulp),
- the endocarp (core).

Each has its own chemical composition: the epidermis consists mostly of lipids, such as cutin and waxes, and the receptacle is high in polysaccharides.

The water content of the fruit exceeds 70% and sugars make up 8–12% of its fresh weight. The sugar content of apple fruits has the following composition: 50% fructose, 18% glucose, 22% saccharose, 4–5% sorbitol, and 5–6% pentosanes and hexosanes. The total sugar content of the epidermis accounts for only 1.2% of the wet weight of the fruit. About 3–5% of the fruit wet weight is accounted for by cell-wall proteins, 0.06–0.12% of which are located in the epidermis; 2% of the wet weight consists of fibers in the form of cell-wall polysaccharides, and 0.6% is accounted for by organic acids, mostly malic acid, followed by citric, quinic, succinic, tartaric, and shikimic acids. The cell-wall polysaccharides of the receptacle account for 70–80% of the dry weight of the fruit. They have a high pectin content, containing about 30% galacturonic acid, and a high cellulose content, exceeding 30% (Massiot and Renard 1997). Lipids account for only 0.3% of wet weight of apple fruits because they are found solely in the epidermis, in which they account for 6% of wet weight, or 30% of dry weight (Campeanu et al. 2009; Colin-Henrion 2008; Massiot et al. 1994; Massiot and Renard 1997; Travers 2002; Veberic et al. 2005; Verdu 2013; Wu et al. 2007). Apple fruits contain little lignin, at only 200 mg per 100 g of unpeeled fruit (Colin-Henrion 2008; Marlett and Vollendorf 1994). The endocarp has a higher lignin content than the rest of the fruit, due to the lignification of the lodges protecting the seeds (Figure 11.1a).

Apple also contains 320 mg of minerals and oligoelements per 100 g of fruit. It also has high vitamin B and E and provitamin A and C contents. Vitamin C content varies from 2 to 25 mg per 100 g, depending on the variety, and the levels of this vitamin are four to six times higher in the peel than in the flesh. Many polyphenols have been detected in apple fruits: phenolic acids, mostly in the form of chlorogenic acid, coumaroylquinic and cafeoylquinic acids, flavanols such as catechins, epicatechins and B1, B2, B3, B5 and C1 procyanidins, flavonols such as quercetin, quercetin-3-rhamnoside, quercitrin, hyperin, isoquercitrin, reynoutrin, avicularin and rutin, dihydrochalcones such as phloretin, phlorizin, phloretin-2′-O-xyloglucoside, anthocyanins such as cyanidin and ideain, and coumarins (Awad et al. 2000; Bureau et al. 2012; Colin-Henrion 2008; Massiot et al. 1994; Travers 2002; Veberic et al. 2005; Verdu 2013; Wu et al. 2007). Most (65%) of the polyphenols present are located in the fleshy receptacle, 24% in the epidermis, 10% in the lignified endocarp (core), and 1% in the seeds (Guyot et al. 1998). Based on dry weight (mg per g dry weight), polyphenols are most concentrated in the epidermis, then the seeds, the endocarp and, finally, the fleshy receptacle (Awad et al. 2000; Henriquez et al. 2010; McGhie et al. 2012; Tessmer et al. 2012). Flavonols and flavanols, in particular, accumulate in the epidermis, whereas phenolic acids are found mostly in the fleshy receptacle. The mesocarp and seeds have a higher dihydrochalcone content than the epidermis.

The chemical composition of plant tissues is particularly complex because it depends not only on plant species and variety, but also on abiotic factors, such as water stress, UV irradiation, etc. as well as infestations of fungi or insects (Massiot et al. 1994).

11.3 Pesticide Inputs

Tree-based production systems, and intensive apple production in particular, initially involves the use of large amounts of pesticides. This pesticide use depends on many different factors, including pest risks (e.g. scab, *Gloeosporium*, blight, fire blight), the resistance of the plant variety, the different harvest periods that some could be rainy, the time interval between treatment and harvesting, the maximum number of applications for each pesticide, and the persistence of the molecules used.

Some of these factors may vary between seasons and geographic locations.

11.3.1 Chemical Composition of Pesticides

Pesticides are not used directly as such, but are applied as complex formulations of one or more active molecules together with additives designed to optimize penetration and the action of the active molecules on the plant surface (Herzfeld and Sargent 2011). The following types of additives are used:

- A carrier to dilute the active molecule, which may be a liquid solvent (often vegetable oils) in liquid formulations, or a solid filler, such as clay or talc in solid formulations (e.g. powders, granulates, pellets),
- Surfactants to optimize the contact between the formulation and the surface treated,
- Stabilizers, adhesives, emulsifiers, antitranspirants, dyes, repulsive agents, emetic agents and, sometimes, antidotes and other substances without biological activity in their own right, but capable of enhancing the activity and ease-of-use of pesticides.

11.3.1.1 Active Molecules

Active molecules have structures containing one or more chemical groups responsible for pesticide activity, and other chemical groups resulting in various degrees of hydrophilicity or lipophilicity, controlling penetration into the target organism. Hydrophilicity and lipophilicity properties are expressed as octanol-water partitioning coefficients.

Pesticides can be classified on the basis of their biological activity as principally insecticides, fungicides or herbicides, or on the basis of their chemical nature as organochlorine compounds, organophosphorus compounds, carbamates, pyrethroids, etc. (IRAC International MoA Working Group 2016; McBean 2012; Office des publications officielles des Communautés Européennes 2003).

11.3.1.2 Surfactants

Surfactants are used (i) to disperse lipophilic active molecules in water, to simplify their use, (ii) to optimize the spreading of droplets over the hydrophobic cuticle, and (iii) to enhance the penetration of active molecules, to protect the plant. Optimal efficiency depends on the nature and concentration of the surfactants added. Anionic surfactants are most effective in formulations for direct contact pesticides. Cationic surfactants must not be used alone because they are phytotoxic, and non-ionic surfactants are frequently used with systemic pesticides because they favor pesticide penetration across the plant cuticle.

11.3.1.3 Other Additives

Many other types of additives are included in pesticide formulations: adhesives, stabilizers, extending agents, plasticizers, buffers, drift control agents, antifoaming agents, thickeners, dyes, emetic agents, repulsive agents, and antidotes. These molecules are added to enhance the adhesion of active molecules to the plant surface, to reduce their leaching and the evaporation of spray droplets, to slow down their photodegradation, to optimize their penetration through the cuticle, to limit their chemical degradation according to pH, to increase spray droplet diameter, to limit foaming during spraying, to increase formulation viscosity, and to protect users from toxic hazards.

11.3.2 Identification of Pesticides Currently Used in French Apple Orchards

In France, in 2011, orchards were sprayed with a mean of 35 treatments, with treatment numbers ranging from 27 to 44 according to the region (Agreste Primeur 2015). More than 70% of the antifungal treatments applied were directed against scab. The risk of scab is particularly high in regions with high rainfall levels, such as the South West and Center/West regions. Codling moth and aphid management accounted for 70% of all insecticide treatments. Codling moth is particularly prevalent in southern regions, whereas the apple trees of the West (from central to southern parts of this region) and those of the Center region are attacked mostly by aphids.

Residues of 27 active molecules were identified in harvested apples from South West France, the leading region of France for apple production, in analyses performed in the 2011–2012 harvest season. Thirteen of these molecules were fungicides: boscalid ($C_{18}H_{12}Cl_2N_2O$), captan ($C_9H_8Cl_3NO$), carbendazim ($C_9H_9N_3O_2$), diphenylamine ($C_{12}H_{11}N$), dithianon ($C_{14}H_4N_2O_2S_2$), dithiocarbamates CS2, dodin ($C_{15}H_{33}N_3O_2$), fludioxonil ($C_{12}H_6F_2N_20_2$), pyraclostrobin ($C_{19}H_{18}ClN_3O_4$), sulfur S_8, tebuconazole ($C_{16}H_{22}ClN_3O$), thiabendazole ($C_{10}H_7N_3S$), and thiophanate-methyl ($C_{12}H_{14}N_4O_4S_2$). Nine were insecticides: four nicotinoids, acetamiprid ($C_{10}H_{11}ClN_4$), flonicamid ($C_9H_6F_3N_3O$), thiacloprid ($C_{10}H_9ClN_4S$), and thiamethoxam ($C_8H_{10}ClN_5O_3S$); two pyrethrinoids, cis-deltamethrin ($C_{22}H_{19}Br_2NO_3$) and esfenvalerate ($C_{25}H_{22}ClNO_3$); flufenoxuron ($C_{21}H_{11}ClF_6N_2O_3$), pirimicarb ($C_{11}H_{18}N_4O_2$), and spinosad ($C_{83}H_{132}N_2O_{20}$). The last five molecules are classified as acaricides: chlorpyrifos-ethyl ($C_9H_{11}Cl_3NO_3PS$), fenazaquin ($C_{20}H_{22}N_2O$), hexythiazox ($C_{17}H_{21}ClN_2O_2S$), propargite ($C_{19}H_{26}O_4S$), and spirodiclofen ($C_{21}H_{24}Cl_2O_4$).

11.4 Pesticide-Fruit Interactions

In apple, 90% of pesticide residues are found in the epidermis (Mota-Sanchez et al. 2012; Rasmussen et al. 2003). Chlorpyrifos-Me, for example, has mostly been detected on the outer surface and in the epidermis of apples (Riccio et al. 2006).

Hydrophilic pesticides have no great affinity for the lipophilic cuticle. They diffuse rapidly across it, to accumulate in the first layer of epidermal cells. Lipophilic pesticides have a higher affinity for the cuticle, in which they tend to remain for longer, delaying their diffusion into the underlying epidermal cells (Figure 11.2) (Liu 2004a).

In their 2012 study, Mota-Sanchez et al. (2012) treated two apple cultivars, Golden Delicious (low cuticular wax content) and Red Delicious (high wax content) with two different doses of pesticide (high and low). In both cultivars, 74% of the residues were located in the cuticle. Thiamethoxam and thiacloprid were concentrated in the cuticle and in the 2 mm of tissue immediately below the cuticle, regardless of the dose applied. However, higher levels of penetration into the 2 mm of tissue underlying the cuticle were reported for indoxacarb at the lower dose. For the higher dose of pesticide, very little

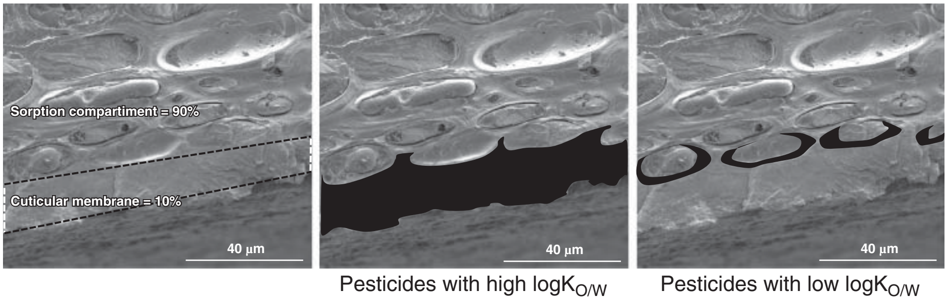

Figure 11.2 Location of lipophilic pesticides (high $\log K_{O/W}$) and hydrophilic pesticides (low $\log K_{O/W}$) based upon (Liu 2004a) on a SEM micrograph of a cross-section of Royal Gala epidermis obtained on a FEI Quanta 250 FEG electron microscope (FEI Company, Eindhoven, the Netherlands) at 2 kV.

residue was detected beyond the first 4 mm under the cuticle. By contrast, for the lower dose, residues were found right through the apple to the endocarp, particularly for thiacloprid.

The cuticle serves as a barrier in the plant, limiting the penetration of pesticides, even if applied at high doses. Most of the pesticide-plant matrix interactions occur during membrane transport in the epidermis.

11.4.1 Epidermis Structure and Function in Apple

Apple peel consists of successive layers of cuticle, epidermis and hypodermis (Figure 11.1b) (Maguire 1998). The cuticle is a complex extracellular layer that covers and protects leaves, primary stems, flowers, petioles, fruits, hairs, and seeds (Lendzian and Kerstiens 1991). It is synthesized by the epidermis of the fruits, primary stems and flower structures (Pollard et al. 2008).

Transmission electron microscopy analyses of apple cuticle have shown that the outer part of the cuticle consists of epicuticular waxes that cover a complex membrane consisting of two distinct parts: the cuticle proper and the cuticular layer (Figure 11.3). This cuticular layer in turn consists of an external layer close to the cuticle and an internal layer close to the epidermis (Konarska 2012, 2014).

Chemically, the cuticle is composed exclusively of lipids (cf. Section 2.3). The lipids present include soluble lipids known as waxes and an insoluble polymer known as cutin. Waxes are dispersed throughout the thickness of the cuticle, from the outer surface, where they are known as epicuticular waxes, to the polymeric cutin matrix and, in some cases, even to the cuticular layers, where they are known as intracuticular waxes (Heredia 2003; Maguire 1998). They are synthesized evenly throughout flowering and fruiting, from initial bud exposure until fruit senescence (Curry 2001).

Cuticle composition and structure depend on many factors:

- Biotic factors, including genetic factors (cultivar), tree-related factors (e.g. nature of the rootstock, health, pruning), the nature of the organ concerned and the stage of fruit development (e.g. size, maturity at harvest),
- Abiotic factors, including topographic factors (e.g. geographic location, elevation, UV exposure) climatic factors (e.g. humidity, temperature, microclimate), nutrients (e.g. water quality, irrigation, tree, and fruit nutrition), chemical factors (e.g. acid

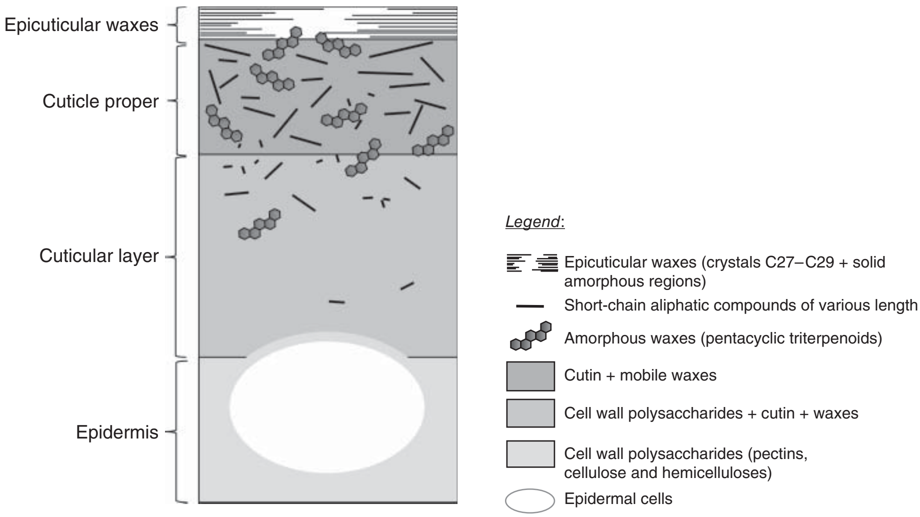

Figure 11.3 Scheme diagram of the molecular structure of apple epidermis. Source: Adapted from (Konarska 2012, 2014; Riederer and Schreiber 1995).

rain, surfactants, xenobiotics such as pesticides sprayed onto the foliage and fruits), the solid particles accumulating on leaf and fruit surfaces (e.g. dust, salts), and storage conditions (e.g. humidity, temperature, proportion of oxygen in the atmosphere) (Curry 2003, 2008; Shepherd and Griffiths 2006; Szakiel et al. 2012). Some of these factors may have a major effect on the cuticle, promoting or inhibiting its development (Curry 2001, 2003). Trichloroacetic acid, for example, inhibits the biosynthesis of cuticular wax (Garrec et al. 1995).

As the outer layer of the skin of the mature fruit, the cuticle serves as a genuine physical and chemical barrier, preventing excessive dehydration of the fruit and the loss of organic and inorganic solutes, and protecting the fruit from mechanical injury, abrasion, the penetration of xenobiotic compounds, such as fertilizers, growth regulators, fungicides, insecticides, and herbicides, UV irradiation and pathogen infection (Szakiel et al. 2012).

11.4.2 Two Diffusion Pathways in the Cuticle

Plant surfaces play a major role in pesticide assimilation (Wang and Liu 2007). Amorphous and crystalline epicuticular waxes are the first, and generally most effective barrier.

Two different pathways of diffusion through the cuticular membrane have been described (Sabljic et al. 1990; Schönherr 2006; Wang and Liu 2007):

– Hydrophilic and lipophilic molecules that are not very volatile and have a high molar volumes, such as polar electrolytes and non-electrolytic organic compounds with a low vapor pressure, including most of the active molecules and additives in pesticide formulations, diffuse through the cuticular membrane via an apolar pathway (Figure 11.4a);

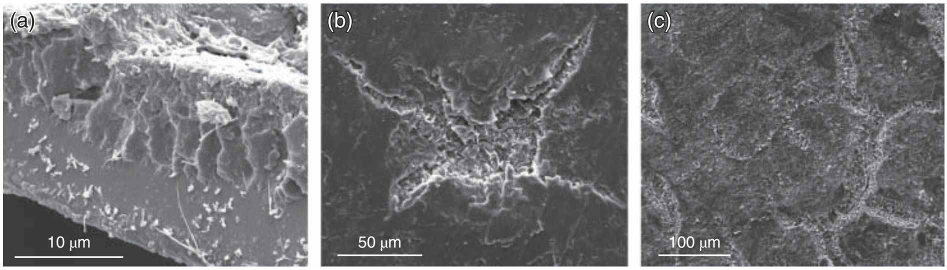

Figure 11.4 SEM micrograph of a cross-section of isolated cuticle at 5 kV (a) and cryo-SEM micrographs of the surfaces of fresh cuticles at 10 kV (b, c) obtained on a FEI Quanta 250 FEG electron microscope (FEI Company, Eindhoven, the Netherlands). Legend: a = enzymatically isolated cuticle from a Fuji apple. b = lenticel on a Pink Lady apple. c = microcracks on a Fuji apple.

– More volatile and less lipophilic solutes and charged analytes follow a polar diffusion pathway of diffusion, via the stomata and lenticels (Figure 11.4b) and microcracks (Figure 11.4c). Pesticide solutions can penetrate into the cuticle if they have a surface tension of less than 23 mN m^{-1}.

The cuticle is a dynamic, ever-changing tissue. The formation of microcracks on the apple surface (Figure 11.4c) (i) depends on fruit growth stage and (ii) is favored by fruit storage after harvest (Curry 2001, 2003; Roy et al. 1999). As the underlying cells of the fruit flesh grow, the tissues expand, leading to a stretching of the cuticle. During storage, high levels of humidity may also promote stretching of the cuticle (Knoche and Grimm 2008; Konarska 2012).

The stretching of the cuticle forces the platelets of epicuticular wax apart, eventually resulting in microcracks. Even the smallest tears of the cuticle surface trigger wax synthesis, with the waxes acting like a chemical sealant, preventing dehydration of the fruit. If this repair mechanism is delayed and the underlying cells are exposed, these cells then begin to synthesize suberin (Curry 2003).

There are no stomata on the surface of mature fruits, but there are lenticels. These apertures do not display the controlled opening and closing typical of stomata (Figure 11.4b), and they are a few hundred microns in diameter. Most develop from stomata on the immature fruit (Curry 2003). Unlike microcracks, the number of lenticels is fixed during the early stages of development, with no possibility of further increase. As the fruit expands, the density of the lenticels thus decreases (Veraverbeke et al. 2003). Lenticels may be open or closed and characterized by the presence of suberin (Veraverbeke et al. 2003). Open lenticels play a key role in gaseous exchanges and the penetration of abiotic active molecules (Wang and Liu 2007). Ionic solutes, such as calcium salts, glyphosate, and nutrients, diffuse through lenticels by the polar pathway to penetrate into the cuticle.

Water diffusion is more complex. With its small molecules, water can follow the polar diffusion pathway (Schreiber 2005). In fact, the diffusion of water vapor depends on both the diffusion properties of the cutin and waxes, and the presence of microcracks and open and closed lenticels (Veraverbeke et al. 2003). However, the cuticle remains the principal site of many of these exchanges (Wang and Liu 2007).

Transpiration via the cuticle and the permeability of the cuticle to lipophilic molecules, such as pesticides, herbicides, and other xenobiotics, are strongly correlated: liquid water and water vapor diffuse across the cuticle in much the same way as lipohilic molecules, via the apolar wax pathway (Schreiber 1995). Some water is also sorbed onto the surface of the fruit, depending on the polysaccharide fraction of the cutin (Dominguez et al. 2011; Veraverbeke et al. 2003).

Schönherr demonstrated the essential role of cuticular waxes in cuticle permeability. Waxes may be amorphous or crystalline (Reynhardt and Riederer 1994; Riederer and Schneider 1990; Riederer and Schreiber 1995). The outer part of the cuticle is characterized by crystalline wax layers, consisting mostly of rigid paraffin chains. These rigid structures contain solid amorphous regions consisting of flexible head groups, substitution groups and small empty interstitial spaces. Short-chain aliphatic compounds with a lower melting point than the crystalline compounds, and compounds subject to steric hindrance, such as cyclic compounds, also form a solid amorphous zone that becomes an amorphous liquid as the temperature increases. Finally, there is a mobile amorphous region, consisting exclusively of the shortest chains, distributed between forming crystals in the solid amorphous regions. When the volume of mobile amorphous compounds exceeds the volume within the solid amorphous region, particularly at higher temperatures, mobile amorphous clusters appear outside the solid amorphous regions. The compounds in the mobile amorphous regions are the most mobile. The occurrence of such regions outside solid amorphous regions greatly improves molecular motion, and these regions display liquid-like behavior.

Partitioning between the amorphous and crystalline regions depends on the length of the hydrocarbon chains, which differs between plant species and depends on wax biosynthesis, which itself depends on many biotic and abiotic factors, as mentioned above. Longer chains ($n > 40$) tend to be more crystalline, and these waxes have a higher melting point.

Most biologically active compounds are unable to penetrate into crystalline structures, due to steric exclusion and low solubility, in particular. The active molecules and water thus diffuse in the solid and liquid amorphous regions of waxes and in liquid regions of cutin, for example (Li and Chen 2009; Reynhardt and Riederer 1994; Riederer and Schreiber 1995).

11.4.3 Study of the Interactions Between Pesticides and Cuticle

Aqueous suspension of pesticides penetrate the plant exclusively through the cuticle (Sabljic et al. 1990). Pesticides penetrate the plant surface through a physical diffusion phenomenon, in response to a chemical potential gradient from the outer layer (i.e. epicuticular waxes) to the cytoplasm of the epidermal cells. This chemical potential gradient is the product of the partitioning coefficient and the concentration gradient (Schönherr and Baur 1994). In addition to solute mobility, this gradient defines the rate of penetration.

Plant surfaces can thus be divided into two distinct parts (Figure 11.2) (Baur et al. 1996; Schönherr and Baur 1994):

- The cuticular membrane, a genuine barrier composed of waxes and cutin making up 10% of the epidermis,
- The sorption compartment, consisting of the polysaccharides of the epidermal cells and accounting for 90% of the epidermis.

Sorption capacity and solute mobility are limited in the cuticular membrane, but much higher in the epidermal cells of the sorption compartment (Schönherr and Baur 1994).

11.4.3.1 Membrane Transport Mechanism for the Active Molecules of Pesticides

The cuticular membrane is a biopolymer in which the membrane transport of water and solutes follows the same general pathway as in other polymers (Buchholz 2006; Cotugno et al. 2016; Wang and Liu 2007). Two-photon excitation microscopy (TPEM) has shown that lipophilic pollutants can penetrate into the plant surface (Wild et al. 2004). TPEM analysis over a period of 96 hours following the contamination of maize leaves with anthracen made it possible to determine the location of anthracen at each stage of membrane transport:

- Dissolution of solutes in the aqueous phase in specific regions on the outer surface of the biopolymer,
- Sorption and diffusion of the solute within the biopolymer,
- Desorption of the solute at the internal surface of the biopolymer, and its release into the sorption compartment.

Active molecules begin to penetrate the plant as soon as the pesticide spray droplet reaches the epicuticular waxes of the outer surface of the cuticular barrier. These waxes have no effect on membrane permeability and the rate of penetration of compounds. Their only role is in controlling the wettability of the plant surface, resulting in the poor retention or diffusion of spray droplets (Buchholz 2006). However, they can help highly lipophilic molecules to penetrate into the plant surface (Baker et al. 1992).

Pesticides dissolve and diffuse in the amorphous regions of the intracuticular waxes and the cutin of the cuticle (Buchholz et al. 1998; Riederer and Schreiber 1995; Schreiber and Schönherr 1993). Cutin is an entirely amorphous biopolymer with an extremely high sorption capacity (Baur et al. 1996; Chen et al. 2008; Li and Chen 2009; Maguire 1998).

Sorption and diffusion depend on a large number of parameters, including the physicochemical properties of pesticides and formulations, abiotic conditions (temperature and humidity), and the characteristics of the plant species. These characteristics determine membrane permeability.

The final step is the desorption of molecules at the internal surface of cuticle, and their release into epidermal cells (Mota-Sanchez et al. 2012). The molecules desorb from the external surface of the epidermal cell walls, crossing the wall to reach the inner surface and the cytoplasm. They are also found in the protrusion of the cuticle between epidermal cells (Wild et al. 2004).

11.4.3.2 Cuticular Membrane Permeability

The cuticle is poorly permeable to water, ionic solutes, polar compounds, nutrients, growth regulators, fungicides, insecticides, and systemic herbicides (Buchholz et al. 1998). This permeability is limited by cuticular waxes, including, in particular, the intracuticular waxes, which are the least penetrable (Buschhaus and Jetter 2011; Chen et al. 2008; Li and Chen 2009; Maguire 1998).

Theoretically, the permeability P of a membrane is proportional to the mobility, assessed with the diffusion coefficient D, and the solubility of the molecule in the membrane, assessed with the sorption coefficient S. It is correlated with the cuticular membrane-water partitioning coefficient $K_{CM/W}$ and is inversely proportional to the thickness of the membrane Δx (Eq. (11.1)) (Baur et al. 1996; Buchholz et al. 1998; Buchholz 2006):

$$P = D \cdot S = D \cdot \frac{K_{CM/W}}{\Delta x} \tag{11.1}$$

D is the speed at which a solute can move in the membrane. It is strongly affected by the molecule size and solute shape.

S is the amount of dissolved solute. It determines the concentration gradient of the solute across the membrane and, thus, the driving force governing mass transport. The solubility of a compound is correlated with its lipophilicity, which can be assessed by calculating the cuticle-water partitioning coefficient $K_{CM/W}$. $K_{CM/W}$ is similar to the octanol-water partitioning coefficient $K_{O/W}$ (Schönherr 2006). The $K_{O/W}$ of solutes can be used to evaluate molecular size. In a given chemical family, $\log K_{O/W}$ is a linear function of molar volume (Hansen 2012).

The molar volume (size, shape, etc.), lipophilicity and chemical structure (e.g. branching, cyclicity) of the solutes affect the partitioning coefficient and, thus, the permeability of the membrane (Sabljic et al. 1990). Molecules with a molecular weight of more than 1 kDa are immediately discriminated because they cannot enter and navigate between plant cells via the plasmodesmata (size exclusion related to plant species). Most pesticide molecules have molecular weights of 100–500 Da. Lipophilicity seems to be an essential parameter governing diffusion.

The chemical nature of the molecule is also important. Chlorinated substituents, the hydrocarbon chains of very long-chain alcohols or fatty acids, and cyclic aromatic compounds have a greater affinity for the cuticle and increase its permeability. Compounds with bonds containing oxygen or nitrogen atoms, the presence of aliphatic hydroxyl groups, non-electrolyte polar compounds, ionic solutes and compounds such as amino acids and glucose have very low partitioning coefficients and solubilities in cuticular waxes and cutin (Buchholz et al. 1998; Sabljic et al. 1990).

11.4.3.3 Identification of the Chemical Compounds of the Cuticle Interacting with Pesticides

The key characteristic of cuticular membranes is their chemical and structural heterogeneity (Figure 11.3). The diffusion coefficient D depends on the real length of the diffusion pathway of the solute through the membrane (Baur et al. 1996; Buchholz et al. 1998). This diffusion pathway depends on the molecular structure of the waxes, i.e. the ratio of amorphous to crystalline waxes: the number, size, spatial conformation and direction of cuticular wax crystals among amorphous permeable waxes. Greater crystallinity leads to a more complex diffusion pathway, with a low solute diffusion coefficient and a lower general mobility. The length of the diffusion pathway therefore generally exceeds the thickness of the cuticular membrane (Bauer and Schönherr 1992; Baur et al. 1996; Buchholz et al. 1998).

During membrane transport, pesticides dissolve and diffuse in the amorphous structures of the cuticle. Many interactions occur between pesticides and the chemical compounds of amorphous intracuticular waxes and cutin.

The cuticle proper consists mostly of lipids with different solubilities: the depolymerizable cutin, the non-depolymerizable and insoluble cutan and the soluble cuticular waxes (Pollard et al. 2008). The flavonoids of the fruit peel are concentrated in the cuticle proper. The cuticle layer, closer to the epidermis, may contain not only cell-wall polysaccharides (cellulose, hemicelluloses, and pectins), but also cutin, and, perhaps, intracuticular waxes (Dominguez et al. 2011; Garrec et al. 1995; Pollard et al. 2008).

Wax compounds are apolar saturated molecules with very long carbon chains (C24-C36) mostly consisting of alkanes, esters of fatty alcohols and acids, primary and secondary alcohols, carboxylic acids, aldehydes, ketones, β-diketones and their derivatives. Many tetracyclic and pentacyclic terpenes with hydroxyl groups, such as sterols (Szakiel et al. 2012; Verardo et al. 2003), lupeol and uvaol, with acid groups, such as ursolic acid, and oleanolic acid, and with ester, ketone and aldehyde groups have also been identified (McGhie et al. 2012; Szakiel et al. 2012). Alkanes, branched acids and esters, alk-1-enes, terpenic hydrocarbons, ω-hydrocarboxylic acids, hydroxyketones, methyl and ethylphenyl esters, benzoic acids and esters, acetates, diesters and some flavonoids are among the more unusual compounds (Bianchi 1995; Buschhaus and Jetter 2011; Pollard et al. 2008; Shepherd and Griffiths 2006; Szakiel et al. 2012).

Chemical compounds are partitioned between the epicuticular and intracuticular waxes, as described below (Buschhaus and Jetter 2011):

- Cyclic compounds, such as triterpenoids, steroids, aromatic compounds, and alkylresorcinols are found exclusively in the intracuticular waxes. Primary alcohols, diols and aliphatic compounds with long chains also tend to accumulate in large amounts in the intracuticular waxes;
- Free fatty acids, alkanes and secondary alcohols with very long chains tend to accumulate in the epicuticular waxes;
- Aldehydes and esters with very long chains are found in both intra- and extracuticular waxes;
- No particular chain length-dependent distribution has been observed for aliphatic compounds with very long chains.

The cuticular waxes of apples characteristically contain C15-C33 alkanes, C16-C30 primary alcohols, C29 secondary alcohols, C20-C28 diols, C6-C30 saturated carboxylic acids and branched carboxylic acids (C16:1–C18:3), hydroxyacids and ursolic acid (Bianchi 1995). Ursolic acid levels can account for 60% of total wax weight. Alkanes and esters account for 15–16% and 11–18% of wax weight, respectively. The cuticular waxes also contain 7–9% alcohols and ketones, and low levels (3–6%) of free acids (Fernandes et al. 1964). Nonacosane (C29) and ursolic acid are the main components of *M. domestica* waxes (Lara et al. 2015). Apple epicuticular waxes are characterized by high levels of hydrocarbons, especially C27 and C29 alkanes and C28:1 and C26:1 alkenes, together with esters (especially C40, C42, and C44 esters, including C16 and C18 esterified acids and C18, C20, C22, C24, and C2 esterified alcohols), aldehydes (especially C30 and C28), secondary alcohols (especially C29), primary alcohols (especially C24, C26, C28, and C30), free fatty acids (especially C16, C18, C18:1, C20, and C22) and less than 1% sterols (Verardo et al. 2003). Following the removal of epicuticular waxes, the extraction and analysis of intracuticular waxes from the Fuji, Royal Gala, Smith, and Granny Smith apple cultivars confirmed that ursolic acid was a major component of intracuticular waxes (Frighetto et al. 2008).

Cutin is a three-dimensional biopolymer. It is amorphous and flexible, but displays some rigidity due to cross-linking. It is insoluble in polar solvents, but can be depolymerized by breaking ester bonds (Buschhaus and Jetter 2011; Pollard et al. 2008). There are several types of cutin monomers: fatty acids functionalized with hydroxyl or epoxyl groups, fatty acids, fatty alcohols, glycerine phenolic compounds. However, the most prevalent monomers of cutin are ω-hydroxylated C16-C18 fatty acids synthesized from oleic or linoleic acid by successive rounds of hydroxylation and epoxidation catalyzed by cytochrome p450 enzymes or by peroxygenases (Dugé de Bernonville 2009; Molina 2010). The C16 and C18 composition of cutin differs between plant species. In *M. domestica*, the main monomer is tetrahydroxystearic acid (Lara et al. 2015). Esterification of the primary alcohol and acid groups of the monomers lead to the formation of a linear polyester. These linear chains often include an oxygenated functional group (epoxyl, oxo, hydroxyl, or diol) in the middle of the chain. Mid-chain secondary hydroxyl groups and hydroxyl groups from glycerin readily form esters with carboxyl groups from other ω-hydroxylated fatty-acid monomers to generate a locally branched polymer (Dugé de Bernonville 2009; Molina 2010). Heredia et al. (2000) and Molina (2010) have proposed partial structures for a section of cutin polymer. However, the precise structure of cutin has yet to be resolved.

After the dewaxing and depolymerization of cutin (i.e. the breaking of ester bonds), cutan is the final solid residue remaining, insoluble and non-depolymerizable. It is amorphous and probably consists of aliphatic compounds linked by ether and C—C bonds. It also contains cell-wall polysaccharides and aromatic compounds. Little is known about cutan biosynthesis, but linoleic acid is thought to be the key precursor (Pollard et al. 2008).

Cutin and cutan levels vary considerably between plant species. Cutan levels are low in apples, from about 3% to 7% of cuticle weight, whereas cutin accounts for 36% of cuticle weight. On average, waxes account for more than 40% of cuticle weight (Chen et al. 2008; Johnson et al. 2007).

In 2012, Li et al. showed that waxes were not the main site of sorption for polar organic pollutants, such as chlorophenols, in apple (Li et al. 2012). Cutin plays a major role, with sorption levels increasing with the octanol-water partitioning coefficient of the solute. The main sorption mechanism is governed by hydrophobic interactions. Higher octanol-water partitioning coefficients reflect a greater involvement of lipophilic components of the cuticle, with cutin playing a greater role than waxes, which in turn play a greater role than cutan and less polar sugars.

In 1997, Schreiber et al. (1997) studied the sorption of two compounds, octadecanoic acid and dotriacontane, in the cuticular waxes of *Hordeum vulgare* leaves. They detected both solutes in amorphous zones of waxes, but not in the same region of these zones. Stearic acid (C18), which has a shorter chain than dotriacontane and also has a polar acid group, was located in an environment of low order within the crystal. By contrast, the much less polar molecule dotriacontane sorbed in an environment very different from that in which stearic acid was found. Several experiments were performed to highlight that dotriacontane sorption could occurred in two environments of different rigidities.

In 2017, Giacinti et al. (2017) highlighted the interactions between triterpenic acids (oleanolic and ursolic acids), the main components of Royal Gala, Fuji, and Pink Lady apple waxes, and pesticides such as boscalid and fludioxonil. These interactions are easily to detect in quantitative analyses of pesticide residues extracted from apple epidermis, as they result in a significant negative matrix effect (Giacinti et al. 2016). An analogous situation undoubtedly exists *in situ*, in the plant matrix. Many pesticide molecules contain pyridine, pyrimidine, and pyrrole rings. These rings have basic

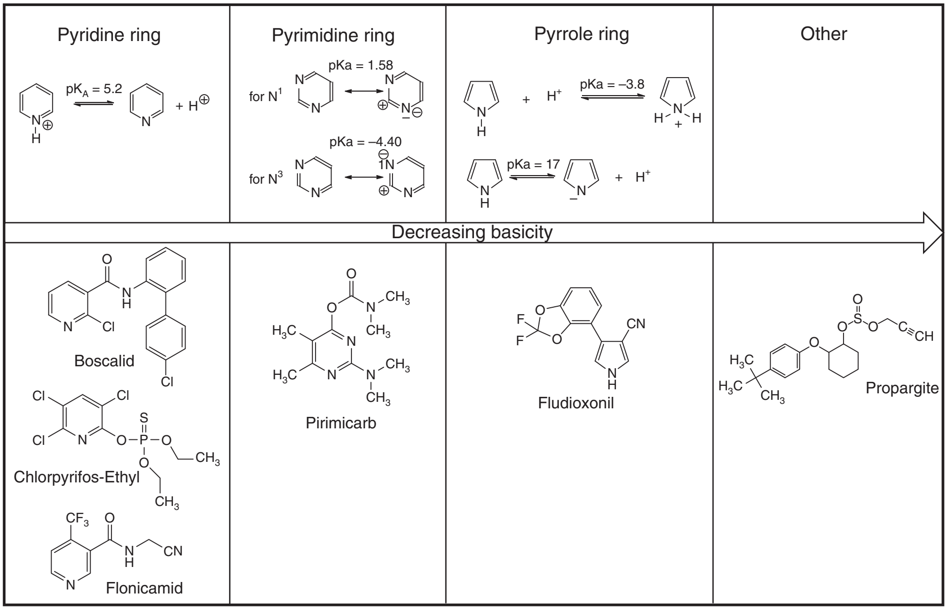

Figure 11.5 Differences in the basicity of pesticides according to their chemical nature. Source: pKa obtained from http://chemicalize.org.

properties, of various strengths, and therefore interact to different degrees with acidic molecules present in the matrix (Figure 11.5) (Adams, 2019; Vicario 2019).

11.4.4 Identification of Factors Likely to Influence Pesticide-Cuticule Interactions

The penetration of a solute depends on (i) spraying parameters, such as pressure and droplet diameter, (ii) the physicochemical characteristics of the plant species, such as size exclusion, tortuosity of the diffusion pathway, and of development stage, leading to different levels of matrix compounds, for example, (iii) the physicochemical properties of the pesticide, such as its lipophilicity, solubility in the membrane (i.e. cuticular membrane-water partitioning coefficient $K_{CM/W}$) or the gradient of pesticide concentration through the diffusion pathway, molar volumes, formulations (i.e. nature and concentration of additives), and (iv) environmental conditions (higher temperatures promote the diffusion of lipophilic molecules) (Lichiheb et al. 2015; Sabljic et al. 1990; Wang and Liu 2007).

Diffusion through plant tissues is essential for the foliar uptake of systemic fungicides and insecticides, growth regulators and defoliants, which are directly applied on leaves. Thus, pesticide formulations (cf. Section 3.1) must combine several basic physicochemical properties to facilitate the penetration of the pesticide into the plant.

11.4.4.1 Pesticide Formulations

Active ingredient concentration and pH are particularly important. Many herbicides are weak acids. At low pH, the non-dissociated form predominates: the pesticide is thus more lipophilic but less soluble in water.

Wet applications are always preferable because the active substance crystallizes if the droplets dry, and is therefore no longer available for membrane transport.

Surfactants, oils and ammonium salts are the most commonly used agents for increasing the penetration of the active ingredients.

Surfactants and Plasticizers The effects of surfactants on pesticide penetration have been studied in detail over the last three decades. The mechanisms involved are complex and may operate at all stages of membrane transport: from deposition on the surface of the plant assisted by humectants, until desorption, through modifications of plasma membrane permeability (Wang and Liu 2007). The nature of the surfactant may increase or inhibit active ingredient uptake, depending on the plant species. For example, organosilicon surfactants, which have a very low surface tension, induce the immediate permeation of aqueous solutions of herbicides, such as glyphosate via the polar diffusion pathway, whereas glyphosate passes through the cuticle in the presence of non-silicon surfactants (Liu 2004b).

Non-ionic surfactants with polar ethoxylated groups (EO) are the most widely used: linear alcohol ethoxylates, alkylphenol ethoxylates and trisiloxane ethoxylates (Wang and Liu 2007).

However, these agents are increasingly being replaced with sugars or polyols, which are considered less damaging to the environment. Ethoxylated surfactants can modify the fluidity of cuticular waxes, the permeability of the cuticle to water, droplet spreading and drying times. They can therefore control wettability (Ramsey et al. 2005). Typically, surfactants with a low EO content enhance the penetration of lipophilic pesticides with a $\log K_{O/W} > 3$, by rendering cuticular waxes more fluid and increasing the permeability of the cuticle to water, whereas those with a higher EO content increase the penetration of more hydrophilic pesticides ($\log K_{O/W} < 0$) by increasing the permeability of the cuticle to water (Ramsey et al. 2005).

Certain compounds are described as active accelerators or plasticizers, because they penetrate the cuticle and increase the mobility of solutes in cuticular waxes: n-alkyl esters, dialkyl esters of phthalic acid, adipic acid and suberic acid, naphthalene, octanoic acid, octanol, and fatty acid esters, including, in particular monoglycerides, and tetraethylene glycol esters (Baur et al. 1996; Buchholz 2006; Mouloungui and Gauvrit 1998; Riederer and Schreiber 1995; Schönherr 1993; Schreiber 2006).

Active accelerators lower the viscosity of the amorphous or simplify the pathway followed by the diffusing solute, without solubilizing the crystalline waxes. They therefore significantly increase the diffusion coefficient D, provided that their own mobility is no greater than that of the solute. The accelerator and the solute act synergistically on the cuticular barrier to enhance solute mobility. Far above the critical micelle concentration (CMC), the accelerator molecules, organized into micelles, form new sorption compartments in the aqueous phase that compete with waxes for the sorption of solutes. The accelerator molecules are adsorbed onto waxes at the same time as the solutes desorb. This phenomenon is completely reversible because elimination of the accelerator restores the prior mobility of the solute. The efficiency of active accelerators depends on their nature and concentration, temperature, solute size, and plant species (Buchholz 2006; Schreiber 1995). For example, tributyl phosphate plasticizes amorphous waxes, thereby increasing the accessible volume for solute diffusion (Buchholz and Schönherr 2000). This greatly decreases the energy of activation, and the solutes are much more mobile. According to a theory advanced in the 1950s (Zielinski and Duda 1992), a solute needs space between the chains of a polymer to diffuse correctly, the required energy being provided by the Brownian motion of the solute itself. The diffusion of a solute is thus limited by the motion of the adjacent chains of the polymer. At a given temperature, a polymer is characterized by a specific distribution of transitory free volumes. As the temperature increases, this specific distribution changes and larger volumes appear due to the breaking of intermolecular bonds, at the expense of smaller volumes (Buchholz 2006). The plasticizer affects the free volume by decreasing the glass transition temperature of the polymer (Tg), thereby increasing its resilience. Inherent components of plant matrices may act as plasticizers. In 2008, Chen et al. (2008) showed that the polysaccharides in apple epidermis acted as plasticizers of cutin but that they had the opposite effect on waxes, increasing their glass transition temperatures. Cutin is a plasticizer of waxes. In the epidermis, the plasticizing or anti-plasticizing effects are complex, due to the large numbers of molecules and their different concentrations. Higher wax contents in the epidermis of a plant are associated with weaker plasticizing effects. Not only is the sorption of the plasticizer low, but its plasticizing effect is weaker than the anti-plasticizing effect of waxes, limiting the sorption of organic pollutants (Li et al. 2009).

In summary, for a surfactant to decrease the glass transition temperature of waxes in apple cuticle, it must (i) penetrate the cuticle, this step being limited by the presence of large amounts of wax, which increase the glass transition temperature of the cuticle, and (ii) have plasticizing properties that outweigh the anti-plasticizing properties of waxes.

Several studies have suggested that the sorption of solutes, such as plasticizers and organic pollutants (e.g. ametryn, polycyclic aromatic hydrocarbons), in cuticular waxes may promote the transition from a solid or even a rigid amorphous phase to a more mobile amorphous phase (Chen and Xing 2005; Shechter et al. 2006).

Oils and Ammonium Salts Plant oils and their esters, which are considered to be environment-friendly and are biodegradable, are used as emulsifiers, at concentrations of 15–20% in concentrated formulations. All oils penetrate into plants. They can increase the efficacy of many herbicides, including aryloxyphenoxy propionates, cyclohexanediones, triazines, bentazone, phenoxy acids, imidazolinones, sulfonyl ureas, and phenmedipham (Wang and Liu 2007).

Ammonium salts have been used for many years and the most widely used is ammonium sulfate. They increase the penetration of many pesticides, but their effects are dependent on plant species (Wang and Liu 2007).

11.4.4.2 Environmental Conditions

The penetration of pesticides into the plant depends heavily on climatic conditions: temperature and humidity (Ramsey et al. 2005). The precise mechanisms underlying the effects of environmental conditions on pesticide uptake are unclear, as there are interactions between pesticides, plant species, temperature, and humidity. High temperature and humidity enhance solute transport. Higher temperatures also have a plasticisizing effect, reversibly modifying the viscosity of amorphous waxes (Buchholz 2006; Schreiber 2006). In the absence of high humidity levels, very high temperatures inhibit pesticide penetration, by drying the droplets of pesticide on the plant surface. Humidity affects the hydration not only of the cuticle, but also of the pesticide droplets, and this effect is much more important for penetration than temperature.

11.4.4.3 Pesticide Molecule Degradation in Plants: New Interactions

Once in the plant, active molecules undergo various degradation processes that may be biotic, induced by the matrix itself (chemical, microbial and/or enzymatic degradation), or correlated with abiotic stresses either in the orchard or during storage after harvesting (e.g. temperature, UV irradiation). Studies of the degradation of active molecules in fruit can be used to determine periods of pest protection and to identify new molecules likely to interact with plant matrix compounds.

Active molecules may be strongly or weakly connected to the matrix. Molecules interacting weakly with the matrix undergo degradation induced by both biotic and abiotic parameters, whereas those interacting strongly with the matrix are subject to degradation induced by biotic factors only (Asensio et al. 1991).

Photodegradation is a key pathway of active molecule degradation immediately after pesticide spraying in orchards (Lichiheb et al. 2015). Radiation of wavelengths exceeding 290–295 nm induces a process of pesticide degradation both *in situ* in fruits and in the environment (water, soils). Photodegradation in plants has been little studied. This process may be direct, with the molecule absorbing light directly, or indirect, with the molecule reacting with light induced radicals. An activation energy of 70–120 kcal mol^{-1} is required to break the chemical bonds in pesticide molecules. This corresponds to irradiation at wavelengths between 250 and 400 nm. Photodegradation in plant depends on three parameters:

- The nature of the active molecules and their formulation,
- The nature of the plant matrix (concentration and composition of cuticular waxes, microbiota),
- The environmental conditions in the field or during post-harvest storage (intensity and spectral composition of light, temperature, humidity).

Riccio et al. (2006) demonstrated the photolysis of chlorpyrifos-methyl in apples and dewaxed apples. They showed that photolysis was slower in apples that still had their waxes. Indeed, waxes absorb UV radiation (Buschhaus and Jetter 2011; Solovchenko and Merzlyak 2003), thereby protecting the pesticide molecules. However, once the molecules penetrate the cuticle, they are finally broken down in the epidermis (Clavijo et al. 1996). Active molecule concentrations on the fruit surface change as follows: penetration (increase in concentration), followed by stabilization (concentrations remain constant) and degradation (phase during which the molecules are exposed to chemical and biological breakdown agents from the fruit). However, not all molecules react in the same way. For example, there is not always a stabilization step (Clavijo et al. 1996).

Many studies have investigated the breakdown of pesticides sprayed on apples, at the laboratory scale and in orchards. Diazinon breaks down much faster than chlorpyrifos in orchards. The kinetics of chlorpyrifos degradation are similar in the laboratory and in orchards (Asensio et al. 1991). Organophosphorus pesticides break down faster in real climatic conditions (UV, rainfall, temperature) than in the laboratory. No degradation of fenitrothion is observed in the laboratory (Barrio et al. 1995). Ethiofencarb, dichlofluanid, fenitrothion, and malathion break down more slowly in the epidermis (17 days) than in the fleshy receptacle (1–4 days) (Clavijo et al. 1996). Acephate breaks down immediately and evenly on the external surface of the epidermis, after 12 days in the epidermis and after 16 days in the fleshy receptacle (Sanz-Asensio et al. 1999). In Melrose apples, only seven of the 21 pesticides sprayed in orchards have been detected at harvest time, and only two remained detectable after five months of monitored storage (Ticha et al. 2008).

Pesticides are very reactive molecules. The extraction of residues for analysis and processing in the food industry may lead to the complete or partial breakdown of pesticide molecules. For example, captan is converted into tetrahydrophthalimide (THPI) during the production of apple puree (Kovacova et al. 2014), and during chromatographic analysis (Banerjee et al. 2010). Carbendazim is not authorized for use in Europe, in accordance with Regulation (EU) No. 1107/2009. Carbendazim is the breakdown product of thiophanate-methyl and benomyl. Thiophanate-methyl is the only one of these molecules approved for use in Europe ("EU Pesticides database", consulted in September 2019). It has very low levels of persistence and rapidly breaks down into carbendazim.

11.5 Conclusion and Future Prospects

Apple fruits are a complex aqueous matrix containing up to 90% water, 8–12% glucides, 3–5% protein, and 2% fiber making up the cell walls (cell-wall polysaccharides, including pectins + lignins), 0.6% organic acids and 0.3% lipids. Lipids account for 30% of the dry weight of the cutinized epidermis. Indeed, the epidermal cells are covered by a protective layer known as the cuticle, which is composed exclusively of soluble lipids, cuticular waxes, and insoluble lipids, cutin. The cuticle constitutes an apolar diffusion pathway that transports small polar uncharged analytes, such as water, and lipophilic analytes with molecule weights of up to 1000 Da, such as pesticides. The exact structural organization of the cuticle remains unclear. The outer layer consists of the epicuticular waxes that serve as a barrier against the external environment (control of water stress, protection against pests, transport of bioactive molecules, such as xenobiotics and pesticides). These waxes protect the cuticle proper, which consists of the cutin biopolymer impregnated with intracuticular waxes. The closer the epidermal cells of the sorption compartment the greater the enrichment of the matrix in cell-wall polysaccharides, such as cellulose, hemicelluloses, and pectins. Polysaccharides play a major role in the desorption of bioactive molecules.

Pesticides are mostly detected in the epidermis of apples. Depending on their physicochemical properties (nature of chemical substituents, lipophilicity, etc.), they interact to different extents with compounds from the plant matrix. During membrane transport, they dissolve and diffuse in amorphous regions of cuticular lipids. The interactions involved are complex and numerous due to the chemical and structural heterogeneity of the cuticular membrane.

Several studies have shown that pesticides that are more basic have a greater tendency to interact with oleanolic and ursolic acids, two key components of the cuticular waxes of apples. It is possible to predict some parameters relating to membrane permeability from the solubility theory developed by Hansen, according to which, two compounds of a similar nature are miscible.

Once they have been transported across the epidermis, pesticide molecules may be broken down by hydrolysis or photolysis *in situ*, catalyzed by plant enzymes or bacteria, or as a result of a state change (vaporization from the plant into the atmosphere). This degradation decreases residual pesticide concentrations from the orchard to the fork. New molecules are generated in this way and take place in new interactions, depending on their physicochemical characteristics.

An understanding of the interactions between pesticides and plant matrix compounds is essential for: (i) the development of analytical methods for trace and ultratrace analysis in complex matrices, and (ii) the development and implementation of extraction processes designed to lower residue levels in foodstuffs such that pesticide residues are below the threshold of detection.

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12

Multiresidue Pesticide Analysis in Cabbage and Cauliflower Using Gas Chromatography Tandem Mass Spectrometry (GC-MS/MS)

Mahadev C. Khetagoudar[1], Mahadev B. Chetti[2], A. V. Raghu[3] and Dinesh C. Bilehal[1]

[1] *Department of Chemistry, Karnatak University, Dharwad, 580003, Karnataka, India*
[2] *Indian Council of Agricultural Research, Pusa, New Delhi, 110 012, India*
[3] *Department of Food Technology, Jain University, Bangalore, 562112, Karnataka, India*

12.1 Introduction

In India, large quantities of pesticides are used for the cultivation of cabbage and cauliflower mainly for the management of various diseases and pests. Due to the stringent rules set by the various developed countries on food safety standards and the regulations on quality parameters, it was found that the residues of the pesticides in food is gaining a lot of attention. Keeping in view the problem of residues of pesticides, the present study was conducted on cabbage and cauliflower of Belagavi District (Karnataka state, India) for the qualitative and quantitative analysis of pesticide residues by GC-MS/MS (Gas Chromatography coupled to Mass Spectrometry).

In recent years, the production and marketing of food has gained topmost priority. This in turn has given rise for the implementation of better agricultural practices and has also prompted a substantial increase in the importance given to pesticide residues and related aspects. It is important to analyze large numbers of samples for residues of pesticide in the food due to their control and regulatory issues. Analytical procedures for pesticide residues are usually time consuming and costly. For this reason multiresidue methods have been devised and regularly applied in regulating pesticide monitoring programs (McMahon and Hardin 1994; Fillion et al. 1995).

There is a difficulty in developing a method for residue analysis mainly due to wider nature of polarity, volatility, and solubility of different pesticides (Sivaperumal et al. 2015). On the basis of different pesticide classes, various methodologies using gas chromatography with various sensitive cum selective detectors viz., Thermal conductivity detector, Nitrogen-phosphorus detector, Electron capture detector and Flame photometric detector have been implemented. Furthermore, several methods have been developed for accurate quantification of residues of pesticides in various consumable food products or commodities. All these seem to be much more complicated because of the use of a large quantity of inert gases which are quite costly and time consuming (Albero et al. 2005; Štajnbaher and Zupančič-Kralj 2003) Therefore, there is a need to develop new methods in the preparation of the sample and the requisite quantification parameters.

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QuEChERS which is a novel quick, easy, cheap, effective, rugged and safe method for the preparation of samples in pesticide residue analysis (Anastassiades et al. 2003). This method has several advantages, firstly, sample throughput is very high, secondly, it does not use chlorinated solvents, third, a very small quantity of solvents are needed which in turn provides a very high recovery percentage for broad spectrum volatility and polarity range of pesticide molecules. Even though this method was developed recently, it has been widely accepted by the international community of pesticide residue analysts. There have been several publications on this topic often replacing the original method with newer and better ones (Lehotay et al. 2005a; Diez et al. 2006; Lehotay et al. 2005b,c; Martínez-Vidal et al. 2006).

Chromatographic system (Gas chromatography or Liquid chromatography) attached to Mass Spectrometry (MS/MS) determination provides us with a method for identifying and quantifying several pesticides in different food matrices (Carneiro et al. 2013). Simple extraction procedure along with very limited cleanup technologies have been employed as a result of the use of more sensitive and selective MS/MS detection. Martinez et al. used gas chromatography mass spectroscopy (GC-MS/MS) with ethyl acetate for extraction of 130 multiclass pesticides (Pihlström et al. 2007). They slightly modified GC-MS/MS the procedure 2004. They reported the use of LC-MS/MS and acetonitrile extraction for the analysis of 73 pesticides in lettuce and oranges (Hetherton et al. 2004). They used both liquid chromatography and gas chromatography attached to mass spectrometry for the simultaneous determination of 446 pesticides in vegetables and fruits (Pang et al. 2006a) and 450 pesticides residues in wine, fruit juice, honey using Solid Phase Extraction (SPE) cleanup (Pang et al. 2006b)

This paper explains an effective and simple experimental procedure for extraction of sample by employing QuEChERS (slightly modified) method and use of gas chromatographic system with mass spectrometric determination for 35 pesticide residues in cabbage and cauliflower samples.

12.2 Experimental Details

12.2.1 Apparatus

(a) *GC MS/MS instrument*. Gas chromatograph (Agilent 6890N) with auto sampler and a Triple Quadrupole Mass spectrometer (Quatro Micro RAB120, Waters) detector was used for the analysis of the pesticides studied. MassLynx Solution software was used for the instrument control and data analysis.
(b) *Chopper and Homogenizer*. Vegetable Chopper was used for chopping and a Homogenizer (Heidolph) was used for proper mixing of the samples.
(c) *Centrifuge*. Centrifuge (Sigma 3K 10) was used for both 2 and 50 ml polypropylene tubes.
(d) *Weighing Balance*. Weighing Balance (Sartorius) was used to weigh the chopped samples and preparation of reference standards reagents.
(e) *Low Volume Concentrator*. Turbovap (Caliper Life Sciences, USA) with inert nitrogen was used for the evaporation of the solvent.

12.2.2 Reagents

(a) *Certified reference materials* (*CRMs*). Certified reference materials (Table 12.2) of pesticides were procured from Sigma-Aldrich/Riedel-de-Haen (Zwijndrecht, The Netherlands).
(b) *Primary secondary amine (PSA)*. SPE sorbent PSA (40 μm, Bondesil PSA) was purchased from Agilent Technologies (Bangalore, India).
(c) *Sodium acetate and Magnesium sulfate*. Reagent grade anhydrous Sodium acetate and magnesium sulfate were procured from Merck (India).
(d) *Ethyl acetate and Acetic acid (Glacial)*. Ethyl acetate and Acetic acid (Glacial) of sufficient quality for pesticide residue analysis were procured from Sigma-Aldrich.
(e) *Cabbage and Cauliflower samples*. Cabbage and Cauliflower samples (2 kg each) were collected from the field in Belagavi District (Karnataka state).

12.2.3 Preparation of Reference Standard Solutions

Individual stock solutions (1000 μg ml^{-1}) were prepared by weighing 10(±0.1) mg of each CRM in amber colored volumetric flask and dissolved in 10(±0.1) ml of ethyl acetate. Stock solutions of these were kept at −20 °C in a deep freezer. Working standards were prepared by diluting the stock solutions accurately. Serial dilution technique was used for the preparation of the calibration curve.

Table 12.1 Optimized GCMS/MS parameters

Gas chromatography	Agilent 6890N with Autosampler (7683)
Mass spectroscopy	RAB120 Waters, Boston, USA (Triple Quadrupole)
Software	MassLynx
GC column	HP-5MS (Length:30 m, 0.25 mm internal diameter, 0.25 μm)
Carrier gas	Helium (Purity 99.999%)
Flow rate	1.3 mL min^{-1}
Injector temp.	280 °C (splitless)
Vol. of injection	1 μl
Oven temp programming	50 °C – 1 min, 25 °C min^{-1} −150 °C
	10 °C /min^{-1} −280 °C (hold time 4 min.)
Mode	Multiple Reaction Monitoring (MRM)
Interface temp.	250 °C
Source	Electron impact (EI+)
Source temp.	250 °C
Total run time	22 min
Electron energy	70 eV
Collision gas	Argon (Purity 99.999%)
Collision gas pressure	$3.5 \times 10\ e^{-3}$
Source penning	$1.75 \times 10\ e^{-3}$

12.2.4 Preparation of Sample

Cabbage and cauliflower samples (2 kg each) were collected from the field at Belagavi District (Karnataka state, India). This area is well known for its good quality cabbage and cauliflowers. These samples were kept at −5 °C in deep freezer until further sample preparation. The cabbage and cauliflower samples (0.5 kg) were then cut and homogenized, further, 10 g of samples were then extracted using acetonitrile with 0.1% acetic acid (10 ml). Next, 1.0 g of sodium acetate and 5 g of magnesium sulfate were added to this sample. The samples were further homogenized by adding acetonitrile followed by centrifugation for three minutes at 2500 rpm. 1 ml of the acetonitrile extract was transferred into a 2 ml extraction tube containing 150 mg of magnesium sulfate and 50 mg of PSA (Primary Secondary Amine). This was then centrifuged at 5000 rpm for five minutes. The supernatant was further transferred to a 1 ml vial and filtered using a 0.2 μm filter paper. Fresh organic cabbage and cauliflower samples with no pesticides spray were used as blanks.

12.2.5 GC- MS/MS Analysis

Optimization was performed using of Gas Chromatography with mass spectrometer and an auto-sampler Table 12.1 depicts the optimized GCMS/MS parameters.

12.2.6 Validation Study

In this method, for the fulfillment of validation criterion, a single laboratory approach was used. The following validation parameters were used:

*Linearity***.** Five calibration levels (1 and 200 ng ml^{-1}) were used for constructing the calibration curve by using pure solvent and matrix.

Selectivity. It was determined by elimination of noise at the retention time of the compound, which is performed by fixing two transitions of MS/MS for individual molecule of analyte by considering the adequate precursor and product ions.

Sensitivity. Detection limit (LOD) in the chromatogram was calculated by using peak signal of the analyte molecule concentration to the three times background noise in the chromatogram. The quantification limit (LOQ) in the chromatogram was set as the lowest concentration with very good recovery range (65–100%) and precision (RSD ≤ 20%). The ion ratio (Q/q) was used for the criterion of confirmation in positive samples. The Q/q is the ratio of the intensity quantification (Q) and confirmation transition (q) (Table 12.2).

12.3 Results and Discussion

12.3.1 Optimization of GC Oven Programming

Optimization of GC oven programme was done to separate the individual test compounds with sharp peak shape, high resolution, less interference of matrix, and high sensitivity (S/N ratio). The initial temperature was set to 50 °C, then increased at the rate of 25 °C min^{-1} from 50 to 150 °C, this in turn reduced the retention time of the

Table 12.2 Average % recoveries (% RSD) of fortified pesticides in cabbage and cauliflower from the QuEChERS extraction method with GC-MS/MS analysis

Sl. No.	Reference standards	tR (Min)	MRM Pre. Ion (Q)	MRM Prod. Ion (q)	CE	LOD (mg/kg)	Fortification levels (mg/kg) Cabbage 0.01	Cabbage 0.05	Cauliflower 0.1	Cauliflower 0.5
1	DEET	7.06	119	65	21	0.001	95(5)	88(2)	95 (6)	91 (3)
2	Propiconazole	7.65	69	41	6	0.01	76(3)	79(10)	75 (13)	79 (10)
3	Phorate	7.85	260	75	5	0.002	90(4)	99(12)	89 (11)	84 (4)
4	Carbofuran	8.35	164	149	8	0.002	91(1)	98(9)	92 (0)	98 (3)
5	Atrazine	8.85	215	58	8	0.005	74(6)	86(8)	87 (12)	87 (4)
6	Lindane	9.04	184	145	10	0.001	86(8)	98(15)	91 (5)	96 (5)
7	Diazinon	9.74	179	137	17	0.0005	96(6)	89(1)	87 (3)	86 (6)
8	Chlorothalonil	9.95	266	133	26	0.004	82 (3)	84 (7)	98 (7)	97 (1)
9	Metalaxyl	10.37	206	59	8	0.002	86(4)	88(5)	93 (2)	92 (9)
10	Fenitrothion	10.64	125	79	11	0.002	92(7)	91(7)	84 (4)	83 (2)
11	Ethion	10.70	231	129	18	0.0001	97(6)	95(9)	90 (7)	85 (2)
12	Aldrin	11.54	263	193	22	0.003	87(13)	91(4)	98 (5)	90 (5)
13	Fenthion	11.99	278	109	12	0.005	99(5)	92 (4)	97 (4)	98 (1)
14	Chloropyrifos	12.05	197	169	16	0.0005	88(4)	97(4)	98 (6)	96 (2)
15	Parathion	12.39	291	109	10	0.003	83(2)	90(5)	97 (14)	96 (2)
16	Triademefon	12.77	208	181	6	0.006	99(3)	99(1)	95 (5)	99(3)
17	Pendimethalin	13.39	252	162	16	0.005	88(2)	96(1)	100 (3)	95(3)
18	Captan	13.95	79	51	20	0.002	90 (3)	84 (4)	84 (4)	83 (2)
19	Phenthoate	14.19	274	121	16	0.0005	97(5)	90 (2)	90(3)	99(0)
20	2,4-DDT	14.61	146	118	7	0.00001	84(3)	97(5)	97 (3)	88 (3)
21	Alfa-endosulfan	14.95	241	170	25	0.004	93(3)	98(9)	96 (5)	95 (3)
22	Butachlor	15.29	176	146	20	0.001	91(7)	93(6)	84 (4)	83 (2)
23	Profenofos	15.76	337	267	8	0.005	91(3)	96(10)	88 (9)	89 (2)
24	2,4-DDD	16.34	235	165	16	0.00001	94(2)	98(3)	97 (3)	100 (2)
25	Endrin	16.85	263	193	22	0.005	88 (5)	94(12)	88(2)	92(10)
26	Chlorfenapyr	17.15	247	75	17	0.02	99 (3)	94 (4)	97 (2)	82(4)
27	Beta-endosulfan	17.41	241	170	25	0.005	98(6)	84(5)	95 (4)	91 (2)
28	Quinolfos	17.87	235	165	15	0.003	93(1)	86(10)	95 (8)	95 (4)
29	Malathion	17.96	173	99	10	0.003	96 (14)	96 (1)	93 (1)	92(2)
30	Triazophos	18.72	161	77	19	0.005	91 (9)	98 (1)	96 (3)	92(4)
31	Iprodione	18.91	314	245	10	0.02	83 (10)	93 (5)	90 (4)	88(2)
32	Beta-cyfluthrin	19.63	165	127	5	0.01	90(1)	98(1)	86 (14)	85 (7)
33	Alfa Cypermethrin	20.17	163	127	6	0.005	92(2)	96(1)	90 (3)	91 (4)
34	Fenvalerate	20.72	167	125	8	0.005	89(3)	98(1)	97 (3)	88 (3)
35	Deltamethrin	21.73	181	152	18	0.008	90(3)	99(0)	97 (3)	93 (4)

tR = Retention Time, MRM = Multiple Reaction Monitoring, CE = Collision Energy, LOD = Limit of Detection.

compound. The temperature of the oven was subsequently increased to 280 °C at the rate of 10 °C min^{-1}. It proved to be helpful in getting a good shape and a larger S/N ratio for all the compounds such as, malathion, parathion, quinalphos, pendimethalin etc., The holding time of four minutes helped in the separation of co eluting cyfluthrin, cypermethrin, fenvalerate and deltamethrin. In case the GCMS/MS full scan mode (50–500 Da) were to be used, then there would have been an uncertainty in the identification of compounds because of closely eluting compounds, thereby resulting in a poor mass spectral purity. However, in the MS/MS mode, such confusion in separation and identification was avoided due to the compound specific selective MRM transition.

12.3.2 Optimization of MS/MS

MS/MS method of optimization was performed on the pesticide standards using ethyl acetate solvent with an EI^+ ionization mode. Optimization was carried out in three steps viz., isolation of precursor (parent) ion, ion excitation and product ion dissociation, and scanning within a certain mass range (Béguin et al. 2006; Nam and Lee 2002). Retention time was fixed for each analyte before MS/MS optimization. After obtaining the full scan spectra of each analyte, precursor ion was selected as base peak of the spectrum. After the selection of a precursor ion, different collision energy (between 4 and 40 eV) were optimized to know the splitting pattern. The main intention of this study was to establish a MRM with two MS/MS transitions.

For each pesticide, product ions with more intensity were selected for the purpose of quantification. Product ions with next intense ion was used for confirmation purpose. In order to find out the highest S/N ratio, the product ion range was maintained at a very narrow level. Table 12.2 shows the product and precursor ions corresponding to the qualitative and or the conformational transitions that were monitored. Optimized collision energy values for all the compounds were maintained between 5 and 35 eV. In order to achieve a satisfactory sensitivity and a good chromatographic peak, the dwell time parameter was set at 0.1 second for each analyte. Matrix matched standards at five different concentration level were used for Q/q ratio calculation, obtaining RSD typically below 16%.

12.3.3 QuEChERS Procedure for Extraction

In order to monitor the residue levels of pesticides in fruits and vegetables, Anastassiades et al. in 2003 developed QuEChERS (quick, easy, cheap, effective, rugged, and safe) method (Anastassiades et al. 2003). It requires acetonitrile (MeCN) for pesticide residue extraction and Na_2SO_4 (anhydrous) and $MgSO_4$ (anhydrous) for partitioning of the acetonitrile extract and water. First, the extract was mixed with PSA, this is called dispersive solid-phase extraction. Polar matrix such as organic acids and color pigments present in the matrix were effectively removed by PSA. This method with a small modification has been included in the official method of AOAC International and the European Standard Organization (CEN) (Anastassiades et al. 2003; Lehotay et al. 2007). Hence QuEChERS method has been found to be widely accepted and very easy to adapt.

12.3.4 Recovery Experiments of Spiked Samples

Usually, the extraction and clean up procedure removes the matrix co-extractives then separates all of the analytes from the matrix. The same does not holds good in most of the matrices during the pesticide residue analysis. As a result, the actual recovery experiments were performed on cabbage and cauliflower samples. The separated peaks with their tR (retention times) are summarized in Table 12.2. Using the linear regression equation recoveries of individual pesticides with different levels of spiking along with replicates were calculated in cabbage and cauliflower matrix. Table 12.2 gives the average recoveries for all spiked pesticide standards at each spiked level in cabbage and cauliflower samples. All the tested 35 pesticides displayed a recovery range between 74% and 100% which is quite acceptable. RSD (relative standard deviation) was used to express the reproducibility and most of the RSD values were found to be less than 15%.

12.3.5 Method Performance

The performance of the method was found to be quite satisfactory for the analysis of pesticide residues in cabbage and cauliflower samples. Those pesticides that are usually difficult to separate chromatographically due to elution problem were resolved by using the MS/MS method (Figures 12.1 and 12.2). The linearity of this method was further checked by establishing the calibration curves for solvent as well as matrix standards with correlation coefficient (R^2) >0.999 for all the standards (Figure 12.3). The recoveries of all of the compounds were found to be between 74% and 100% with the RSD below 15%. The confirmations of the detected pesticide residues were done on the basis of the qualities to target MRM ratio (European Commission Decision 2002/657/EC of 12 August 2002). When we analyzed the samples in the full scan mode of GC MS/MS, it

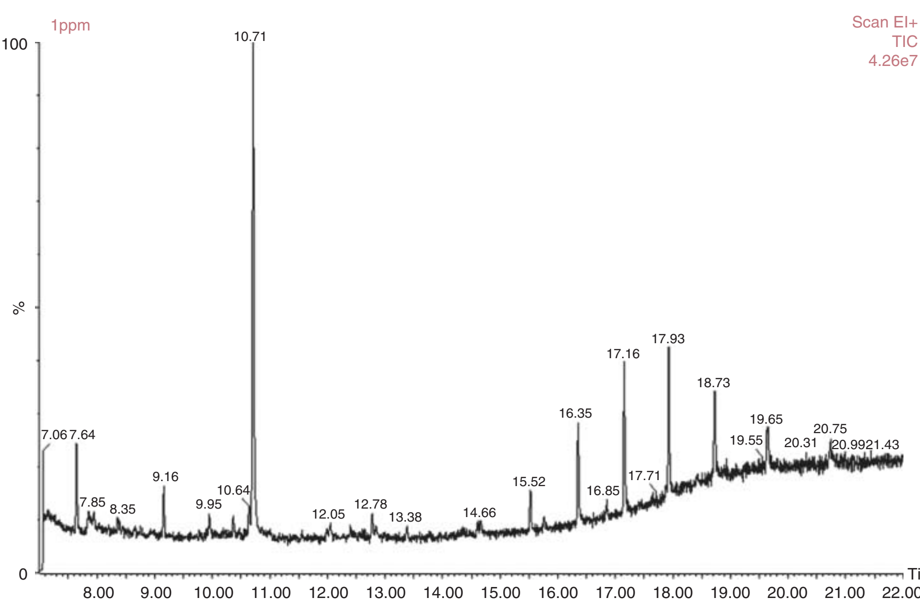

Figure 12.1 Typical total ion chromatogram of certified reference standards.

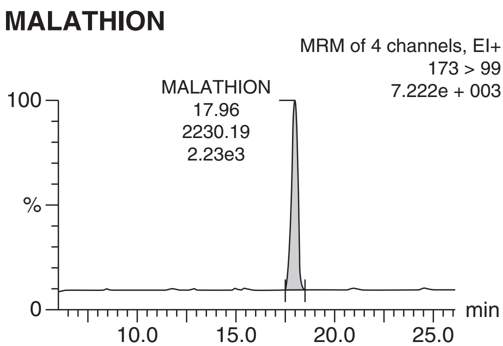

Figure 12.2 Typical chromatogram of Malathion.

Compound name: MALATHION
Correlation coefficient: r = 0.996487, r^2 = 0.992986
Calibration curve: 30.6769* x + –90.3545
Response type: External Std, Area
Curve type: Linear, Origin: Include, Weighting: 1/x, Axis trans: None

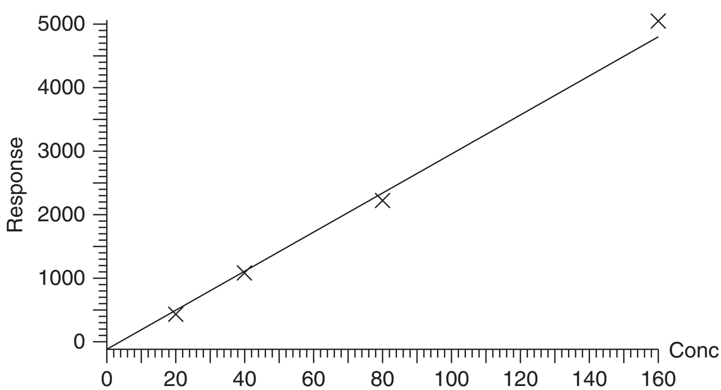

Figure 12.3 Malathion linearity over the concentration range of 20–160 ppb.

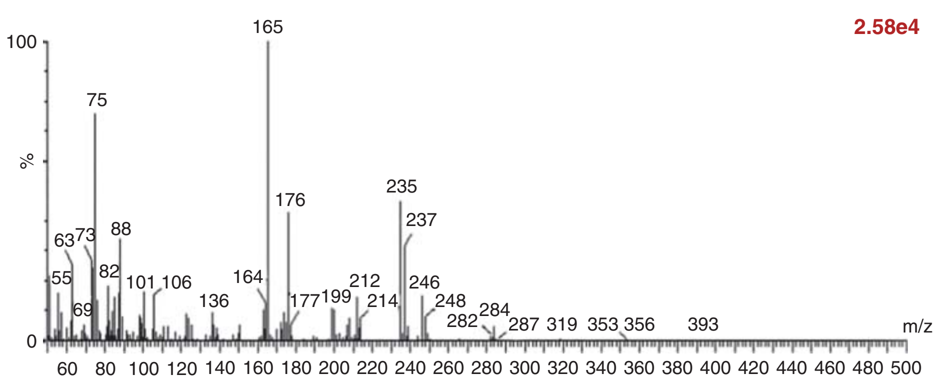

Figure 12.4 Typical mass spectrum.

gave only qualitative data which matched the NIST library (Figure 12.4). The results were found to match with an accuracy of more than 60% of the standard. Due to poor peak shape and poor peak area, it was not possible to quantify the compound in a full scan mode. However, this problem established a very high sensitivity and superior selectivity for target oriented mass spectrometry for trace level detection of residues of pesticides in agricultural commodities.

12.4 Applicability of the Developed Method

12.4.1 Sampling

Cabbage and cauliflower samples were collected from farmers fields in Belagavi district (Karnataka state, India). These areas are very popular for the production of cabbage and cauliflower and also use of excessive pesticides. The developed analytical method was used for the determination of residues of pesticides in cabbage and cauliflower samples and were analyzed in triplicate. The results confirmed that the cabbage and cauliflower samples contained pesticide residues well above the prescribed level viz., carbofuran, chlorfenapyr, fenvalerate, and malathion in cabbage and chlorfenapyr, fenvelarate, quinalphos in cauliflower samples (Table 12.3). Cabbage and cauliflowers which were analyzed in the present study mainly contributed to the major dietary intakes of the citizens in India. It is evident that most of the samples exceeded the MRL values, hence rejection in the international market which is unfit for human consumption. By careful monitoring of the dosage of pesticides, we can check the residue level within the acceptable limit.

Table 12.3 Results of cabbage and cauliflower sample analysis collected from Belagavi district (n = 50)

Sl. No.	Name of the pesticides	MRLs exceeded in samples	Residue content (ppm)	EU MRLs (ppm)
Cabbage				
1	Carbofuron	4	0.04	0.02
2	Chlorfenapyr	6	0.10	0.03
3	Fenvelarate	2	0.14	0.01
4	Malathion	3	0.08	0.02
Cauliflower				
1	Chlorfenapyr	8	0.10	0.03
2	Fenvelarate	4	0.14	0.01
3	Quinolphos	3	0.08	0.02

MRL, Maximum Residue Limit; **ppm,** parts per million; EU, European Union.

12.5 Conclusion

Cabbage and cauliflower contaminated with residues of pesticides pose a major health hazard. Therefore, there is a need to develop an effective method for the detection of contaminated pesticides. Hence, for the simultaneous confirmation and quantification of 35 pesticides in cabbage and cauliflower samples, a multi-residue method has been developed and validated. For multi-class pesticide residue determination, GC-MS/MS with triple quadrupole analyzer played an important role. Within 22 min of run time all the closely eluted and co-eluted peaks were separated with higher sensitivity. The two MRM transitions, one for confirmation another for quantification, achieved very good sensitivity and selectivity for possible safe identification by the use of Q/q ratio parameter. The limit of detection was lower than the MRL prescribed. SPE with acetonitrile solvent was employed. Finally, the method was successfully validated for two concentrations viz., 0.01–0.05 mg kg^{-1} for cabbage and cauliflower sample. The validated method reduces the overall cost of analysis and also offers low uncertainty measurement. Further, this method was successfully employed for the analysis of real world cabbage and cauliflower samples.

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13

Pesticide Toxicity Amelioration in Plants by Plant Hormones

Palak Bakshi[1], *Shagun Bali*[1], *Parminder Kaur*[1], *Anjali Khajuria*[2], *Kanika Khanna*[1], *Bilal Ahmad Mir*[3], *Puja Ohri*[2] *and Renu Bhardwaj*[1]

[1] *Plant Stress Physiology Lab, Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar 143005, India*
[2] *Department of Zoology, Guru Nanak Dev University, Amritsar 143005, India*
[3] *Department of Botany, School of Life Sciences, Satellite Campus Kargil, University of Kashmir, Jammu and Kashmir, 190006, India*

13.1 Introduction

Rising population enhances the demand of food and to meet the ever increasing demand of a country agricultural productivity is one of the important objectives to meet. So in order to increase gross productivity of agriculture, the use of fertilizers, pesticides, etc. becomes a prime tool to protect the plant from pest attack which results in reduction of annual food production. The word pesticide has the suffix "cide" which means to kill, i.e. is called as "Pest Killer" and is also referred to as a biocide which is classified differently (Figure 13.1 and Table 13.1) The use of pesticides in Asia is on an alarming level in which the most consumptive countries are China followed by Korea, Japan, and India (Uqab et al. 2016). Developing countries of Asia mostly use Organochlorine pesticides such as Aldrin, Dieldrin, DDT, Hexachlorocyclohexane (HCH), etc. because of their cost effectiveness and action on multi pests (FAO 2005; Gupta 2004).

In the developing world, the use of pesticides work in self-poisoning as reported by Eddleston et al. (2002) and every year worldwide around three million cases of pesticide poisoning have been reported. Schulz (2001) shows pesticides as the main source of contamination which passes to the environment by their use in agriculture and viticulture. They directly or indirectly effect humans and plant life present in aquatic ecosystem (Moore et al. 2007) as well as the natural ecosystem (Awasthi et al. 2001). A study by Abhilash and Singh (2009) shows the National and International status of pesticides in which per year approximately two million tons of pesticides are consumed, out of which 24%, 45% and 25% is consumed by USA, Europe, and rest of the world respectively. The government of India, 2007 ranked India on twelfth position in the world and largest producer of Asia.

Pesticides have to be biologically active to control unwanted living species and is different from other chemicals, i.e. they have different degree of toxicity. According to Moretto and Colosio (2011) the use of pesticides affects target as well as non-target

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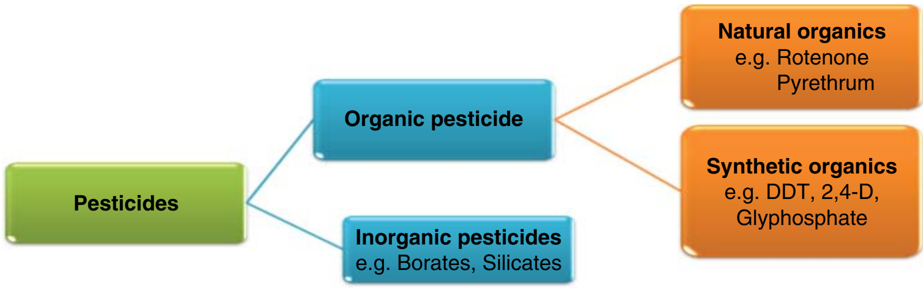


Figure 13.1 Classification based on chemical nature.

Table 13.1 Classification based on target

Pesticides	Target
Avicides	Birds
Algicides	Algae
Bactericides	Bactria
Insecticides	Insects
Nematicides	Nematodes
Fungicides	Fungi
Molluscicides	Mollusk
Rodenticides	Rodents
Acaricides	Mites
Virucides	Viruses

species. Frequent use of pesticides results in development of resistance in pests for it therefore new chemical compounds and higher doses were given, causing a large number of side effects in plants. In developing countries still uses the DDT, HCH, and Lindane as they are cost effective compounds, but are environment persistent and are banned in developed countries. As a result of this, these compounds remain in environment for long period and affect the biotic component of nature, contaminate food, health hazards, etc. In plants, pesticide toxicity results in chlorosis, necrosis, vein discoloration which affects plant growth and development negatively (Kana et al. 2004). A study done on rice seedlings with Chlorpyrifos and Imidacloprid by Sharma et al. (2012, 2013) shows a reduction in biomass, and degradation of photosynthetic pigments. Xia et al. 2006 reported various phytotoxic effects of pesticide in cucumber plant. The effect of pesticides on plants has been described by several workers as represented in Table 13.2.

Productivity in agriculture depends on many conditions and pests like pods borers and sucking bugs, seed borers, etc. have a catastrophic effect in net productivity. Damage in pods result in production of low level of seeds or no seeds, therefore pesticides protect them from getting attacked by the bugs and for better yield. A study done by Parween (2012) which shows that on application of 0.3 mM chlorpyrifos in

Table 13.2 Pesticidal effect on various plant species

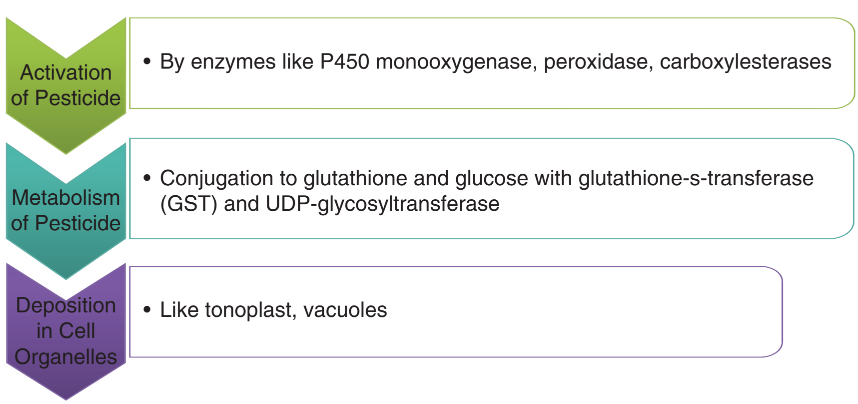
S. No	Pesticide Class	Plant species	Effect of pesticides	References
1.	Herbicide	*Triticum aestivum* L.	Loss of Chlorophyll, Carotenoid content	Ekmekci and Terzioglu 2005
2.	Insecticide	*Lens culinaris* L.	At 0.1% biomass, growth parameters shows increase and after that decreases	Bashir et al. 2007
3.	Fungicide	*Catharanthus roseus* L.	Up regulation of aminoacid, proline, glycine betaine content	Jaleel et al. 2007
4.	Herbicide	*Zea mays*L.	C_4 cycle enzymes like Rubisco, MDH,etc. shows inhibition	Nemat Alla et al. 2007
5.	Fungicide	*Withaniasomnifera* L.	Root length increases but shoot length shows decrease.	Jaleel et al. 2008
6.	Insecticide	*Vigna unguiculata* L.	At 200 ppm significant reduction in shoot length, root length, biomass however at lower concentration above studied parameters shows increase	Mishra et al. 2008
7.	Herbicide	*Saccharum officinarum*L.	Enhancement in SOD, APX, Lipid peroxidation	Chagas et al. 2008
8.	Insecticide	*Momordica charantia* L.	Root length and shoot length, biomass shows significant decrease at 200 ppm concentration	Mishra et al. 2009
9.	Herbicide	*Oryza sativa* L.	Reduction in plant biomass.	Huang and Xiong 2009
10.	Insecticide	*Cenchrussetigerus* Vahl, *Pennisetum pedicellatum* Tan.	At concentration of 75 mg kg^{-1} and 100 mg kg^{-1}, delayed as well as decrease ingermination was examined.	Dubey and Fulekar 2011
11.	Insecticide	*Oryza sativa* L.	Significant increase in sugars, proteins, and amino acid in rice leaves but in leaf sheaths phenol content reduces	Suri and Singh 2011

(*continued*)

Table 13.2 (Continued)

S. No	Pesticide Class	Plant species	Effect of pesticides	References
12.	Insecticide	*Vigna radiata* L.	Plant height, leaves per plant, plant biomass shows significant increase but further increase in dose of insecticide shows downregulation of above studied parameters	Parween et al. 2011
13.	Herbicide	*Triticum aestivum* L., *Secale cereale* L., and *Zea mays* L.	Increase in Total antioxidant activity, Catalase, Ascorbate peroxidase, lipid peroxidation	Lukatkin et al. 2013
14.	Insecticide	*Oryza sativa*	Production of Reactive oxygen species like Superoxide anions, hydrogen peroxide results in oxidative burst	Sharma et al. 2015

Vigna radiata L. seedlings results in increase in yield by enhancing number of pods, seed count, physiological parameters. In the experiment of Rehim et al. 2010 pesticide application increases growth and development of *Zea mays* and bean plant which enhances the productivity. Similar results found in Chibu et al. 2002 in *Glycine max* and *Oryza sativa* and Boonlertnirun et al. (2005) in rice. But imprudent and inadvisable use of pesticide leads to pesticide pollution and also accumulation of residue of pesticide in food at high levels. A report by Boobis et al. (2008) mentioned that safe level of pesticide amount in food cannot be predicted as large number of messengers work at very low amount in our body. Plants itself have a defense mechanism in order to protect them from pesticides which includes three step enzymatic detoxification strategy by Coleman et al. (1997); Cherian and Oliveira (2005) as shown below.



Taking into account the serious pesticide pollution problem, research is being carried out in order to reduce the use of pesticides for sustainable agriculture and finding out the alternative strategies of pesticides (Awang et al. 2015). Various methods are present today in order to remediate or ameliorate the pesticide from nature which includes various physico-chemical methods, enzymatic methods and amelioration with the help of plant hormones.

13.2 Physico-Chemical Methods

13.2.1 Chemical Detoxification and Disposal Methods

a) *Hydrolysis*. It involves the breakdown of ester linkages found in pesticides like carbamates, acetaniledes, pyrethroids, and organophosphates under acidic conditions at high temperature. However, the way for different compounds varies according to the nature of that compound. Various compounds were used for hydrolysis such as calcium hydroxide, sodium hydroxide, potassium hydroxides, sodium perbo- rate, etc. Badawi and Ahmed (2010) reported that addition of a copper (II) ion complex effectively promote the hydrolysis of the carbaryl, diazinon, and cypermethrin.. Theriot and Grunden (2011) reported the hydrolysis of various organophosphorous compounds by the use of various microbial enzymes.
b) *Ozonation*. It involves the oxidation of various aromatic compounds which gets boosted with the help of ozone in presence of UV radiations. The need of UV light is to form the hydrogen radicals playing an important role of oxidizing agents. It is an easy process with rapid effect but cost effective and high energy utilization. Whangchai et al. (2011) reported the use of ozone (O_3) to reduce deposition of chlorpyrifos in fresh lychee fruits. Xiong et al. 2011 reported that when ozonation is combined with ultrasonication the results are more effective to eliminate the heterocyclic pesticides present in water. Pengphol et al. (2012), reported the detoxification of chlorpyrifos which is extensively used on vegetable and fruit crops. They have reported the oxidative degradation of chlorpyrifos by ozonation along with ultrasonic treatments.
c) *Oxidation and reduction*

   Simple oxidation. It is defined as the process which involves the use of H_2O_2 for production hydroxyl radicals which react with the pollutant molecules present in the liquid. This process was rather cheap but not much effective.

   Cavitation. It is a phenomenon which involves the formation, growth, and following crumpling of cavities or microbubbles appearing in milliseconds of time, giving out large amount of energy. Further divided into two types: acoustic cavitation (achieved by passage of high frequency ultrasound waves, 16 kHz–1 MHz)) and Hydrodynamic cavitation (achieved by adjusting the flow and pressure). Under unfavorable conditions the water molecules present in the cavities break down into $H^{\cdot}$ and $OH^{\cdot}$ radicals. Reactive $OH^{\cdot}$ radicals extend into the liquid and causes oxidation of pollutant molecules (Patil et al. 2014).

   Advanced oxidation process (*AOPs*). It is the process in which chemical oxidants (such as H_2O_2 and O_3), catalyst (TiO_2) and salts in presence of UV irradiation with hydrogen peroxide leads to production of hydroxyl radicals, an effective oxidants with extremely oxidation potential of 2.8 V as compared to the molecular ozone (Saritha et al. 2007; Gogate and Patil 2015). These processes are called as AOPs and are basically employed for treatment of effluent water.

1) *UV*. In this process use of light in addition to water, to carry out breakdown of molecules into fragments takes place.
2) *UV/H_2O_2*. Since hydrogen peroxide leads to generation of OH^* radicals, thus it requires a relatively high amount of H_2O_2 in presence of UV exposure.
3) *Photo catalysis*. It involves the use of a semiconductor like ZnO or TiO_2 and an artificial UV light. It is time consuming process when compared with other AOPs. Reaction was initiated by the absorption of the light along with production of electron-hole pairs. The reducing power of generated electrons causes reduction of metal with the generation of the super oxide radical – while left behind holes are competent of oxidizing adsorbed H_2O or HO^- to reactive HO radicals.
4) *Fenton process*. In this process, $OH^{\cdot}$ radicals are generated by using Fenton reagent. It is accompanied by the use of H_2O_2 to Fe^{2+} salts where it acts as a catalyst. However, this process is not much effective to cause mineralization of organic compounds.
5) *Photo-Fenton*. It is an advancement over Fenton process where light is used (UV-VIS wavelength > 300 nm). Fe^{2+} complexes are produced by photolysis of Fe^{3+} and Fenton reactions are carried out by the H_2O_2.

13.2.2 Physical Detoxification and Disposal Methods

a) Adsorption

   It is process in which a substance adsorbs at the surface of another substance called as adsorbent. It is low cost process and based on porosity, sites available, and adsorbent surface area. There are various types of adsorbents available which were used for removal of different pesticides. Generally, activated carbon is used as adsorbent for removal of different pesticides from wastewater and water because of its versatility, high porous nature and surface area. Different kinds of activated carbon are available commercially like powdered activated carbon (PAC), granular activated carbon (GAC), carbon fibers, carbon clothes, activated carbon, black carbon, etc. (Ahmad et al. 2010). Recently, Organo-phosphorous such as diazinon which was used as insecticide was removed by the use of activated carbon from an aqueous solution (Akbarlou et al. 2017). Similarly, Njokua et al. (2014) investigated the removal of herbicide called bentone by sky fruit husk activated carbon (SFHAC) which was used as adsorbent. In 2014, Ke L. et al., reported the use of activated carbon obtained from rice straw for removing carbofuran from aqueous solution. According to this report, it was effective and low-cost adsorbent. Felsot et al. 2003 reported the removal of various pesticides in rinse water like chlorpyrifos, malathion, dimethoate, diazinon and propoxur by using the Carbolator. It decreases the amount of waste produced by many folds due to efficient absorbtion of pesticides. Similar findings were also reported by many different researchers (Foo and Hameed 2010; Seyhi et al. 2014; Velo-Gala et al. 2015).

b) Incinerations
It is the oxidation process carried at very high temperature which converts the toxic pesticide into ash and inorganic gases (volatile acids, water vapor, particles, CO_2, and oxides of metal). Incineration of pesticide should be done at temperatures more than 1000 °C so that within the first two seconds the pesticide gets treated. At high temperatures, smoke production does not take place and produced combustion gases which are similar to those produced by wood burning. Low temperature may lead to production of toxic intermediates products.
c) Open Burning
It is an inexpensive and easy method for the degradation of toxic pesticides, in which piling of empty paper and plastic containers containing pesticides are carried and setting all of them on fire. However, this approach is very much hazardous to workers, flora and fauna. It leaves toxic residue as well as emits various gases, smoke, and fumes directly into the atmosphere.
d) Land Cultivation
It is a method, used for discarding the pesticidal waste by dumping in $15 \times 15 \times 1$ m of unlined soil evaporation pit. This system may cause leaching thus plastic lined pits were constructed so that to avoid leaching. The site of degradation should be in an area which prevent the toxicity of surface as well ground water sources. Construction of concrete pit like plastic lined pit should be carried on leveled ground area with length varies from 8–10 m, depth of 0.5–1 m, and a width of 3.5 m and strengthened with 0.20 m thick concrete walls. It must possess the roof top to prevent rise in water level from climatic activities (rain, snow) but remain open to the atmosphere in order to allow evaporation of water.
e) Land Filling
Here deep soil pits were constructed, and the degradation of contaminated soil was done by microorganisms which changes the composition of the toxic elements. These micro flora effectively breakdown the pesticide components into non harmful elements.

13.3 Enzymatic Methods

Enzymes can be used as a good alternative for decontamination of pesticides because of following features (Karam and Nicell 1997; Alcalde et al. 2006):

a) They act as effectors in transformations of toxicological contaminants.
b) They complete the conversions of contaminants into inorganic end products.
c) They function as active catalysts with broad or narrow spectrum specificity which can be applied to different compounds.

Moreover, enzymes possess many advantages over other methods such as they are not repressed by inhibitors produced in microbial metabolism. Also, under extreme conditions they can be used and are highly active in presence of microbial antagonists (Sheldon and van Rantwijk 2004). Due to all these properties enzymes act as eco-friendly catalysts for treatment of pollutants in environment. Enzymes can undergo pesticide degradation intracellularly or extracellularly (Scott et al. 2008). The prime classes of enzymes involved in pesticide degradation are oxidoreductases, dehalogenases, hydrolases, and transferases and they are mainly produced by microorganisms (Coppella et al. 1990).

13.3.1 Oxidoreductases

These is a wide group of enzymes catalyzing bioremediation reactions. Glyphosate oxidase (Gox) is the best type of enzyme involved in bioremediation of pesticides and is involved in the degradation of glyphosate. It is a flavoprotein amine oxidase produced by *Pseudomonas* and catalyzes oxidation of glyphosate to aminomethylphosphonate (AMPA) and generates keto acid glyoxylate (Scott et al. 2008). In addition, it is also produced by *Agrobacterium* with more efficient degrading activities (Settembre et al. 2003). Moreover, monoxygenases are also involved in xenobiotics degradation through addition of oxygen atom (Joosten and van Berkel 2007). One of the most important members of monooxygenase family in pesticide degradation is two-component flavin diffusible monoxygenase family (TC-FDM) (Galan et al. 2000). Ese and Esd, are well known members of this family involved in the breakdown of polychlorinated insecticides endosulfan and endosulfate (Sutherland et al. 2004). Ese undergoes oxidation of methylene group of endosulfan or endosulfate that produces an intermediate product which is highly unstable; and further leads to dehydration and generation of endosulfan (s-containing intermediate) (Weir et al. 2006). After this, it forms monoalcohol through desulfurization. Another family of oxidoreductases involves Cytochrome P450 oxidoreductases having broad substrate range that catalyzes many recalcitrant reactions (Werck-Reichhart et al. 2000). One of the best example in this category includes bioremediation of herbicide by cytochrome CYP1A1 that degrades atrazine, norflurazon, and chlorotoluron (Kawahigashi et al. 2005, 2007; Yamada et al. 2002). Further cytochrome CYP76B1 also catalyzes oxidative dealkylation of phenylurea herbicides like linuron, chlortoluron, and isoproturon (Didierjean et al. 2002). Also, $P450_{cam}$ produced by *Pseudomonas putida* is highly active against chlorinated pollutants like hexachlorobenzene and pentachlorobenzene (Chen et al. 2002). However, toluene dioxygenases; the member of dioxygenases is an ideal enzyme in the catalysis of monocyclic, fused, linked aromatics, and aliphatic olefins (Whited and Gibson 1991; Bui et al. 2001). They also enable sulfoxidation reactions by conversions of compounds such as methyl-p-nitrophenyl sulfide, ethyl and methyl phenyl sulfide, and p- methoxymethyl sulfides into sulfoxides. They actively function in the detoxification of BTEX residues (p-xylene, benzene, toluene, ethylbenzene), polychlorinated hydrocarbons and chlorotoluenes.

13.3.2 Hydrolases

Another wide category of enzymes are hydrolases which are usually involved in pesticide detoxification. Hydrolysis of different pesticides such as urea, thioesters, esters, carbon-halide bonds, peptide bonds, etc. are stimulated and work without redox cofactors (Scott et al. 2008). Carboxylesterases are type of enzymes which fall under this category helps in detoxification of organophosphorous insecticides (Campbell et al. 1998). In addition, they also degrade pyrethroid insecticides (Heidari et al. 2005). The type of mutation G137D converts this enzyme into phosphoesterase, hindering its carboxyl esterase activity and thus facilitating the breakdown of pesticides such as diazinon, malathion, etc. (Newcomb et al. 1997). It has been further reported that phosphoesterases (OPH, Opd A) produced by *Pseudomonas diminuta, Flavobacterium*, and *Agrobacterium radiobacter* are involved in catalysis of organophosphorous triesters (Harcourt et al. 2002). Apart from this, haloalkane dehalogenases (LinB, AtzA, TrzN) are very effective in detoxification of hexachlorocyclohexane (HCH), a well-known insecticide used against various pests (Kutz et al. 1991). LinB is a haloalkane dehalogenase and is widely useful in degradation of HCH and is produced from *Xanthobacter autotrophicus* and *Sphingomonas paucimobilis*. In addition to this, AtzA and TrzN belonging to amidohydrolase family is produced by *Pseudomonas* and is involved in atrazine catabolic pathway.

13.3.3 Lyases

These are small category of enzymes that undergo catalysis in absence of water or redox factors. The basis for its action is haloelimination which degraded the insecticide γ- hexachlorocyclohexane by lindane dehydrochlorinase (Scott et al. 2008). Haloalkane dehydrochlorinases (LinA) are involved in breakdown of γ- HCH insecticide (Nagata et al. 1993) which are further breakdown by enzyme LiB encoded by lin operon. Therefore, both LinA and LinB possess the strategy to completely remove γ- HCH from environment. However, the method by which it could be achieved is the use of bacteria encoding these enzymes such as *Sphingobium indicum* that contains natural lin operon for degradation of pesticide residues (Raina et al. 2007).

Some of the reports of pesticide detoxification with the help of enzymes are tabulated below (Table 13.3).

13.4 Plant Growth Regulators

13.4.1 Auxins

Auxin, first phytohormone identified and has been broadly researched and focused for many decades (Taiz and Zeiger 2002). Since its existence, our perceptive of auxin actions has significantly enhanced. However, its biology of activities is extremely complicated and difficult to explain. Synthetic analogues which have similar structure to auxins were analyzed for auxinic activity consequently lead to the auxinic herbicides discovery, the initial selective synthetic herbicides (Sterling and Hall 1997). Auxinic herbicides effectively employed to manage broadleaf weeds for more than 60 years with minimum expansion of auxinic herbicide-resistant weeds (Heap 2007). At low concentrations, auxinic herbicides have similar properties to natural auxin. Whereas at high doses, they cause diverse growth irregularities in sensitive dicots such as thickening of roots, stems, leaf epinasty and/or cupping and stem twisting, chlorosis and necrosis (Sterling and Hall 1997; Kelley et al. 2005).

Understanding of auxin signal transduction machinery and auxin-conjugating enzymes may provide new illumination on herbicidal activity. Genes are identified which are induced in reaction to auxin may offer a new path for recognition of non-target herbicide damage in crop plants. In soybean, the auxin-responsive gene (GH3) is particularly stimulated in response to auxinic herbicides and may provide a novel technique for detecting auxinic herbicide damage. Development in our perceptive

Table 13.3 Enzymes involved in Pesticide detoxification

S·No	Enzymes	Pesticides	Source of enzymes	References
1.	Dahalogenases	Organochlorine pesticides (DDT, DDD, HCB, hexachloro-benzene, cyclodiene)	*Aerobacter aerogenes*	Ghosh et al. 2010
2.	Esterases	Organophosphates	*Pseudomonas, Agrobacterium radiobacter, Alteromonas, Plesiomonas, Burkholderia, Hyphomicrobium*	Rosman et al. 2009
3.	Peroxidases	Mecoprop, Isoproturon	*Actinomycetes sp.*	Torres-Duarte et al. 2009
4.	Dehalogenases	Lindane	*Pseudomonas paucimobilis*	Gianfreda 2008
5.	Gox	Glyphosate	*Agrobacterium, Pseudomonas*	Scott et al. 2008
6.	Oxidoredutases (LiP)	Pyrene	*Phanerochaete chrysosporium*	Gianfreda 2008
7.	Chloroperoxidases	Tetra and Polychlorinated phenols and Anilines	*Caldaromyces fumago*	Gianfreda 2008
8.	Laccase	Anthracene benzo pyrene	*Trametes versicolor*	Dodor et al. 2004
9.	Laccase	Bromoxyme, Dichlofenthron, Dichlorophen, Dinoterb, Diuron, Linuron, Niclosamide, Propanil, Picloram, Pentachlorophenol	*Coriolopsis gallica*	Fogg et al. 2003
10.	Hydrolases	Carbofuran, Carbyl or Parathion, Diazinon, Coumaphos	*Ahromobacter, Pseudomonas, Flavobacterium, Nocardia, Bacillus cereus*	Sutherland et al. 2002a,b
11.	Monooxygenases, Hydrolase	Pyrethroids, Organophosphates, Endosulfan, Carbamates,	*Agrobacterium*	
12.	Peroxidases	Anilines, Herbicides, Polyaromatics	*Artromyces ramosus*	Duran and Esposito 2000
13.	Cellulases, phosphatases	Herbicide (Brominal), 3,5-dibromo-4-hydroxybenzonitrile	*A. versicolor, A. Sydowii*	Omar and Abdel-Sater 2001

(*continued*)

Table 13.3 (Continued)

S·No	Enzymes	Pesticides	Source of enzymes	References
14.	Phenol oxidases	Chlorinated compounds	*Trametes versicolor, Phanerochaete chrysosporium*	Duran and Esposito 2000
15.	Aryl sulfatases, Phosphatases	Brominal (3,5-dibromo-4-hydroxybenzonitrile), Insecticide (Selecron) (O-(4-bromo-2-chloro-phenyl) O-ethyl S-n-propyl phosphorothioate)	*Aspergillus, Emericella nidulans*	Omar and Abdel-Sater 2001
16.	Laccase, Lignin degrading enzyme systems (LDSs)	PAHs, Phenanthrene, Chrysene, benzopyrene, benzophenanthrene	*Phanerochaete chrysosporium, Trametes versicolor*	Bumpus 1989, Bogan and Lamar 1996.
17.	Lignin peroxidases (LiP), Lignosulfate	Polychlorinated biphenyls (PCBs)	White rot fungi (*Pleurotus ostreatus, Trametes versicolor*)	Zeddel et al. 1993; Novotny et al. 1997
18.	Laccases (Lacc), Lignin peroxidases (LiP), Mn-peroxidases (MnP)	Anthracene, Pyrene, Phenanthrene, Fluoranthene	*Nematoloma forwardii*	Guenther et al. 1998
19.	Parathion, paraoxon	Organophosphates	*Escherichia coli*	Richins et al. 1997
20.	Hydrolyases	Carbofuran, diazinon, coumaphos	*Achromobacter, Pseudomonas, Flavobacterium, Nocardia, Bacillus*	Reddy 1995; Serdar et al. 1982

of auxin biology will offer many novel opportunities for futuristic application of auxinic herbicides in agriculture (Kelley and Riechers 2007).

13.4.2 Abscisic Acid

Abscisic acid (ABA) is a well-known plant growth hormone reported to get accumulated under water deficit condition (Zhang et al. 2006). ABA not only play important role in plant developmental processes (seed dormancy, embryo maturation, stomatal closure, and senescence) but also in promoting tolerance against abiotic and biotic stresses. ABA plays role in management of various stresses e.g. drought (Al Muhairi et al. 2015; Hussain et al. 2015), temperature (Liu et al. 2016), heavy metal (Pompeu et al. 2017). The function of ABA in detoxification of pesticide is still a field of exploration. The role of ABA in mediating pesticide detoxification was studied with herbicide safener cyprosulfamide (CSF) in *O. sativa* plants (Dashevskaya et al. 2013). CSF exposure to plant (either alone or in along with ABA) protected the plants but, the combined treatment of CSF and

ABA allowed plants to uphold increased growth characteristics and shows involvement of CSF and ABA signaling pathways.

ABA is also reported to increase the resistance of cotton cotyledon to herbicide endothall as studied by Rikin and Rubin 1983. Endothall (10^{-4} M) was exposed to *Gossypium hirsutum* for 24 hours resulted in enhanced level of polyphenols and browning of tissues. The pre-exposure with 10^{-5} ABA effectively lowers the electrolyte leakage and other apparent damaging effects. The treatment with ABA many hours before the exposure to endothall lowered the harmful effects and induces resistance in plants.

The uniconazole induced ABA accumulation in *Phaseolus vulgaris* is another example of resistance to environmental stress which was reported by Mackay et al. 1990. The 14 day old plants were given 100 ml of Unicanazole treatment ($10\,mg\,l^{-1}$) and ABA ($3\,mg\,l^{-1}$). These plants were kept in observance and analyzed at alternate days. Results suggest that concentration of ABA, proline, and total amino acids got enhanced which is responsible for environment stress resistance in *P. vulgaris* also suggests that this accumulation of ABA might be due to unicanazole treatment. The role of ABA in tolerance to a non- selective herbicide Phosphinothricin (PPT) (a glutamine synthetase inhibitor) was studied by Hsu and Kao 2004. The Enzyme-linked immunosorbent assay (ELISA)was used to determine the content of ABA in PPT-tolerant and PPT-sensitive rice cultivars. Decline in and chlorophyll and protein content were observed to assess the PPT toxicity. The exposure to PPT results in major enhancement in ABA level in PPT tolerant cultivar (cv. Taichung 67, TNG67) however no alteration in the content of ABA was examined in PPT sensitive cultivar (cv-Taichung Native 1, TN1). Exposure to PPT results in minor decline in transpiration rate of TN1 in comparison to TNG67 seedlings. Pretreatment of ABA to TN1 plants increase the PPT tolerance and results in rescued accumulation of NH_4^+ induced by PPT. Application of ABA biosynthesis inhibitor i.e. fluridone, declined tolerance to PPT and also the content of NH_4^+ in the leaves of TNG67 plants. The effect was antagonized on application of ABA. All these experiments showed that enhancement in the endogenous levels of ABA upregulate the tolerance of rice seedlings to PPT.

13.4.3 Brassinosteroids

Brassinosteroids (BR)s are recognized as protecting agents in crop plants from fungicides, herbicides, and insecticides toxicity. Exogenous application of BRs reduced the pesticidal residue of common organochlorine, carbamate, and organophosphorus pesticides by 30–70% in rice, tomato, cucumber, strawberry, celery, Chinese chives, garlic Chinese cabbage, etc. In tomato plants, genomic microarray studies revealed that co-application of fungicide (chlorothalonil [CHT]) and BR shows enhanced expression of 301 genes and also genes expressing oxidoreductase, cytochrome P450, hydrolase and transferase. BRs increased pesticide degradation by elevating glutathione-S-transferase (GST) activity and glutathione metabolism through a Respiratory burst oxidase homologue1 (RBOH1)-dependent pathway. Gene silencing technique demonstrated that brassinosteroids reduced the concentration of pesticides in plants possibly by stimulating their signaling pathway induced metabolism associated brassinosteroids-triggered production of H_2O_2 and change in redox potential at cellular level. The study gives a new approach for reducing pesticide remains in crop plants by utilizing their own detoxification process (Zhou et al. 2015).

Exogenous treatment of 24-epibrassinolide (24-EBL) hastened the metabolism of different pesticides (chlorpyrifos, β-cypermethrin, chlorothalonil, and carbendazim) resulted in decrease in the level of residues in *Cucumis sativus* L. Chlorpyrifos is an extensively used pesticide, in *Cucumis sativus* L. it cause considerable decrease in quantum yield of PSII (ΦPSII) and net photosynthetic activity (Pn).. Treatment of 24-EBL improved Pn and ΦPSII under chlorpyrifos toxicity, and this effect of 24-EBL was related with decrease in chlorpyrifos residues. 24-EBL stimulated the actions of GST, POD (Guaiacol peroxidase), and GR (Glutathione reductase) in chlorpyrifos treated plants. It also increased the expression of P450 (encodes for P450 monooxygenase) and MRP (Multidrug resistance-associated protein) (which encodes for ABC type transporter). 24-EBL has also stimulatory effect on pesticides (cypermethrin, chlorothalonil, and carbendazim) metabolism. The results revealed that brassinosteroids could be effective and eco-friendly natural substances appropriate for wide application to decrease the hazardous effect of pesticides on human and environment (Xia et al. 2009). BRs stimulate activities of various plant P450s and increase conjugation of glutathione concerned in the herbicide biodegradation (Hatzios and Burgos 2004). It has been reported that phytohormones are involved in plant induced defense and detoxification mechanisms in response to various pesticides (Hatzios and Burgos 2004).

Stress amelioration is assisted by BR through diverse regulatory mechanisms such as osmolytes accumulation, increased pigment content, reduction in MDA (Malondialdehyde) content, decreased production of reactive oxygen species (ROS), increased the expression of antioxidative defense genes and their activity. Improvement in these attributes provided a protective mechanism to combat the toxic effects caused by pesticides in rice plants. The study elucidated the ameliorative role of BRs in response to stress is significant for its innovative application in agriculture (Sharma et al. 2015).

Application of EBL reduced the concentration of hydrogen peroxide (H_2O_2) and superoxide anion (O_{2-}) followed by increased activities of SOD (superoxide dismutase), CAT (catalase), GR, GST, POD, and also enhanced the GSH content. The expressions of SOD, CAT, GR, POD, NADH (Nicotinamide adenine dinucleotide), NADH-ubiquinone oxidoreductase, GSH-S (glutathione synthase), GSH-T (glutathione transporter-1), P450 (cytochrome P450 monooxygenase), CXE (carboxylesterase) and GST1-3,5-6 genes were increased in the seedlings treated with EBR under IMI (Imidacloprid) toxicity. Treatment of EBL reduced the expression of RBO (respiratory burst oxidase) in IMI stressed seedlings. In *Brassica juncea* seedlings, EBR declined IMI residues by more than 38%. From the study, it has been concluded that seed soaking treatment of EBR significantly decreased oxidative stress and Imidacloprid (IMI) residues by modulating the expression of antioxidative specific genes of *B. juncea* under IMI stress. Application of EBR may protect plants from pesticide toxicity (Sharma et al. 2017).

Sharma et al. 2016a reported that EBL application improved growth, content of photosynthetic pigments, total phenols, polyphenols, and organic acids under IMI toxicity in *B. juncea*. The gene expression of key enzymes of pigment, polyphenols, phenols, and organic acid biosynthetic pathways were analyzed such as *CHLASE* (chlorophyllase), *PSY* (phytoene synthase), *CHS* (chalcone synthase) and *PAL* (phenylalanine ammonialyase), *CS* (citrate synthase), *SDH* (succinate dehydrogenase), *SUCLG1* (succinyl Co-A ligase), *MS* (malate synthase) and *FH* (fumarate hydratase). It has been concluded that application of 24-EBR improved growth and altering the gene expression of key enzymes

(*CHLASE, PSY, CHS, PAL, CS, SDH, SUCLG1, MS and FH* in *B. juncea* seedling under IMI stress (Sharma et al. 2016a).

Extensive use of pesticides protects the crop plants from several insect pests. But, this results in toxicity to plants which lead to growth retardation. IMI treated plants showed a significant decrease in shoot length, number of leaves, chlorophyll content and gaseous exchange parameters such as photosynthetic rate, stomatal conductance, inter-cellular CO_2 and transpiration rate as compared to untreated plants. Carotenoids, anthocyanins, and xanthophylls were enhanced with IMI stress. Application of EBL reduced the harmful effects of IMI and enhanced the growth, photosynthetic pigment contents and gaseous exchange parameters in *B. juncea* plants under IMI stress (Sharma et al. 2016b).

Chlorpyrifos (CPF) caused negative influence on growth and protein content of rice seedlings but it enhanced the contents of MDA and proline. Antioxidative enzymatic activities (SOD, APX, CAT, GPX, and MDHAR) were enhanced under CPF application. 24-epibrassinolide (EBL) treatment improved growth, protein and proline contents and the activities of antioxidant enzymes however there was reduction in MDA content under CPF toxicity in rice. Treatment of EBL increased the transcript level of Fe-SOD and CAT in CPF treated seedlings. EBL ameliorated the stress induced by CPF in rice seedlings (Sharma et al. 2012).

Treatment of EBL improved growth, protein and proline content and also upregulated Fe-SOD, Cu-Zn SOD, APX, and CAT in the rice seedlings under IMI stress (Sharma et al. 2013). In plants, pesticide toxicity caused oxidative stress and their remains accumulate in their parts, which is a chief issue for the environment and human health. BRs provide protection to the plants against pesticide toxicity.

13.4.4 Salicylic Acid

Salicylic acid (SA) is an important plant growth regulator. It plays vital role in plant defence and pathogen attack (Loake and Grant 2007). In response to biotic and abiotic stress, SA acts as a signaling molecule in plants and elicits specific responses. The signaling transduction pathway of SA during defence action involved its interaction with ROS. Studies of Ding et al. 2002; Yang et al. 2003; Zhou et al. 2009 have reported the role of SA in plant adaptive response to abiotic stress. SA has also been reported to modulate proline level in plants which is an important precursor of abiotic stress in plants (Matysik et al. 2002). Hence, SA is supposed to have important role in pesticide detoxification.

It was found by Cui et al. 2010 that SA decreased Napropamide (herbicide) toxicity by reducing its accumulation in *Brassica napus*. The plants given treatment of 8 mg kg^{-1} of napropamide resulted in toxic symptoms like stunned growth and oxidative damage. However, treatment with 0.1 mM SA not only helps in promoting plant growth but also reduce oxidative damage i.e. reduced $O_2^{-\cdot}$, H_2O_2 and activities of antioxidative enzymes. Similarly, exogenous application of SA to *Vicia faba* L. alleviates the toxic effects of insecticides like alphamethrin (AM) and endosulfan (ES). Application of different concentrations (1.50, 3.0, and 6.0 ppm) of insecticide on *Vicia faba* showed significant decline in mitotic index (MI) and formation of various chromosomal abnormalities in the meristematic tissue. However, pre-treatment of seeds with SA leads to enhanced MI and significant decline in the formation of chromosomal abnormalities. SA treatment also reduces the accumulation of proline and enhances the carotenoid content in plants (Singh et al. 2013).

Ananieva et al. (2002, 2004) reported that SA enhances tolerance to paraquat (Pq.) in barley plant. Plants given treatment of 500 μmol l^{-1} of SA along with 10 μmol l^{-1} of Pq. Exposure to 10 μmol l^{-1} declined the activity of APX and GR and increases the activity of CAT. However, the pretreatment of 500 μmol l^{-1} of SA for 24 hours increased the activity of SOD, POD, and CAT suggesting the role of SA in antagonizing the effect of paraquat. The adverse effect of quizalofop-p-ethyl (a post emergent aryloxyphenoxy propionate herbicide) exposure to *Helianthus annus* L. cv. was observed by Bayram et al. 2015. The treatment to *Helianthus annus* with (0.3–3.1 mM) quizolofop-p-ethyl results in enhanced antioxidative enzymes, pigment system, and total phenolic compounds after 1, 5, 10, and 15 consecutive days. Treatment of 0.5 mM SA resulted in increased herbicide resistance by enhancing the activity of certain enzymes (PPO and APX).

The role of SA in inducing protection in *V. faba* plants against Ridomil MZ attacked by fungi (*Botrytis fabae*) causing chocolate spot disease was studied by Soliman 2015. The use of ridomil MZ and infection by fungi cause significant reduction in plant growth parameters. However, exogenous and pre-soaking treatment with SA improved plant growth and yield components and content of biochemical compounds. Salicylic acid and its mixture with different insecticide treatments (i.e. cyhalothrine, imidacloprid, and profenofos) was given to cotton plant. The plants were first given treatment with only these insecticides as well as in reduced doses by combination with 1 mol l^{-1} of SA. The mixed treatment of SA and insecticides showed enhanced pesticide potency against *Spodoptera littoralis* larva and also enhances yield characteristics (Ali 2008).

13.4.5 Jasmonic Acid

The profound use of pesticides had become a hazard to human life as well as natural ecosystem (Awasthi et al. 2001). Taking into account this serious problem research is being carried out for reduction in the use of pesticides for sustainable agriculture and finding out the alternative strategies of pesticides (Awang et al. 2015). Different studies have been done in order to understand the impact of Jasmonic acid (JA) in plants against different type of stresses (Avanci et al. 2010). Although JA is involved in many physiological processes such as stomatal opening, root growth, seed germination, tendril coiling, fruit ripening, leaf senescence, tuber formation, and also act as defensively in response to pathogenic attack (Wasternack 2007). It was demonstrated that JAs along with their derivatives modulates gene expression activities concerned with defensive actions (Rosahl and Feussner 2005). These defensive responses can be initiated because of necrotrophic pathogenic infections (Trusov et al. 2006; Avanci et al. 2010). Moreover, studies have been reported in which JA improved resistance to herbivore attack by inducing defensive genes (Lorenzo and Solano 2005; Howe and Jander 2008). Therefore, there is a need to use such elicitors which could act as an alternative strategy of pesticides. By the use of JA, plant resistance could be enhanced in response to natural herbivores without altering the natural processes associated with plants (Thaler et al. 2001). The trend of use of JA against insect herbivore pests is been considerably increasing McConn et al. 1997; Wasternack and Parthier 1997). They lead to accumulation of endogenous levels of JA, thus enabling the direct link of JA against herbivory (Doares et al. 1995). Along with this, JA when applied exogenously, enhances the defensive pathway in tomato against damage caused by herbivores (Thaler et al. 1996). This damage caused by *Helicoverpa zea* further induces various enzymatic activities such as peroxidases, lipoxygenase, phenylalanine ammonia lyase, proteinase inhibitors, and polyphenol oxidases. Several studies were examined to study the effect of JA-induced responses against herbivory attack in tomato plants and it was found that JA was toxic to herbivores and it reduced the number of aphids, caterpillars, thrips, beet armyworm larvae and flea beetles (Thaler et al. 2001).

In order to use JA as pest control technique, it not only reduced the herbivore attack but also improved the yield of the crop. In addition, jasmonate pathway being highly conserved in plants could be a functional tool to control pests in various plants taxa (Constabel and Ryan 1998). Studies were further conducted to compare the use of pesticides and JA against different pests in chili plants. The results showed that JA treated chili plants reduced the disease incidence caused by pesticides and improved its growth and yield characteristics. Therefore, they concluded that exogenously applied JA (0.5 mM) in chili plants could be the most effective and possible substitute of pesticide application (Awang et al. 2015). It was further demonstrated that the expression of enzymes involved in defense such as lipoxygenases and peroxidases followed by polyphenol oxidases were stimulated in plants exposed to ventral eversible gland intact (VEGI) caterpillars. Further, it was observed that genes encoding these enzymes were also involved in JA biosynthesis and terpene synthases (Zebelo et al. 2014). Considerable evidence implicates the role of JA against *Bradysia impatiens* as observed in *Arabidopsis* (McConn et al. 1997) and spinach (Schmelz et al. 2002). They demonstrated that this mutant will be the most effective genetic model for studying the defensive genes as they observed that exogenously applied methyl jasmonate (MeJA) significantly reduced the mortality rate up to 12% which shows that plants are able to protect themselves against the herbivory attack (Ballare 2011). The nature of these studies shows that JA-induced resistance against herbivore community could be linked in developing the alternate methods to pesticide application in plants in near future.

13.4.6 Polyphenols

Polyphenols are plants secondary metabolites with one phenolic ring (OH attached with aromatic hydrocarbon chain), produced in the phenylpropanoid pathway exclusively through the shikimmic acid pathway. There basic structure is devoid of nitrogen based functional group (Quideau et al. 2011). These are categories into three main classes: Flavonoids (C6–C3–C6 backbone), Tannins and Phenolic acids. Green leafy vegetables are rich in antioxidants in the form of polyphenols (Datta et al. 2013). These antioxidants are free radical scavengers and possess the capability to inhibit lipid peroxidation and prevent oxidative damage caused by ROS. Present investigation deals with the study of their role in detoxification of various pesticides. It has been reported that polyphenols have anti-stress properties and lower the damage caused by various pesticides. These polyphenols neutralize the various ROS and do not leads to formation of singlet oxygen that cause huge damage in cell membrane. Various enzymes that produce these secondary metabolites are activated during stress caused by various pesticides like carbamates, acetaniledes, pyrethroids, carbaryl, diazinon, cypermethrin, and organophosphates. Pesticides are chemical substances that include various herbicides, insecticides, weedicides, and used globally to control pests in household and agricultural environments (Diwedi and Flora 2011). Several findings have been reported by various scientists who showed protective role of polyphenols during pesticides stress. Mallick et al. 2009 revealed the protective role of polyphenols content in-vitro in *Enydra fluctuans* during adverse effects of acephate, an organophosphate insecticide. The methanolic extracts of *E. fluctuans* have proved to be a strong reduction power, possess significant DPPH (2,2-Diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity. Datta et al. 2010 reported similar results in rats by polyphenols under acephate an organophosphorous Acephate (O,S-dimethyl acetylphosphoramidothioate), used against various pests like aphids, wasps, thrips, jassids, ants, which affects crops such as wheat, wheat, etc. Similarly, polyphenols of *Ipomoea aquatic* are reported to have protective role in Charles Foster rat against carbofuran toxicity.

The effects of polyphenols were measured in terms of various enzyme activities which was found to be significantly increased with *I. aquatic* extracts treatment in carbofuran treated individuals when compared to carbofuran treated species. Similarly, Sharma et al. 2016a results were concomitant with these findings. In this report, application of 24-epibrassinoslide in *B. juncea* under imidacloprid (IMI) toxicity induce the polyphenol and total phenol content as well as the phenylalanine ammonialyase (PAL) activity in such plants, revealed the indirect involvement of polyphenols in stress tolerance which could be due to activation of phenylpropanoid pathway (Ahammed et al. 2013; Xie et al. 2013). All these findings revealed the protective role of polyphenols during stress induced by different pesticides.

13.5 Conclusion

Pesticides play key role in enhancing the agriculture productivity which also results in high profits to farmers by controlling diseases but their adverse effects now increase more than their benefit given to human race. These effects raised in the form of pest resistance, reduction in beneficial component of nature like pollinators, predators, earthworms, micro flora, etc. Persistent nature of most of the pesticides and their frequent use in large scale results in its entry in higher trophic levels. Plants also show toxicity symptoms of it, non target plants also get affected. To protect our environment, plants, animals including human its necessity to proper use of pesticides, alternative strategies like IPM (Integrated Pest Management), resistant genotype, physical and mechanical control, alternate cropping system, proper spraying equipment. Educational programs for farmers to encourage them and to make sure proper use of new techniques to reduce the pesticide pollution from our environment.

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14

Transgenic Strategies to Develop Resistant Plant Against the Pathogen and Pest

Neeraj Kumar Dubey, Kapil Gupta, Pawan Yadav, Jogeswar Panigrahi and Aditya Kumar Gupta

Department of Biotechnology, School of Life Sciences, Central University of Rajasthan, Ajmer, 305817, India

14.1 Introduction

Plants are sessile and grow across the diverse agro-climatic conditions. Many biotic and abiotic factors including pathogens, pests, drought, heavy metals, UV-B, etc. (Tripathi et al. 2012a, b; Singh et al. 2014; Rejeb et al. 2014; Singh A et al. 2015; Singh S et al. 2015; Tripathi et al. 2015, 2016; Singh et al. 2017; Tripathi et al. 2017; Bakhat et al. 2018) influence several morpho-physiological, biochemical, and molecular traits which ultimately affect the net crop yield and the sustainable agro-ecosystems. The majority of crop plants provide a conducive habitat for pests and pathogens. The battle between plants and herbivorous pathogens/pests has been continuing since the first crops grown by ancient people. Therefore, the farmers developed many strategies to control these pests and pathogens, which evolved through time with the development of scientific knowledge regarding plant–pest interaction. Pest management strategies generally aim to augment the net crop yield by reducing loss and minimum input costs. Innumerable efforts have been made to develop suitable techniques for the protection of crops from pests and pathogen invasion, and most of these techniques broadly employ two approaches, viz. conventional and biotechnological approaches. Conventional approaches include cultural practices, physiochemical control measures, and biological/phytochemicals mediated controls. Chemically synthesized, biological, as well as phytochemical derived pesticides seem to be promising in reducing the yield loss; but the development of resistance against these pesticides and targeting to beneficial insects/pests are one of the major drawbacks of these pesticides. Thus, the best strategy will be the introducing insecticide/pesticide property in growing crops without harming its productivity.

Innate resistance to attacking pathogens and pests is only available for some crops and induced resistant property can be obtained by approaches like transgene introgression and molecular breeding employing host resistance. Although molecular breeding technique is considered as less expensive and ecofriendly, however, host resistance for the concerned biotic stress among the compatible genotype are not available as reported in the case of many borers, folder, sheath blight and rot diseases in rice where no germplasm possessed the resistant alleles for the concerned traits (Grover and Pental

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2003). Thus, transgene approach arises as novel tool to introduce new insecticidal genes in desired crops (Koziel et al. 1997). In this approach, one or multiple genes or gene products inhibiting the metabolic and physiological process of the targeted pest were used to combat the pests. Many gene integrated plants generated belonging to different crops and plant species by using a combination of variety of genes and promoters to control the insect pests (Silva and Klessig 1998; Gatehouse 2008). Even transgenic strategy helps the farmers to manage the crop productivity by growing herbicide and insect resistant transgenic plants (James 2003), and this approach can meet the food requirement of the growing population that has estimated upto 6 billion by 2050 (James 2003). Further, the integrated genes can be transferred to desired varieties through conventional breeding strategy (Koziel et al. 1997).

The development of transgenic plant is multi step process. It includes identification of pest specific toxic genes, development of expression cassette of the gene for plant using suitable promoter, genetic transformation technique for generation of transgenic plants, insect bioassay, comparison of economic value of transgenic lines in terms of yield and field trial. Two major systems were mostly used in transgenic development for pest resistant viz. use of bacterial insecticidal genes (δ-endotoxin) to provide protections from pest damage and exploiting the endogenous plant protection mechanisms. In addition, other plant insecticidal genes including inhibitors like protease inhibitors, amylase inhibitors, lectins etc. which have inhibitory activity against the pests were also used successfully. Transgenic crop expressing δ-endotoxin was the first crop commercialized. Further, the RNAi mediated pathogen/pest resistant transgenic strategy has also been applied to control the pathogen/pest attack. In this chapter different strategies and approaches used to control pathogen and pest has been discussed along with use of the insecticidal genes for the development of transgenic crops.

14.2 Techniques Used for Transgenic Plant Development

Gene transfer methods during transgenic plant development provides versatile platform not only to introduce the genes controlling agroeconomic traits for cultivar development but also for studying their functional and regulatory property. Since the initial report of Agrobacterium mediated transformation to produce transgenic plants (Veluthambi et al. 2003; Gelvin 2000; Darbani et al. 2008) large number of gene transfer methods have been developed and propounded in literature (Table 14.1). These gene transfer methods are broadly categorized into three groups, viz. biological vector based, physical or direct DNA delivery and combination of both biological and physical methods.

The biological vector based methods includes mostly the Agrobacterium based transformation and vectors whereas the direct gene delivery techniques include the chemical alterations or application of physical forces such as pressure or electric discharge for delivery of desired gene into the host cell (Darbani et al. 2008). In combination of biological and physical methods are used in such cases where biological and physical methods are not much efficient. Physical methods like sonication or Agrolistics approach facilitate entry DNA into host and beneficial for plants which are recalcitrant in transformation (Hansen and Chilton 1996; Trick and Finer 1997; Pathak and Hamzah 2008). Among all the techniques Agrobacterium mediated and biolistic mediated methods are the methods of choice for the development of transgenic plants aiming

Table 14.1 Gene transfer methods used for plants

Approach	Description of technique	References
Biological delivery methods		
Agrobacterium rhizogenes	Carrot disks, tobacco, and morning glory stem segments inoculated	Chilton et al. (1982) and Tepfer (1984)
Agrobacterium tumefaciens	Tobacco stem segments, leaf disks	Barton et al. (1983) and Fraley and Horsch (1983)
Agroinfection	Leaves or fruits inoculated with Agrobacterium containing foreign viral DNA	Grimsley and Bisaro (1987) and Fu et al. (2005)
In planta	Agrobacterium suspension applied by vacuum infiltration or dipping floral parts, meristems or embryo axis	Bechtold et al. (1993) and Clough and Bent (1998)
Other microorganism	Used Rhizobium, Sinorhizobium, and Mesorhizobium bacterium containing disarmed Ti plasmid for transformation of tobacco and Arabidopsis	Broothaerts et al. (2005)
Physical delivery methods		
Bioactive beads	Immobilized DNA on calcium alginate microbead- for transfer to protoplasts	Sone et al. (2002), Liu, H.B. et al. (2004), Liu, Y.J. et al. (2004), and Murakawa et al. (2008)
Electroporation	Electric pulses delivered to protoplasts, mesophyll cells, intact tissues	Fromm et al. (1985), Lorz et al. (1985), Li et al. (1991), and Arencibia et al. (1995)
Laser micropuncture	Laser-mediated holes in cells and tissues allow uptake of foreign DNA	Guo et al. (1995) and Badr et al. (2005)
Liposomes	Liposome containing DNA is taken up by protoplasts	Deshayes et al. (1985)
Microinjection	Injecting foreign DNA into protoplasts, intact cells	Morikawa and Yamada (1985), Crossway et al. (1986), and Griesbach (1987)
Nanoparticles	Nanoparticles coated DNA is taken up by protoplasts	Torney et al. (2007)

(Continued)

Table 14.1 (Continued)

Approach	Description of technique	References
Particle bombardment	Acceleration of tungsten and gold particles coated with foreign DNA by vaccum pressure	Klein et al. (1988), Sanford (1991), and Vasil et al. (1991)
Electric discharge	Gold particle acceleration via electric expulsion to transfer DNA to target cells (ACCELLTM technology)	McCabe and Christou (1993)
PEG mediated DNA uptake	DNA transferred to protoplasts, pollen/plant or cut styles just after pollination	Uchimiya et al. (1986) and Ohta (1986)
Silicon carbide whiskers	Vigorous shaking silicon carbide fibers along with DNA and suspension cells or embryogenic callus	Kaeppler et al. (1992) and Petolino et al. (2000)
Combination of physical and biological methods		
Agrolistics	Combination of biolistic and Agrobacterium approach, delivering virD1 and virD2 genes biolistically for in planta transfer of T-DNA	Hansen and Chilton (1996)
Sonication assisted with Agrobacterium	Plant tissue is sonicated for short periods in Agrobacterium suspension	Trick and Finer (1997) and Pathak and Hamzah (2008)

genetic augmentation. However, due to exploration of plant genomes and emergence of the functional genomics as frontier area in plant research there is requirement for high throughput plant transformation methods. Thus, at present the plant genetic transformation methods receive renewed emphasis and the development of nanoparticles for DNA delivery into plant cell is emerging and is likely to integrate with either Agrobacterium and the biolistic method of DNA delivery system fulfills the requirements in area of plant research.

14.3 Transgenic Plants Developed Against Pathogens and Pests

14.3.1 Virus

Viruses are important pests attacking the plant and reducing crop productivity. Due to the green revolution, growing of homogenous crop variety in wide area led them more prone toward viral diseases (Dasgupta et al. 2003). Viruses utilize machinery of host plant for their multiplication and assembly. Targeting the machinery, resistant plants can be developed against viral disease. Generally, two strategies have been utilized to generate transgenic plant against vial disease. In first one pathogen derived resistance is achieved by using viral coat protein (CP), replicase (Golemboski et al. 1990), movement protein (Lapidot et al. 1993; Cooper et al. 1995), and satellite RNA. In another strategy non pathogen derived resistance through post transcriptional gene silencing, ribosomal inactivating protein (Lodge et al. 1993), protease inhibitors (Gutierrez-Campos et al. 1999), interferon like system, etc. have (Dasgupta et al. 2003) used (Table 14.2). CP-mediated resistance has been used to develop several virus resistant plants (Beachy 1999; Dasgupta et al. 2003). Gene for CP of tobacco mosaic virus (TMV) expression in tobacco led to development of resistance against TMV (Abel et al. 1986). The expression of zucchini yellow mosaic virus (ZYMV) and/or watermelon mosaic virus 2 (WMV 2) coat protein (CP) in Hybrid squash ZW-20 showed resistance against ZYMV and/or WMV 2 (Fuchs and Gonsalves 1995). Expression of Cymbidium mosaic virus (CymMV) coat protein in Orchid resulted to resistance against Cymbidium mosaic virus (Koh et al. 2014). Expression of sugarcane mosaic virus (SCMV) coat protein in sugarcane showed resistant against SCMV (Yao et al. 2017).

Gene-silencing strategy has also been applied to virus resistant transgenic plants development program (Leibman et al. 2015; Prins et al. 2008). Expression of double-stranded RNA (dsRNA) or RNAi against virus showed resistance in Cassava cultivar KU50, sugarcane, rice, Jatropha and tomato showed resistant against Sri Lankan cassava mosaic virus (SLCMV) (Ntui et al. 2015), Potyvirus SCMV (Guo et al. 2015), Rice black-streaked dwarf virus (RBSDV) (Ahmed et al. 2017), Indian cassava mosaic virus (ICMV) (Ye et al. 2014), tomato yellow leaf curl virus (TYLCV) and Geminivirus (Leibman et al. 2015; Singh A et al. 2015; Singh S et al. 2015) respectively. Further expression of RNA-dependent DNA methylase (RdDM) in *Vigna mungo* and GmAKT2 potassium channel in soybean showed resistant against *V. mungo* yellow mosaic virus (VMYMV) (Poggin et al. 2003) and Soybean mosaic virus (SMV) (Zhou et al. 2014) respectively. The expression of eukaryotic translation initiation factor 2B-beta (eIF2Bβ) in Mustard (*Brassica juncea*) showed resistance against Potyvirus Turnip mosaic virus (TuMV) (Shopan et al. 2017).

Table 14.2 Genes used for virus resistant program development through transgenic strategies

S.-No.	Genes used	Promoter used	Crop plants	Target viruses	References
1	Coat protein (CP) gene of tobacco mosaic virus (TMV)	CaMV35S	Tobacco	Tobacco mosaic virus	Abel et al. (1986)
2	Replicase of TMV	CaMV35S	Tobacco	Tobacco mosaic virus	Golemboski et al. (1990)
3	Movement protein of TMV	CaMV35S	Tobacco	Tobacco mosaic virus	Lapidot et al. (1993)
4	Pokeweed antiviral protein (PAP, a ribosome-inhibiting protein)	CaMV35S	Tobacco and tomato	Multiple viruses	Lodge et al. (1993)
5	Defective movement protein of TMV	CaMV35S	Tobacco	Multiple viruses	Cooper et al.(1995)
6	Zucchini yellow mosaic virus (ZYMV) and/or watermelon mosaic virus 2 (WMV 2) coat protein (CP)	—	Hybrid squash ZW-20	Zucchini yellow mosaic virus (ZYMV) and/or watermelon mosaic virus 2	Fuchs and Gonsalves (1995)
7	Rice cysteine proteinase inhibitor	CaMV35S	Tobacco	Potyviruses	Gutierrez-Campos et al. (1999)
8	RNA-dependent DNA methylase (RdDM)	CaMV35S	*Vigna mungo*	*Vigna mungo* yellow mosaic virus (VMYMV)	Poggin et al. (2003)
9	Overexpression GmAKT2 potassium channel	CaMV 35S	Soybean	Soybean mosaic virus (SMV)	Zhou et al. (2014)
10	Cymbidium mosaic virus (CymMV) coat protein	—	Orchid	Cymbidium mosaic virus	Koh et al. (2014)
11	double-stranded (ds) RNA with sequences homologous to five key genes of ICMV-Dha strain DNA-A	CaMV 35S	*Jatropha curcus*	Geminivirus -Indian cassava mosaic virus (ICMV)	Ye et al. (2014)

12	Targeted to three conserved sequences (the intergenic region [NCR], V1-V2 and C1-C2 genes of genome of (TYLCV)	CaMV35S	Tomato	tomato yellow leaf curl virus (TYLCV)	Leibman et al. (2015)
13	RNA interference (RNAi) against AV2 and AV1 of DNA	CaMV 35S	Cassava cultivar KU50	Sri Lankan cassava mosaic virus (SLCMV)	Ntui et al. (2015)
14	RNA interference (RNAi) against Sorghum mosaic virus (SrMV) coat protein (CP)	CaMV 35S	Sugarcane cultivar ROC22	Potyvirus sugarcane mosaic virus (SCMV) or Sorghum mosaic virus (SrMV)	Guo et al. (2015)
15	RNAi against proteins (AC2 and AC4)	—	Tomato	Geminivirus	Singh A et al. (2015), Singh S et al. (2015)
16	RNA interference (RNAi) against genes S7-2 or S8	RbcS	Rice	Rice black-streaked dwarf virus (RBSDV)	Ahmed et al. (2017)
17	Bacteriophage CP933 endolysin	Transient expression	Tobacco	Antimicrobial activity	Kovalskaya et al. (2016)
18	Eukaryotic translation initiation factor 2B-beta (eIF2Bβ)	CaMV 35S	Mustard (*Brassica juncea*)	Potyvirus Turnip mosaic virus (TuMV)	Shopan et al. (2017)
19	Sugarcane mosaic virus coat protein	Maize ubiquitin	Sugarcane	Sugarcane mosaic virus (SCMV)	Yao et al. (2017)

14.3.2 Bacteria

Phytopathogenic bacteria are a serious pest for agricultural productivity. Cultivated major crops are generally affected by at least one pathogenic bacterium during their life period (De La Fuente and Burdman 2011). Several strategies have been tried to develop transgenic crops like cotton, tomato, rice, citrus, etc. by expression of antibacterial proteins, inhibiting pathogenicity or increasing natural plant defenses (Mourgues et al. 1998) (Table 14.3). Transgenic expression of T4 bacteriophage Lysozyme, *Aspergillus niger*'s Glucose oxidase, *Erwinia carotovora*'s Pectate lyase in Potato lead to development of resistance against *E. carotovora* (During et al. 1993; Wu et al. 1995; Wegener et al. 1996). Transgenic expression of Resistance protein Xa21, maize's Rxo1, and Rxo1 with wild rice's Xa23 gene lead to development of resistant against *Xanthomonas oryzae* (Wang et al. 1996; Zhao et al. 2005; Zhou et al. 2009). Expression of lytic protein attacin E in transgenic apple (*Malus domestica*) lead to resistant against *Erwinia amylovora* (Norelli et al. 1994). Further expression of Reverse peptide of indolicidin (Rev4), barley's Thionin, human's Lysozyme, *Pseudomonas syringae's* Tabtoxin protein in transgenic tobacco lead to resistant against *E. carotovora* and *P. syringae* (Xing et al. 2006; Carmona et al. 1993; Nakajima et al. 1997; Anzai et al. 1989). Over expression of a putative receptor-like kinase gene (GbRLK) of *Gossypium barbadense* cv. Hai7124 in cotton and Arabidopsis led to development of resistance against *Verticillium dahliae* (Jun et al. 2015). Transgenic expression of antimicrobial peptide SP1-1 in tomato led to development of resistance against *Xanthomonas campestris* pv. vesicatoria (Diaz et al. 2016). Expression of FLS2 Receptor of *Nicotiana benthamiana* in transgenic citrus led to resistance against *Xanthomonas citri* (Hao et al. 2016). Further Overexpression of *OsMYC2*, *OsWRKY51*, and *OsWRKY45* in transgenic rice led to resistance against *X. oryzae* pv. oryzae (Xoo) (Uji et al. 2016; Hwang et al. 2016; Goto et al. 2016). Although many of the transgenic crops have been developed to avoid losses by phytopathogenic bacteria during pre- and post-harvesting stages, the best strategy still needs to come to avoid the loss by these pests.

14.3.3 Fungi

Fungi mediated yield loss in the agriculture and horticulture sectors is rated second position (Grover and Gowthaman 2003). Several transgenic approaches by using genes encoding Pathogenesis-related proteins (PR), Ribosome-inactivating proteins (RIPs), Small cystein-rich proteins, Lipid transfer proteins, Storage albumins, etc. were followed to generate transgenic plant against fungal disease (Grover and Gowthaman 2003) (Table 14.4). The plants expressing the Defensin protein in tomato, pepper (*Capsicum annuum*), eggplant led to development of resistance against fungi like Fusarium, *Colletotrichum gloeosporioides* and *Alternaria solani* respectively (Abdallah et al. 2010; Darwish et al. 2014; Seo et al. 2014). Transgenic expression *Thinopyrum intermedium* MYB transcription factor (TiMYB2R-1), Potato antimicrobial peptide SN1 and wheat AGC kinase gene (TaAGC1) in wheat led to development of resistant against take-all disease (*Gaeumannomyces graminis*) and *Rhizoctonia cerealis* respectively (Liu et al. 2013; Rong et al. 2013; Zhu et al. 2015). Transgenic expression of BvGLP-1 (germin-like protein of sugar beet), 3-deoxy-7-phosphoheptulonate synthase (GhDHS1) gene of *Gossypium hirsutum*, GbSBT1 gene of *G. barbadense* in *Arabidopsis thaliana* led to

Table 14.3 Genes used for bacteria resistant program development through transgenic strategies

S.-No.	Genes used	Promoter used	Crop plants	Target bacteria	References
1	Reverse peptide of indolicidin (Rev4)	Peanut chlorotic streak caulimovirus (PClSV)	Tobacco and Arabidopsis	*Erwinia carotovora*	Xing et al. (2006)
2	Thionin of barley	CaMV 35S	Tobacco	*Pseudomonas syringae* partial	Carmona et al. (1993)
3	Lytic protein attacin E	—	Apple (*Malus domestica*)	*Erwinia amylovora*	Norelli et al. (1994)
4	T4 bacteriophage lysozyme	CaMV 35S	Potato	*Erwinia carotovora*	Düring et al. (1993)
5	*Aspergillus niger*'s glucose oxidase	CaMV 35S	Potato	*Erwinia carotovora* partial	Wu et al. (1995)
6	*Erwinia carotovora*'s Pectate lyase	CaMV 35S	Potato	*Erwinia carotovora* (partial)	Wegener et al. (1996)
7	Resistance protein Xa21	—	Rice	*Xanthomonas oryzae* (total)	Wang et al. (1996)
8	Human lysozyme	CaMV 35S	Tobacco	*Pseudomonas syringae*	Nakajima et al. (1997)
9	*Pseudomonas syringae* derived Tabtoxin protein	CaMV 35S	Tobacco	*Pseudomonas syringae*	Anzai et al. (1989)
10	Maize gene, *Rxo1*	CaMV 35S	Rice	*Xanthomonas oryzae*	Zhao et al. (2005)
11	Maize resistance gene Rxo1 and wild rice Xa23 gene	CaMV 35S	Rice	*Xanthomonas oryzae* pv. oryzae and oryzicola	Zhou et al. (2009)
12	Overexpression of a putative receptor-like kinase gene (GbRLK) from *Gossypium barbadense* cv. Hai7124.	CaMV 35S	Cotton and Arabidopsis	*Verticillium dahliae*	Jun et al. (2015)
13	Antimicrobial peptide SP1-1	Inducible 4XW2/4XS promoter	Tomato	*Xanthomonas campestris* pv. vesicatoria.	Diaz et al. (2016)
14	Overexpression of OsMYC2	CaMV 35S	Rice	*Xanthomonas oryzae* pv. oryzae (Xoo)	Uji et al. (2016)
15	Over expression of rice transcription factor OsWRKY51	CaMV 35S	Rice	*Xanthomonas oryzae* pv. oryzae	Hwang et al. (2016)
16	FLS2 receptor of *Nicotiana benthamiana*	CaMV 35S	Citrus	*Xanthomonas citri*	Hao et al. (2016)
17	WRKY45	PR1b	Rice	*Xanthomonas oryzae* pv. oryzae	Goto et al. (2016)

Table 14.4 Genes used for fungi resistant program development through transgenic strategies

S.-No.	Genes used	Promoter used	Crop plants	Target fungi	References
1	Defensins	CaMV 35S	Tomato	Fusarium wilt	Abdallah et al. (2010)
2	Expression of BvGLP-1 (germin-like protein of sugar beet)	CaMV 35S	*Arabidopsis thaliana*	*Verticillium longisporum* and *Rhizoctonia solani*	Knecht et al. (2010)
3	SniOLP (*Solanum nigrum* osmotin-like protein) and Rs-AFP2 (*Raphanus sativus* antifungal protein-2) genes	CaMV 35S	Peanut (*Arachis hypogaea* L.)	Leaf spot disease (*Cercospora* spp)	Vasavirama and Kirti (2012)
4	*Thinopyrum intermedium* MYB transcription factor (TiMYB2R-1)	Maize ubiquitin (Ubi) promoter	Wheat	Take-all disease (*Gaeumannomyces graminis*)	Liu et al. (2013)
5	Potato antimicrobial peptide SN1	Maize ubiquitin (Ubi)	Wheat	*Gaeumannomyces graminis* var. tritici	Rong et al. (2013)
6	Arabidopsis NPR1 gene	CaMV 35S	Cotton	*Thielaviopsis basicola*	Kumar et al. (2013)
7	Wasabi defensin gene of *Wasabia japonica*		Eggplant	*Alternaria solani*	Darwish et al. (2014)
8	Defensin, J1-1	CaMV 35S	Pepper (*Capsicum annuum*)	Anthracnose disease, (*Colletotrichum gloeosporioides*)	Seo et al. (2014)
9	Overexpression of wheat AGC kinase gene (TaAGC1)	Maize ubiquitin (Ubi)	Wheat (*Triticum aestivum*)	*Rhizoctonia cerealis*	Zhu et al. (2015)
10	Expression of double stranded RNA (dsRNA) against FOW2, FRP1, and OPR	CaMV 35S	*Arabidopsis thaliana*	*Fusarium oxysporum*	Hu et al. (2015)

11	Overexpression of 3-deoxy-7-phosphoheptulonate synthase (GhDHS1) gene of *Gossypium hirsutum*	CaMV 35S	*Arabidopsis thaliana*	*Verticillium* wilt.	Yang et al. (2015)
12	A rice gene homologous to Arabidopsis AGD2 (ABERRANT GROWTH AND DEATH2)-LIKE DEFENSE1	CaMV 35S	Rice	*Magnaporthe oryzae*	Jung et al. (2016)
13	Over-expression of the Pikh gene	CaMV 35S	Rice	Blast disease (*Magnaporthe oryzae*)	Azizi et al. (2016)
14	WRKY45	PR1b	Rice	*Magnaporthe oryzae*	Goto et al. (2016)
15	GbSBT1 gene from *Gossypium babardense*	CaMV 35S	Arabidopsis	*Fusarium oxysporum* and *Verticillium dahliae*	Duan et al. (2016)
16	The knock-down of MdMLO19	CaMV 35S	Apple (*Malus domestica*)	Powdery mildew (*Podosphaera leucotricha*)	Pessina et al. (2016)
17	Rice OsVAMP714 fungal pathogen	CaMV 35S	Rice	Rice blast fungus (*Magnaporthe oryzae*)	Sugano et al. (2016)
18	Arabidopsis L-type lectin receptor kinase genes LecRK-I.9 and LecRK-IX.1	—	*Nicotiana benthamiana*	*Phytophthora resistance*	Wang et al. (2016)
19	Overexpression of pathogen-induced grapevine TIR-NB-LRR (VaRGA1)	CaMV 35S	*Nicotiana benthamiana*	*Phytophthora parasitica*	Li et al. (2017)

development of resistance against *Verticillium longisporum* and *Rhizoctonia solani*, *Verticillium* wilt, *Fusarium oxysporum*, and *V. dahliae* respectively (Knecht et al. 2010; Yang et al. 2015; Duan et al. 2016).

Transgenic expression of AGD2 (Aberrant growth and death2)-like defense1, Pikh Gene, WRKY45, and OsVAMP714 in rice led to development of resistant against Blast Disease (*Magnaporthe oryzae*) (Jung et al. 2016; Azizi et al. 2016; Goto et al. 2016; Sugano et al. 2016). Transgenic expression of Arabidopsis L-type lectin receptor kinase genes LecRK-I.9/LecRK-IX.1 and TIR-NB-LRR (VaRGA1) in *N. benthamiana* led to development of resistance against *Phytophthora* pathogens (Wang et al. 2016; Li et al. 2017). Further transgenic expression of SniOLP (*Solanum nigrum* osmotin-like protein) and Rs-AFP2 (*Raphanus sativus* antifungal protein-2) genes in peanut (*Arachis hypogaea* L.) led to the development of resistance against leaf spot disease (*Cercospora* spp) (Vasavirama and Kirti 2012). Similarly, the transgenic expression of Arabidopsis NPR1 gene in cotton led to resistant development against *Thielaviopsis basicola* in cotton (Kumar et al. 2013). RNAi strategy has also applied to generate transgenic against fungal phytopathogenes. Like expression of double stranded RNA (dsRNA) against FOW2, FRP1, and OPR of *F. oxysporum* in *A. thaliana* showed resistance against target fungi (Hu et al. 2015). Similarly the knock-down of *MdMLO19* via small interfering RNA (siRNA) in transgenic Apple (*M. domestica*) showed resistant to *Podosphaera leucotricha*; Powdery mildew causing fungus (Pessina et al. 2016).

14.3.4 Nematodes

Similar to other pest, nematodes are also serious pest for crops having well establishes root and root-oriented crop productivity. There are about 4100 species of phytopathogenic nematodes identified (Decraemer and Hunt 2006). Their distribution varies from geographically restricted, e.g. *Nacobbus* spp. to globally cosmopolitan nature, e.g. *Meloidogyne* spp. (Nicol et al. 2011). Several transgenic plants like Arabidopsis, eggplant, tomato, maize, soybean, cotton, sweet potato have generated to develop resistance against phytopathogenic nematodes (Table 14.5). Transgenic soybeans expressing HGCP prodomain of cysteine proteinase, salicylic acid methyltransferase, and (E,E)-α-farnesene synthase showed resistance against cyst nematode (*Heterodera glycines*) (Marra et al. 2009; Lin et al. 2013; Lin et al. 2017). Transgenic cotton (*G. hirsutum*) expressing *Glycine max* homolog of Non-race specific disease resistance 1 (Gm-NDR1-1) and MIC-3 showed resistant against Root-knot nematode (*Meloidogyne incognita*) (Wubben et al. 2015; McNeece et al. 2017). Similarly, transgenic tomato expressing candidate root-knot nematode resistance gene (designated as CaMi) of resistant pepper line PR 205 showed resistant against *Meloidogyne* spp. (Chen et al. 2007). Eggplant (*S. melongena*) and tomato plants expressing Mi-1.2 gene showed resistant against *Meloidogyne javanica* (Goggin et al. 2006). RNAi strategy also has been applied to develop resistant plant against nematodes. Expression of RNAi against parasitism gene 16D10 of *Meloidogyne* species in Arabidopsis, dsRNA against V type ATPase of Corn rootworm (western corn rootworm [WCR]) *Diabrotica virgifera* in maize, and siRNAs against unc-15 gene of Stem nematode (*Ditylenchus destructor*) in sweet potato (*Ipomoea batatas*) showed resistance against attacking nematodes (Huang et al. 2006; Baum et al. 2007; Fan et al. 2015).

Table 14.5 Genes used for nematodes resistant program development through transgenic strategies

S.-No.	Genes used	Promoter used	Crop plants	Target nematode	References
1	RNAi against parasitism gene 16D10	CaMV 35S	Arabidopsis	Meloidogyne species	Huang et al. (2006)
2	Mi-1.2 gene	CaMV 35S	Eggplant (*S. melongena*) and tomato	*Meloidogyne javanica*	Goggin et al. (2006)
3	dsRNA directed against V type ATPase to	CaMV 35S	Maize	Corn rootworm (WCR) *Diabrotica virgifera virgifera*	Baum et al. (2007)
4	Candidate root-knot nematode resistance gene (designated as CaMi) of resistant pepper line PR 205	CaMV 35S	Tomato	Root-knot nematode (*Meloidogyne*)	Chen et al. (2007)
5	Over expression of HGCP prodomain	CaMV 35S	Soybean	*Heterodera glycines*	Marra et al. (2009)
6	Overexpression of a soybean salicylic acid methyltransferase gene	CaMV 35S	Soybean	Soybean cyst nematode	Lin et al. (2013)
7	Overexpression of MIC-3	CaMV 35S	*Gossypium hirsutum* L. (Coker 312)	Root-knot nematode (RKN; *Meloidogyne incognita*)	Wubben et al. (2015)
8	Small interfering RNAs (siRNAs) against unc-15 gene	CaMV 35S	Sweet potato (*Ipomoea batatas*)	Stem nematode (*Ditylenchus destructor*)	Fan et al. (2015)
9	*Glycine max* homolog of NON-RACE SPECIFIC DISEASE RESISTANCE 1 (Gm-NDR1-1)	CaMV 35S	*Gossypium hirsutum*	*Meloidogyne incognita*	McNeece et al. (2017)
10	(E,E)-α-farnesene synthase gene	CaMV 35S	Soybean	Soybean cyst nematode (SCN)	Lin et al. (2017)

14.3.5 Insects

Due to their cosmopolitan presence, herbivorous insects are one of the biggest threats to standing as well as post-harvest crop production loss. About 50–60% staple crops are lost during postharvest operations including storage stages (Kumar and Kalita 2017). Insects damage the crops by chewing or sucking. Targeting normal physiology of insects like digestion, movement, mating etc. their population and damages to crop can be controlled. Several transgenic plant of *A. thaliana*, *B. juncea*, castor, chickpea, maize, peanut, peas, poplar, potato, rice, tobacco, and cotton have generated to cope the attacking insects (Table 14.6). The inhibitors targeting the digestive activity were used to generate the transgenic plants against attacking insect. α-amylase inhibitor of common bean was used to control the bruchid beetles in transgenic pea (Shade et al. 1994) as well as pea weevil (*Bruchus pisorum*) (Schroeder et al. 1995). Tobacco and rice plants expressing potato proteinase inhibitor 2, showed resistant against *Spodoptera exigua* (Jongsma et al. 1995). Transgenic expression of tyrosine-derived cyanogenic glucoside dhurrin, Photorhabdus toxin, Aphid alarm pheromone and terpene synthase (TPS10) in *A. thaliana* showed resistant against flea beetle (*Phyllotreta nemorum*), tobacco hornworm (THW) (*Manduca sexta*), aphid (*Myzus persicae*) and *Spodoptera littoralis* (Tattersall et al. 2001; Petell et al. 2004; Liu et al. 2003; Beale et al. 2006; Schnee et al. 2006).

Discovery of Bt (*Bacillus thuringiensis*) derived Crystal (Cry) proteins have been shown to be excellent insecticides with no hazard to humans. Many transgenic plants like castor, cotton, peanut, etc. have been generated and showed excellent resistance property against chewing insects. Plants of castor (*Ricinus communis* L.) expressing Cry1Ec protein showed resistant against tobacco caterpillar (*Spodoptera litura*) and castor semilooper (*Achoea janata* L.) (Sujatha et al. 2009). Transgenic expression of single Cry1Ec protein in peanut (*A. hypogaea* L.) showed resistant against *S. litura* (Tiwari et al. 2008) while combination of Cry1Ec with rice chitinase showed resistant to both *S. litura* and fungal pathogen *Phaeoisariopsis personata* (Beena et al. 2008). Transgenic tobacco, tomato, and rice plants expressing Cry1Ab Cry1Ac, Cry3A showed resistance against THW, Colorado potato beetle and *Scirpophaga incertulas* (Perlak et al. 1991, 1993; Anoop et al. 2007). Transgenic tobacco and cotton expressing Cry1Ec showed resistant against *S. litura* (Singh et al. 2004).

The new strategies have also been adapted for developing resistance against multiple insects. An example is fusion of Cry1Ac with the nontoxic ricin B-chain (RB) galactose-binding domain of Ricinus, confers resistance to *S. littoralis* and *Cicadulina mbila* (Mehlo et al. 2005). Further expression of single or different combination of genes like Cry1Ab/Cry1Ac, Cry1Ab-Cry1Ac hybrid, Avidin NlHT1, Nlcar, Nltry, Snowdrop lectin (*Galanthus nivalis* agglutinin, GNA), and *Allium sativum* leaf agglutinin (ASAL) genes in rice showed resistant against yellow stem borer (*S. incertulas*), *Tribolium confusum*, *Sitotroga cerealella*, brown planthopper (*Nilaparvata lugens*), *Aphis craccivora*, *M. persicae*, and leafhopper (*Nephotettix virescens*; GLH) (Tu et al. 2000; Ramesh et al. 2004; Yoza et al. 2005; Zha et al. 2011; Rao et al. 1998; Saha et al. 2007; Foissac et al. 2000).

Although Bt technology has created success stories and the major problem with lepidopteron pests have largely been taken care of, however, sap sucking insects are also a major threat for crop cultivation. To control sap sucking insects, different lectins have been used. Transgenic expression of *A. sativum* agglutinin (ASA) in

Table 14.6 Genes used for insect resistant program development through transgenic strategies

S. No.	Genes used	Promoter used	Crop plants	Target insect	References
1	Pathway for tyrosine-derived cyanogenic glucoside dhurrin biosynthesis	—	*Arabidopsis thaliana*	Flea beetle (*Phyllotreta nemorum*)	Tattersall et al. (2001)
2	Photorhabdus toxin			Tobacco hornworm (*Manduca sexta*)	Petell et al. (2004) and Liu et al. (2003)
3	Aphid alarm pheromone	CaMV35S		Aphid (*Myzus persicae*)	Beale et al. (2006)
4	Terpene synthase (TPS10)	CaMV35S		*Spodoptera littoralis*	Schnee et al. (2006)
5	Wheat germ agglutinin (WGA)	CaMV35S	*Brassica juncea*	Mustard aphid (*Lipaphis erysimi*)	Kanrar et al. (2002)
6	cry1EC	CaMV35S	Castor (*Ricinus communis* L.)	Tobacco caterpillar (*Spodoptera litura* Fabr) and castor semilooper (*Achoea janata* L.)	Sujatha et al. (2009)
7	ASA: *Allium sativum* agglutinin (Mannose)	CaMV35S	Chickpea	Chickpea aphid (*Aphis craccivora*)	Chakraborti et al. (2009)
8	Avidin		Maize	Stored-produce insect pests	Kramer et al. (2000)
9	Insecticidal protein from Pseudomonas IPD072Aa	—	Maize	Western corn rootworm (WCR) (*Diabrotica virgifera virgifera* LeConte)	Schellenberger et al. (2016)
10	Insect chitinase cDNA from cotton leaf worm (*Spodoptera littoralis*)	—	Maize	Corn borer (*Sesamia cretica*)	Osman et al. (2015)
11	Cry1EC	CaMV	Peanut (*Arachis hypogaea* L.)	*Spodoptera litura*	Tiwari et al. (2008)
12	Cry1EC and rice chitinase	CaMV		*Spodoptera litura* and fungal pathogen *Phaeoisariopsis personata*	Beena et al. (2008)
13	δ-endotoxin Cry1EC	Pathogenesis responsive promoter PR-1a		*Spodoptera litura*	Tiwari et al. (2011)

Table 14.6 (Continued)

S. No.	Genes used	Promoter used	Crop plants	Target insect	References
14	alpha-Amylase inhibitor	CaMV	Peas (*Pisum sativum*)	Cowpea weevil	Shade et al. (1994)
15	alpha-Amylase inhibitor	—	Peas (*Pisum sativum*)	Bruchus beetles	Schroeder et al. (1995)
16	Rice OZC-1	CaMV35S	Poplar	Beetle (*Chrysomela tremulae*)	Leple et al. (1995)
17	Concanavalin A (ConA)	CaMV35S	Potato	Green peach aphid (*Myzus persicae*) tomato moth (*Lacanobia oleracea*)	Gatehouse et al. (1999)
18	*Galanthus nivalis* agglutinin (GNA)	CaMV 35S and ST-LS1 promoters	Potato	Aphid	Mi et al. (2017)
19	Cry1Ab/Cry1Ac	Actin1	Rice (*Oryza sativa* L.)	Leaf folder and yellow stem borer (*Scirpophaga incertulas*)	Tu et al. (2000)
20	Cry1 Ab - Cry1Ac hybrid	CaMV35S		Yellow stem borer	Ramesh et al. (2004)
21	Avidin	GluB-1 endosperm-specific promoter		*Tribolium confusum* and *Sitotroga cerealella*	Yoza et al. (2005)
22	NlHT1, Nlcar, Nltry	Ubiquitin1		Brown planthopper (*Nilaparvata lugens* Stål)	Zha et al. (2011)
23	Snowdrop lectin (*Galanthus nivalis* agglutinin; GNA)	RSs1 and maize ubiquitin			Rao et al. (1998)
24	*Allium sativum* leaf agglutinin (ASAL) gene	RSS1 and rolC		*Aphis craccivora*, *Myzus persicae*	Saha et al. (2007)
25	GNA snowdrop lectin (*Galanthus nivalis* agglutinin; GNA)	RSS		leafhopper (*Nephotettix virescens*; GLH)	Foissac et al. (2000)

26	RNAi against MsCYP6B46	CaMV35S	Tobacco	*Manduca sexta*	Kumar et al. (2012)
27	Caffeine biosynthetic pathway	—		*Spodoptera litura*	Kim et al. (2006)
28	Ribosome-inactivating proteins	CaMV 35S		*Helicoverpa zea*	Dowd et al. (2003)
29	PTA: *Pinellia ternata* agglutinin (Mannose)	CaMV35S		Peach potato aphid (*Myzus persicae* Sulzer)	Yao et al. (2003)
30	Cry 1Ie	Ubiquitin		Corn borer	Liu, H.B. et al. (2004), Liu, Y.J. et al. (2004)
31	*Allium sativum* leaf lectin (ASAL)	CaMV 35S		*Myzus persicae*	Dutta et al. (2005)
32		ASus1		*Myzus nicotianae*	Sadeghi et al. (2007)
33	Proteinase inhibitors I and II	CaMV 35S		*Manduca sexta*	Johnson et al. (1989)
34	Si-RNAs	CaMV35S		White fly (*Bemisia tabaci*)	Thakur et al. (2014)
35	Cholesterol oxidase	Figwort mosaic viruses promoter		Cotton boll weevil (*Anthonomus grandis grandis* Boheman)	Corbin et al. (2001)
36	Cry1Ab Cry1AC, Cry3A	CaMV35S	Tobacco and tomato	Tobacco hornworm (THW), Colorado potato, beetle	Perlak et al. (1991), (1993)
37	Potato proteinase inhibitor 2	CaMV35S	Tobacco and rice	*Spodoptera exigua*	Jongsma et al. (1995)
38	Cry1Ec	CaMV35S	Tobacco and cotton	*Spodoptera litura*	Singh et al. (2004)
39	Tma12 protein from *Tectaria macrodontal*	CaMV35S	Cotton	White fly (*Bemisia tabaci*)	Shukla et al. (2016)

chickpea showed resistance against chickpea aphid (*A. craccivora*) (Chakraborti et al. 2009). Transgenic expression of Wheat germ agglutinin (WGA) in *B. juncea* showed resistant against mustard aphid (*Lipaphis erysimi*) (Kanrar et al. 2002). Transgenic expression of Concanavalin A (ConA) and *Galanthus nivalis* agglutinin (GNA) in potato showed resistance against sap sucking pest Green peach aphid (*M. persicae*) and moth (*Lacanobia oleracea*) (Gatehouse et al. 1999; Mi et al. 2017).

Transgenic expression of Avidin, IPD072Aa protein from *Pseudomonas*, and insect chitinase of leaf worm (*S. littoralis*) in maize showed resistant against stored-produce insect pests, WCR (*D. virgifera virgifera* LeConte), and corn borer (*Sesamia cretica*) (Kramer et al. 2000; Schellenberger et al. 2016; Osman et al. 2015). Transgenic expression of Rice OZC-1 gene in poplar showed resistant against Beetle (*Chrysomela tremulae*) (Leple et al. 1995).

Due to easy regeneration processes, maximum transgenic lines of tobacco have been generated to prove their insect resistant property. Transgenic tobacco expressing Caffeine, RIPs, *Pinellia ternata* agglutinin (PTA), Cry 1Ie, *A. sativum* leaf lectin (ASAL), Proteinase inhibitors and Cholesterol oxidase showed resistance against different insects like *S. litura*, *Helicoverpa zea*, peach potato aphid (*M. persicae* Sulzer), corn borer, *Myzus nicotianae*, *M. sexta* and cotton boll weevil (*Anthonomus grandis* grandis Boheman) (Kim et al. 2006; Dowd et al. 2003; Yao et al. 2003; Liu, H.B. et al. 2004; Liu, Y.J. et al. 2004; Dutta et al. 2005; Sadeghi et al. 2007; Johnson et al. 1989; Corbin et al. 2001).

Expression of RNAi targeting metabolic genes of insects like *M. sexta* and white fly (*Bemisia tabaci*), showed development of resistance against these insects in transgenic tobacco (Kumar et al. 2012; Thakur et al. 2014). For the source of insecticidal genes used for insect resistance in transgenic plant generation, different bacterial originated insecticidal protein, plant originated lectins, and enzyme inhibitors have been discovered to generate transgenic plant against attacking insects. Recently an insecticidal protein named prTma12 from fern (*Tectaria macrodontal*) has been isolated and transgenic cotton expressing this protein showed resistant against white fly (*B. tabaci*) (Shukla et al. 2016).

14.3.6 Parasitic Weeds

Parasitic plants attack the host plant either above ground (dodders-*Cuscuta* sp. and mistletoes) or below ground like *Orobanche* and *Striga* host plant parts (Parker and Riches 1993). Several transgenic plants like tobacco, lettuce, tomato, and *Medicago truncatula* have been generated for resistance against attacking weeds (Table 14.7). The transgenic tobacco expressing Sarcotoxin IA and Chlorsulfuron showed resistance against *Orobanche aegyptiaca* and *Orobanche ramosa* (Hamamouch et al. 2005; Slavov et al. 2005). RNAi strategies have also been proved successful in development of resistant plants against parasitic weeds. RNAi against Mannose 6-phosphate reductase (M6PR) of *O. aegyptiaca* in tomato, SHOOT MERISTEMLESS-like (STM) gene of parasite dodder (*Cuscuta pentagona*) in tobacco (*Nicotiana tabacum*), Acetyl-CoA Carboxylase of *Triphysaria versicolor* in *M. truncatula*, and carotenoid cleavage dioxygenases (CCD7 and CCD8) of *Phelipanche aegyptiaca* in *N. benthamiana* showed development of resistance against attacking weeds (Aly et al. 2009; Alakonya et al. 2012; Bandaranayake and Yoder 2013; Aly et al. 2014). Recently multiple genes silencing of

Table 14.7 Genes used for parasitic weeds resistant program development through transgenic strategies

S..No.	Genes used	Promoter used	Crop plants	Target weeds	References
1	Sarcotoxin IA (40-residue peptide from the fly- *Sarcophaga peregrina*)	HMG2	Tobacco	*Orobanche aegyptiaca*	Hamamouch et al. (2005)
2	Chlorsulfuron resistant	—	Tobacco (*Nicotiana tabacum* L.)	Broomrape (*Orobanche ramosa* L.)	Slavov et al. (2005)
3	RNAi against GUS gene (hpGUS) of transgenic *Triphysaria versicolor*	CaMV35S	Lettuce	*Triphysaria versicolor*	Tomilov et al. (2008)
4	RNAi gainst Mannose 6-phosphate reductase (M6PR)	CaMV35S	Tomato	*Orobanche aegyptiaca*	Aly et al. (2009)
5	Against SHOOT MERISTEMLESS-like (STM) gene	SUCROSE-PROTON SYMPORTER2 (SUC2)	Tobacco (*Nicotiana tabacum*)	Parasite dodder (*Cuscuta pentagona*)	Alakonya et al. (2012)
6	RNAi against acetyl-CoA carboxylase	—	*Medicago truncatula*	*Triphysaria versicolor*	Bandaranayake and Yoder (2013)
7	RNAi against carotenoid cleavage dioxygenases (CCD7) and (CCD8)	CaMV35S	*Nicotiana benthamiana*	*Phelipanche aegyptiaca*	Aly et al. (2014)
8	Silencing of three *Phelipanche aegyptiaca* genes PaACS, PaM6PR and PaPrx1	CaMV35S	Tobacco and tomato	*Phelipanche aegyptiaca*	Dubey et al. (2017)

parasitic plant has also showed enhanced resistance against parasitic weed in compare to targeting single genes (Dubey et al. 2017).

14.4 Regulation of Insecticidal Gene Expression

Virus derived constitutive promoters have been used to regulate the expression of desired genes for generating insect resistance. However constitutive expression of insecticidal products caused abnormal effect on plant development as well targeting of beneficial insect. For example to control *S. litura* attack in cotton, groundnut, pigeon pea, and castor, a hybrid δ-endotoxin Cry1EC expression under the control of CaMV 35S promoter was employed (Singh et al. 2004; Tiwari et al. 2008). These works gave satisfactory results like transgenic maize expressing terpene synthase (TPS10) gene under CaMV35S promoter attracts the parasitoid of *S. littoralis* and gives protection (Schnee et al. 2006). Expression of *Photorhabdus luminiscences* protein A under the control of Cassava vein mosaic virus (CVMV) promoter in *A. thaliana* gives the resistance against *M. sexta* (Liu et al. 2003). Caffeine production in tobacco under CaMV35S promoter gives protection against *S. litura* and *Pieris rapae* (Kim et al. 2006). Similarly, the expression of *A. sativum* leaf lectin under the control of CaMV35S promoter gives protection to rice plant against sap sucking insect brown planthopper (*N. lugens*; BPH) and green leafhopper (*N. virescens*; GLH) (Saha et al. 2006). Transgenic tobacco expressing cholesterol oxidase under the different constitutive promoters like figwort mosaic virus promoter, enhanced CaMV35S promoter, and rubisco small sub unit; provide resistance against cotton boll weevil (*A. grandis* grandis) (Corbin et al. 2001).

Transgene silencing may occur for the foreign promoters and can lead to shutdown of a promoter activity (Kloti et al. 2002) and thus constitutive promoters from a plant origin were used (Potenza et al. 2004). For example, different transgenic plant lines expressing RNAi under RbcS promoter (Ahmed et al. 2017), virus coat protein (Yao et al. 2017), *T. intermedium* MYB transcription factor (TiMYB2R-1) (Liu et al. 2013), Potato antimicrobial peptide SN1 (Rong et al. 2013), wheat AGC kinase gene (TaAGC1) under maize ubiquitin (Ubi) promoter (Zhu et al. 2015), Cry1a(b)/Cry1a(c) under Actin1 promoter (Tu et al. 2000) showed resistance against their target insects. But the use of constitutive promoter expresses the transgene in all tissues of plants leading to exhaust plant resources even when they are not required. Thus, people had tried to fine tune the expression of insecticidal proteins by using tissue specific promoters for example endosperm-specific promoter (Yoza et al. 2005), phloem specific promoter like RSs1 (Rao et al. 1998), RSS1 and rolC (Saha et al. 2007), RSS (Foissac et al. 2000), ASus1 (Sadeghi et al. 2007), SUC2 (Alakonya et al. 2012) at specific tissue preferred by attacking insect and pest. To make insecticidal molecule expression finer and to control their response to pest infestation to avoid the disadvantage of constitutive nature of tissue specific promoter, there is indeed need of insect inducible promoters. Different wound inducible promoter, like AoPR1 (Gulbitti-Onarici et al. 2009), 4XW2/4XS (Diaz et al. 2016), pathogenesis responsive PR1a and b (Tiwari et al. 2011; Goto et al. 2016), and HMG2 (Hamamouch et al. 2005) have been used to confirm the insecticidal expression at the wound site of pest attack. So the final target to express desired insecticidal molecule at site and at the time of insect pest attack will be achieved by using insect and wound specific promoters.

14.5 Advantages

Advantages of transgenic strategy are because of its reliability in increment of food production, yields, quality, and pharmaceutical farming (Grover and Pental 2003; Darbani et al. 2008). The transgenic plants also have the capacity to tolerate and resist against incoming pathogens and pests. Due to dependency of agricultural production on agrochemicals and increasing input cost due to pesticide, fungicides and herbicide uses, the use of transgenic plants would be a great approach in reducing damages to environment and human health (Sharma et al. 2003). Several history like generation of insect-resistance crops of corn, soybean etc., via expressing *B. thuringiensis* (Bt) toxin, cotton, mungbean, and tomato etc. resistant to viruses (Sharma et al. 2005), herbicide resistance crops with chemicals to kill surrounding weeds (Ferber 1999), increasing shelf life of Flavr-Savr tomato (Moffat 1998), increasing vitamin A precursor (beta-carotene) in golden rice (Ye et al. 2000) etc. have made. Proper use of Biotechnology can support the growing population. Another benefit of transgenic strategy would be in better utilization of genetic resources and reducing the breeding time period (Chand and Pal 2003).

14.6 Disadvantages

In spite of the bright future of transgenic strategy there are some drawbacks like safety of transgenic material (Sharma et al. 2003), use of antibiotic-resistance marker genes to generate transgenic plant can produce antibiotic resistance in pathogens, spreading of herbicide resistant gene to other weeds via horizontal or vertical gene transfer, development of some potential allergens from genetically modified crops. Apart from this killing of non-target beneficial insects can occur, accumulation of toxic product to environment and development of resistance against insecticides and pesticides in insect pests may occur. Further clearance from government body to grow transgenic plants is also a limitation because it takes a long time to get approval from government in countries like India. Another drawback is ethical issues related to alteration of biological systems (Chand and Pal 2003).

14.7 Future Strategies

The war between farmer and herbivorous pest will continue. Sometimes the farmer wins, but suddenly development of new resistant insects is observed. In a better strategy to use insect inducible promoters for expression of insecticidal molecules should be preferred to avoid the emergence of resistance in pests and other cons. Regulated expression of insecticidal molecules at the site of insect feeding will pose minimal load on the plant as well; and it is also desired for safety related issues. It is essential to carefully identify the target pest and consider all control options regarding that. In future integration/combination of many strategies like transgenic, biopesticides, traditional breeding programs, and tools like crop rotation, use of greenhouse or net house will reduce the use of pesticides and will lead to optimal pest and disease control and the goal of new research should be oriented toward effective, durable, and environmentally friendly technique (Freeman and Mwang'ombe 2009). Furthermore, the antibiotic resistant gene can be removed and a marker free plant can be generated either by site specific recombination strategy or by co-transformation method (Veluthambi et al. 2003).

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