Khalid Rehman Hakeem
Mohd Sayeed Akhtar *Editors*

Plant, Soil and Microbes

Volume 2: Mechanisms and Molecular Interactions

Springer

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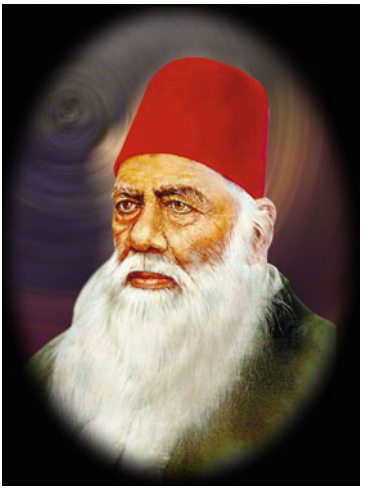

Sir Syed Ahmad Khan
(1817-1898)

A great visionary, statesman, Muslim reformer of the 19th century and founder of Aligarh Muslim University, India

Foreword

Worldwide considerable research in the area of belowground plant-microbe interaction is quite important to enrich soil fertility and in the enhancement of crop productivity. It seems imperative to understand the modifications of belowground interactions under specific plant and microbe communication system accomplished by molecular dialogues. It is, therefore, essential to decode and explore this molecular language so as to establish a successful tripartite relationship among plant, soil and microbes. The soils are the product of rocks, minerals and organic matters with a pivotal role in ecology. Most of the plants depend on soil, but plants and their associated microorganisms are also important component together in the formation and sustainability of rhizospheric ecology. The understanding about plant, soil and microbe interaction is limited; molecular mechanisms and other several consequences may reveal with other process yet to be explored. This book entitled *Plant, Soil and Microbe, Volume 2: Mechanisms and Molecular Interactions* deals with how plant-microbe interactions occur using molecular pattern and applied in environment scavenging such as pesticide degradation and polycyclic aromatic hydrocarbons (PAHs) remediation. The importance of fungal symbiosis, tripartite interactions among plant-*Trichoderma*-pathogen with special reference to proteomic tools in biocontrol, has been described. The mechanism of plant growth promoting rhizobacteria-soil-root interaction, their ability towards growth, secondary metabolite production and nutrient uptake in medicinal and aromatic plants has been suitably mentioned. The bacterial determinants and plant defence induction in sustainable agriculture have an added advantage to strengthen the concept of biocontrol of deleterious phytopathogens. The elaborative description on the molecular identification of phytoplasma diseases in ornamental plants is itself appealing. The introductory account on allelochemicals from ascocarp of *Tuber* species is a point of difference. Besides, mycorrhizal associations, the biocontrol potential of *Bacillus thuringiensis*, genomics of plant-soil microbial diversity and the importance of root exudates in rhizosphere ecosystem and phytohormones in abiotic stress tolerance of plants have been elaborated. It is oceanic to gain update in the quest for knowledge of plant-soil-microbial interactions and their applications in a befitting manner. The editors *Khalid Rehman Hakeem* and *Mohd. Sayeed Akhtar* have put some

outstanding efforts to compile subject experts' contribution in a very attractive manner with an understanding of sequences of the chapters. *Plant, Soil and Microbe, Volume 2: Mechanisms and Molecular Interactions* includes broad contributions from all dimensions of agronomy. Specifically, this volume describes a holistic view of plant-microbe interactions, and its recent molecular mechanism emerged from studying multi-tropic interaction. The editors have immensely provided a solid foundation of the subject interesting for the researchers involved in soil microbiology, plant pathology, ecology and agronomy.

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D.K. Maheshwari

Preface

Plants are exposed to a huge diversity of microbes in the environment. Owing to the broad range of microbes, a complex set of molecular mechanisms mediates the plant-microbe interactions. These interactions have been seen to possess both negative and positive effects on either or both the members. Considering the importance of these ground rhizospheric microorganisms in the plant disease protection, it came into highlight from research that the combined application of these microorganisms is more beneficial than the use of a single agent and provides a better management against the soil-borne plant pathogens. The interaction of these microorganisms also provides an overview about the biological functions of soil and its interaction with the plant-microbe system, nutrient management, biogeochemical cycling, water various environmental condition in response to biotic and abiotic stresses, signalling of molecules during host-pathogen interaction, role of phytohormones against the environmental stresses and the major challenges in the formulation of microorganisms for the biocontrol products. The molecular approach of these microorganisms is also the basis for understanding the mechanism involved in disease suppression by these hidden underground beneficial microbes.

This volume with 18 chapters from experts on the subject describes a holistic view of plant-microbe interactions and its recent molecular mechanism emerged from studying multi-tropic interaction. It is imperative to understand the modifications of belowground interactions under specific plant and microbe communication system accomplished by molecular dialogues. We hope that the book will be helpful for the graduate students, teachers, researchers and industry persons, who are interested in soil microbiology, plant pathology, ecology, environmental sciences and agronomy.

We are highly grateful to all our contributors for readily accepting our invitation for not only sharing their knowledge and research but for venerably integrating their expertise in dispersed information from diverse fields in composing the chapters and enduring editorial suggestions to finally produce this venture. We greatly

appreciate their dedication. We are also thankful to Prof. (Dr.) D. K. Maheshwari for his suggestions and writing the foreword for this volume. We also thank Springer-International team for their generous cooperation at every stage of the book production.

Serdang, Malaysia — Khalid Rehman Hakeem
Shahjahanpur, Uttar Pradesh, India — Mohd Sayeed Akhtar

Contents

The original version of the book frontmatter was revised. The spelling of the second editor, Mohd Sayeed Akhtar's name was corrected in the Table of Contents. The Erratum to the book frontmatter is available at 10.1007/978-3-319-29573-2_19

About the Editors

Khalid Rehman Hakeem, PhD is working as a fellow researcher at the Faculty of Forestry, Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia, and also visiting professor at Fatih University, Istanbul, Turkey. He has obtained his MSc (Environmental Botany) as well as PhD (Botany) from Jamia Hamdard, New Delhi, India, in 2006 and 2011, respectively. He did his postdoctorate in the fields of forest dynamics and biotechnological studies from Universiti Putra Malaysia from 2012 to 2013. Dr. Hakeem has more than 8 years of teaching and research experience in plant ecophysiology, biotechnology and molecular biology as well as in ecological and environmental sciences. Recipient of several fellowships at both national and international levels, Dr. Hakeem has so far edited and authored more than 15 books with international publishers. He has also to his credit more than 100 publications in peer-reviewed international journals, including 35 book chapters with International publishers. He is also an editorial board member and reviewer of several high-impact international journals. Dr. Hakeem is currently engaged in studying the plant processes at ecophysiological as well as proteomic levels.

Mohd. Sayeed Akhtar, PhD is working as an assistant professor in Gandhi Faiz-E-Aam College, Shahjahanpur, affiliated to M.J.P. Rohailkhand University, Bareilly, U.P., India. He has received his PhD degree from Aligarh Muslim University (AMU), India, in 2008. He has conducted his postdoctoral research at the Botanical Institute, University of Basel (BIB), Switzerland (2008–2010), and Chonbuk National University (CBNU), Republic of Korea, in 2011, respectively. He also works as an assistant professor, Department of Biology, College of Natural Sciences, Jimma University, Jimma, Ethiopia (form 2011 to 2014), and fellow researcher UDQ9 at the Institute of Tropical Agriculture, Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia (from 2014 to 2015). Dr. Akhtar has more than 12 years of research and teaching experience in soil microbiology, applied microbiology, environmental microbiology, molecular biology, plant pathology and plant nanobiotechnology. Dr. Akhtar has received several prestigious fellowships at national and international levels. His promising approach and dedication stands him in the row of foremost scientists in the field of plant-microbe interaction and plant

nanobiotechnology. He is author and coauthor of about 50 research articles in peer-reviewed journals, contributed 12 book chapters in the books published by Springer-Verlag and also edited 4 books with international publishers. He is serving the scientific community as editorial board member and reviewer of several high-impact international journals. His current research is focused on the rhizospheric plant-microbe interactions and their molecular biotechnology, bioremediation, biomineralization, nano-fertilizers and nanobiotechnology.

Plant-Microbe Interactions: A Molecular Approach

Mustafeez Mujtaba Babar, Sumayyah Fareed Khan, Muhammad Kazim Zargaham, Najam-us-Sahar Sadaf Zaidi, and Alvina Gul

Abstract Plants thrive in a complex environment comprising of various biotic and abiotic agents. Like all biological systems, these agents tend to interact with the plant body. Microorganisms form a major portion of the ecosystem and have been found to inoculate or infect members of all the kingdoms. Plants and microbes have developed molecular mechanisms to interact with one another and attain the maximum benefit from the interactions. This mutualistic relationship provides benefit not only to the microbes but also to the plants. Based upon this complex molecular interplay, a number of mechanisms have been studied and are currently being employed for the agricultural, environmental, and health benefits. The principles of biofertilization and bioremediation utilize the plant-microbe interactions for the survival of the two players along with contributing to the food chain and the ecosystem. Similarly, the secondary metabolites obtained from these organisms contribute to human medical and agricultural welfare. These processes are regulated by a variety of biological, physical, chemical, and environmental factors, the study of which can be helpful in exploiting better outcomes from the interaction. The advent of modern techniques has helped in deciphering the role of various molecular players of the plant-microbe interactions. Moreover, they can be employed for regulating the plant-microbe interaction for improved efficiency. The current chapter discusses the molecular mechanisms involved in the plant-microbe interactions exhibited in biofertilization, bioremediation, biocontrol, and induced systemic resistance. Afterwards, the factors affecting the molecular machinery involved in these pathways have been discussed. Toward the end, a brief introduction of the genetic

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manipulative techniques and their applications in the plant-microbe interactions has been presented.

Keywords Molecular interaction • Rhizosphere • Biofertilization • Bioremediation • Genetic engineering

1 Introduction

Plants and microbes are the two most abundant organisms in the biosphere. They exist even in the geographical regions that are otherwise not inhabited by humans and other larger animals. The interaction between these organisms has, hence, long been established. This relationship has been seen to possess both negative and positive effects on either or both the members. Infection of plants with bacterial, viral, or fungal pathogens often leads to disease and death of the plant. Similarly, in response to the microbial infections, plants have developed ways and means to protect themselves by producing products that kill these microbes. Contrarily, a number of microbial and bacterial species interact with one another in a positive manner for the benefit of both the organisms. Plants, being larger in size with a well-developed nutrient processing mechanism, can offer both support and nutrition to the microbes. On the other hand, bacterial species in close contact with plants can offer exogenous metabolites and supplements that would, otherwise, not be available to the plant. Moreover, many beneficial microbes defend the host plants against the infection from pathogenic bacteria by releasing agents that specifically kill the invading microbes (Thomashow and Bakker 2015). Such a strong interaction requires a highly efficient communication pathway involving multiple molecular players. A continuous trans-cellular and inter-organismal control of molecules ensures the systematic coordination of signals to "detect, regulate, and affect" in response to particular stimuli (Trapet et al. 2015). The response is, hence, observed in the form of a defensive, regulatory, or conducive outcome. The developments in the field of molecular sciences have exhibited the involvement of a complex interplay of physical, chemical, biological, and environmental signals to regulate the plant-microbe interactions including biofertilization, bioremediation, antibiosis, and biocontrol. The recent understanding in the rapidly developing field of plant-microbe molecular interactions has been presented here.

2 Biofertilization

An important application of the microbe-plant interaction is the biofertilization process in which the symbiotic relationship between the two partners is exploited for promoting the growth of the plants. In the process, the bacteria utilize the nitrogen from the environment to produce nitrogen-containing compounds that are useful for the plants.

In turn, plants provide harbor and carbon source to the bacteria. This natural process accounts for nearly 65 % of the nitrogen fixation for the agricultural crops worldwide (Prasad et al. 2015). Many legumes are used as fertilizers as they tend to promote the nitrogen fixation process by harboring the bacteria necessary for nitrogen fixation. The most common strains of bacteria utilized for the process belong to the *Rhizobium*, *Azorhizobium*, and *Sinorhizobium* genera and are collectively referred to as plant growth-promoting rhizobacteria, or PGPR (Berrada and Fikri-Benbrahim 2014).

The interaction initiates when the bacteria secrete the signaling molecules, mainly lipooligosaccharides, that result in an increase in the growth of stem and root nodules (Mabood et al. 2014). Thereafter, the bacteria penetrate the cortical tissue, divide there, and differentiate into nitrogen-fixing bacteroids. The plant develops a low oxygen status, resulting in the activation of nitrogenase—an enzyme that converts atmospheric nitrogen to ammonia. The genes mainly involved in the process are nitrogen fixation (*nif*) and nodule formation (*nod*) (Gomes et al. 2015). Though the pathway of bacterial strains involved in the signaling process is well known, efforts are underway to decipher the genomic and proteomic machinery of the plants involved in nitrogen fixation. Understanding these mechanisms can potentially help in introducing similar pathways in nonleguminous plants in order to induce a greater capability to fix atmospheric nitrogen. This can, hence, be helpful in increasing the yield of widely cultivated food and cash crops like cotton and rice.

Much similar to the bacterial strains that reside on various plant parts, a number of free-living bacterial species are also involved in the nitrogen fixation process. These rhizobacteria include *Acetobacter*, *Azotobacter*, *Azospirillum*, and *Azoarcus*. They function in a manner much similar to the endophytic bacteria present on the roots and shoots of the plants. At a low oxygen concentration, the nitrogenase complex present in the bacterial proteome converts the atmospheric nitrogen to plant-usable ammonia (Ipata and Pesi 2015). The genetic machinery employed by the free-living microbes is much similar to that utilized for nitrogen fixation, utilization, and regulation by the endophytic bacteria. For agricultural purposes, both the endophytic and free-living bacteria are coated on the seeds and then introduced in the soil. For an efficient biofertilization process, the bacteria are expected to achieve a cell count that is capable enough to survive and establish their presence for an extended time period. A high number does not only help in developing a better source for plant nourishment but also aids in warding off the microbes that might adversely affect the plants (Rogers and Oldroyd 2014). In response, the healthy plant provides nutrients and helps the bacteria to colonize the root and shoot systems of the plant.

Recent findings of the molecular players involved in the biofertilization process have provided a new avenue for exploiting this symbiosis for increasing the agricultural yield. Many bacteria in the rhizosphere have been found to contain certain genes on the chromosome, within the symbiotic island or the symbiosis plasmid (*sym*), that mimic the rhizobial type III secretion system (Ji and Dong 2015; Almario et al. 2014). This genetic machinery is involved in the secretion of certain "symbiosis-forming proteins" which have been isolated from a number of plant-beneficial bacterial strains including *Pseudomonas fluorescens*. Similarly, *nif* genes

have also been isolated in a number of free-living nitrogen-fixing bacteria including *Azoarcus* species (Devi and Momota 2015).

An understanding of the genes and gene products involved in various steps of this process can potentially be helpful in replicating the same mechanisms in plants that, otherwise, do not establish a symbiotic relationship with nitrogen-fixing bacteria. Attachment of the bacteria to the seeds is generally the first step of the inoculation process. In a recent study on *Pseudomonas putida* strains, gene products homologous to calcium-binding protein and multidrug efflux have been found to be actively involved in the interaction with seeds (Molina-Santiago et al. 2014). Similarly, molecular dissection of *Pseudomonas fluorescens* has shown the presence of a number of *rhi* genes (rhizosphere genes) that are involved in the process of nutrient absorption from the plants, bacterial metabolism, and secretion of active agents (Moreno and Rojo 2014; Pizarro-Tobías et al. 2015). A number of genes involved in root colonization have also been identified in other species of *Pseudomonas* and *Bacillus* and other bacterial genera including the ones taking part in biofilm formation and lipopeptide and polyketide metabolite production. Similar studies have shown the plant-induced activation of ABC transporters and porins in various PGPRs (Wisniewski-Dyé and Vial 2015). One additional advantage of introducing PGPRs is their ability to retard the growth of other microbes. Insertion of gene segments (operons), genes, and genetic cassettes has yielded the expression of secondary metabolites that help in protecting the PGPRs and, ultimately, the host plants from unwanted microbial infections. PCA (phenazine-1-carboxylic acid), PCN (phenazine-1-carboxamide), and ACC (1-aminocyclopropane-1-carboxylic acid) are some of the metabolites that aid the colonization of beneficial bacterial strains on the root surface (LeTourneau et al. 2015; Shahverdi et al. 2014; Kim et al. 2014). Microbe-plant interactions are dependent upon a number of abiotic factors as well. Of chief importance in this respect are the soil pH, presence of organic acids, stress factors, and the availability and nature of carbon source.

Biofertilizers, hence, provide a highly efficient and environment-friendly means for providing the plants with the required nutrition to supplement their growth requirements. Many bioinoculants, including *Rhizobium* and *Bradyrhizobium*, are available in the market for the purpose. Alternate bacterial strains including *Bacillus*, *Pseudomonas*, and *Streptomyces* are now being promoted commercially as well. Though significant progress has been made in deciphering the underlying mechanisms, further investigation in this regard can help in improving the overall efficiency of the naturally as well as the artificially induced biofertilization process.

3 Rhizoremediation by Plant-Microbe Cleaning Team

The application of microbe-plant interaction is not only limited to the use of microorganisms for the development and production of useful metabolites for agricultural purposes. A relatively newer, yet highly established, technique is the exploitation of this interaction for the bioremediation process. Pollutants, from

both organic and nonorganic sources, deteriorate the ecological and biogeographical status of various areas. They are generally deposited in certain areas of the ecosystem which, thereafter, act as nuclei for environmental distress. As per recent estimates, the cost to restore the polluted sites in the USA alone would cost nearly two trillion dollars (Knight et al. 2015). The methods generally adopted for the removal of waste matter include incineration and landfilling which further contribute to increasing the air, land, and water pollution. In contrast to these techniques, bioremediation offers a cheaper, safer, and smarter environmentally friendly alternate for treating the waste material. The underlying principle of bioremediation is the use of biological system, generally microbes or plants, for converting the highly toxic agents to safer products. The genoproteomic machinery of the biological organisms is highly adaptive in nature and, hence, when exposed to the new environmental and/or nutritional conditions, tend to utilize these pollutants and convert them into less toxic products. This process is referred to as natural attenuation and has been employed on large scale for the treatment of polycyclic aromatic hydrocarbons (PAH) polluted sites (Srivastava 2015; Juhasz 2014). Moreover, many industrial solvents including toluene, benzene, and ethylene have also been rendered nontoxic by the same phenomenon (El-Naas et al. 2014). Another similar term, phytoremediation, defines the process, whereby the pollutants are detoxified by plants (Bisht et al. 2015). Plants carry out the process by absorbing the pollutants from the soil, transporting them to the shoot, and/or converting them into relatively safer products. Mustard, sunflower, tobacco, and maize have been found to attain these goals in a number of studies (Prasad 2015).

A combination of natural attenuation and phytoremediation is observed in the case of rhizoremediation in which the interaction of microbes and plants is exploited for detoxifying the pollutants. Rhizosphere—comprising the symbiotic bacteria in the roots of plants—has been related to the degradation of many pesticides, herbicides, and PAH-containing pollutants (Hou et al. 2015; Fulekar 2014). The effectiveness of rhizoremediation process depends upon a number of factors including the plant species, microbial strains, and type of pollutant among other pedological and botanical factors. Legumes and grasses are considered to be the most suitable species that can contribute to the process (Brígido and Glick 2015). They have an extensive branching in their roots which usually homes a large number of bacteria. This mechanism provides an efficient means to detoxify the pollutants. At the molecular level, the pollutants are generally subjected to metabolism by the microbial or botanical enzymatic systems. In certain cases, however, they are mineralized, thereby resulting in the immobilization of these agents (Helbling 2015). The resulting products formed by either one of the symbionts can also be utilized by the other partner for its benefit.

A number of methods have been employed for utilizing the rhizoremediation process for achieving maximum efficiency. Some researchers have reported the introduction of a bacterial strain along with the plant seed. This strategy can help in the propagation of the bacterial strain along with the growing root system of the plant. Such interaction has been exploited for the degradation of naphthalene—a toxic organic compound (Agarry and Oghenejoboh 2015). Introducing a consortium

of diverse bacterial strains has been found to degrade a number of pollutants as each strain tends to employ a different catabolic pathway to detoxify the respective pollutant (Fuentes et al. 2014). In addition to these strategies, genetically engineering the microbes and plants to produce transgenic varieties with greater or diverse ability to detoxify the environmental pollutants can be even more useful (Peng et al. 2014; Mouhamad et al. 2014; Jagtap and Bapat 2015). Transgenically produced tobacco plants, expressing the mammalian cytochrome P450 system, have been found to metabolize a number of pollutants including chloroform and trichloroethylene (Renault et al. 2014). The molecular tools can, hence, be employed for improving the activity of this efficient microbe-plant interaction to attain maximum ecological and biological benefits.

4 Biocontrol and Antibiosis

Microbe-plant interactions may be presented in a number of ways. In the case of a symbiosis, both the members benefit. However, in the case of parasitism, the host organism has to bear the adverse consequences of the interaction. Most microorganisms tend to possess a number of mechanisms to defend themselves and their symbiotic partners from pathogenic microbes. The use of microbes or microbe-derived products for inhibiting the growth of pathogenic agents is covered under the scope of biocontrol and has been investigated in a number of recent studies. The environmental and health concerns associated with the conventional tools have shifted the focus toward the use of these environmentally friendly, efficient, and reproducible biocontrol methods.

A number of mechanisms are employed by the microbes to defend themselves and their symbiotic partners, the plants, from pathogenic microorganisms. The chief technique among these biocontrol strategies is the upregulation of antibiosis process by the production of secondary metabolites that either kill the pathogenic bacteria or retard their growth (Clay 2014). *Pseudomonas*, for instance, produces DAPG for attaining effective biocontrol (Weller 2015). Similarly, nonpathogenic strains of *Agrobacterium* produce a highly effective antibiotic agrocin that specifically kills the pathogenic species of bacteria (Hooykaas 2015). Apart from producing secondary metabolites, a number of beneficial microbes compete with other organisms for nutrients and space, thereby inhibiting the secondary infections. Moreover, in certain stress conditions, the microbes produce entities that absorb nutrients and trace elements from the environment protecting the growth of pathogenic organisms. In this respect, siderophores and pyoverdines are released by microbes to sequester the essential elements. A number of antibiotics have been reported that play a significant role in the biocontrol process. Table 1 represents a few examples of the agents generally involved in antibiosis and, therefore, biocontrol process.

The application of the antibiotics in the biocontrol process has been characterized by employing various techniques of genetic engineering. Using these techniques, a detailed insight of microbe-plant interactions has been attained. Mutants

Table 1 Secondary metabolites produced by plant-microbe interactions for attaining effective antibiosis

Metabolite	Activity
Ammonia	Bactericidal, fungicidal
Butyrolactones	Fungicidal
Kanosamine	Fungicidal
HCN	Bactericidal, fungicidal
Oligomycin	Fungicidal
2,4-diacetylphloroglucinol	Fungicidal
Viscosinamide	Bactericidal, fungicidal
Zwittermicin	Bactericidal, fungicidal

lacking the production of antibiotics and those overexpressing their production are employed for studying the effects of antibiotics on the biocontrol process. Conversely, probes or reporter genes have also been utilized for the identification and characterization process. These molecular techniques have provided a thorough understanding of the operons, genes, and gene clusters that are involved in protecting the plants from the adverse effects of pathogenic bacteria (Singh and Singh 2014). Many of these techniques have established an increased presence of the bacterial strains to the improved biocontrol process.

Among the organisms that are generally employed for achieving biocontrol, *Pseudomonas* strains are considered to be one of the most important candidates. This bacterial species is relatively fast growing with ideal lab handling properties. Moreover, its genome can be easily manipulated. *Pseudomonas* is competitive with a number of nutritional sources and can easily thieve in new nutritional and environmental conditions (Mercado-Blanco 2015). In nature, pseudomonads have been found to carry a strictly regulated antibiosis controlling system. It is found to be composed of two main parts: the sensor and the cytoplasmic unit. The environmental sensor system is generally a membrane protein that is activated or triggered by certain environmental factors. Though these factors have not been fully characterized at the molecular level, yet they are considered to be either the part of the chemical exudates from the host plants, chemicals from the pathogenic bacteria, or the products produced in the soil as a result of the presence of pathogenic bacteria (Carvalhais et al. 2015). The second component, the cytoplasmic response factor, initiates the signaling process that results in the production and release of the antibiotic agent thereafter. An imbalance in either of the components results in the inactivation or discrepancy in the antibiosis. For instance, a mutation in membrane or the cytoplasmic genes lead to the incapability of the cells to produce *Phl*, *Plt,* HCN, protease, and phospholipase, the agents generally used for inhibiting the propagation of pathogenic bacteria (Almario et al. 2014; Sharma et al. 2014; Llamas et al. 2014). Moreover, among the fungal species that have been exploited for attaining biocontrol, *Trichoderma* species is the established choice (Srivastava et al. 2014). The fungus can be easily grown and has a wide host range. Similarly, nonpathogenic species of *Pythium* and *Fusarium* are also being used for biocontrol (Gerbore et al. 2014; Zarafi et al. 2015). Unlike bacterial biocontrol agents which

have been effective in controlling both bacterial and fungal infections, fungi are effective in controlling the infection by other fungal species only. The molecular mechanisms of fungus-based biocontrol are not very well understood and, hence, are under investigation by a number of research groups worldwide.

The mechanisms adapted by the microbes are effective in preventing the growth of bacterial and fungal pathogens. One such example is the biocontrol of a few varieties of *Gaeumannomyces graminis* which are involved in the take-all disease of wheat by *Pseudomonas aureofaciens* (Panwar et al. 2014). The bacteria produce a toxin PCA that specifically acts as a fungicidal agent (Borriss 2015). Much similar to the molecular mechanism mentioned earlier, the infection of the roots by the fungus causes the exudation from the roots. This exudate causes an increase in bacterial propagation of both pseudomonads and other beneficial bacteria. Within the bacterial cells, there is an upregulation of the expression of *phzI* gene, resulting in an increased activity of *N*-acyl-L-homoserine lactone or generally referred to as HSL (Khabbaz et al. 2015; Schenk et al. 2014). This ultimately leads to an increase in the production of PCA which inhibits the further colonization and propagation of the fungus on roots. Similarly, the other bacteria in and near the infection region also cause an increase in the production of PCA aiding the biocontrol against pathogenic fungi. Apart from the *phzI* gene, two sigma factors rpoS and rpoD have also been found to control the transcription and, thereafter, expression of the PCA (Duca et al. 2014). In certain cases as in *Pythium debaryanum* infections, there is an upregulation of trehalose signaling (Smeekens 2015). This ultimately signals the activation of bacterial systems in pseudomonads to provide an effective biocontrol cover to plants including sugar beet. Therefore, a multicomponent system is involved, directly or indirectly, in activating the antibiosis process in bacterial population (Fig. 1).

Another mechanism adopted by the beneficial microbes to inhibit the growth of pathogenic organisms on the host plant is the direct interaction of the bacteria with the parasite followed by the release of cell-degrading enzymes. Actinomycetes, a gram positive group of soil bacteria, tend to parasitize the fungal pathogen and degrade the spores of the fungi. The bacteria interrupts the transport of nutrients from plant to the fungus and an increase in bacterial colonization that leads to an increased biotic stress on the fungus or a complete degradation of the fungus, owing to the release of extracellular enzymes. Cellulases and glucanases are the enzymes that have been established to cause the degradation of cellulose and chitin component of the fungal pathogens (Kubicek et al. 2014; Turrà et al. 2014). Similarly, molecular intervention studies have also identified the involvement of endochitinase and other proteases in the prevention of fungal infections in cotton, wheat, and berries (Nagpure et al. 2014).

The competition for nutrition and space within the rhizosphere also plays a major part in the biocontrol process. An increase in the number of "beneficial" microbes causes the pathogenic bacterial and fungal species to compete for space, carbon source, nitrogen source, micronutrients, and the infection/inoculation sites on the host plant. The growth of pathogenic strains of *Fusarium oxysporum*, generally associated with *Fusarium* wilt in tomato, legumes, tobacco, and banana, is suppressed by the presence of nonpathogenic strains of the same fungal species (Jiménez-Díaz et al. 2015). Apart from the competition for space, certain microbial

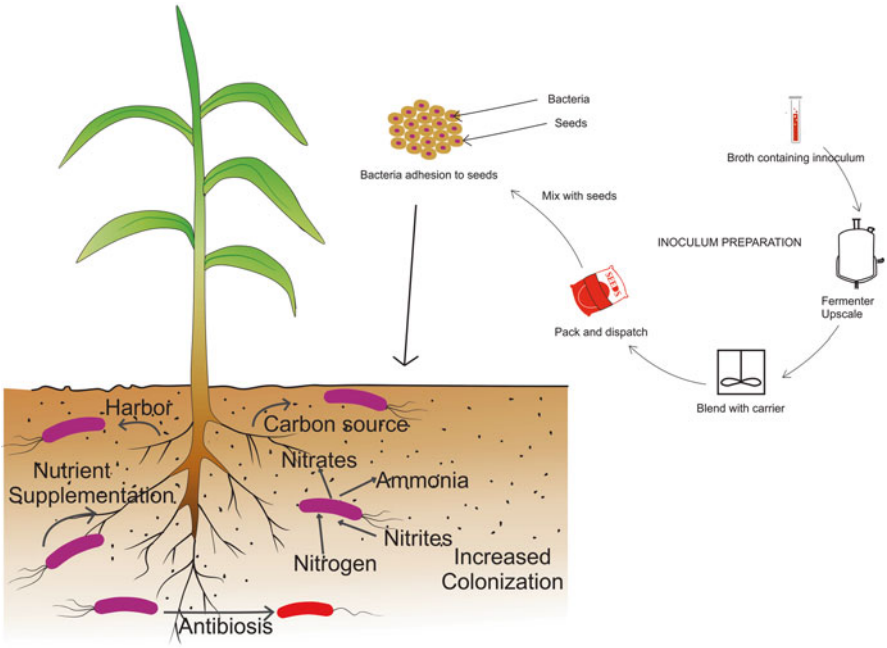

Fig. 1 Inoculation of microbes for biofertilization and biocontrol process

species require a specific nutrient for their survival. Unavailability of the appropriate amounts of the nutrient causes the ultimate death of the pathogenic microbes. An interesting example of this phenomenon is the biocontrol achieved in the wheat rhizosphere against the pathogenic varieties of *Gaeumannomyces* species due to the limited availability of thiamine (Kemen 2014). The microorganisms involved in the biocontrol process have developed a number of means to increase their survival. Modification of the transport proteins to cause an enhanced absorption of the nutrients, faster growth cycles, and rapid induction of inoculation at the preferred sites of infection provides them with a major edge over the pathogenic forms. One such example is the interaction of *Idriella bolleyi* fungi with the cortical cells, leading to the prevention of infection from pathogenic fungi. The fungus occupies the upper region of the root system and travels down in growing nodules to establish its role as an effective biocontrol agent. Though the competition provides adequate defense to the plant from the pathogenic bacteria, further research in the molecular mechanisms involved in the process is still needed.

An interesting example of the biocontrol process that employs the production of secondary metabolites along with the principle of nutrition competition is the production of siderophores or iron-chelating agents (Xia et al. 2014). Under iron-stress conditions, microbial species produce the molecules which sequester the limited

amount of iron available in the plant rhizosphere, making it unavailable to the pathogenic microbes. The siderophores tend to chemically interact with the oxidized form of iron, ferric ions, and aid in their mobilization into the cell. Pseudomonads, for instance, produce pyoverdine and pyochelin, which transport the iron intracellularly preventing the attack of bacterial and fungal organisms on the roots of various crops (Cézard et al. 2015; Cunrath et al. 2015). Siderophores may be used only by the bacteria that produces them, or once released, they may also be utilized by other bacterial species. Apart from the nonspecificity of the bacteria, siderophores also cause the chelation of other metals like aluminum, copper, zinc, manganese, lead, and cadmium (Braud et al. 2015). Additionally, these molecules might also cause the induction of resistance within the plants to a number of pathogens.

Though most of the biocontrol mechanisms involve the use of a single microbe, the involvement of multiple organisms for protecting the plants from infection can be very effective. These microbes can act in harmony and provide synergistic benefits. Addition of multiple microbial species including different species of bacteria or fungi or a bacterial-fungal consortium can be highly effective in inhibiting the growth of pathogenic microbes. A variety of mechanisms can, hence, be exploited for the purpose. Various combinations of *Bacillus* species have been found to protect cucumber and tomato plants from different pathogens (Xu et al. 2014; Hao et al. 2014; Singh and Siddiqui 2015). Similarly, *Trichoderma* and *Pseudomonas* strains have been used in combination for protecting the plants from *Pythium* infection (Kumar et al. 2014). Employing microbial consortia not only helps in providing effective biocontrol, but reports have also suggested an improvement in plant health owing to the induction by PGPRs. The interaction between the indigenous microbial population and the inoculated biocontrol agents has to be considered while devising an effective biocontrol strategy.

In general, the beneficial microbes are introduced in the plant rhizosphere by coating the seeds or the roots of plant saplings with the microbial inoculum. In addition to exogenously introducing the microbes, certain agricultural practices also tend to promote the growth of beneficial bacteria within the rhizosphere. Of prime importance among these are the crop rotations, introduction of soil amendments, soil fumigation, and solarization (Chandel 2015; Sun et al. 2015). These methods are considered safe by the farmers as no genetic or microbial manipulation is involved. A deeper understanding of the plant-microbe interaction is necessary to integrate the molecular techniques with the current agricultural practices. Currently, enhanced efforts are being made to produce transgenic microbes that can provide effective biocontrol. Moreover, the role of environmental conditions, local fauna, and cropping systems is also being studied to help in improving the overall effectiveness of the process.

5 Systemic Resistance

Microbial infection of plants generally causes either of the two presentations: an infection exhibited in the form of plant disease or the development of resistance to the pathogenic agent. In case of an infection, the pathogen or the pathogenic metabolites target various parts of the plant and are then spread throughout the

plant body, ultimately, resulting in the phenotypic changes and finally death. Conversely, for inducing the systemic resistance, following a microbial infection, there is an oxidative stress condition at the local site, resulting in the apoptosis of these cells (Baxter et al. 2014). The rapidly dividing pathogens are entrapped within the cells and, hence, do not mobilize to other parts of the plant body. At the same time, a number of secondary metabolites, including phytoalexins, pathogenesis-related proteins (PR proteins), and proteases, are produced that help in the prevention of the pathogenic attack at any secondary site (De Coninck et al. 2015). The release of these metabolites, especially PR proteins, has been related to the phenomenon known as "induced systemic resistance"—*the vaccination of the plants* (Pieterse et al. 2014a). Moreover, the cell morphology at the local site is also changed in order to prevent the colonization and exaggerated infection at various secondary sites.

The main molecular players involved in ISR are the PR proteins which are generally classified into two main classes: the acidic PR proteins and the basic PR proteins (Golshani et al. 2015). The first category includes the proteins that are generally found in the intercellular spaces, while the basic PR proteins are mainly concentrated in the intracellular regions, for instance, within the vacuole of the plant. Both the classes are functionally similar but vary mainly in the amino acid sequences and, therefore, the molecular weights as well. A number of PR proteins possess chitinase or glucanase activity which helps the plant to defend themselves against the pathogenic fungal species (Ng and Wong 2013). These proteins hydrolyze the cell wall of the fungus, thereby preventing the plant from fungal infection. The role of a large number of PR proteins is, however, still unknown. Generally, they have been isolated in biotic stress conditions from a number of plants and, therefore, serve as excellent markers for correlating the microbial infection. On similar grounds, phytoalexins, in particular camalexin, have been related to ISR and hence help in promoting plant defense against the microbes (An and Mou 2014). Both PR proteins and phytoalexins induce the nonspecific protection of the plants, i.e., the induction of systemic resistance by any one type of the microbe can help in the prevention of attacks by other microbial species.

The mechanism of ISR initially involves the priming or sensitization process in which the microbial pathogen is "sampled" by the plant (Fu and Dong 2013). This results in a complex molecular interplay, causing the activation of the signaling and effector functions, thereafter. The chief among the chemical molecules acting as the inducers for ISR is β-aminobutyric acid (Justyna and Ewa 2013). The activation is followed by the secondary changes including the morphological adaptations like lignification or regulation of other functions including the overexpression of PR proteins and phytoalexins. The exact molecular mechanisms of the priming pathways are, however, still under investigation. After the priming has occurred, the signaling pathways are activated in a manner to prepare the whole plant for protection against the pathogen attack. Salicylic acid (SA)-mediated pathways are considered the most active way to effectively establish the ISR (An and Mou 2014). Infection at the primary site leads to an increase in the endogenous production of SA adding the metabolite into the vascular system of the plant. SA is produced and released from

sites other than that of primary infection as well, causing thereby a "SA storm" within the plant body. The role of SA has been established in a number of plants including wheat, rice, potato, and certain legumes mediated by salicylate synthase (SAS) as well as naphthalene hydroxylase G (*nahG*) gene systems (Kobayashi 2015; Puntus et al. 2015). However, genetic engineering experiments have also lead to the identification of other non-SA systems in the activation and maintenance of ISR. The exact nature of molecules, however, still remains to be established.

As discussed, the effects of the molecules produced during ISR may be in the form of morphological, physiological, or metabolic changes. A diverse set of signaling pathways and chemical reactions are generally involved in the process. These pathways might synergize or antagonize the effects of one another. An in-depth understanding of the molecular players involved in inducing ISR is necessary to devise means to protect the host plants. A number of research groups have investigated the role of various plant metabolites in the ISR regulation. The production of jasmonic acid (JA) is one such pathway adopted by many plants to defend themselves from the microbial and even animal attacks (Carvalhais et al. 2015). In response to the wounding at the primary site of infection/ingestion, the membrane lipids are converted to linolenic acid ultimately transforming to JA. Systemin is another molecule that has been indicated to play a role in the activation of these pathways (Huffaker 2015). The release of JA, hence, results in the overexpression of genes encoding proteinase inhibitors and other enzymes involved in the production of volatile oils, phenolics, and alkaloids, all of which play an important role in the biodefense process. These mechanisms have been well characterized in the case of legumes in response to fungal infection by *Trichoderma viride* (Ruocco et al. 2015). In this case, cellulysin released by the fungus induces the production and release of JA and downstream volatile compounds (Pushpalatha et al. 2013) (Fig. 2).

Whether mediated by SA or JA, ISR has been effective in protecting the plants from subsequent microbial and herbivore attacks. In certain cases, it has been observed that the metabolites produced by one pathway can confer resistance against the pathogens that, otherwise, induce the metabolite production by other pathways. In the case of watermelon, for instance, the attack of the crop by thrips and other insects leads to the protection against fungal attack by *Colletotrichum* species (Lima et al. 2014). Similarly, beetle attacks can help in preventing attacks from *Rumex* species of fungal pathogens (Hejcman et al. 2014). A number of research groups have also reported the antagonistic responses when the plants are attacked by different organisms or have been induced to produce ISR against pathogens. In the case of tomato, for example, chemically induced ISR causes a decrease in the ability of the plant to respond to wound-induced proteinase inhibitors. Moreover, treating the plants with salicylic acid, acetyl salicylic acid, or other chemical inducers also leads to a partial reduction in the systemic resistance. These compounds interact with the octadecanoid-signaling cascade, upstream pathway for the production of JA, and inhibit it in a competitive, dose response manner (Vidhyasekaran 2015). In general, the pattern of ISR involves either of the three mechanisms: the induction by a biological agents against a biological agent, a chemical agent against a biological agent, or a biological agent against a chemical agent. A detailed investigation of these methods needs to be

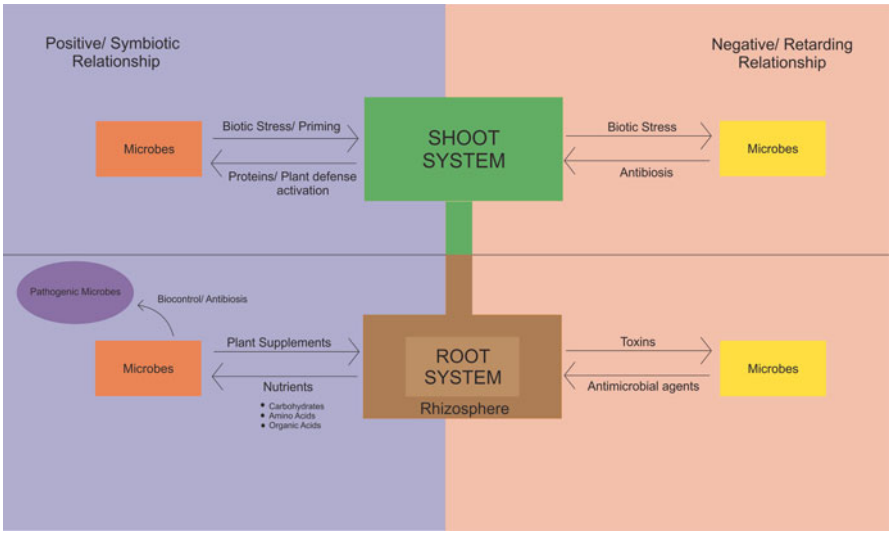

Fig. 2 Biodefense mechanisms in plants based upon plant-microbe molecular interactions

done in order to establish an appropriate means to induce systemic resistance against various pathogenic organisms.

One of the major disadvantages of the ISR mechanism, however, is that the plants are not protected until they have not been exposed to the pathogen at least once. Therefore, in case the primary microbial attack is quite significant, there would be a total failure of resistance mechanisms. Moreover, predictive information needs to be attained in order to prevent the plants from subsequent attacks from the microbes. Concurrent, biotic, and abiotic stress factors should also be considered as either of these can also cause an increase in the propensity of the microbes to cause infection. Therefore, a complex molecular interplay is involved in the induction and sustenance of ISR.

6 Factors Affecting Plant-Microbe Interactions

6.1 Mechanical Signals

Plants respond to a number of physical stimuli. Certain plants possess highly sensitive mechanosensory cells that respond to the external stimuli very rapidly. This can be observed in the case of *Mimosa* and *Dionaea* species of plants (Johnson et al. 2014). In a large variety of plants, however, the response is not as fast, yet there is always a change in the morphological, cellular, or molecular machinery owing to the mechanical simulation. Jasmonic acid, for instance, is

known to be involved in inducing plant resistance against various microbial pathogens (Pieterse et al. 2014b). An increase in the jasmonic acid generally mimics the mechanical simulation and vice versa. This phenomenon coordinates an exaggerated response to bacterial and fungal organisms. Similarly, calcium levels are also changed in response to any mechanical stimuli, causing the activation of a variety of downstream defense response players (Stael et al. 2015). These stimuli are known to upregulate the touch-inducible genes encoding the calmodulin protein that is associated with the development of immunity against the pathogens. It is, therefore, evident that on exposure to these mechanical stimuli, specific molecules are activated within the plant body that mimic the microbial infection aiding the development of resistance, thereafter.

Microbes exploit mechanical simulation to interact with the plants. Many fungi produce a penetration peg to aid its entry into the cell as well as for providing the support to the pathogen (Wang and Qi 2015). After gaining a strong support, the hyphae utilize shear mechanical force to breach further into the underlying tissue. This phenomenon has been observed in the case of *Colletotrichum* and *Magnaporthe grisea* species. After this initial physical interaction, the fungus causes an imbalance in the molecular and chemical homeostasis leading to various presentations within the cells including the nuclear repositioning as observed in the case of an abiotic stress response in plants as well. The response has been well characterized in *Gigaspora* and *Phoma medicaginis species* infection in plants. In case of plant-microbe infection, the nuclear repositioning is supported by the development of a penetration apparatus that facilitates the entry of the fungus in to the plant body. Membrane reorganization, cytoplasmic streaming, microtubule reaggregation, and other mechanical responses are also observed in combination with the molecular reactions in response to a microbial infection (Ueda et al. 2015). Extracellular ATP or eATP acts as a signaling molecule to cause the activation of a number of effector molecules including lectin nucleotide phosphohydrolase (LNP) and other kinases through G-protein complexes (Fliegmann and Bono 2015).

Elictins are another group of molecules that are produced as a result of microbial infection. Following an increase in calcium concentration and oxidative stress, certain kinases are activated. These kinases, for instance, in *Phytophthora* species, cause the activation of nucleotide-binding-site Leucine-rich repeats, or NBS-LRR proteins (Reddy et al. 2015). These proteins cause a hypersensitive reaction that further warrants the activity of self-defense mechanisms. Among other instances of use of mechanical forces for establishing the microbial infection in plants is the development of infection thread. The rhizobacterium is located on the root nodules. The presence of a small colony of microbes induces the production of root hair curl (Fournier et al. 2015). The structure tends to curl onto itself resulting in an increased force and, hence, plant response to both biotic and abiotic stress factors. Based on the induction of host responses to both biotic and abiotic stress factors, it is imperative that the physical and mechanical stimuli act in a similar way as other induction molecules.

6.2 Chemical Signals

The exposure of a plant to the microbial attack leads to a variety of chemical modifications. These changes are observed in response to certain enzyme-activated reactions or release of certain metabolites that ultimately lead to the generation of new chemical entities. The chief among the chemical modifications are the redox reactions and the generation of oxygen moieties. Pathogen-triggered immunity (PTI), for instance, is the resistance developed as a result of microbial recognition and involves multiple players for the generation of ultimate response (Pieterse et al. 2014a). The microbes are identified by means of representative pathogen-associated molecular patterns, or PAMPs (Macho and Zipfel 2014). This is followed by the induction of PR genes and an outburst of reactive oxygen species, or ROS (Corpas et al. 2015). These mechanisms lead to the ability of plants to effectively defend themselves against a microbial attack. Microbes have, in response, developed mechanisms to establish their pathogenicity by the release of effector molecules that interfere with the intracellular PTI responses. Plants have developed another mechanism, referred to as effector-triggered immunity or ETI, which can detect the effector molecules or the accompanying alterations within the plant cell, leading to the generation of a secondary immune response against the pathogen (Cui et al. 2015). Both ETI and PTI are mediated by the generation of large amounts of ROS that are involved in the direct killing of the microbes, retardation of their growth, or generation of mechanism to induce apoptosis in order to reduce the microbial spread within the plant body. Microbes, with their high genetic adaptability, have developed means to prevent the effects of ROS and establish their infection in the host plants.

The chemical interplay between microbes and host plants should, therefore, be deciphered in order to develop effective means to utilize the genetic engineering technologies for the benefit of plants. The nature of the stress molecules helps in determining the pathways involved in the interactions. Plant-derived ROS, for instance, not only lead to the development of resistance against the microbe but also act as a trigger for the generation of microbial responses against the ROS release. In *Magnaporthe* species, glutathione peroxidase, catalases, transcription factors, and serine-rich proteins are all involved in mounting a successful response against the host ROS. The genes including *HYR*, *AP1*, *DES1*, *CATB*, *NOX1*, and *NOX2* are upregulated for the generation of an effective response (Demidchik 2015). In other pathogenic fungi like *Podospora* and *Epichloe*, these changes have been related to the modifications in their growth patterns represented by alterations in hyphae growth, spore-forming ability, and establishment of infection. Based upon similar studies, it is established that the ROS are not solely effective in inducing or neutralizing the pathogen infection on plants. Contrarily, in certain microbial species, these chemical signals act as triggers for enhancing the microbial attack. The exaggerated response involving catalases, ROS, and, the ultimate generation of, hydrogen peroxide might be effective in inhibiting a localized attack (Lindermayr and Durner 2015). However, to effectively control the infection, other molecular and biological players are also required, for instance, the upregulation of proteins and certain mechanical changes.

6.3 Environmental Factors

Apart from the intrinsic factors of both the microbes and the plants, a number of environmental aspects do have a significant effect on the manner in which these organisms interact. Among these, the biotic and abiotic stress factors play an important role. The time of exposure of the microbes and the plants to these agents varies widely. However, they do regulate the molecular machinery within the organism which ultimately leads to the modification in the interaction patterns between the two players. As discussed earlier, in iron-stressed conditions, the bacteria produce certain agents known as siderophores that chelate the iron from the surroundings aiding the survival of the bacteria under these conditions (Ahmed and Holmström 2014). This property, additionally, serves as a bactericidal mechanism depriving the pathogenic microbes from iron, ultimately leading to the diminished colonization of the pathogenic bacteria in the rhizosphere. The interaction between these biotic and abiotic players is not one sided only. The secondary metabolites of microbes also modify their environment. Antibiosis, for instance, is a general mechanism adapted by many microbes to survive and colonize the plant surface (Wackett 2014). Pseudomonads release a number of antibiotics to suppress the growth of pathogenic bacteria in the rhizosphere and maintain the environment in a manner that ensures their long-term survival.

The physical and nutritional characteristics of the soil are one of the main drivers in regulating plant-microbe interactions. The availability of carbon and nitrogen sources, for instance, determines the kind and density of microbial population around the plant. A combination of responses known as plant-soil feedback, or PSF, comprises a set of reactions that are observed after the interaction of plants with the soil microbiota and vice versa (Kos et al. 2015; Baxendale et al. 2014). Many functional traits of the plants act as the modulators of PSF which might adversely affect the plant growth. Soil humidity and partial pressures of entrapped gases also contribute to the selective growth of certain microbes in the ecological sphere (Valentín-Vargas et al. 2014). Among other environmental factors contributing to the regulation of plant-microbe interaction are agricultural practices, cropping systems, exposure to sunlight, temperature variation, and infestation with herbivorous agents.

7 Applications of Plant-Microbe Interactions

The significant importance of plant-microbe interactions under natural circumstances leads to the adaptability of these mechanisms to obtain greater agricultural, biological, and environmental benefits. Microbes tend to initiate both beneficial and adverse effects in the plant body. Bioremediations, biofertilization, and biocontrol are some of the examples of beneficial aspects of plant-microbe interactions. Conversely, microbial diseases in plants have been associated with famine and poverty over the past several decades. In extreme cases, for instance, in the case of ergot poisoning caused by the

fungus *Claviceps purpurea*, humans and animals present with neurological and metabolic symptoms, leading even to death (Young et al. 2015). However, with modern agricultural practices and increased awareness about these interactions, various aspects of the plant-microbe interactions are well understood.

The advent of genetic engineering tools has provided efficient means to study plant-microbe interactions. Therefore, many signaling pathways and molecular players are now very well characterized. The treatment of roots of young plants with rhizobacterial species is now a common practice for the biofertilization purpose. Similarly, a number of bacterial and fungal species are inoculated along with the plants to attain effective biocontrol against the pathogenic microbes. The chief among the biocontrol agents include pseudomonads and *Bacillus* species. The complex plant-microbe interaction is also utilized in order to attain effective phytoremediation in which the organic pollutants are utilized by microbes and plants, thereafter, for the metabolic activity of these organisms.

Recent progress in agricultural processes not only utilizes the whole organisms, i.e., bacteria and fungus, for the improvement of crop yield, but also molecular and submolecular units of the microbes have been used for the purpose. At the molecular level, an interesting example is the utilization of the tumorigenesis capability of the fungus *Agrobacterium tumefaciens* (Bourras et al. 2015). The fungus infects a large number of plant species, and, hence, the genetically modified vectors based on the fungal genome are used for attaining successful gene transfer in many plants. Similarly, small interfering RNA molecules, designed on the basis of plant genome, have been employed to prevent the plants from viral attacks (Cullen 2014). These viral suppressors of RNA (VSRs) integrate into the molecular machinery and inhibit the viral propagation. MAPK, or mitogen-activated protein kinases, are involved in the signaling pathways for the defense of plants against the pathogens (Hettenhausen et al. 2015). These molecules are often induced artificially to obtain an exaggerated response against the pathogens. Similarly, using a spectrum of physical and chemical inducers, plants can be used to produce certain secondary antibiotics that would have otherwise been produced only in response to microbial infections. Chief among these metabolites are various forms of alkaloids, volatile oils, and antibiotics (Maag et al. 2015). On similar basis, antibiotics are produced by the microbes for defending themselves and their plant hosts from pathogen attacks. Antibiotics and siderophores are the chief examples in this regard. Moreover, using the molecular pharming techniques, overexpression of the secondary metabolites has been helpful in gaining a variety of medical and economic benefits. It is, therefore, well established that a deeper understanding of the plant-microbe interactions is necessary for improving agricultural, ecological, and medical gains obtained from the system.

8 Conclusion and Future Perspectives

The interactions between plants and microbes contribute to the improvement in plant health, microbial colonization, and the surroundings. A number of complex molecular mechanisms are involved in this process. Though the advent of modern genetic techniques has helped in improving the understanding of plant-microbe

interactions, efforts need to be made to decipher the exact mechanisms and molecules involved. The application of latest techniques of molecular biology, including NGS and multispecies transcriptomics, can be helpful in improving the outcomes of the research into the plant-microbe interactions. The studies and outcomes, thereafter, would help in improving the quality of food, medicine, and environment. New techniques and technologies based upon the genoproteomic, transcriptomics, and metabolomics understanding would, therefore, provide an opportunity to exploit maximum benefits of the plant-microbe relationship.

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Interaction Between Pesticide and Soil Microorganisms and Their Degradation: A Molecular Approach

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Abstract The use of pesticide in crops is meant to protect plants against harmful insects and increases crop yields. Pesticides are biologically active compounds, and an unintended consequence of its application may influence physicochemical proprieties of soil and lead to significant changes in microbial populations and activities influencing microbial ecological balance affecting soil fertility and metabolic activity of soil microbial communities. Inactivation of nitrogen-fixing and phosphorus-solubilizing microorganisms is observed in pesticide-contaminated soils. Recent studies show that some pesticides disturb molecular interactions between plants and N-fixing rhizobacteria and consequently inhibit the vital process of biological nitrogen fixation. Similarly, many studies show that pesticides reduce activities of soil enzymes that are key indicators of soil health. The applied pesticides may also influence many biochemical reactions such as mineralization of organic matter, nitrification, denitrification, ammonification, redox reactions, methanogenesis, etc. The fate of pesticides applied in agricultural ecosystems is governed by the transfer and degradation processes and their interaction with soil microorganisms. The increasing reliance of sustainable agriculture on pesticide has led to concern about their ecotoxicological effects influencing microbial populations and enzyme activities, which may serve as indicators of soil quality. In this chapter, we attempt to analyze the impacts of pesticides on soil microbial communities, soil biochemical reactions, and molecular approach of degradation of pesticide by soil microorganisms.

Keywords Pesticides • Microorganism • Nitrogen fixation • Ecological balance • Soil microbial community

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1 Introduction

The use of pesticide in crops against a range of pests infesting agricultural crops is meant to protect plants against different groups of pests. The amount of applied pesticides reaching the target organism is about 0.1 %, while the remaining bulk contaminates the soil environment (Carriger et al. 2006) which can alter the chemical and biological proprieties of soil and affect their metabolic activities. The effects of pesticides on soil microorganisms consist in the decrease of the number of microorganisms, alterations in biochemical activity, and quantitative and qualitative decrease of the microbial community (Filimon et al. 2015). Microbial biomass is an important indicator of microbial activities and provides direct assessment of the linkage between microbial activities and the nutrient transformations and other ecological processes.

Pesticide applied in field practices is not neutral for the soil environment (Choudhary et al. 2008). Improper application of chemical preparations which often have the ability of long-term persistence is frequently the cause of their excessive accumulation in the soil. Agricultural chemicals undergo various transformations in the soil. The intensity and directions of those transformations depend largely on the kind of chemicals applied, its properties, and rate of decomposition and also on the properties of the soil environment (Onet 2009). This constitutes a serious threat to the natural environment. Such chemical may interfere with the run of microbiological and biochemical processes which play a key role in the correct functioning of ecosystems. There are also reports documenting the ability of soil microorganisms to degrade pesticides in the soil environment (Hussain et al. 2007). The degradation products of these pesticides are assimilated by soil microorganisms resulting in increased population sizes and activities of microorganisms (Das and Mukherjee 2000b). Recently, molecular techniques have been used to elucidate the impact of pesticides on microbial community structure and functioning (Widenfalk et al. 2008).

This chapter reviews research on pesticide and its degradation in two sections. The first section reviews recent research concerning pesticide effect on microbial population and biochemical reaction in soil, whereas the second section addresses the molecular basis of degradation of pesticide by soil microbes.

2 Effect of Pesticide on Soil Microbial Activities

2.1 Population

Pesticides undergo various degradative, transport, and adsorption/desorption processes in soil depending on the chemical nature of the pesticide (Laabs et al. 2007) and soil properties (Weber et al. 2004). Microbial population is an important indicator of microbial activities and provides direct link between its activities and transformations of nutrient and other ecological processes (Schultz and Urban 2008). The

negative impacts of pesticides on microbial biomass and respiration of soil have been studied by Pampulha and Oliveira (2006). Generally, a decrease in soil respiration reflects the reduction in microbial biomass (Klose and Ajwa 2004). For some microbes, applied pesticide may act as a source of nutrients and energy, whereas other group of pesticides may be toxic to other organisms (Johnsen et al. 2001). Sometimes, initially microbial population is affected by pesticide application, but with time after a certain period of acclimation, the microbial population returns to normal or even increases (Fliessbach and Mader 2004). This is an indication of changes in microbial catabolic capabilities that may be either due to induced pesticide degradation capabilities or due to a change within the microbial community. In the work of Bacmaga et al. (2015), they indicate that the diflufenican + mesosulfuron-methyl + iodosulfuron-methylsodium mixture had a generally stimulating effect on oligotrophic bacteria, spore-forming oligotrophic bacteria, organotrophic bacteria, and actinomycetes. A detailed description of impacts of various pesticides on soil microbial communities is summarized in Table 1.

In addition to the persistence, concentration, toxicity of the applied pesticide, and its bioavailability, various other factors are responsible for the impact of pesticides on soil microbial population. Bioavailability of applied pesticides in soil environment contributes a major role on soil microbes' population. Adsorption and desorption processes regulate concentration of a contaminant in soil solution (Bonczek and Nkedi-Kizza 2007; Katagi 2008) and hence its bioavailability, bioactivity, and degradability in soil environment. Inhibitory effect of insecticide chlorpyrifos and quinalphos in the loamy sand was found greater than in the sandy loam soil due to greater bioavailability (less sorption) of the pesticides in loamy sand which result in lower organic carbon and clay content (Menon et al. 2004). Effect of insecticides, viz., monocrotophos, quinalphos, and cypermethrin, on microbial density in a black clay soil has been reported by Gundi et al. (2005) and observed that the presence of organic matter and vegetation influences the pesticide toxicity to the soil microbes. By using integrated approaches of soil microbial biomass analysis and community-level physiological profiles (CLPPs), Wang et al. (2006) evaluated the effect of methamidophos and urea on microbial diversity in soil and concluded that agrochemicals enhanced functional diversities of soil microbial communities and reduced microbial biomass that some bacterial species might be enriched in soils under methamidophos stress. Demanou et al. (2006) investigated the effect of a combined application of copper and mefenoxam on the functional diversity of soil microbial communities determined by structural and metabolic profiling (arbitrarily primed and RNA arbitrarily primed PCR). After pesticide spray to agricultural fields, pesticides undergo bio- and physiochemical transformations and form different metabolites which are either more persistent or lethal to target and nontarget organisms or harmless depending upon the victims and metabolites formed.

Virag et al. (2007) studied the effects of pesticides and their degradative products, produced by UV treatment, on microbiological activity. They selected five photosensitive pesticides (carbendazim, acetochlor, simazine, EPTC, and chlorpyrifos) and six representative soil microbes (*Bacillus subtilis*, *Pseudomonas*

Table 1 Effect of pesticide on soil microorganisms

S. no.	Pesticide	Microbial community	Result	References
1	Sulfentrazone @ 0.7 μg g^{-1} soil	Bacteria and actinomycetes	Increase in the counts of bacteria and actinomycetes	Martinez et al. (2008)
2	Diuron herbicide at doses of 1.5, 7.5, and 150 mg kg^{-1} soil	Heterotrophic bacteria	An increase in the size of heterotrophic bacterial populations in loamy sand	Cycon and Piotrowska-Seget (2009)
3	Apyros 75 WG at doses of 8.9, 89.0, and 890.0 μg kg^{-1} containing sulfosulfuron	Bacteria and actinomycetes	Inhibited the proliferation of bacteria and actinomycetes, and bacteria were most sensitive to the tested compound	Kucharski and Wyszkowska (2008)
4	Atrazine, primextra, paraquat, and glyphosate	Azotobacter and fungi	Inhibitory effects of herbicides on soil-dwelling microorganism herbicide inhibited the proliferation of *Azotobacter* and fungi	Sebiomo et al. (2011)
5	Apyros 75 WG herbicide	Fungus	Fungal proliferation was inhibited	Kucharski and Wyszkowska (2008)
6	Mesotrione applied to soil at 0.45–45 mg kg^{-1}	Fungus	A stimulatory effect of herbicides on fungal counts	Crouzet et al. (2010)
7	2,4-dichlorophenoxyacetate applied to soil at 1–10 mg kg^{-1}	Fungus	A stimulatory effect of herbicides on fungal counts	Zabaloy et al. (2010)
8	Metazachlor doses of 0.333–213.312 mg kg^{-1} soil DM	Bacteria, fungus	Changes in the structure and biodiversity of microbial communities	Bacmaga et al. (2014a)

fluorescens, *Mycobacterium phlei*, *Fusarium oxysporum*, *Penicillium expansum*, and *Trichoderma harzianum*) for their experiments. Among them, acetochlor and its degradation products were more toxic to bacteria than fungi. All bacterial strains were sensitive to the parent compound and its degradation products as well. End product of carbendazim was moderately toxic against *P. fluorescens* and *B. subtilis* but strongly toxic against *T. harzianum*. Chlorpyrifos and its metabolites did not inhibit the test organisms. They concluded that the pesticide photo degradation could result in significant changes in soil microbiota in addition to the formation of biologically harmful degradation products.

To evaluate the effect of agrochemicals, various molecular techniques are used to study the change in the structure and functions of microbial community. Long-term effects of methyl parathion contamination were investigated by Zhang et al. (2006) on soil microbial diversity estimated by 16S rRNA gene cloning. In the control soil, a member of bacterial division, the *Bacillus* genus, and a member of a *Proteobacteria*, while in methyl parathion-contaminated soil, the dominant phylotypes included a member of the *Flexibacter–Cytophaga–Bacteroides* division, and two members of the g-proteobacteria were observed. This study provided the evidence to assess the long-term environmental toxicological effects of methyl parathion on microbial diversity. Hoshino and Matsumoto (2007) investigated the effect of chloropicrin and 1,3-dichloropropene and spinach growth on fungal community structure in a field by developing a nested PCR-DGGE method with a new combination of primer pairs. They showed that the chloropicrin treatment changed DGGE profiles drastically and reduced the diversity index H0 in both bulk and rhizosphere soils after 2 months of fumigation. The profiles and diversity index did not recover completely even after a period of 1 year. The DGGE profile of 1, 3-dichloropropene demonstrated a smaller change during 2 months of fumigation which became indistinguishable from the control plots after 6 months of fumigation. The authors concluded that the rhizosphere may contribute to minimizing the effect of chemical fumigation. Paul et al. (2006) demonstrated by using 16S rRNA gene sequences that the community structure of the pesticide-contaminated soil was mainly constituted by *Proteobacteria* and *Actinomycetes*. Borzı et al. (2007) investigated the impact of the fungicide fenhexamid (FEX) on the genetic structure of soil bacterial communities already having pcaH sequence. The use of FEX increased the number of the gene copies which implied microbial population of the contaminated soil adapted to the presence of FEX with an increase in degradation potential.

Su et al. (2007) investigated the toxic effects of acetochlor, methamidophos, and their combination on *nif*H gene in soil. The acetochlor decreased the *nif*H gene diversity (gene richness and diversity index values) and changed the *nif*H gene composition with increasing its concentrations. The methamidophos also reduced *nif*H gene richness in first 4 weeks. The medium concentrations of methamidophos (150 mg kg^{-1}) changed *nif*H gene diversity in first week, but higher concentrations (250 mg kg^{-1}) demonstrated prominent effects on *nif*H gene diversity in next weeks. Wang et al. (2006) conducted an experiment to examine the effect of continuous input of methamidophos for 4 years on the biochemical, catabolic, and genetic characteristics of soil microbial communities through characterizing microbial biomass,

PLFA profiles, and CLCPs and ARDRA patterns. They reported that high methamidophos inputs significantly decreased total microbial biomass carbon (Cmic) and fungal biomass, but enhanced Gram-negative bacteria biomass and catabolic activity with no significant effects on the Gram-positive bacteria. These studies support the applications of molecular techniques in studying the response of soil microbes to applied pesticides.

2.2 Biochemical Reaction in Soil

Microorganisms that inhabit in the soil have the ability to carry out biochemical transformations like nitrogen (N), phosphorus (P), sulfur (S), and carbon (C). Agrochemicals may directly or indirectly affect biochemical reactions such as nitrogen fixation, nitrification, denitrification, and ammonification by activating/deactivating specific soil microorganisms and/or enzymes (Kinney et al. 2005). Information on possible effects of pesticides on all biochemical processes is sparse; however, a description of pesticides' effects on soil biochemical reactions is summarized in Table 2.

Table 2 Selected lists of genes involved in the degradation of pesticides

Gene	Organism
Bacteria	
Opd	*Pseudomonas diminuta*
opaA	*Alteromonas* spp.
opdA	*A. radiobacter*
adpB	*Nocardia* sp.
pepA	*Escherichia coli*
hocA	*Pseudomonas monteilii*
pehA	*Burkholderia caryophylli*
Phn	*Bacillus cereus*
ophB	*Burkholderia* sp. JBA3
ophC2	*Stenotrophomonas* sp. SMSP-1
OpdB	*Lactobacillus brevis*
Imh	*Arthrobacter* sp. scl-2
Mpd	*Ochrobactrum* sp. Yw28, *Rhizobium radiobacter*
Oph	*Arthrobacter* sp.
Mph	*Arthrobacter* sp. L1 (2006)
MpdB	*Burkholderia cepacia*
opdE	*Enterobacter* sp.
Fungi	
A-opd	*Aspergillus niger*
P-opd	*Penicillium lilacinum*

Modified from Singh and Walker 2006

2.2.1 Nitrogen Fixation

An efficient and natural source of nitrogen is the biological nitrogen fixation (BNF). The total BNF has been estimated twice (175 million tons) as compared to the total nitrogen fixation by nonbiological processes. Legumes and rhizobial symbioses contribute nearly half the annual quantity of BNF entering soil ecosystem (Singh and Walker 2006). Pesticide may influence the nodulation and BNF in legumes either by affecting virulence of attacking nodular bacteria, the root fibers of the plants in which the infection occurs, or both. Some agrochemicals not only inhibit the nitrogen fixation process in nitrogen-fixing bacteria but also reduce the bacterium's respiration rate (Tate 1995; San-Tos and Flores 1995). Atmospheric nitrogen gas (N_2) is fixed by the enzyme nitrogenase. Application of pesticides affects the efficiency and activity of nitrogenase enzyme. A decrease in total nitrogenase activity (measured from pots sown with *Pisum sativum* plants) with the application of herbicides was reported (Singh and Wright 1999). There is clear evidence that nontarget soil bacteria are influenced by pesticides, but the impacts of these pesticides vary from stimulatory to highly inhibitory. Effects may be direct or indirect and are dependent upon several interacting factors that relate to the mode of application and the soil environment. A number of factors, viz., chemical nature of pesticides, concentration used, microbial community structure, type of soil, and soil conditions, can contribute to divergent research findings (Digrak and Ozcelik 1998). Thus, previous studies attributed such differences to the dual behavior of pesticides, both harmful and beneficial for soil microorganisms. The vital process of nitrogen fixation is coordinated and regulated by phytochemical signaling to *Rhizobium* (Baker 1998). Some pesticides interfere with plant–Rhizobium signaling and affect symbiotic nitrogen fixation by inhibiting nodulation by *Rhizobium* (Fox et al. 2007). Detailed description of pesticides' effects on plant–*Rhizobium* signaling has been given by Fox et al. (2001). Variation among legume species with regard to nodulation and N_2 fixation under pesticide treatment may depend on the type and dose of the pesticide, species of *Rhizobium* and legume, and stage of development of the *Rhizobium*–legume symbiosis. Khan et al. (2006) reported that herbicides applied in sandy clay loam soil had an adverse phytotoxic effect on chickpea vitality and subsequently the *Mesorhizobium*–chickpea symbiosis. Similarly, Madhaiyan et al. (2006) studied the influence of different pesticides on the growth and survival of *Gluconacetobacter diazotrophicus* strain PAL5. The monocrotophos, lindane, and dichlorvos proved lethal to *Gluconacetobacter*, while endosulfan, chlorpyrifos, and malathion effects were intermediate. The herbicides did not affect the growth and survival of *Gluconacetobacter* in the medium except for the concentrations exceeding recommended rates while it had a slight effect on the growth of *Gluconacetobacter* at recommended dose except Ridomil. Most of the pesticides affected the cell morphology to a larger extent resulting in larger number of pleomorphic cells.

Gulhane et al. (2015) studied the effect of pesticides Hilcyperil and Nuvan on desired nitrogen-fixing bacteria, *Rhizobium* spp. and *Azotobacter* spp., which are very essential for the growth of plant as well as for more yields. Nuvan pesticide greatly inhibited the growth of both the nitrogen-fixing bacteria as compared to

that of Hilcyperil and concluded that pesticides which are under field condition possibly due to its high toxic nature reduced the population of these bacteria under field condition.

2.2.2 Mineralization of Organic Compounds and Availability of Nitrogen, Phosphorus, and Potassium in Soil

Assessing pesticide effects on organic compounds such as carbon and nitrogen mineralization is a standardized component of testing pesticides for nontarget effects in the registration process by USEPA and OECD in various countries. Organic matter is one of the most critical properties of soil that affects soil quality, productivity of soil, and emission of trace gases to atmosphere. Much changes in the levels and the dynamics of organic matter is controlled by biological activities in soil and the quantity and quality of plant residues returned to the soil. Understanding the effects on both processes is important in understanding pesticide interactions in soil and their role in supporting plant growth and overall ecosystem health. Walia et al. (2014) evaluated the effect of mancozeb at different concentration (0–2000 ppm) and concluded that 0, 10, and 100 ppm of mancozeb had little effect on the average amount of available phosphorous released. Above 100 ppm, the amount of phosphorous increased significantly with increase in mancozeb concentrations, and maximum value was obtained at 2000 ppm mancozeb (97.60 ppm). The effect of incubation period revealed significant increase in average phosphorous after 1 week of incubation (71.34 ppm). Further incubation had inhibitory effect on phosphorous solubilization as average phosphorous decreased to 49.43 ppm at the end of 3 weeks. Some scientists reported that in soils treated with different insecticides at recommended doses (BHC, phorate, carbofuran, and fenvalerate representing the organochlorine, organophosphate, carbamate, and pyrethroid groups of insecticide), the rate of mineralization of organic C was higher compared to the control. This indicated that biodegradation of insecticides stimulated the growth and activities of heterotrophic microorganisms which favor mineralization of organic matter and biological transformation of other plant nutrients in soil to derive energy, carbon, and other elements for microbial metabolism for their cellular constituents, resulting in lower retention of organic C in soil (Debnath et al. 1994; Das and Mukherjee 2000a). Application of insecticides resulted in an increase in the amounts of mineralized N (NH_4^+ and NO_3^-) in soil. Insecticides probably stimulated the growth and activities of ammonifying and nitrifying bacteria which were mainly responsible for mineralization of organic N. Chen and Edwards (2001) studied the effect of captan, benomyl, and chlorothalonil on N-dynamics in soils. All fungicides enhanced rates of net N-mineralization and nitrification initially, but reduced the rates after 20 days. They attributed the increase in N-mineralization to the death of certain fungi and increase in certain bacteria that most likely increased the rates of N-mineralization. Demanou et al. (2004) studied the effect of Ridomil Gold copper fungicide on N-ammonification and P-mineralization and found higher mineralization of both elements, probably occurred through killing of a part of microflora and increasing NH_4^+–N by surviving

part of the microflora. The inhibition of nitrification could be another way of NH_4^+–N accumulation in soil. Das and Mukherjee (2000b) also reported higher mineralization of P with the incorporation of insecticides suggesting that insecticides significantly increased the phosphate-solubilizing/phosphate-mineralizing microorganisms. Different results that have been reported by Sardar and Kole (2005) observed significant reduction in N-, P-, and K-mineralization with the application of chlorpyrifos (organophosphate insecticide). The inhibitory effect on available N, P, and K was attributed to the primary and secondary metabolites of chlorpyrifos metabolism rather than parent chlorpyrifos itself. However, the average N and P status was recovered at 120 days after the disappearance of the metabolites. Arginine deaminase catalyzes the mineralization of nitrogenous compounds in soil to release ammonium and nitrate. Menon et al. (2004) reported that arginine ammonification activity of rhizospheric microbes was inhibited after seed treatment with chlorpyrifos and quinalphos and their principal metabolites.

A more accurate assessment of P-solubilizing ability is revealed by the solubilization index (SI). Ahemad and Khan (2012a) evaluated the effect of selected herbicides (quizalofop-p-ethyl, clodinafop, metribuzin, and glyphosate) at one, two, and three times the recommended field rates on the plant-growth-promoting (PGP) traits of *Pseudomonas putida* strain PS9 isolated from the mustard rhizosphere. The effect of 1× and 2× of all herbicides on SI of the *P. putida* PS9 was less deleterious, while the highest concentration (3×) had the maximum adverse impact on P-halo formation. The order of toxicity of herbicides at 3× on SI was quizalofop-p-ethyl > clodinafop > metribuzin = glyphosate. Another study by Ahemad and Khan (2012c) was designed to explore beneficial plant-associated rhizobacteria exhibiting substantial tolerance against fungicide tebuconazole vis-a-vis synthesizing plant growth regulators under fungicide stressed soils and to evaluate further these multifaceted rhizobacteria for protection and growth promotion of green gram [*Vigna radiata* (L.) Wilczek] plants against phytotoxicity of tebuconazole. The *P. aeruginosa* strain PS1 solubilized phosphate significantly and produced indole acetic acid, siderophores, exo-polysaccharides, hydrogen cyanide, and ammonia even under tebuconazole stress. Generally, tebuconazole at the recommended two and three times the recommended field rate adversely affected the growth, symbiosis, grain yield, and nutrient uptake in green gram in a concentration-dependent manner. In contrast, the *P. aeruginosa* strain PS1 along with tebuconazole significantly, increased the growth parameters of the green gram plants. The inoculant strain PS1 increased appreciably root nitrogen, shoot nitrogen, root phosphorus, shoot phosphorus, and seed yield of green gram plants at all tested concentrations of tebuconazole when compared to the uninoculated plants treated with tebuconazole. Dubey et al. (2012) studied the effect of different pesticides such as carbofuran, phorate, carbosulfan, and thiamethoxam on soil microflora and resulted that the viable count of rhizobia and phosphate-solubilizing bacteria from rhizospheric soil of leguminous crop ranged between 107 and 105 Cfu/g soil.

2.2.3 Nitrification, Denitrification, Ammonification, Sulfur Oxidation, and Respiration

Walia et al. (2014) studied the impact of fungicide mancozeb at different concentration (0–2000 ppm) on different soil biological processes. Nitrification of diammonium hydrogen phosphate in soil revealed that average nitrate–nitrogen (NO_3–N) decreased significantly with the increase in mancozeb concentrations from 0 (34.74 ppm NO_3–N) to 2000 ppm (20.63 ppm NO_3–N). However, the difference in NO_3–N between 250 and 500 ppm and 1000 and 2000 ppm was nonsignificant among themselves. The effect of mancozeb (0–2000 ppm) on the production of ammoniacal nitrogen (NH_4^+–N) from applied peptone was studied in apple orchard soil. The findings showed significant decrease in the average NH_4^+–N in mancozeb-treated soil as compared to control. The amount of average ammoniacal nitrogen was at par among 250 and 500 ppm and 1000 and 2000 ppm of mancozeb concentrations. Average ammoniacal nitrogen decreased with increase in incubation period irrespective of mancozeb concentrations.

One of the primary indices used in the estimation of the status of the soil environment is the respiratory activity which indicates the level of general activity of soil microorganisms (Dutta et al. 2010). This process is related to the degradation and oxidation of organic compounds, in which CO_2 is evolved. The disturbances exhibit in carbon transformation processes in soil due to the effect of pesticides can be measured by the respiratory activity. Tys and Rutkowska (2013) studied the long-term effect of Reglone 200 SL and Elastiq 550 EC on the respiratory activity in soil. They demonstrated that the amount of emitted CO_2 and the content of ammonium and nitrate ions depended largely on the periods of the analysis and the type of chemical agent. The optimal dose of 200 SL and Elastiq 550EC applied caused periodic statistically significant changes in the respiratory activity. Araujo et al. (2003) observed 10–15 % increase in the amount of evolved CO_2 in the soil studied under glyphosate treatment. Ahemad and Khan (2012b) assessed the effect of selected pesticides [herbicides (metribuzin and glyphosate), insecticides (imidacloprid and thiamethoxam), and fungicides (hexaconazole, metalaxyl, and kitazin)] at recommended and higher dose rates on plant growth-promoting activities of the *Mesorhizobium* sp. isolated from chickpea nodules. Among these strains, the *Mesorhizobium* sp. strain MRC4 was specifically selected due to the highest tolerance levels for all selected pesticides and the maximum production of plant growth-promoting substances. Strain MRC4 produced indole acetic acid (44 lg ml^{-1}), siderophores [salicylic acid (35 lg ml^{-1}) and 2,3-dihydroxy benzoic acid (19 lg ml^{-1})], exo-polysaccharides (21 lg ml^{-1}), HCN, and ammonia. Under pesticide stress, pesticide-concentration-dependent progressive decline in all plant growth-promoting traits of the *Mesorhizobium* sp. strain MRC4 exposed was observed except for exo-polysaccharides which consistently increased with exceeding the concentration of each pesticide from recommended dose.

Soil Enzymes

For the biodegradation of natural and anthropogenic organic compounds in soil, an enzyme plays a vital role and is often used as indicators of changes that occur in the soil environment in response to pesticides and fertilizers (Bacmaga et al. 2012). Dehydrogenases, similarly as catalase, are intracellular. Enzyme activity is strongly correlated with microbial activity (Bello et al. 2013). In the experiment of Bacmaga et al. (2015), soil enzymes showed different sensitivity to a mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methylsodium on soil enzymatic activity, compared with uncontaminated samples. They observed that dehydrogenase enzyme was most sensitive to soil contamination with the tested pesticide at the dose of 36.480 mg kg^{-1} and catalase activity was significantly reduced on day 60 in response to herbicide doses of 2.280–9.120 mg kg^{-1}. Cycon et al. (2013) demonstrated that dehydrogenases were strongly inhibited by napropamide doses of 2.25 and 22.5 mg kg^{-1}. The inhibitory effect produced by the 2.25 mg kg^{-1} dose was transient, and on incubation day 28, dehydrogenase activity was similar to that of control soil. Doses tenfold higher than those recommended by the manufacturer significantly inhibited dehydrogenase activity throughout the experiment. A decrease in phosphatase activity was also noted by Yao et al. (2006) in soil contaminated with acetamiprid, Wyszkowska and Kucharski (2004) in a study of Triflurotox 250 EC, and by Wyszkowska (2002) in an experiment with Treflan 460 EC. Bacmaga et al. (2012) observed a positive effect of the Aurora 40 WG herbicide containing carfentrazone-ethyl on the activity of the above enzymes. In the study of Bacmaga et al. (2015), the tested herbicide had a neutral effect on the activity of urease and β-glucosidase. In treatments subjected to the highest herbicide dose, urease activity increased by 7.5 % on day 30 and decreased by 13.2 % on day 60. On day 30, the activity levels of β-glucosidase were fairly similar across the analyzed treatments, but on day 60, they decreased by 13.8 % in treatments exposed to herbicide doses of 1.140 and 2.280 mg kg^{-1}. Saha et al. (2012) observed higher levels of β-glucosidase activity in soil samples treated with alachlor, butachlor, and pretilachlor. Walia et al. (2014) studied the effect of mancozeb (0–2000 ppm) on soil enzymes, viz., amylase, invertase, and phosphatase that showed adverse and disruptive effect when mancozeb used was above 10 ppm in unamended soil. Khudsar and Askar (2013) studied the effect of some pesticides on growth, nitrogen fixation, and *nif* genes in *Azotobacter chroococcum* and *Azotobacter vinelandii* isolated from soil. They investigated the effect of Dimethoate (insecticide), Bayleton (fungicide), and Imazetapir (herbicide) on nitrogenase activity through amplification of *nif*H1, *nif*H2, *nif*H3, *nif*U, *nif*V, and FV genes for *A. chroococcum* and *nif*H, *nif*D, *nif*K, *nif*M, and FV genes for *A. vinelandii* by PCR technique. Band generation of PCR-amplified *nif* gene fragments was used to evaluate the effect of tested pesticides on *A. chroococcum* and *A. vinelandii* isolated from soil cultivated with wheat plants and applied with pesticides. The number of bands present or absent is used to estimate the influence of pesticides on the bases of the number of shared amplification products. Both insecticide- and fungicide-treated pots

affected negatively the *nif* genes, and *nif*H1, *nif*H2, *nif*H3, *nif*U, and *nif*V, while herbicide did not affect the *nif* genes, and the bands appeared as normal sample of *A. chroococcum*.

3 Molecular Basis of Degradation of Pesticide by Soil Microbes

In order to investigate molecular basis of pesticide biodegradation by soil microorganisms, several works had been reported on the role of catabolic genes and the application of recombinant DNA technology. Genes responsible for pesticide degradation such as plasmids and transposons of only a few microorganisms have been shown to encode enzymes responsible for the degradation of several pesticides.

In comparison with standard microbiological methods, molecular techniques give more comprehensive interpretation for in situ microbial community. RFLP, dot blot, Southern blot, PCR amplification, subsequent analysis of bacterial rRNA genes by sequencing, preparing metagenomic libraries, denaturing gradient gel electrophoresis (DGGE), and microarrays are several techniques which are applied for degradation (Sinha et al. 2009). In this study, colonies were hybridized by entire plasmids as probes to compute the cells containing catabolic plasmids, and then they observed positive relationship between plasmid concentrations and the rates of mineralization. These techniques were used to monitor the xylE and ndoB genes involved in creosote degradation in soil communities. Amplicon length heterogeneity PCR (LH-PCR) and terminal restriction fragment length polymorphism (T-RFLP) technique were used to monitor the effect of nutrient amendments on microbial community during bioremediation of petroleum-contaminated soils (Mills et al. 2003). The gene encoding TCE-RDase required for PCE biotransformation, *tceA*, has been cloned and sequenced by an inverse PCR approach. Sequence comparisons of *tce*A to proteins in the public databases revealed weak sequence similarity confined to the C-terminal region, which contains the eight-iron ferredoxin cluster binding motif (Magnuson et al. 1998). Direct DNA hybridization techniques have been made to monitor TOL and naphthalene-degrading plasmid (NAH) (Sayler and Layton 1990). Polychlorinated biphenyl (PCB) catabolic genes have been used to measure the level of PCB-degrading organisms in soil microbial communities with the help of dot-blot technique (Walia et al. 1990). To quantify the degradation of 2,4-dichlorophenoxyacetic acid (2, 4-D), *tfd*A and *tfd*B gene probes have been used and identified with the help of Southern hybridization technique (Holben et al. 1992).

The degradation of organochlorine pesticides by pure cultures has been proven to occur in situ. One of the works published in nature by Matsumura et al. (1968) was able to evidence the breakdown of dieldrin in the soil by a *Pseudomonas* sp. Most of the xenobiotic-degrading bacteria harbor plasmids which code for catabolic genes. To characterize the appropriate genes and to enhance the process of degradation through improved constructed strains, a proper management is required, and

degradation technology is spanning the spectrum from environmental monitoring ultimately to biodegradation as well as bioremediation (Eyers et al. 2004). Currently, molecular approaches are being used to characterize the nucleic acids of various bacteria from environmental samples (Hurt et al. 2001). Comparing with standard microbiological methods, the molecular techniques provide us with a more comprehensive interpretation of the in situ microbial community and its response to both engineered bioremediation and natural attenuation processes (Brockman 1995). Different microbial enzymes with the capacity to hydrolyze pesticides have been identified (Yan et al. 2007) such as **organophosphorus hydrolase** (OPH, encoded by the *opd* gene). This gene has been found in bacterial strains that can use organophosphate pesticides as carbon source. These plasmids show considerable genetic diversity, but the region containing the *opd* gene is highly conserved. **Methyl-parathion hydrolase** (MPH; encoded by the *mpd* gene), Are *Pseudaminobacter*, *Achromobacter*, *Brucella* and *Ochrobactrum* genes, they were identified by comparison with the gene *mpd* from *Plesiomonas* sp. M6 strain (Zhongli et al. 2001), the gene for the organophosphorus hydrolase has 996 nucleotides, a typical promoter sequence of the promoter TTGCAA N17 TATACT from *E. coli* (Zhang et al. 2005). In various isolates of microorganisms capable of degrading pesticide, several genes have been described in Table 3.

Another powerful molecular technique known as metagenomic libraries has been flourished for the identification of the desired catabolic genes. Basically, metagenomic is a culture-dependent genomic analysis; it is either function-driven approach or sequence-driven approach of total microbial communities, which provides access to retrieve unknown sequences (Schloss and Handelsman 2003). Though the technique is applicable, yet contains certain drawbacks. One of the major drawbacks is less recovery of desired clones that however can be overcome. The metagenomic libraries are particularly promising for locating denitrifying genes (Eyers et al. 2004). The sequence-driven approach which is primarily based on conserved regions in bacterial genes has also been studied. It is reported that certain hybridization probes (screen out clone libraries for specific DNA sequences) may identify the required genes for degradation. For example, in the denitration of 2,4,6-trinitrophenol, the *ndp*G and *ndp*I genes were identified in *Rhodococcus erythropolis HL-PMI* (Heiss et al. 2003). In order to search out diverse degrading genes in relation to bacterial ecology, fingerprinting techniques are also used which are tagged to a PCR reaction to amplify selected sequences. For example, the amplified segment of *nah*Ac genes from a miscellaneous bacterial population may be of related size when amplified with a particular set of *nah*Ac-specific degenerate primers; nevertheless, they contain minute differentiation within the PCR-amplified products (Schneegurt and Kulpa 1998). To investigate the PCB degradation, polymorphism and PCR amplification of *bph*C gene have been done, but no significant restriction polymorphism has been observed. Through DGGE and terminal restriction fragment length polymorphism (T-RFLP), a comparative analysis of PCB-dechlorinating communities in enrichment cultures has been reported (Watts et al. 2001). To evaluate the restriction fragments of PCR-amplified products, matrix-assisted laser desorption ionization time-of-flight mass spectrophotometry (MALDI-TOF-MS) has been

Table 3 List of pesticides and their degrading bacterial species

Pesticides	Bacteria	Isolated sites	References
DDT (dichlorodiphenyltrichloroethane)	*Pseudoxanthobacter liyangensis* sp. Nov.	Bacterial strain, DDT-3T, was isolated from DDT-contaminated soil in Liyang, PR China	Liu et al. (2014b)
	Novosphingobium Arabidopsis sp. Nov.	Bacterium, designated strain CC-ALB-2T, was isolated from the *Arabidopsis thaliana* rhizosphere	Liu et al. (2014a)
	Alcaligenes sp. Strain DG-5	Bacteria were isolated from DDT-contaminated sediment	Gao et al. (2011)
	Serratia marcescens DT-1P	Bacteria were isolated by long-term enrichment of soil samples collected from DDT-contaminated fields	Bidlan and Manonmani (2002)
HCH/lindane (1,2,3,4,5,6-hexachlorocyclohexane)	*Sphingobium czechense* LL01^T	Bacterial strain was isolated from (HCH) contaminated soil at Spolana Neratovice, a former Czech producer of lindane	Niharika et al. (2013)
	Sphingomonas sp. NM05	Studied by surfactant (rhamnolipid, sophorolipid, and trehalose) mediated enhanced biodegradation	Manickam et al. (2012)
	Streptomyces sp. M7	Studied by the use of lindane as the only carbon source	Cuozzo et al. (2009)
	Pseudomonas strains	Strains isolated from agricultural soil possess c-hexachlorocyclohexane-degrading ability	Nawaz et al. (2003)
2, 4-D (2,4-dichlorophenoxyacetic acid)	*Maribacter* sp. AMSU	Bacterial strain was isolated from aquaculture effluent by enrichment culture technique	Sankaralingam et al. (2013)

Pesticides	Bacteria	Isolated sites	References
	Delftia sp.	Bacterial strain was isolated from a polluted river in Buenos Aires, Argentina	Gonzalez et al. (2012)
	Pseudomonas putida SM1443	Studied by fed-batch microcosm system and a lab-scale sequencing batch reactor (SBR) to enhance degradation capacity of 2,4-D	Quan et al. (2010)
	Comamonas koreensis strain CY01	Anaerobic reductive dechlorination of 2, 4-D and the role of humic substances in the degradation	Wang et al. (2009)
Diuron DCMU (3-)3,4-dichlorophenyl)-1,1-dimethylurea)	*Arthrobacter* sp. BS2 and *Achromobacter* sp. SP1	Bacterial strain was isolated from enrichment culture of buffer strip (BS) soil and in the sediments (SED) of the Morcille river in the Beaujolais vineyard where diuron was found	Devers-Lamrani et al. (2014)
	Micrococcus sp. strain PS-1	Bacterial strain was isolated from diuron storage site	Sharma and Suri 2011
	Pseudomonas sp. and *Stenotrophomonas* sp.	Diuron-degrading bacteria were isolated from enrichment culture of lotic surface water	Batisson et al. (2007)
	Streptomyces sp.	17 *Streptomyces* strains, obtained from agricultural soils, were determined in the laboratory	Castillo et al. (2006)
	Arthrobacter sp.	A bacterial strain was isolated from a soil by enrichment procedures	Widehema et al. (2002)

reported (Taranenko et al. 2002). Probes with DNA microarrays have been designed to identify key genes involved in the degradation of 2, 4-dichlorophenoxyacetic acid in the presence of 2,4-D with *Ralstonia eutropha*, a 2,4-D degrading bacteria (Dennis et al. 2003). Replicative limiting dilution PCR (RLD-PCR), an alternate quantitative PCR for environmental application, is based on RLD analysis and the pragmatic trade-offs between analytical sensitivity and practical utility (Chandler 1998). This method has been used to detect and quantify specific biodegradative genes in aromatic-compound-contaminated soil. The catabolic genes *cdo*, *nah*Ac, and *alk*B were used as target genes (Chandler 1998). The biotransformation of pyrene by *Mycobacterium* KMS is extensively studied and confirmed with the aid of proteomics by identifying almost all the enzymes required during the initial steps of the degradation of this pericondensed PAH compound (Liang et al. 2006).

4 Conclusion and Future Prospects

The application of molecular tools in the field of microbial ecology is undergoing unprecedented changes. With the postgenomic molecular approaches, we simply dented the surface of the genetic and metabolic diversity of the prokaryotes. Several questions on a number of microbes and microbial communities that are governed by biological, chemical, and physical factors remain to be understood. Understanding the functional roles of uncultured organisms still remains a daunting task, as most of the genes identified have no homologous representatives in databases. Although considerable progress has been made in the characterization of microbial communities by the application of metagenomic, metatranscriptomic, and proteogenomic approaches, many technical challenges remain including DNA, RNA, and protein extraction from environmental samples, mRNA instability, and low abundance of certain gene transcripts in total RNA. The next-generation sequencing techniques are still developing, and many technological innovations particularly tuned for environmental samples are expected in these techniques. Development in bioinformatics tools is also needed for whole-genome analysis and metagenomic and metatranscriptomic approaches. Quantitative assessment of microbial communities is the greatest challenge due to significant biases associated with nucleic acid isolation and PCR and requires more advanced DNA/RNA extraction techniques for environmental samples. All of the molecular approaches available for community structure and function analysis have advantages and limitations associated with them, and none provides complete access to the genetic and functional diversity of complex microbial communities. A combination of several techniques should be applied to interrogate the diversity, function, and ecology of microorganisms. An interdisciplinary systems approach embracing several “omics” technologies to reveal the interactions between genes,

proteins, and environmental factors will be needed to provide new insights into environmental microbiology.

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In Silico Functional Analyses of SWEETs Reveal Cues for Their Role in AMF Symbiosis

Muhammad Sameeullah, Tijen Demiral, Noreen Aslam, Faheem Shehzad Baloch, and Ekrem Gurel

Abstract SWEETs are novel class of sugar effluxers, which have unique functional role in plant biology. Besides nectar production, freezing tolerance, and transport of hexoses across tonoplast and growth-supporting role of pathogens, these SWEETs could have potential role in establishing powerful symbiotic relationship at the root interface and also in feeding to arbuscular mycorrhizal fungi (AMF) symbionts. The microarray or transcriptome expression of SWEET genes from colonized roots revealed that out of 28 *Medicago* SWEETs, three genes (*MtSWEET1b*, *MtSWEET3c*, and *MtSWEET12*) were induced specifically due to AMF symbiosis. The root type specific expression of these three genes was also enhanced by AMF colonization in rice. The degree of expression of *OsSWEET1b*, *OsSWEET3b*, and *OsSWEET12* was increased in colonized large lateral roots (LLRM) and crown roots (CRM), while *OsSWEET3b* and *OsSWEET12* were induced in fine lateral roots (FLRM) and CRM, respectively. Promoter regions of these SWEETs represent critical motif elements (MYCS, PB1S, and PHR1) which play critical role in establishment of AMF symbiosis and phosphate starvation-induced responses, respectively. Taken together, these SWEETs have potential to be explored via functional genomics tools to understand feeding mechanisms to symbionts.

Keywords AMF symbiosis • Phylogenetic tree • Promoter analyses • Sugar effluxers • SWEET

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1 SWEET Proteins

SWEET transporters or sugar efflux transporters were identified (Chen et al. 2010; Braun 2012) by integrated tools of genetics, genomics, cell biology, and biochemistry by using fluorescence resonance energy transfer (FRET), an optical sensor that could detect the level of sugars in cytoplasm (Lager et al. 2006; Braun 2012). SWEETs do not show strong tendency to pH dependence and function in both uptake and efflux assays (Eom et al. 2015). These proteins play crucial role in the efflux of sugars. For example, AtSWEET11 and AtSWEET12 localized to the plasma membrane of mesophyll parenchyma cells are involved in efflux of sucrose (Chen et al. 2012; Eom et al. 2015) to the cell walls between parenchyma and companion cells. The sucrose transporter localized at the plasma membrane of companion cell import sugar into companion cells and then finally through plasmodesmata load into the phloem (Fig. 1). Le Hir et al. (2015) very recently reported that AtSWEET11 and AtSWEET12 to transport glucose and fructose in addition to sucrose and their expression localized not only in phloem parenchyma cells but also in xylem cells close to the cambium region. Furthermore, the double *Arabidopsis* mutants *sweet11-sweet12* exhibited greater freezing tolerance than the wild type and both single mutants by modification of the chemical composition of the xylem

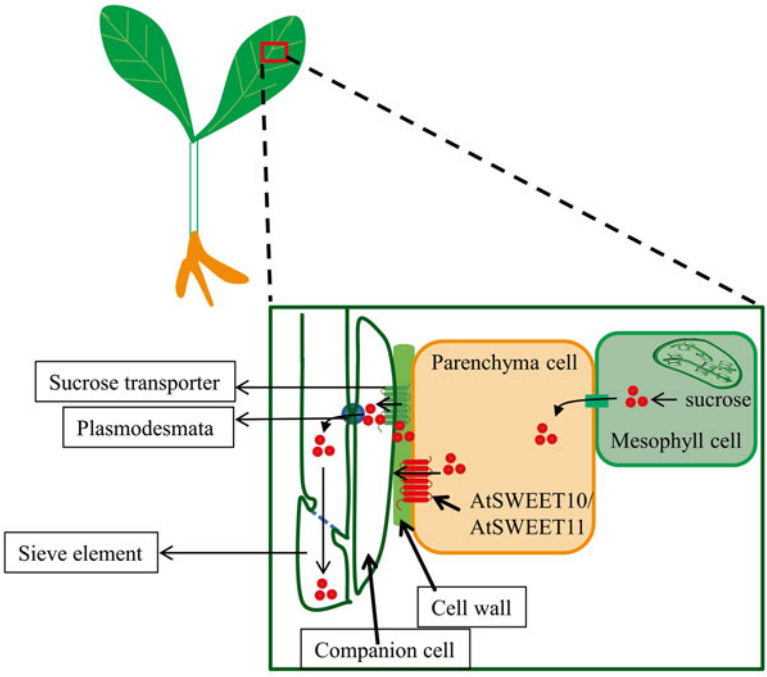

Fig. 1 A key role of SWEETs for translocation of sucrose from the site of synthesis (mesophyll cell) to phloem

cell walls (Le Hir et al. 2015). These findings suggested that AtSWEET11 and AtSWEET12 have a role as sugar exporters delivering carbon-containing skeletons to developing xylem cells to support secondary cell wall formation besides their role in contributing phloem loading in source leaves (Le Hir et al. 2015). SWEET9 is involved in the secretion of nectar (Lin et al. 2014) that is essential as food for pollinating insects (Sargent 2004). AtSWEET5, AtSWEET8, and AtSWEET13 are essential in pollen feeding. AtSWEET11, AtSWEET12, and AtSWEET15 which are expressed in seed coat and endosperm have a role in healthy seed development, while triple knock-out mutants exhibited extremely procrastinate embryo development and seed phenotype was wrinkled (Eom et al. 2015). AtSWEET16 and AtSWEET17 are tonoplast-localized hexose transporters. Overexpression of AtSWEET16 improved the seed germination and freezing tolerance (Klemens et al. 2013). AtSWEET17 controls the fructose content of *Arabidopsis* leaf (Chardon et al. 2013) and also facilitates fructose content across the tonoplast of leaf and roots of *Arabidopsis* (Guo et al. 2014). AtSWEET2 functions on the root tonoplast of the cortex and epidermis cells by sequestering glucose into root vacuoles, thereby limiting carbon loss into the rhizosphere (Chen et al. 2015). Reduced sugar availability in the rhizosphere due to SWEET2 activity thus provides *Arabidopsis* seedlings with resistance against soilborne pathogen *Pythium irregulare* (Chen et al. 2015).

Maintenance of sustainable food production is seriously hampered by plant pathogens worldwide, and exploitation of disease resistance (R) genes seems the best bet to combat the adverse effects of the diseases. One of the most common phytopathogens causing blight disease in rice (*Oryza* sp.) is *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Mansfield et al. 2012), and virulence of most *Xoo* strains mainly relies on the presence of individual transcriptional activator-like (TAL) effectors (Streubel et al. 2013). TAL effectors recognize and bind to the effector-binding elements (EBEs) at the promoter region of disease susceptibility (S) genes in host DNA, which are thus induced to provide advantage to the pathogen. Hutin et al. (2015) screened a germplasm of 169 rice accessions for polymorphism in the promoter of the major bacterial blight susceptibility (S) gene *OsSWEET14* which encodes a sugar transporter targeted by numerous strains of *Xoo* and identified a single allele (*xa41*(t)) conferring resistance against half of the tested *Xoo* strains. They showed that this allele with a deletion of 18-bp conferred resistance against *Xoo* strains by possibly preventing the binding of several TAL effectors known to target *OsSWEET14* (Hutin et al. 2015). Out of 21 SWEETs, five SWEETs from clade III have been shown that these can support the growth of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in rice. Among these five SWEETs, OsSWEET14 was induced by bacterial pathogen transcription activator-like 5 (TAL) which is a novel TAL effector. These artificial TAL effectors, which targeted specifically to the SWEETs, could support the growth of pathogens (Streubel et al. 2013). A SWEET from citrus CsSWEET1 could express upon infection of *Xanthomonas citri* in a TAL effector-dependent manner (Hu et al. 2014) also another example of feeding role of SWEETs to pathogens. To date, the feeding role of SWEETs to symbiotic microbes is still missing, and the horizons of SWEET interaction with symbionts to develop efficient symbiotic relationships with plants need to be explored. The detailed studies of SWEET interaction with symbionts could play a role in sustainable agriculture and healthy crop management under abiotic and biotic stresses. However, SWEET proteins could be involved in establishment of

symbiotic interactions between arbuscular mycorrhizal fungi (AMF) and plant roots (Udvardi et al. 1990). Thus, SWEET protein might be responsible for transport of carbohydrates from roots to AMF cells to flourish its growth and thus make AMF capable of continuous supply of phosphate for the host plant as a feedback. The phosphates can be acquired by low- or high-affinity phosphate transporters in roots, which can be then translocated to shoot region to meet the demands of the plant (Ceasar et al. 2014). Here, we present the SWEET proteins, which were upregulated due to inoculation with AM fungi in *Medicago truncatula* and *Oryza sativa*.

1.1 Phylogenetic Tree

SWEET genes were classified into four clades following the classifications described previously (Chen et al. 2010; Streubel et al. 2013). Purple color lines and SWEETs belong to clade I, teal color represents clade II, green color for clade III SWEETs, and olive color for clade IV and from *Arabidopsis*, *Medicago*, and rice. Red color-labeled SWEETs were induced by arbuscular mycorrhizal fungi (AMF) symbiosis in *Medicago* and rice plant roots. MtSWEET1b, OsSWEET1b OsSWEET3b, and MtSWEET3c belong to clade I, and MtSWEET12 and OsSWEET12 fall in clade III (Fig. 2).

1.2 SWEETs Induced by AMF Symbiosis in Medicago

Gomez et al. (2009) employed Affymetrix GeneChip® *Medicago* Genome Array for transcript profiling which was associated with AMF (*Glomus intraradices*) symbiosis. Their focus was to determine the gene expression related to AMF symbiosis in cortical cells. However, SWEET genes were unattended since SWEETs were reported later (Chen et al. 2010, 2012). SWEET expression log intensity according to values of genome array of control roots and AMF symbiotic roots revealed that out of 12 SWEETs, three-SWEET expression was prominently elevated due to AMF symbiosis. Expression level of *MtSWEET1b*, *MtSWEET3c*, and *MtSWEET12* was AMF symbiotic dependent manner, while rests of SWEETs were

Fig. 2 (continued) MtSWEET12 (Medtr8g096320), MtSWEET13 (Medtr3g098910), MtSWEET14 (Medtr8g096310), MtSWEET15a (Medtr2g007890), MtSWEET15b (Medtr5g067530), MtSWEET15c (Medtr7g405730), MtSWEET15d (Medtr7g405710), MtSWEET16 (Medtr2g436310), *Oryza sativa* SWEETs: OsSWEET1a (LOC_Os01g65880), OsSWEET1b (LOC_Os05g35140), OsSWEET2a (LOC_Os01g36070), OsSWEET2b (LOC_Os01g50460), OsSWEET3a (LOC_Os05g12320), OsSWEET3b (LOC_Os01g12130), OsSWEET4 (LOC_Os02g19820), OsSWEET5 (LOC_Os05g51090), OsSWEET6a (LOC_Os01g42110), OsSWEET6b (LOC_Os01g42090), OsSWEET7a (LOC_Os09g08030), OsSWEET7b (LOC_Os09g08440), OsSWEET7c (LOC_Os12g07860), OsSWEET7d (LOC_Os09g08490), OsSWEET7e (LOC_Os09g08270), OsSWEET11 (LOC_Os08g42350), OsSWEET12 (LOC_Os03g22590), OsSWEET13 (LOC_Os12g29220), OsSWEET14 (LOC_Os11g31190), OsSWEET15 (LOC_Os02g30910), and OsSWEET16 (LOC_Os03g22200)

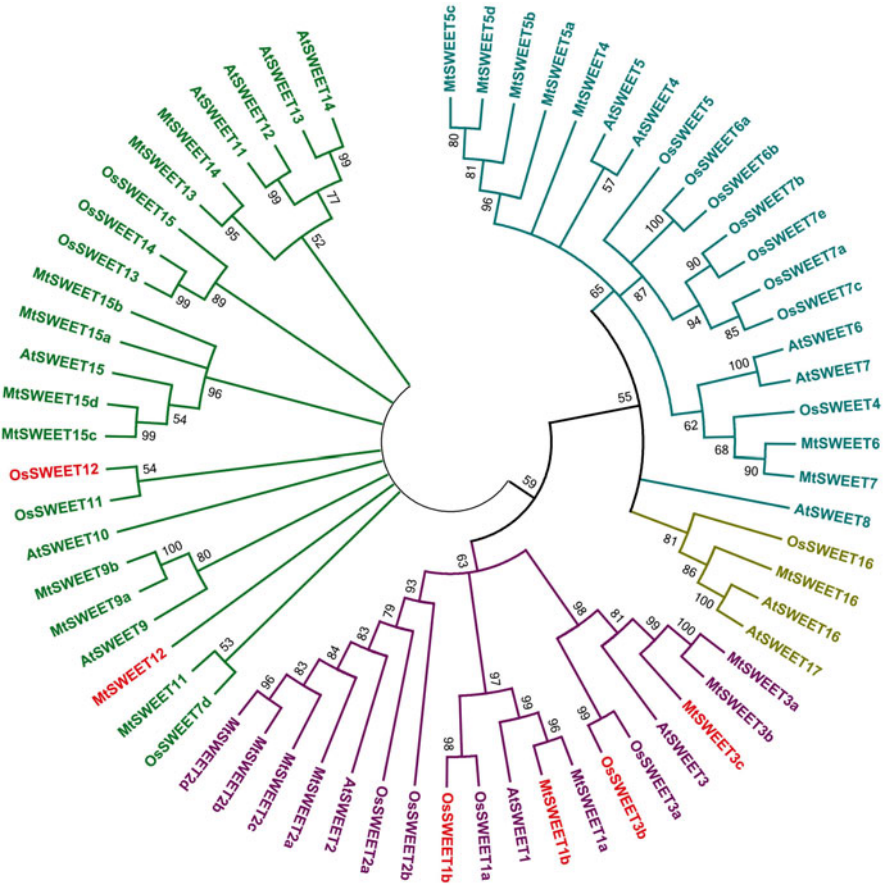

Fig. 2 Phylogenetics of SWEETs from *Arabidopsis*, *Medicago*, and rice. The phylogenetic tree was constructed by amino sequences of SWEETs from *Arabidopsis thaliana*, *Oryza sativa*, and *Medicago truncatula*. Protein sequences were aligned using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/), and phylogenetic tree was developed using MEGA7 assuming a Poisson substitution model, uniform rates among sites, and partial deletions for missing data and gaps with 1000 replicates. *Red color*-labeled SWEETs were induced by arbuscular mycorrhizal fungi (AMF) in *Medicago* and rice plant roots. SWEET accessions were followed from Lin et al.(2014), and MtSWEET16 accession was retrieved from *Medicago truncatula* gene expression database (http://mtgea.noble.org/v3/). Protein sequences of *Arabidopsis* were obtained from https://www.*Arabidopsis*.org, *Oryza sativa* protein sequences from http://rice.plantbiology.msu.edu, and *Medicago truncatula* protein sequences from http://plantgrn.noble.org/LegumeIP/ or http://phytozome.jgi.doe.gov/pz/portal.html. *Arabidopsis* SWEET gene names and accession are AtSWEET1 (At1G21460), AtSWEET2 (At3G14770), AtSWEET3 (At5G53190), AtSWEET4 (At3G28007), AtSWEET5 (At5G62850), AtSWEET6 (At1G66770), AtSWEET7 (At4G10850), AtSWEET8 (At5G40260), AtSWEET9 (At2G39060), AtSWEET10 (At5G50790), AtSWEET11 (At3G48740), AtSWEET12 (At5G23660), AtSWEET13 (At5G50800), AtSWEET14 (At4G25010), AtSWEET15 (At5G13170), AtSWEET16 (At3G16690), AtSWEET17 (At4G15920), *Medicago truncatula* SWEETs: MtHext1 (Medtr1g104780), MtSWEET1a (Medtr1g029380), MtSWEET1b (Medtr3g089125), MtSWEET2a (Medtr8g042490), MtSWEET2b (AC235677_9), MtSWEET2c (Medtr6g034600), MtSWEET2d (Medtr2g073190), MtSWEET3a (Medtr3g090940), MtSWEET3b (Medtr3g090950), MtSWEET3c (Medtr1g028460), MtSWEET4 (Medtr4g106990), MtSWEET5a (Medtr6g007610), MtSWEET5b (Medtr6g007637), MtSWEET5c (Medtr6g007623), MtSWEET5d (Medtr6g007633), MtSWEET6 (Medtr3g080990), MtSWEET7 (Medtr8g099730), MtSWEET9a (Medtr5g092600), MtSWEET9b (Medtr7g007490), MtSWEET11 (Medtr3g098930),

downregulated or insensitive to response of AMF symbiotic relationship (Fig. 3). Doidy et al. (2012) explored sucrose transporters in *Medicago truncatula* (MtSUTs) and their implication in carbon partitioning toward the fungal symbiont by inoculation with the arbuscular mycorrhizal fungus *Glomus intraradices*. Their results proved the involvement of MtSUT1-1 in roots and MtSUT4-1 in leaves upon AMF inoculation, implying a role of these transporters in phloem unloading toward AMF-colonized sink tissues during plant symbioses (Doidy et al. 2012). But the role of SWEETs in sugar partitioning in AMF symbiosis still remains to be explored.

1.3 SWEET Expression Depends upon AMF Symbiosis in Rice

Transcriptome of AMF symbiosis of *Oryza sativa* was reported by Gutjahr et al. (2015). *Rhizophagus irregularis* (previously called *Glomus intraradices*) was inoculated with *Oryza sativa* ssp. Japonica cv. Nipponbare. They investigated different root types and associated transcript phytohormones and secondary cell wall, but SWEETs still remain elusive. OsSWEET1b transcript expression upon AMF symbiosis was highly upregulated in large lateral roots (LLRM), and degree of expression was relatively lower in other colonized roots such as fine large lateral roots (FLRM) and crown roots (CRM). However, overall expression of *OsSWEET1b* was higher in colonized roots compared to non-colonized roots (NC). The expression of *OsSWEET3b* was highly upregulated in FLRM roots than FLRNC. Expression level was also increased in LLRM than in LLRNC. However, expression of *OsSWEET3b* was downregulated in CRM compared to CRNC that suggest the tissue specificity of *OsSWEEt3b*. Expression of OsSWEET12 was slightly upregulated in FLRM and LLRM compared to FLRNC and LLRNC, respectively. However, degree of

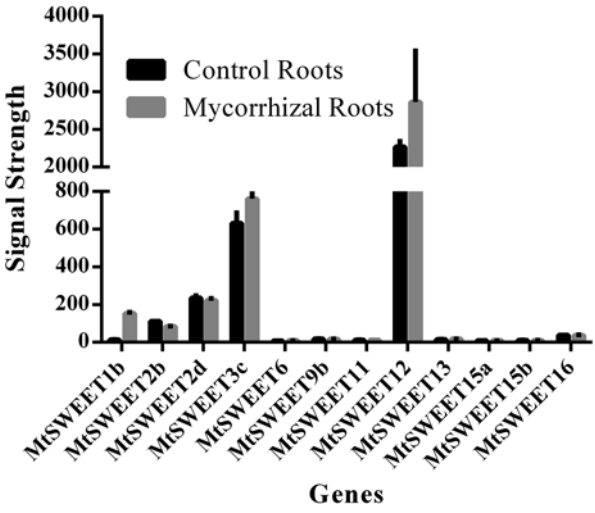

Fig. 3 *Medicago truncatula* SWEET expression regulated by AMF symbiosis. Three microarray expression values from three independent repeats of control or treated roots with AMF symbiosis of *Medicago truncatula* were used. Data represent mean ± SE

expression of OsSWEET12 was higher in CRM than CRNC suggesting the tissue specific role of this SWEET gene (Fig. 4).

In silico prediction of SWEET genes was performed which were induced by AMF colonization in *Medicago* or rice as shown in Figs. 3 and 4. The protein sequences were used to predict subcellular localization that was performed by Wolf PSORT, Plant-mPLoc, and RiceNetDB. Localization of MtSWEET1b and 3c at the chloroplast, vacuole, and cell membrane depicts multicellular functionality; however, MtSWEET12 is predicted to be localized in the cell membrane and extracellular. OsSWEET1b and 3b localized in the cell membrane predominantly, while OsSWEET12 localization is predicted in extracellular and the nucleus (Table 1).

1.4 Prediction of Transmembrane Helices of AMF-Induced SWEETs

Transmembrane helices and topology of SWEETs were conducted by using the TMHMM2.0 program http://www.cbs.dtu.dk/services/TMHMM/. As a representative dicot and a monocot, topologies of MtSWEET1b and OsSWEET1b were predicted, respectively. SWEET proteins consist of seven transmembrane helices and with peptide sequences of 200–250 (Fig. 5).

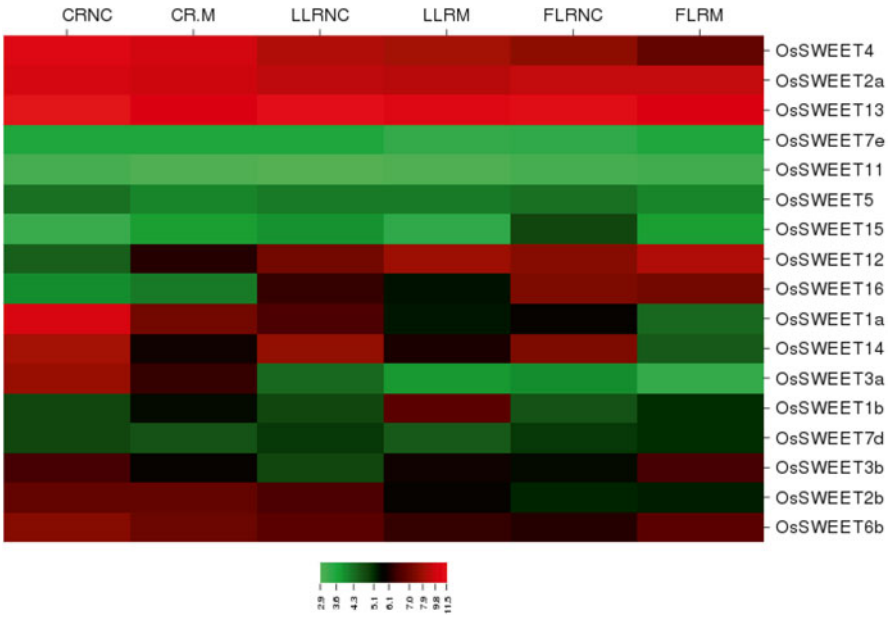

Fig. 4 Heat map expression of rice SWEET genes induced in different root types. Crown roots (CR), large lateral roots (LLR), fine lateral roots (FLR), non-colonized (NC), mycorrhizal (M)

Table 1 In silico subcellular localization prediction of SWEETs induced by AMF

Genes	Accession no.	Length	Subcellular prediction
MtSWEET1b	Medtr3g089125	247	Chloroplast, vacuole, cell membrane
MtSWEET3c	Medtr1g028460	256	Chloroplast, vacuole, cell membrane
MtSWEET12	Medtr8g096320	255	Cell membrane, extracellular
OsSWEET1b	Os05g35140	261	Cell membrane, Golgi
OsSWEET3b	Os01g12130	252	Cell membrane
OsSWEET12	Os03g22590	300	Extracellular, nucleus

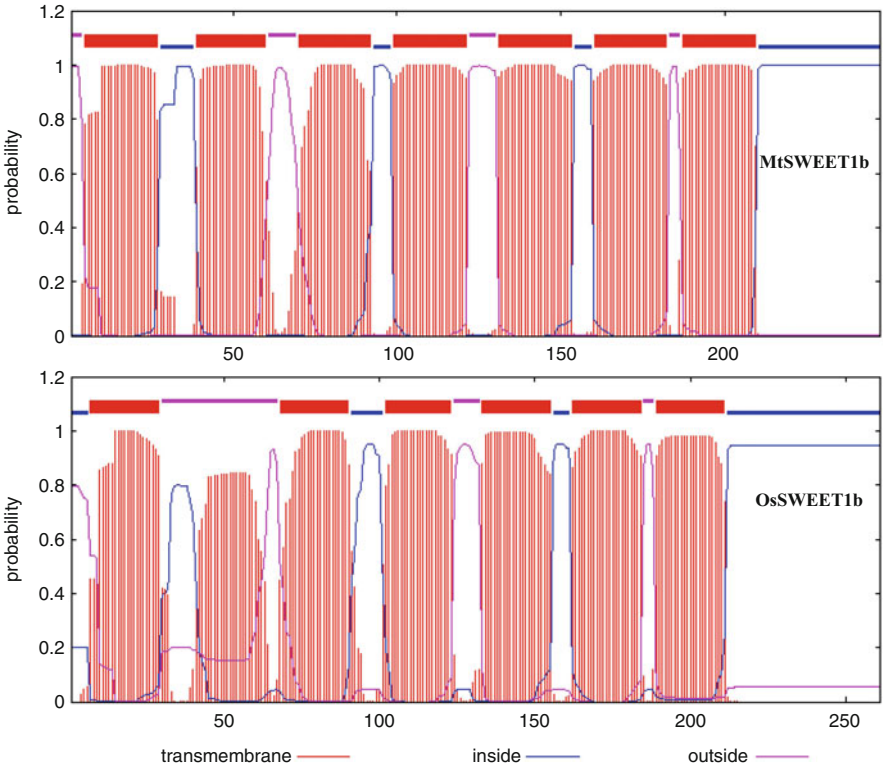

Fig. 5 Predicted transmembrane helices of AMF-inducible SWEETs from *Medicago* and rice

1.5 Promoter Analyses of AMF-Inducible SWEETs

In silico analysis of putative promoters regions of SWEETs (*MtSWEET1b*, *MtSWEET3c*, *MtSWEET12*, *OsSWEET1b*, *OsSWEET3b*, and *OsSWEET12*) of *Medicago truncatula* and *Oryza sativa* revealed several known *cis*-acting elements which are responsible for phosphate starvation (PHR1 or P1BS) or transcriptional activators involved in AMF-mediated inorganic phosphate transporters genes

(MYCS). Specifically, MYCS *cis*-acting elements were found in *MtSWEET1B*, *OsSWEET1B*, *OsSWEET3b*, and *OsSWEET12* but not in *MtSWEET3c* and *MtSWEET12*. The MYCS element is an 11 bp sequence (TTTCTTGTTCT) which is present in many other phosphate transporters LePT4, StPT4, SmPT4, NtPT4, SmPT5, NtPT5, MtPT4, StPT3, SmPT3, and NtPT3 (Chen et al. 2011). There are two MYCS *cis*-elements in promoter regions of *OsSWEET1b* and *OsSWEET3b* and one in *MtSWEET1b* and *OsSWEET12* (Table 2). P1BS elements were found in phosphate transporters LePT4, StPT4, SmPT4, NtPT4, SmPT5, NtPT5, MtPT4, StPT3, SmPT3, NtPT3, OsPT11, OsPT2, SmPT2, and AtPT8. The P1BS elements (GNATATNC) were reported for the first time by Rubio et al. (2001). These phosphate starvation response elements were also observed in promoter regions of *MtSWEEt1b* (two elements), *MtSWEET3c* (three elements), *MtSWEET12* (two elements), and *OsSWEET1b* (three elements). These P1BS elements could be involved in the regulation of AMF-inducible SWEETs in a similar manner as reported previously (Chen et al. 2011). PHR1, a MYB transcription factor, to which P1BS element interacts to regulate the expression of phosphate starvation-inducible genes (PSI) (Daram et al. 1998; Rubio et al. 2001; Schachtman and Shin 2007) also found in the promoters of SWEETs of rice and *Medicago truncatula*. The promoter *cis*-acting elements (P1BS, PHR1, and MYCS) play essential role for the induction and maintaining of high expression of AMF-activated PHT genes (Chen et al. 2011) that also suggest a similar putative role for SWEETs in monocots and dicots. The *cis*-acting elements nodulin consensus 1 and 2 have similar function but are variable in the SWEETs. Promoter of *MtSWEET1b* contains two elements of nodulin consensus 1 and 11 of consensus 2. *MtSWEET3c* promoter has four and eight, *MtSWEET12* has six and eight, *OsSWEET1b* has three and nine, and *OsSWEET12* has six and two nodulin consensus 1 and consensus 2, respectively. *OsSWEET3b* has three elements of nodulin consensus 2 (Table 2). Nodulin consensus were activated specifically in nitrogen-fixing nodules and also in mycorrhizal colonized root cells as well suggest a critical associated role of these elements in symbiosis (Vieweg et al. 2004; Fehlberg et al. 2005). By considering all these data, we predict a presumptive model in Fig. 6 that shows possible function of SWEETs in supplying carbohydrates to arbuscular mycorrhizal roots during infection.

2 Future Perspective

SWEETs are potential sugar effluxers, which offer unique ability to feed AMF symbionts at root soil interface. However, these recently reported novel classes of sugar effluxers need to be explored functionally in detail either using forward or reverse genetic approach. CRISPR/Cas9, a new and a novel genome editing strategy, can be employed to characterize these AMF-inducible SWEET candidates from *Medicago* and rice. Subcellular localization prediction either using homologous or heterologous approach will also help in depicting clear picture of these SWEET expression sites and will be helpful in revealing their feeding role to symbionts. Interaction of

Table 2 Presence of *cis*-acting elements related to phosphate starvation and AM symbiosis in promoter regions of SWEETs

Gene name	*Cis*-element	Sequence	Function	Copies/promoter	Upstream location of *cis*-elements
MtSWEET1b	PHR1/MYB transcription factor	GCATATCC	Phosphate starvation response	2	−68, −2298
	MYCS	TTTCTTGTTCT	Mycorrhizal transcription factor	1	−2283
	P1BS	GNATATNC	Responsiveness to phosphate deprivation	2	−1742, −1747
	Nodulin consensus 1	AAAGAT	Activation in infected cells of mycorrhizal colonized roots and nodulation	2	−556, −1611
	Nodulin consensus 2	CTCTT	Activation in infected cells of mycorrhizal colonized roots and nodulation	11	−43, −84, −840, −1000, −1024, −1621, −1796, −1868, −1996, −2248, −2796
MtSWEET3c	PHR1/MYB transcription factor	GCATATCC	Phosphate starvation response	1	−2735
	P1BS	GNATATNC	Responsiveness to phosphate deprivation	3	−1977, −2250, −2303
	Nodulin consensus 1	AAAGAT	Activation in infected cells of mycorrhizal colonized roots and nodulation	4	−325, −1556, −2644, −2683
	Nodulin consensus 2	CTCTT	Activation in infected cells of mycorrhizal colonized roots and nodulation	8	−60, −176, −384, −767, −1074, −1812, −2392, −2935
MtSWEET12	P1BS	GNATATNC	Responsiveness to phosphate deprivation	2	−608, −1771
	Nodulin consensus 1	AAAGAT	Activation in infected cells of mycorrhizal colonized roots and nodulation	6	−682, −1371, −1503, −1953, −2567, −2837
	Nodulin consensus 2	CTCTT	Activation in infected cells of mycorrhizal colonized roots and nodulation	8	−128, −693, −796, −908, −1154, −1257, −1995, −2228

OsSWEET1b	PHR1/MYB transcription factor	GCATATCC	Phosphate starvation response	6	−1353, −1362, −1367, −1798, −1825, −2255
	MYCS	TTTCTTGTTCT	Mycorrhizal transcription factor	2	−132, −406
	P1BS	GNATATNC	Responsiveness to phosphate deprivation	3	−778, −783, −2291
	Nodulin consensus 1	AAAGAT	Activation in infected cells of mycorrhizal colonized roots and nodulation	3	−1776, −2134, −2719
	Nodulin consensus 2	CTCTT	Activation in infected cells of mycorrhizal colonized roots and nodulation	9	−297, −565, −639, −656, −778, −1637, −1837, −2354, −2814
OsSWEET3b	PHR1/MYB transcription factor	GCATATCC	Phosphate starvation response	5	−155, −160, −198, −642, −845
	MYCS	TTTCTTGTTCT	Mycorrhizal transcription factor	2	−273, −1914
	Nodulin consensus 2	CTCTT	Activation in infected cells of mycorrhizal colonized roots and nodulation	3	−2012, −2303, −2358
OsSWEET12	PHR1/MYB transcription factor	GCATATCC	Phosphate starvation response	3	−1569, −1570, −2369
	MYCS	TTTCTTGTTCT	Mycorrhizal transcription factor	1	−2427
	P1BS	GNATATNC	Responsiveness to phosphate deprivation	2	−1761, −1781
	Nodulin consensus 1	AAAGAT	Activation in infected cells of mycorrhizal colonized roots and nodulation	6	−891, −912, −1093, −1592, −2060, −2578
	Nodulin consensus 2	CTCTT	Activation in infected cells of mycorrhizal colonized roots and nodulation	2	−998, −1642

These motifs are located between −1 and −3100 bp upstream of ATG start codon. The analysis of SWEET promoters was performed using Genomatix Software Suite v3.4 (http://www.genomatix.de/?s=6d6a837b2130461f14fb6272ffae52b5) and PLACE (https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?lang=en&pj=640&action=page&page=newplace)

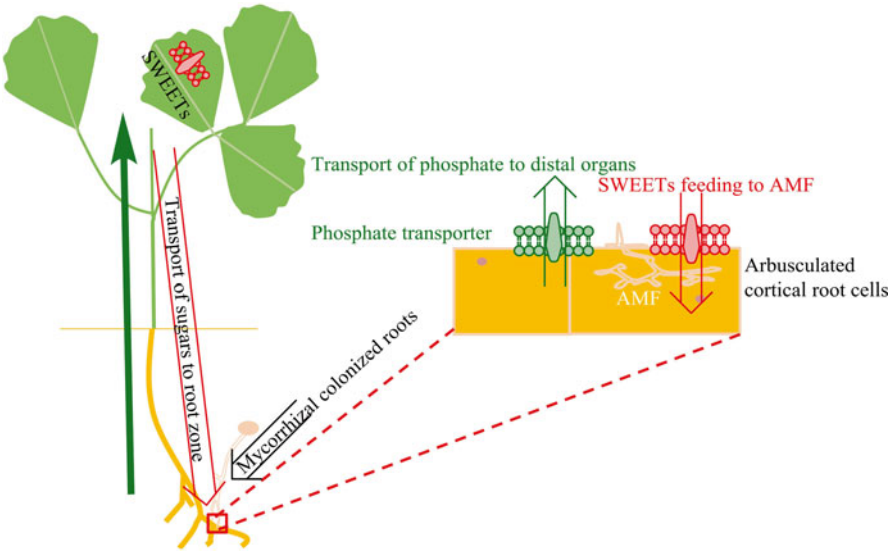

Fig. 6 A presumptive model elaborating the putative feeding role of SWEETs to AMF at root soil interface

SWEETs with other transporters and metabolic invertase activities in different subcellular compartments need to be studied more to clarify the role of these transporters in sugar secretion from plant roots to feed AMF symbionts and to enlighten the intricate sugar flux pathways in both infected and uninfected roots. Furthermore, these SWEETs can be a source of crop breeding tools for breeding better roots with excellent ability of establishment of symbiosis.

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Root Exudates and Their Molecular Interactions with Rhizospheric Microbes

Mallappa Kumara Swamy, Mohd. Sayeed Akhtar, and Uma Rani Sinniah

Abstract Biologically important plant-microbe interactions are mediated by a wide array of signal compounds rhizodeposited from both plant and microbial species. Root exudates are some of the potentially important low molecular weight compounds secreted from plant roots. They are involved in building a network of biointeractions through several physical, chemical, or biological interactions. Application of bioinoculums has significantly improved growth parameters and yield of many economically valued crops. Root exudates mediate the plant-microbe interactions by colonizing the roots and promoting root growth. Also, root exudates improve chemical and physical characteristics of the rhizospheric soil. Some of the beneficial plant-microbe associations include nitrogen fixation by rhizobium, symbiotic biointeractions with AM (arbuscular mycorrhizal) fungi, and PGPR (plant-growth-promoting *Rhizobacteria*). These interactions improve plant growth and quality, stress tolerance, and plant defense responses. Root exudates constitute a wide variety of secondary metabolite constituents that help plants to guard against microbial infections, insects, or herbivore attack. Root exudates secreted by plants act as antimicrobial agents to curb various harmful rhizospheric pathogens. In this chapter, we provide a summary of literatures on the significance of plant-microbe interactions in the improvement of plant morphological and biochemical features. Further, detailed information on various types of root exudates and their role in mediating plant-microbe interactions and possible exploration of root exudates as a novel antimicrobial compounds are also discussed.

Keywords Soil microbes • PGPR • Mycorrhizae • Signal molecules • Antimicrobials

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The original version of this chapter was revised: The spelling of the second author's name was corrected. The erratum to this chapter is available at 10.1007/978-3-319-29573-2_19

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1 Introduction

In nature, plants exhibit variety of biotic interactions between rhizospheric soil microbes by means of extremely complex mechanisms mediated by a wide array of signals produced from both plants and microbial species (Badri et al. 2009; Huang et al. 2014). Plants secrete root exudates as key signals into their surroundings to facilitate its better survival by establishing positive interactions with microbial community in the rhizosphere (Haichar et al. 2008; Bonfante and Anca 2009; Xie et al. 2012). However, the complex molecular interactions occurring between the soil microbes and plant roots are mainly modulated by exudates of roots. These exudates are known to build a network of interactions with plant roots and their surrounding rhizospheric microbes through various physical, chemical, or biological interactions (Huang et al. 2014; Haichar et al. 2014). Over past few years, researchers have suggested that the application of plant-growth-promoting rhizobacteria (PGPR) is sustainable in agricultural practices and development of agricultural biotechnology products such as biofertilizers, phytostimulators, biopesticides, and bioremediators. PGPR are extensively utilized all over the world, and their application rate is extremely rising due to numerous advantages. The growth and yield of many agriculturally important crops have been significantly increased by the application of PGPR (Akhtar and Siddiqui 2010; Bhattacharyya and Jha 2012; Ahemad and Kibret 2014).

Some past research studies have clearly stated that the microbial associations are very specific to plant species (Figueiredo et al. 2011; Haichar et al. 2014). Most of the legume plants are well known to associate with bacterial strains of rhizobacteriaceae family to fix the atmospheric nitrogen. Sugiyama and Yazaki (2012) reported that mutual symbiotic association fixes annually about 40–60 million metric tons of atmospheric nitrogen. However, the rhizobial bacterial species are also reported to secrete indole acetic acid. Changes occurring due to these auxin levels are shown to influence root nodule organogenesis and development. Similarly, it is reported that the auxin and cytokinin ratio play a key role in the regulation of nodule development (Figueiredo et al. 2008).

Since, PGPR are not only used as yield stimulators but also as bioprotectors in the management of plant pathogens or diseases (Figueiredo et al. 2008). Similarly, the symbiotic association of arbuscular mycorrhizal (AM) fungi with plants improves the uptake of water and mineral nutrients and also provides resistance toward stresses and pathogens (Akhtar and Siddiqui 2008; Akhtar and Panwar 2011; Akhtar et al. 2015). In this symbiotic association, both fungi and plants benefited each other. For instance, mycorrhizal fungi help the plants in uptake of nutrients from soil and in return provide carbohydrates to plants. However, the molecular mechanisms of nutrient exchange between the host plant and fungi are yet to be understood clearly (Thompson and Cunningham 2002; Bonfante and Anca 2009). The mycorrhizal fungi and their propagules, hyphae, and rhizomorphs form a network or a bridge between plant roots, fungi, and soil through which movement of nutrients is believed to occur (Bonfante and Anca 2009; Akhtar et al. 2011).

This network formation is mainly mediated by numerous signaling incidents involving low molecular weight compounds secreted from both plant and fungi (Paszkowski 2006; Parniske 2008).

Plant and soil fungal association used is agricultural practices to improve the soil fertility known as biofertilizers. This biological approach improved the soil fertility as well as prevents the environment by the hazardous effect of synthetic fertilizers. Also, this emerging practice has totally reduced the application of chemical products for controlling various plant diseases as eco-friendly approach (Figueiredo et al. 2008; Bais et al. 2008), as concluded by several past investigators that the plant roots secrete numerous types of compounds which are believed to facilitate the possible interactions between the plant root and the surrounding environment especially during symbiotic interactions (Bais et al. 2008; Bonfante and Anca 2009; Sugiyama and Yazaki 2012; Rashid et al. 2015). Over the last few years, rhizospheric investigation has witnessed the existence of these interactions between root and root, root and microbe, or root and insects (Badri and Vivanco 2009; Shukla et al. 2013; Haichar et al. 2014). Some of the examples of root exudates include amino acids, sugar molecules, organic acids, mucilage (polysaccharides), various proteins, phenolic acids, and secondary metabolite compounds (Bais et al. 2008; Badri et al. 2013; Haichar et al. 2014; Rashid et al. 2015). The secretion of root compounds is a normal process of plant root rhizodeposition to release major source organic carbon into soil (Nguyen 2003; Badri and Vivanco 2009). However, more research efforts are still required to understand the molecular mechanisms of root secretions. Root exudation mediates the plant-microbe interactions by colonizing the roots and promoting root growth. The rhizosphere soil provides an environment for diverse class of microbial community which is useful as well as harmful to the plants. Some of these microbes associate to form beneficial interaction with the plants. In contrast, plant interactions with pathogenic bacteria can be harmful to the plants. Studies have revealed the existence of rich microbial community around the rhizospheric soil of different plant species.

Hence, plant-microbe interactions might be both positive and negative approaches depending on the other factors of its rhizosphere vicinity (Mougel et al. 2006; Micallef et al. 2009; Sugiyama and Yazaki 2012; Haldar and Sengupta 2015). Extensive research reports accumulated over the past last decade have witnessed the new understanding on these beneficial interactions of plant exudates and microbial flora of rhizospheric soil. These studies have unlocked the possible exploration and application of plant microbial interactions by using various biotechnological tools and techniques for better crop production (Yedidia et al. 2001; Woo et al. 2006). Some of the negative plant-microbe interactions modulated by root exudates are the association with microbial pathogens, parasitic plants (Shukla et al. 2013). Rhizospheric bacterial strains utilize root exudates as nutrient source and mediate in the process of contaminant elimination and also can degrade various ecological pollutants (Bais et al. 2008; Shukla et al. 2013). Few researchers have emphasized on the aspect of understanding the possible functions of root exudates and the competent microbes in the process of phytoremediation and rhizoremediation (Gleba et al. 1999; Shukla et al. 2010, 2013). Also, root exudates act as antimicrobial molecules

to provide tissue-specific resistance against various pathogenic bacterial strains (Bais et al. 2005). Hence, knowledge on the mechanisms of interactions is very crucial in exploring the applications of plant-microbe interactions in many ways in the modern-day agricultural practices. This chapter describes the importance of root exudates, applications, and their role in understanding various mechanisms of interactions.

2 Root Exudates and Their Characteristics

The rhizospheric soil surrounding the plant roots is characterized by many kinds of distinctive biochemical, ecological, and physical interactions that are largely mediated by various chemical compounds released by plant roots into their immediate vicinity. These wide arrays of chemical compounds that are exuded to the rhizosphere by the plant roots are generally known as root exudates (Walker et al. 2004; Huang et al. 2014). The quantity of root exudate secretion depends mainly on plant species, age, cultivar type, plant root metabolic attributes, root system architecture, and environmental conditions that come across during plant growth (Bertin et al. 2003; Haichar et al. 2008; Compant et al. 2010; Haldar and Sengupta 2015). Secretion of plant root exudates into the soil requires large amounts of energy (5–21 % of fixed carbon). Primarily, root exudates are the low molecular weight carbon-containing chemical compounds that are derived mainly from the products of photosynthesis (Bertin et al. 2003). Root exudates function as potent chemical messengers to facilitate rhizobacterial chemotaxis process and mediate biological interactions through wide array of complex molecular networks (Walker et al. 2004; Bais et al. 2006; Glick et al. 2007; Cheng et al. 2009; Xie et al. 2012; Haichar et al. 2014). Root exudates are known to perform various functions such as the regulation of plant-microbe association, encouragement for various symbiotic interactions, prevention from herbivores attack, and inhibition of other competent plant growth in their surroundings (Haldar and Sengupta 2015). Moreover, it also improved the chemical and physical characteristics of the rhizospheric soil (Walker et al. 2003; Haichar et al. 2014; Yadav et al. 2015).

Root exudates encompass a wide array of chemical constituents including primary and secondary metabolites, ions, mucilage, free oxygen molecules, and water molecules (Hejl and Koster 2004; Bais et al. 2006), while other arrays of signal molecules include amino acids (glutamine, arginine, cystine, asparagine, aspartic acid, cysteine), enzymes, peptides, sugars (oligosaccharides, fructose, arabinose, glucose, mannose, maltose), vitamins, nucleotides, organic acids (ascorbic acid, acetic acid, benzoic acid, ferulic acid, malic acid), fungal stimulators, plant inhibitors, chemoattractants, growth regulators, sterols (campestrol, cholesterol, sitosterol, stigmasterol), fatty acids (palmitic, stearic, linoleic, linolenic, oleic), tannins, phenolic compounds, and few other miscellaneous chemicals. Some of the examples of primary root exudates comprise amino acids, enzymes, proteins, organic acids, sugars, lipids, flavonoids, allelochemicals, siderospores, coumarins,

and aliphatic and aromatic chemical metabolites (Bertin et al. 2003; Shukla et al. 2013; Huang et al. 2014; Haldar and Sengupta 2015). Among all these root exudates, organic acids play a significant role by serving as energy source for microbial cellular metabolism and also act as intermediate in biogeochemical cyclic reactions in the rhizospheric soil (Shukla et al. 2013).

Legume plants are widely consumed throughout the world, and hence, metabolic profiling studies and other basic research have been mainly focused on the same plant species in order to understand the types, characteristics, and functions of root exudates. Moreover, legume plants exhibit some of the biologically significant property, viz., fixation of atmospheric nitrogen through symbiotic association with rhizobacteria. Some of the commonly explored plants for root exudates and other metabolites include *Medicago sativa*, *Trifolium repens*, *Pisum sativum*, *Lotus japonicus*, *Medicago truncatula*, *Phaseolus vulgaris*, and *Glycine max* (Desbrosses et al. 2005; Farag et al. 2009; Hernandez et al. 2009; Rispail et al. 2010; Sugiyama and Yazaki 2012). Most of the characteristic properties and primary metabolic activities involved in symbiotic nitrogen fixation have been discovered through various classical methods involving studies on plant biochemistry, genetics, and molecular biology. Use of genetic approaches, transcriptomics, proteomics, and other functional genomics studies has provided better understanding of metabolic activities of nodule formation in some of the model plants such as *Lotus japonicas* and *Medicago truncatula* (Desbrosses et al. 2005; Sugiyama and Yazaki 2012; Xie et al. 2012).

In Arabidopsis plant, many reports have identified numerous root exudates such as sugars, amino acids, fatty acids, and an assortment of proteins (De-la-Pena et al. 2008; Badri et al. 2009; Badri and Vivanco 2009; Chaparro et al. 2013). Flavonoids and other phenolics are the most common compounds looked for in majority of the metabolomic studies (Abdel-Lateif et al. 2012; Badri et al. 2013). Use of GC-MS (gas chromatography-mass spectrometry) profiling has revealed the possible plant metabolites such as asparagine, octadecanoic acid, glutamate, cysteine, putrescine, homoserine, mannitol, gluconic acid, threonic acid, glycerol-3-P, and glyceric acid-3-P to be involved in root nodulation process in legume plants (Desbrosses et al. 2005). Some of the key signal molecules exuded from the legume plants that are involved in the interaction of plant microbes includes isoflavonoids derived from phenylpropanoids, and also they act as defensive compounds. The process of symbioses in legume and rhizobacterial nodulation is mediated by multiple actions of flavonoids which act as a signal molecule (Cooper 2007; Subramanian et al. 2007; Farag et al. 2008; Abdel-Lateif et al. 2012). The intracellular and extracellular secondary metabolome compounds of *M. truncatula* were analyzed by Farag et al. (2008) by using HPLC (high-performance liquid chromatography) analysis coupled with UV (ultraviolet) photodiode array detection method and mass spectrometry. The study revealed three novel methylated isoflavones (6-hydroxy-7,4′-dimethoxyisoflavone, 7-hydroxy-6,4′-dimethoxyisoflavone, and 5,7-dihydroxy-4′,6-dimethoxy isoflavone). Their study also highlighted the flexibility involved in the metabolic isoflavonoid biosynthetic pathways which depend on the nature of external stresses or elicitations. It has been reported that strigolactone secreted by plants like *L. japonicus* are involved in facilitating the arbuscular mycorrhizal symbiosis (Steinkellner et al. 2007). Root exu-

dates (vestitol) of *L. japonicus* function as chemical barriers to suppress the invasion of *Striga hermonthica* (a parasitic weed) into its roots (Ueda and Sugimoto 2010).

The complex interaction between rhizobium and roots is because of definite genetic as well as metabolic signals communicating between both symbionts (Geurts et al. 2005; Rispail et al. 2010). The signal compounds communicate between both plant host and rhizobia to form symbiosis. Also, rhizobium produces a large number of signaling compounds including Nod factor and many other surface polysaccharides which are involved in mediating the process of host-specific symbioses. Likewise, specific root exudates are secreted by host plant to mediate the preinfection events by triggering Nod factor biosynthetic pathways. The synthesized Nod factors then stimulate the accumulation of flavonoids by inducing flavonoid-encoding gene expressions (Cooper 2007; Haichar et al. 2014). Some of the reports suggest that flavonoids regulate the transport and accumulation of auxins inside the cortical cells to mediate root nodule development (Wasson et al. 2006; Subramanian et al. 2007). In another study by Rispail et al. (2010), inoculation of a symbiont *M. loti* to *L. japonicas* induced diverse alterations in the quantity of phenolic compounds secreted by the roots, while the compounds vestitol, sativan, and phytoalexin were not observed in the root zone after inoculation (Rispail et al. 2010; Badri et al. 2013). The identified coumestan and two other unidentified flavanones increased after inoculation of *M. loti* are described to be involved in nod gene stimulation. Proteomic and metabolomic approaches are being effectively used to study the targeted root exudate compounds in legume plant species. Plant-microbe interactions such as plant-rhizobia, plant-PGPR, and plant-arbuscular mycorrhizal fungi are described well in legume plants, and these interactions have significantly enhanced plant growth and yield (Sugiyama and Yazaki 2012; Haichar et al. 2014). The exudation of root compounds takes place through different processes such as passive transport, active transport, and transporter-mediated processes (Bais et al. 2006; Badri and Vivanco 2009).

3 Beneficial Plant-Microbe Interactions Mediated by Root Exudates

3.1 Root Exudates and Plant-Rhizobacteria Interactions

Biological interactions between plant and microbes occur through various molecular mechanisms and benefit the plant directly or indirectly. Root exudates modulate positive plant-microbe interactions and thereby regulate the plant growth, development, and yield. Some of these beneficial interactions include fixation of atmospheric nitrogen through root nodule formation by rhizobia in legume plants, providing tolerance against biotic as well as abiotic stresses and interactions with PGPR to improve plant growth and quality (Gray and Smith 2005; Bais et al. 2006; Badri et al. 2013; Huang et al. 2014). Moreover, biofilms, antibiotics, and other metabolites produced by bacteria interact with plants positively to impart

protection against likely pathogens, insects, and herbivores. Few root compounds secreted into rhizospheric soil exhibit allelopathic effect (Bais et al. 2004, 2006; Foley and Moore 2005; Ueda and Sugimoto 2010). Over the past few decades, many research studies on molecular interactions between legumes and *Rhizobium* spp. to form root nodule have been well documented. The root nodules are unique organs occurring in legume plant roots, and it harbors rhizobacteria involved in fixing atmospheric nitrogen. This specialized structure allows plants to utilize fixed nitrogen directly, and bacterium obtains its nutrients for its survival, and thus this is a mutual association benefitting both the species. Rhizospheric root soil favors the increased microbial activity, and plant-microbe symbioses are usually initiated by colonization of these soil-borne microbes. This is due to the fact that plant roots release abundant organic carbon that favors microbial ecology (Hartmann et al. 2009; Haldar and Sengupta 2015). It appears that rhizobacteria are attracted toward plants due to signal compounds and nutrients released by its roots (Bais et al. 2006). These bacteria form networks with plant roots through recognizing signal molecules produced by roots and further induce colonization by producing more signals. These signals are recognized by microbes to initiate symbioses with plants through physical interaction mediated by pili, fimbriae, adhesins, flagella, Type III and Type IV secretion system, and signal proteins (Lugtenberg et al. 2002; de Weert et al. 2002; Bais et al. 2006). Many studies have stated that flavonoids in the exudates act as a major chemical compound to attract rhizobia (Faure et al. 2009; Badri et al. 2013). About 4000 different types of flavonoids have been recognized and characterized in plants. Interestingly, isoflavonoids are observed only legume plants (Bais et al. 2006). Flavonoids are believed to induce several nod genes of the rhizobium spp. to produce nod factors (lipochitooligosaccharides) that cause curling of root hairs, form infection thread, and finally initiate bacterial colonization to form nodules. Nod factors could be modified with the substitution of acetate, carbamoyl, sulfate groups, and sugars (Bais et al. 2006; Haldar and Sengupta 2015). The familiar nod genes of rhizobia include nod A, nod B, and nod C. Also, there are species-specific nod genes. In addition, lipooligosaccharides released from bacteria were shown to stimulate plant genes responsible for flavonoid biosynthesis. It has been reported that these nod genes are involved in the synthesis of nod factors, and the expression of genes specific to species modifies nod factor structure (Perret et al. 2000; Riely et al. 2004; Badri et al. 2013). Horiuchi et al. (2005) reported that the interaction between *Medicago truncatula* (a legume) and *Sinorhizobium meliloti*, a root exudate, dimethylsulfide, attracts nematodes (*Caenorhabditis elegans*), and these nematodes carry *Sinorhizobium meliloti* to the vicinity of plant roots. Xanthones, isovanillin, and vanillin are some of the other non-flavonoid-related molecules which induce the expression of nod D gene clusters. However, they are needed in large quantity compared to flavonoids (Cooper 2007; Badri et al. 2009). The interaction of a rhizobium, *Mesorhizobium tianshanense*, with *Glycyrrhiza uralensis* (licorice) plants revealed the secretion of canavanine, a chemical compound commonly observed in the root exudates and seed coats of many legume plants (Cai et al. 2009). Canavanine was found to be toxic to various soil bacteria but nontoxic to rhizobacterial strains as they

possessed a mechanism to detoxify it. This research supports the exudation of specific antimetabolites by legume plants in order to select right rhizobia for a successful symbiosis. Nod genes of *Bradyrhizobium japonicum* are induced by the exudates such as isoflavonoids, genistein, and daidzein that are released by *Glycine max*. However, these compounds inhibit the expression of nod gene in *Sinorhizobium meliloti*, a nitrogen-fixing bacterium. Luteolin is another common flavonoid which can encourage the expression of nod genes in *S. meliloti* (Bais et al. 2006). Malic acid secreted in *Arabidopsis* plant root has shown to regulate the defensive responses activated by pathogens and effectively recruits *Bacillus subtilis* FB17, a beneficial rhizobacterial strain (Rudrappa et al. 2008). Similarly, Neal et al. (2012) have reported that benzoxazinoids (root exudates), such as 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one, attract beneficial rhizobacteria (*Pseudomonas putida*) to the site of rhizosphere. Badri et al. (2013) have revealed that *A. thaliana* plant root exudates phenolic compounds which serve as signal compound to attract soil bacteria. The amount of root exudates and their composition differs with the environmental changes and surrounding soil microbial flora. Also, pH of the rhizospheric soil encourages the growth of microbial community in the root surrounding (Bravin et al. 2009; Haldar and Sengupta 2015).

3.2 Root Exudates and Plant-Mycorrhizal Interactions

About 80 % of plant species including terrestrial plants, ferns, angiosperms, woody gymnosperms, and grasses are found to have symbiotic interactions with soil mycorrhizal fungi (ectomycorrhizae, endomycorrhizae, vesicular arbuscular mycorrhizae (AM), ericoid and orchid mycorrhizae). This symbiotic association enhances plant growth by increased uptake of nutrients, while fungi are benefitted with nutrients (carbohydrates and lipids) of the host plant roots (Bais et al. 2008; Haldar and Sengupta 2015). AM fungi associate with plants in a similar way as observed in plant-rhizobia interaction. Both mycorrhizae and rhizobia make use of similar signal molecules and proteins to regulate their associations with plants. Similar to rhizobia, AM fungi also recognize host plant species based on the available root exudates in the soil. Therefore, it is hypothesized that both AM fungi and rhizobia associations share a common origin of plant-microbe interaction and probably originated from a fungi (Nagahashi and Douds 1999, 2003; Levy et al. 2004; Bais et al. 2006; Akhtar et al. 2011). However, the exact mechanism of mycorrhizal association with specific host plant is yet to be recognized. As AM fungi are found in the rhizosphere soil, their propagules such as hyphae, rhizomorphs, and also spores are known to form a network of connections or a bridge between plant roots, fungi, and soil through which movement of nutrients is believed to occur (Simard et al. 1997; Bonfante and Anca 2009). This network formation is mainly mediated by numerous signaling incidents involving low molecular weight compounds secreted from both plant and fungi (Paszkowski 2006; Besserer et al. 2008; Parniske 2008). Flavonoids

present in low quantity are often proposed to stimulate the initial symbiotic association of AM fungi (Vierheilig and Piche 2002; Besserer et al. 2006; Haichar et al. 2014). Nevertheless, it is very well understood that the main signal factor involved in bridging plant-mycorrhizal symbiosis is considered to be strigolactone, a root exudate released from the plant (Akiyama et al. 2005, 2010; Haldar and Sengupta 2015). Strigolactones are carotenoid pathway-derived plant hormones which are generally produced when there is a nutrient deficiency. They also regulate plant growth and developmental processes by inhibiting shoot branching or by modifying plant structure (Akiyama et al. 2010). Strigolactones when released into the soil specify the host plants to symbiotic fungal species or plant parasites and stimulate the branching of hyphae during the symbiotic association between AM and host plant species (Lopez-Raez et al. 2008; Smith 2014; Al-Babili and Bouwmeester 2015). Root exudates of tomato, sorghum, pea, *L. japonicas* contained strigolactones, while they were absent in the root exudates of tobacco, carrot, and alfalfa. This indicates that there are many other signal compounds in the root exudates which are also responsible for the activation of fungal hyphae branching (Garcia-Garrido et al. 2009; Sugiyama and Yazaki 2012). Strigolactones were also observed in the root exudates of non-host plants of AM such as white lupin (*Lupinus albus*) and *Arabidopsis thaliana* (Yoneyama et al. 2008; Goldwasser et al. 2008). *Lupinus albus* was shown to produce pyranoisoflavones that inhibit the fungal hyphae growth and development (Akiyama et al. 2010). Similarly, Oba et al. (2002) have reported that root exudates of *Lupinus* species (*L. luteus*, *L. aridus*, *and L. cosentini*) inhibited growth of AM fungal hyphae (*Gigaspora margarita*). This could be a competitive strategy to suppress the possible mycorrhizal associations in other plant species or to strengthen their competing fitness.

Studies on gene expression, RT-PCR (reverse transcriptase polymerase chain reaction), and blotting techniques have indicated that during the initial phase of hyphal penetration, many signal molecules from plants are released to chemoattract mycorrhizal fungi. Several early nodulin genes such as Psam5, PsENOD12A, MsENOD2, MtENOD11, MsENOD40, and leghaemoglobin VfLb29 are shown to get induced during the early development of symbioses (Fruhling et al. 1997; Albrecht et al. 1998; Kosuta et al. 2003). In *Pisum sativum* plants, gene expression findings have suggested that induction of *PsENOD12A* and *Psam5*genes is found during appressorium formation and hyphal penetration into the root cortex (Albrecht et al. 1998; Roussel et al. 2001). In *Oryza sativa* plants, Blilou et al. (2000) have reported that appressorium formation is due to expression of *Ltp* (lipid transferase protein) gene in epidermal cells by using gene-promoter β-glucuronidase (GUS) fusion studies. However, in *Medicago truncatula*, *ENOD11* gene was found to get activated transcriptionally in cortical and epidermal cells where hyphae penetrate during *Gigaspora rosea* infection (Chabaud et al. 2002). In physically separated culture of AM and *Medicago truncatula*, Kosuta et al. (2003) have demonstrated that root signal molecules induce hyphae to secrete fungal factors which further induce the expression of the *MtENOD11* (nod factor-inducible gene). This study was confirmed by using a p*MtENOD11-gusA* reporter gene expression system. The study also reports that in all the tested AM (*Gigaspora rosea*, *Gigaspora margarita*,

Gigaspora gigantean, and *Glomus intraradices*), transgene expression was initially observed at the root cortex and later extended from the root hair emergence region to the matured root hair region. This suggests that though AM infection occurs in cortex zone of roots, its proliferation is restricted mainly to root tissues, and this mechanism is highly regulated by host plant. According to García-Garrido and Ocampo (2002), plant-mycorrhizal symbiotic establishment triggers plants to activate various defensive mechanisms such as degradation of elicitors, control of signal compound concentration, defensive regulation through nutrition and hormone, and regulation of symbiotic genes and pathogen-related gene expression. However, activation and regulation of these defensive responses during symbiosis are yet to be understood. In many cases, host plant defenses are very weak, and it differs from the responses that are usually noticed during plant-pathogen relations. The enzymes such as chalcone synthase and phenylalanine ammonia lyase responsible for flavonoid biosynthesis were induced in *M. truncatula* cells which contained arbuscules. However, the defense-specific enzyme isoflavone reductase was not induced. This suggests that mycorrhizal fungi growth is stimulated by flavonoid biosynthesis and not the phytoalexins (antimicrobials) (Harrison 2005; Bais et al. 2006). The induction of *lpt* (lipid transfer protein) gene was found to regulate the appressoria formation and hyphal penetration of *Glomus mosseae* during colonization with *Oryza sativa* roots (Blilou et al. 2000). In a study by Lanfranco et al. (2005), it was found that when spores of *Gigaspora margarita* (BEG 34) were exposed to *L. japonicus* and *M. truncatula* root exudates, induction level of *GmarCuZnSOD* gene was found to increase. This gives evidence on the involvement of fungal reactive oxygen species-scavenging systems in plant-fungi interactions. Further studies have suggested that reactive oxygen species and SOD produced from *Oidiodendron maius* and *Glomus intraradices* play a pivotal role in mycorrhizal symbiosis (Abba et al. 2009; Gonzalez-Guerrero et al. 2010). A necrotrophic fungus, *Sclerotinia sclerotiorum*, was found to repress defensive mechanisms of host plants such as *Lycopersicon esculentum*, *Nicotiana benthamiana*, or *N. tabacum* (Veluchamy et al. 2012). Though many researchers have identified the role of root exudates in mediating plant-fungal associations, still many chemical communications in the rhizosphere are yet to be documented at the molecular level for better exploitation of symbioses for agricultural benefits.

3.3 Root Exudates and Plant-PGPR Interactions

PGPR are a group of naturally occurring useful rhizobacteria that colonize with a plant root system and exhibit positive synergistic effect by stimulating plant growth, development, and yield. PGPR trigger the production of growth hormones as well as facilitate uptake of nutrients effectively by plants from their surroundings. Moreover, they release inhibitor compounds that guard plants against diseases or other environmental stresses (Jahanian et al. 2012; Ipek et al. 2014). Application of chemically synthesized fertilizers, pesticides, and plant nutrients

has significantly reduced with the application of PGPR in modern agricultural practices (Bhattacharyya and Jha 2012). It is believed that PGPR establish association with plants through plant root signals. However, meager information is available on the involvement of root exudate compounds in mediating the process of plant-PGPR interactions and their regulatory acts. Root exudates of plants containing chemical signals are contemplated to communicate with signal molecules of PGPR during their interactions. de Weert et al. (2002) have reported the chemotactic reaction of *Pseudomonas fluorescens* WCS365 during its root colonization in tomato plants. The major chemoattractive root exudates of tomato for *P. fluorescens* were found to be malic acid and citric acid. All nonmotile mutants of *P. fluorescens* (cheA mutants) showed no chemotactic response. Likewise, other root exudates including amino acids and carbohydrates are also reported to have dominant chemoattractive ability for PGPR population in rhizospheric soil (Somers et al. 2004; Huang et al. 2014). Arabinogalactan proteins are complex plant cell wall proteins unique to plant organs and root exudates. These fascinating sets of macromolecules are also involved in facilitating the interactions of plant roots with rhizobacteria (Nguema-Ona et al. 2013; Huang et al. 2014). Cannesan et al. (2012) have reported that arabinogalactan proteins of *Pisum sativum* and *Brassica napus* roots induced encystment formation and inhibited germination of *Aphanomyces euteiches* zoospores. A study by Vicre et al. (2005) suggests the positive role of arabinogalactan proteins in *A. thaliana* root colonization with PGPR. Likewise, Xie et al. (2012) report the chemotactic ability of arabinogalactan proteins to beneficial microbial species. According to them, root exudate of pea, wheat, legumes, and *Arabidopsis* showed a novel mode of arabinogalactan-induced polar attachment with *Rhizobium leguminosarum*. These reports thus suggest that arabinogalactan proteins play a major role in the attachment of rhizobacterial strains to root surfaces. Bacilio-Jiménez et al. (2003) characterized the rice plant root exudates and studied the chemotaxis of *Corynebacterium flavescens*, *Bacillus pumilus*, *Azospirillum brasilense*, and *Bacillus* sp. isolated from the rice rhizosphere. The study revealed the positive chemotactic nature of root exudates for all the rhizobacterial strains tested. The major outer membrane proteins (MOMPs) of rhizobacteria share a homology with bacterial porins. These MOMPs possess cell surface-exposed domains where adhesion process might take place to initiate plant-bacterial interaction (Burdman et al. 2000). In another study by Burdman et al. (2001), MOMPs of *Azospirillum brasilense* were shown to act as an adhesion to assist in bacterial cell aggregation and root attachment in sweet corn, forage corn, sorghum, wheat, tomato, common bean, and chickpeas. Many phytostimulants (cytokinins, auxins, and gibberellins) are secreted by PGPR to improve plant development. Customarily, plants also release root exudates that serve as nutrients for PGPR around the rhizosphere. Some of the root exudates such as tryptophan also serve as precursors for phytohormone synthesis in plants (Steenhoudt and Vanderleyden 2000; Bais et al. 2006; Lawal and Babalola 2014). PGPR produce 1-aminocyclopropane-1-carboxylate deaminase, a precursor for the biosynthesis of a phytohormone, ethylene which is involved in root growth regulatory mechanisms (Glick et al. 2007). The volatiles (acetoin and 2,3-butanediol) secreted by

Bacillus spp. were shown to improve *Arabidopsis* plant growth. This suggests that plant-rhizobacterial association may not always require physical attachment (Ryu et al. 2003; Doornbos et al. 2012).

4 Root Exudates as Antimicrobials Confer Plant Protection

A diverse class of microbial population prevalent in the rhizosphere soil is mainly influenced by plant root exudates. However, some of these bacterial and fungal strains are pathogens which cause diseases and hence can be detrimental to plants. These damages are managed by plants through defensive responses like suppressing pathogenic microbial strains or recruiting helpful microbial strains. Moreover, plant exudates constitute a wide array of secondary metabolites that help plants to guard against microbial infections, insects, or herbivores attack (Foley and Moore 2005; Doornbos et al. 2012; Haichar et al. 2014). Root exudates secreted by plants act as antimicrobial agents to curb the harmful rhizospheric pathogens. In response to pathogens, plant releases root exudates (defensive proteins, phytoalexins, and other unnoticed chemicals) into their surroundings. Root exudates which act as antimicrobial compounds include indole, benzoxazinone, terpenoids, flavonoids, phenolics, and isoflavonoids. These antimicrobials are observed in plants such as rice, *Arabidopsis*, soybean, corn, and a legume, *Medicago truncatula* (Bais et al. 2004, 2006; Perry et al. 2007). The secretions of *A. thaliana* roots function as antimicrobials provided tissue-specific defensive response to various pathogenic bacterial strains (Bais et al. 2005). But, *Pseudomonas syringae* strain showed resistance to these antimicrobials and infected the plant roots. This resistance ability was proposed to be dependent on the secretory system (type III). Similarly, *P. aeruginosa* forms root colonization, and eventually biofilm is formed which resists antimicrobials secreted from roots (Walker et al. 2004). Hairy roots of soybean induced the biosynthetic pathway of phenylpropanoids, to secrete isoflavones when challenged with a pathogen, *Fusarium solani* (Lozovaya et al. 2004). It is well described that phenylpropanoid pathway is activated in response to pathogenic fungi or other biotic stresses (Lanoue et al. 2010; Miedes et al. 2014). Rosmarinic acid (a caffeic derivative) was produced from hairy root cultures of basil (*Ocimum basilicum*) when challenged with a plant pathogen (*Pythium ultimum*) that causes root rot diseases. Rosmarinic acid was shown to possess a strong antimicrobial property against several rhizospheric microbes (Bais et al. 2002). In a study by Rudrappa et al. (2008), infected *Arabidopsis* plants with *Pseudomonas syringae* (a bacterial leaf pathogen) pv. Tomato DC3000 (Pst) was shown to recruit *Bacillus subtilis* FB17 as a biocontrol agent to infected plant roots. In their study, the rhizospheric strain *B. subtilis* FB17 showed chemotaxis to signal molecule, malic acid secreted by infected plant roots. Plant roots secrete many defense proteins in addition to antimicrobials that also confer root resistance or mediate plant-microbe interactions (De-la-Pena et al. 2008; Denance et al. 2013). Likewise, Lanoue et al. (2010) investigated the likely secretion of defensive root exudates in barley (*Hordeum vulgare*) when

challenged with a fungal pathogen, *Fusarium graminearum*. The results revealed that root exudates inhibited the germination of *F. graminearum* macroconidia. The identified root exudates included *t*-cinnamic, ferulic, *p*-coumaric, vanillic, syringic, 4-hydroxyphenylacetic, indoleacetic, and benzoic acids. In other ways, harmful microbes can be repressed by recruitment of biocontrol bacteria such as *Pseudomonas* spp. that establishes efficient root colonization. Root colonization by *Pseudomonas* spp. can result in suppression of wide range of plant pathogens (Akhtar and Siddiqui 2010; Lanoue et al. 2010). When plants are infected by pathogens, they secrete natural compounds called glucosinolates that are later hydrolysed by an endogenous thioglucosidases enzyme called myrosinases to yield several antimicrobial compounds such as isothiocyanates, thiocyanates, and nitriles (Halkier and Gershenzon 2006). A diterpene compound, rhizathalene A produced by noninfected *A. thaliana* plants, is considered to give defense against herbivorous insect attack (Denance et al. 2013; Haichar et al. 2014). Plant defense responses against pathogens and pests are mainly regulated by signaling networks of the major phytohormones such as jasmonic acid, jasmonates, salicylic acid, and abscisic acid (Robert-Seilaniantz et al. 2011). Researchers have proposed that strigolactone compounds provide plant defenses by regulating jasmonic acid signaling pathway to secrete defense-related hormones (Dor et al. 2011; Denance et al. 2013). Irrespective of numerous advanced studies carried out to understand natural compounds of root exudates and their role as antimicrobials or defensive molecules, their significance in the rhizosphere is yet be established completely. Hence, these findings could pave a way for future scientists to focus on the direction to discover novel new lead molecules as antimicrobials from root exudates.

5 Conclusion and Future Prospects

We described an overview of research information on the importance of biologically important plant-microbe interactions. Also, a wide range of root exudates and their significant role in mediating various plant-microbe interactions are discussed in detail. This chapter mainly focuses on the symbioses of plants with rhizobia, AM fungi, and PGPR as they are widely considered for developing agricultural biotechnology products such as biofertilizers, phytostimulators, biopesticides, and bioremediators. Moreover, these beneficial microbial inoculums are used in the sustainable agricultural practices worldwide. Volatile compounds of root exudates play a significant biological role in establishing communications between plant roots and the rhizospheric microbial flora. Through these biointeractions, plants are benefited through increased nutrient uptake from soil and better defensive responses against unfriendly surroundings. However, these biointeractions mediated by root exudates are yet to be understood clearly due to the fact that all biological interactions occur below ground. Hence, there is a need to establish new methodologies to explore their interactions under lab conditions. Literature survey has witnessed that root exudates function as signal molecules during plant-microbe interactions. Many study reports have

identified several genes and their regulatory expressions to produce root exudates for establishing biointeractions. Yet, more research efforts are needed to understand these interactions in detail at molecular level. Understanding about other root exudate genes, regulatory aspects of these genes and their expression under different environments, gene manipulation studies to modify root exudate products, alterations in the biosynthetic pathways of root exudates, and factors effecting root exudation are some of the research areas for the coming years. Progress in these research areas could be beneficial in developing economically valued crop plants with a capacity to produce higher useful root exudates. As plant interaction studies are mainly restricted to only few rhizosphere microbes, future research should focus on the understanding of other possible plant-microbe interactions in the complex rhizosphere environment. Also, chemical characterization of these rhizodeposits will pave a way in the discovery of novel metabolites with antimicrobial activity.

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A Proteomic Approach to Understand the Tripartite Interactions Between Plant-*Trichoderma*-Pathogen: Investigating the Potential for Efficient Biological Control

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Abstract Efficient biological control of plant diseases involves successful interactions among plant, biocontrol agents, and pathogens. *Trichoderma* spp. being the most popular and successful biocontrol agents are predominantly used to protect plants against a broad range of phytopathogens. However, a better understanding of the tripartite relationship established among *Trichoderma*-plant-pathogen is necessary in order to advance the practical applicability in agroecosystems and to unveil the cross talk involved in this beneficial association. Moreover, comprehensive knowledge of this three-way association is also required to identify the effective strain of *Trichoderma* to be used for efficient plant disease control. In this regard, several approaches have been adapted to study these tripartite interactions at molecular level such as transcriptomics, proteomics, and metabolomics. Although transcriptomic approach generates huge data, the study is incomplete without involving proteomic aspect, as it is directly responsible for cellular activity. Therefore, implication of proteomics in studying plant-pathogen interaction is now gaining noteworthy attention. Recently, proteomic approach has been found to contribute successfully in recognizing and characterizing the major proteins playing key role in inducing the defense mechanism in plants against pathogen attack. Nevertheless, empathizing proteomics of *Trichoderma* spp. can be used to discover novel determinants that would be helpful in developing new biocontrol formulation with enhanced biocontrol potential. Moreover, strain improvement using such determinants could also be achieved. In addition, proteomic study of the pathogen in this interaction is of great interest, as it would give insight into two aspects: firstly, the major factors contributing to the

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pathogenicity and secondly, targeting such factors for diminishing the pathogenicity. Therefore, in this chapter we focus our attention on highlighting the recent advances and findings regarding the proteomic approach used to study tripartite interaction between *Trichoderma*-plant-pathogen.

Keywords *Trichoderma* • Proteomics • Host-pathogen interaction • Secretomics • Molecular communication

1 Introduction

Conventional techniques used to prevent plant diseases have been based on the application of synthetic chemicals. Chemical fungicides can have hazardous effects on the environmental and human health (Naseby et al. 2000). A decline or elimination of chemical pesticide applications is highly desirable in present agricultural system. Among various alternatives, one of the most potent approaches is the use of biocontrol agents (BCAs) for disease control. Plethora of biocontrol agents are available in global markets, including bacteria such as *Pseudomonas*, *Bacillus*, *Agrobacterium*, and *Streptomyces* and fungi such as *Trichoderma*, *Gliocladium* (Singh et al. 2012; Romero et al. 2007), *Ampelomyces*, *Beauveria bassiana*, *Metarhizium*, *Coniothyrium* (Whipps et al. 2008), and *Candida* (Torres et al. 2003).

Filamentous ascomycetes *Trichoderma* (teleomorph *Hypocrea*) is a free-living saprophytic fungus frequently found in rhizosphere. These fungi are avirulent plant symbionts and antagonists to many soilborne phytopathogenic fungi. Currently, *Trichoderma* spp. are the most frequently investigated fungal biocontrol agents, and more than 60 % of the commercially available registered biofungicides worldwide are based on different formulations of *Trichoderma* (Keswani et al. 2013). About 250 products are commercially available for field applications in India (Singh et al. 2009). Application of *Trichoderma* strain in agriculture can provide various advantages: (1) rhizosphere competence that helps in root colonization and quick establishment of BCA in soil microbial community, (2) biological control of plant pathogenic fungi by an array of mechanisms, and (3) plant growth promotion (Keswani et al. 2013).

2 *Trichoderma*-Pathogen Interaction

Trichoderma is a fast-growing opportunistic fungus and produces an array of antibiotics and cell wall-degrading enzymes such as chitinases, cellulases, glucanases, etc. Most of the strains of *Trichoderma* are "rhizosphere competent" and easily degrade polysaccharides, chlorophenolic compounds, hydrocarbons, and in some case even

pesticides residues (Mishra et al. 2015; Harman et al. 2004). The main mechanisms that *Trichoderma* utilizes in interaction with fungal pathogens are antibiosis (Keswani 2015) and mycoparasitism (Keswani et al. 2013). The direct interactions between *Trichoderma* spp. and other phytopathogenic fungi are usually expressed as mycoparasitism or necrotrophic hyperparasitism (Bisen et al. 2015). Various cell wall-degrading enzymes and lytic enzymes are secreted by *Trichoderma* during the mycoparasitism which hydrolyze the host fungal cell wall and result in liberation of oligomers from the host cell wall (Table 1) (Woo et al. 2006; Kubicek et al. 2001; Sarma et al. 2014).

Table 1 Some major hydrolytic enzymes secreted by *Trichoderma* spp.

Species	Hydrolytic enzymes	Total amino acids	Accession no.
T. reesei	Cellobiohydrolase, beta-glucan	490	1003195A GI: 223874
	Endoglucanase I	371	1EG1_C GI: 2392307
	Chitinase 18-5	277	AEV54218.1 GI: 359720067
	Glycoside hydrolase family 39	440	XP_006969975.1 GI: 589115905
T. virens	Hydrophobin	45	ABE60727.1 GI: 91771573
	Endochitinase 42	252	ADP37575.1 GI: 31089309
	Extracellular serine protease	409	AAO63588.1 GI: 29421423
	Beta-1,6-glucanase precursor	429	AAL84696.1 GI: 19072999
T. atroviride	Endochitinase	344	AAM77132.1 GI: 33413585
	Chitinase 18-5	270	AJD86858.1 GI: 747037110
T. harzianum	Cellobiohydrolase I Cel7a	426	2YOK_A GI: 414145321
	Endochitinase 42	265	ADN93313.1 GI: 307752707
	Class V chitinase	397	AAT47713.1 GI: 48928004
	Secreted aspartic proteinase	530	ABK64120.1 GI: 118161444
	Chitinase 18-5, partial	245	ACY68414.1 GI: 262478801
T. viride	Cellobiohydrolase I	514	AAQ76092.1 GI: 34582632
	Endoglucanase III	418	AAQ21383.1 GI: 33521680
	Cellobiohydrolase	470	ADJ10628.1 GI: 299033169
	Exocellulase	515	ACX42576.1 GI: 260513728
	Endoglucanase VIII	438	ACD36972.1 GI: 187766738
	Endo-1,4-beta-xylanase	229	ABK59833.1 GI: 117959724
	Beta-D-glucoside glucohydrolase	74	AAQ76093.1 GI: 34582634
	Cellobiohydrolase II	471	ABH01083.1 GI: 110826033
T. koningii	Endochitinase 42	242	ADN93307.1 GI: 307752695
	Cellulose 1,4-beta-cellobiosidase	513	S45380 GI: 630421
	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)	337	S29814 GI: 422228
	Cellobiohydrolase II	471	AAK01367.1 GI: 12667268
	Endoglucanase	234	AAM77712.1 GI: 218421

Table 2 Various antimicrobial secondary metabolites produced and secreted by *Trichoderma* spp.

Species	Secondary metabolites	References
T. aggressivum	Mellein	Krupke et al. (2003)
T. lignorum	Lignoren	Berg et al. (2004)
T. atroviride	Atroviridins A, B, and C, α-phellandrene, ferricrocin, β-bisabolene, α-terpinene, β-phellandrene, γ-curcumene, α-bergamotene, α-curcumene	Oh et al. (2000), Vinale et al. (2008), Stoppacher et al. (2010)
T. longibrachiatum	LGB II and LGB III, Longibrachin, Mevastatin, Trichogin GA IV	Leclerc et al. (2001), Degenkolb et al. (2006), Mohamed-Benkada et al. (2006)

Currently, 287,808 proteins in *T. reesei*, 12,712 in *T. virens*, 12,237 in *T. atroviride*, 1181 in *T. harzianum*, 215 in *T. viride*, and 121 in *T. koningii* have been characterized (http://www.ncbi.nlm.nih.gov/).

Trichoderma spp. produce plethora of biologically active secondary metabolites (Keswani et al. 2014). Antibiotic and secondary metabolites' production is well associated with biocontrol ability, and the use of purified antimicrobial compounds was found effective against various fungal plant pathogens (Jain 2012) (Table 2). Secondary metabolite harzianopyridone displayed strong antifungal activity against *B. cinerea*, *R. solani* (Dickinson et al. 1989), and *Pythium ultimum* (Vinale et al. 2006), while harzianolide, T39 butenolide, and T22 azaphilone displayed in vitro inhibition of *P. ultimum* and *R. solani* (Vinale et al. 2006). In tomato and canola seedlings, incidence of *B. cinerea* and *Leptosphaeria maculans* was significantly reduced when same metabolites were applied at concentration of 1–10 mg/ml (Vinale et al. 2008). Cerinolactone at 100 µg/plug concentration showed 100 and 28 % inhibition in growth of *P. ultimum* and *B. cinerea* (Vinale et al. 2011). Competition for growth factors such as nitrogen, carbon, and space is also a defense strategy employed by *Trichoderma* spp. to control plant pathogens (Singh 2012). In addition, *Trichoderma* spp. have an enhanced ability to mobilize soil nutrients, thus making it more competitive and efficient than any other rhizhosperic microorganism (Rakshit et al. 2015).

3 *Trichoderma*-Plant Interaction

In addition to the biocontrol potential against phytopathogens, many *Trichoderma* species are potent plant root colonizers and cause considerable alteration in plant physiology and metabolism (Harman et al. 2004). It is well accepted that some

strains of *Trichoderma* promote plant growth by increasing nutrient availability to the roots, improve crop production, and induce systemic resistance in plants against various phytopathogens (Fig. 1) (Harman et al. 2004). Trichokonin secreted by *T. koningii* when applied as foliar spray at concentration of 100 nM resulted in improved resistance response in *Cucumis sativus*. In 57 % reduction in lesion size, 54 % lesion inhibition was recorded in *Nicotiana tabacum* against tobacco mosaic virus (TMV) infection (Luo et al. 2010). Yedidia et al. (1999) observed the physical interaction between plant and *Trichoderma* under electron microscopy and reported that hyphae of *Trichoderma* penetrate the root cortex but colonization is checked by deposition of callose. *Trichoderma* mediated induction of defence responses in various plants have been well documented (Table 3). Increased resistance against pathogen attack was shown by a vast range of plants species, when seeds were primed with *Trichoderma* spp. (Bisen et al. 2015; Singh et al. 2011). Plant growth promotion and inhibition property of antimicrobial secondary metabolites secreted by *Trichoderma* spp. have been reported (Vinale et al. 2009a, b; Luo et al. 2010). Application of harzianic acid at concentration of 100 µg/seed resulted in 45 % inhibition in stem length in canola seedlings, while when applied at concentration of 100 ng/seed, 42 %

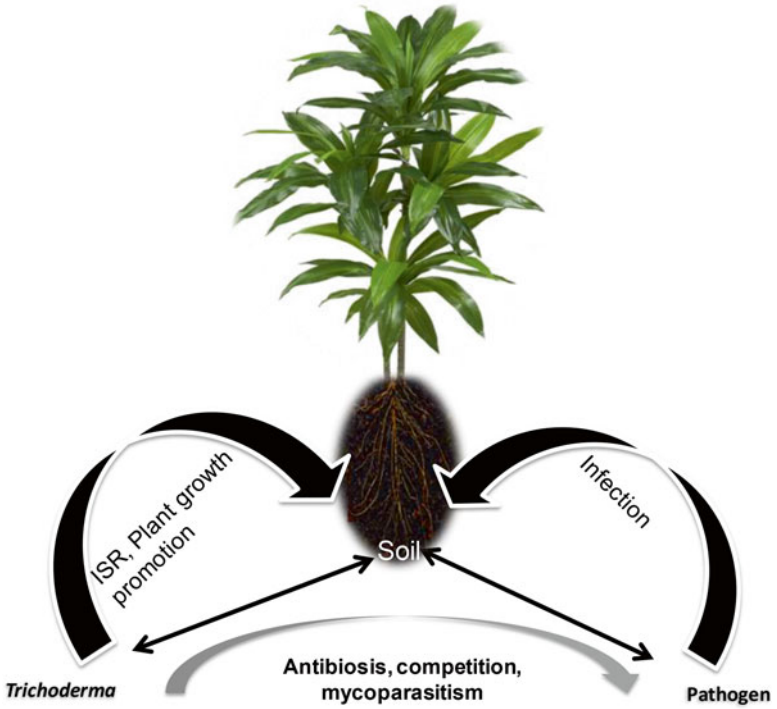

Fig. 1 Three-way interaction between *Trichoderma*-plant-pathogen

Table 3 Factors involved in *Trichoderma*-induced resistance in plants

Trichoderma strain	Target pathogen	Factors involved in induced resistance	References
Trichoderma spp.	*P. capsici*	Various defense-related genes and ESTs	Bae et al. (2011)
T. atroviride, *T. harzianum*	*B. cinerea*	SA, JA	Tucci et al. (2011)
T. arundinaceum IBT40837	*B. cinerea*, *R. solani*	SA, JA	Malmierca et al. (2012)
T. hamatum T382	*B. cinerea*	Phenylpropanoid compounds	Mathys et al. (2012)
T. harzianum T39	*Plasmopara viticola*	Jasmonic acid (JA), ethylene (ET), reactive oxygen species (ROS), callose, various defense-related proteins	Perazzolli et al. (2012)
T. asperellum SKT-1	*Pseudomonas syringae* pv. *tomato*	Salicylic acid (SA), JA, ET	Yoshioka et al. (2012)

increase in stem length was recorded (Vinale et al. 2009a, b). Similarly, positive effects of harzianolide on defense response and plant health were reported in *Solanum lycopersicum* and *Brassica napus* seedlings (Vinale et al. 2008).

Species of *Trichoderma* have been characterized as avirulent plant symbiont (Harman et al. 2004). Recent studies have revealed that colonization of *Trichoderma* spp. is not only limited to plant root tissues and rhizosphere but also as endophytes (Druzhinina et al. 2011). Most of the newly discovered endophytic *Trichoderma* were previously unknown, e.g., *T. stromaticum*, *T. evansii*, *T. amazonicum*, *T. theobromicola*, *T. martiale*, and *T. taxi* (Mukherjee et al. 2012) (Table 4). The endophytic *Trichoderma* spp. are reported to protect hosts from various biotic and abiotic stresses through transcriptomic changes (Bailey et al. 2006; Bae et al. 2009).

T. harzianum strain T22 has shown enhanced level of growth hormone in cherry plant. In vitro inoculation of strain T22 in cherry resulted in 76 % increases in root length and 61 % increases in shoot length. Although *T. harzianum* T22 did not release any phytohormones in the medium, 143 % increase in gibberellic acid (GA3) and 40 % increase in indole acetic acid (IAA) were recorded in the roots (Sofo et al. 2011). Similarly, 71 % and 49 % increase in gibberellic acid and indole acetic acid (IAA) concentration was recorded in cherry leaves, respectively (Sofo et al. 2011). Concentration of five phytohormones including zeatin, salicylic acid, ethylene precursor 1-aminocyclopropane-1-carboxylic acid, IAA, and jasmonic acid in melon plants inoculated with *T. harzianum* CECT 20714 was analyzed. Up to 30 % increase in zeatin, 40 % increase in IAA, and 40 % increase in ACC were recorded and correlated with increased root and shoot length (Martinez-Medina et al. 2011).

Table 4 Endophytic *Trichoderma* spp. isolated from various hosts

Trichoderma spp.	Plant	Effect on biotic and abiotic stress	Reference
Trichoderma sp.	*H. brasiliensis*	–	Santamaria and Bayman (2005)
T. viride	*Azadirachta indica*	–	Verma et al. (2005)
T. theobromicola	*Theobroma cacao*	–	Samuels et al. (2006)
T. paucisporum, *T. caribbaeum* var. *aequatoriale*	*Theobroma* spp.	–	Samuels et al. (2006)
T. taxi	*Taxus mairei*	–	Zhang et al. (2007)
T. lieckfeldtiae, *T. martiale*	*Theobroma* spp.	Biological control of black pod rot caused *Phytophthora palmivora*	Hanada et al. (2008)
T. hamatum DIS 219b	*Theobroma cacao*	Root growth and delayed drought response	Bae et al. (2009)
T. evansii	*Theobroma* spp.	–	Samuels and Ismaiel (2009)
T. koningiopsis	*Hevea brasiliensis*		Gazis and Chaverri (2010)
T. amazonicum	*Hevea brasiliensis*, *H. guianensis*	–	Chaverri et al. (2011)
T. asperellum, T. virens, *T. brevicompactum*, *Hypocrea lixii*	*Musa paradisiaca*	–	Xia et al. (2011)
T. atroviride CAE_{210}, *T. atroviride* CSE_{114}, *T. koningii* CSE_{32}	*Cupressus arizonica*, *Thuja orientalis*, *C. sempervirens* var. *cereiformis*	Biocontrol of *Pyricularia oryzae*	Hosseini-Moghaddam and Soltani (2014)

4 Deciphering the Intricacies of Tripartite Interaction Through Proteomics

The tripartite interactions involving plant, *Trichoderma*, and phytopathogen have received less attention in contrast to the plant-*Trichoderma*, plant-pathogen, and *Trichoderma*-pathogen interactions. The major focus of the studies on the three-way interactions has been related to molecular changes during pathogen attack and/or plant responses (Dangl and Jones 2001; Hammond-Kosack and Parker 2003; Suzuki et al. 2004). Various defense factors, signaling molecules, and virulence and avirulence factors have been identified in plants (Cánovas et al. 2004; Rep et al. 2002)

and in microbes (Kazemi-Pour et al. 2004; Smolka et al. 2003). Furthermore, the influence of biocontrol agents on plant and pathogen has not been a extensively studied at proteomics level, even though this technique offers an effective alternate to decipher such communication networks (Woo et al. 2006).

Though structural genomics has revealed significant information on the identity and structure of genes expressed in plant-microbe interactions (Singh et al. 2004; Talbot 2003), proteomics allows a large-scale exploration of gene expression and has been studied for protein profiles produced from varied interaction (Pandey and Mann 2000; Lim and Elenitoba-Johnson 2004).

In order to analyze the differential protein produced during the tripartite interactions between plant-*Trichoderma*-pathogen, Marra et al. (2006) investigated the interactions of *Trichoderma*, plant, and different fungal pathogens by using proteomic techniques. Alteration in the proteomes of each partner during the three-way cross talk was studied, and the most appealing differential spots were analyzed via PMF. Several differentially expressed proteins were found in the *T. atroviride* proteome during the tripartite interactions with foliar pathogen *B. cinerea* and bean leaves. Results showed that the tripartite interaction may be regulated by disease-related factors and plant proteome-specific PR proteins. A protein with PPIase activity was earlier identified by LC-MS/MS in the proteome of *T. harzianum* (Grinyer et al. 2005). The in silico study of data from plant-*Trichoderma* and plant-*Botrytis* interactions exposed many homologues to PR proteins. Conserved domains like nucleotide-binding sites (NBS), leucine-rich repeats (LRR), and SGS domains along with conserved sequences of Bet v I PR and Barwin-protein families were found. For example, thaumatin-like protein and tobacco PR-4 family with a Barwin domain involved in the plant defense response to *Magnaporthe grisea* (Kim et al. 2004) were accumulated in the host. Various proteins from the *T. atroviride* interaction showed similarities with ABC transporters and fungal hydrophobins. In *M. grisea* proteome, virulence factors such as cyclophilins were also regulated in the interaction with the antagonist and with the plant. The role of cell wall-degrading enzymes secreted by *Trichoderma* spp. in the vicinity of the fungal phytopathogens including *Pythium ultimum* and *Rhizoctonia solani* is also well known (Keswani et al. 2013). The significance of endochitinases and exochitinases (*nag1* and *chit42*) of *T. atroviride* in the mycoparasitism of soilborne phytopathogens is also well documented (Lu et al. 2004).

5 Two-Dimensional Polyacrylamide Gel Electrophoresis

In 2D-PAGE, proteins are separated according to net charge present on them by isoelectric focusing in first dimension, and in second dimension, proteins are resolved according to their molecular weight (Gorg et al. 2004). Protein spots can be identified by staining the gels with different stains such as Coomassie brilliant blue, colloidal silver stain, and SYPRO Ruby stain (Nat et al. 2007; Patterson and Aebersold 2003; Duley and Grover 2001). 2D-PAGE images are analyzed using

various software packages such as PDQuest BioRad, Melanie, Progenesis, Phoretix, Z3, and Z4000 (Righetti et al. 2007). Identification of excised spots is done by mass spectroscopy (Zhu et al. 2003). 2D-PAGE gels also offer information on various biochemical and biophysical parameters including isoelectric point, molecular weight, and posttranslational modifications (PTMs) (Gorg et al. 2004; Wittmann-Liebold et al. 2006). 2D-PAGE is a reproducible, sensitive, and steadfast technique for studying comparative alterations in proteome of an organism (Somiari et al. 2003; Hakeem et al. 2012).

Comparative analysis of differential protein pattern in *T. atroviride* grown on different substrates including *R. solani* cell wall residues and glucose was done by Grinyer et al. (2005). In *T. atroviride* grown on glucose, a total of 67 proteins were observed in comparison to 56 proteins from that of grown on *R. solani* cell wall. Proteins observed in *T. atroviride* grown on *R. solani* cells range from proteins involved in antagonistic activity of *T. atroviride* to novel proteins. Of the different proteins, *N*-acetyl-β-D-glucosaminidase and 42-kDa endochitinase have been found to play an important role in antagonism against various fungal pathogens. *T. atroviride* secretes both enzymes in order to hydrolyze the cell wall of the fungal pathogen (Mach et al. 1999).

6 Fluorescent Two-Dimensional Difference Gel Electrophoresis (2D-DIGE)

It allows the analysis of two differential protein regulations between target samples and control by treating the samples with fluorescent dyes and run on single 2D gel. Thus, protein present in one sample can be compared with its differentially labeled counterpart from other sample. Later, the gel is scanned at two different wavelengths that stimulate dye molecules and reveal whether spot is coupled with one or two dye molecules (Unlu et al. 1997). DIGE offers comparing of many protein samples under comparable electrophoresis conditions and thus ensure accuracy of quantification and better reproducibility (Van den Bergh and Arckens 2005; Zhou et al. 2005). An alternative approach is the gel-free proteomic technology, which is more suitable for the analysis of proteins with low abundance in complex samples (Lambert et al. 2005; Nat et al. 2007; Zhu et al. 2010).

7 Multidimensional Protein Identification Technology (MudPIT)

In this process protein samples are digested with sequence-specific enzymes including endoproteinase lyase and trypsin and separated by reversed-phase (RP) high-performance liquid chromatography (HPLC) and strong cation exchange (Issaq et al. 2005; Washburn et al. 2001). MudPIT technique offers fast, sensitive,

and reproducible analysis while generating an extensive list of existing proteins in given sample (Rose et al. 2004; Washburn et al. 2001). MudPIT is used in different proteomic experiments such as protein profiling of cell membrane, cell organelle, and analysis and identification of protein expression (Yates et al. 2005; Speers and Wu 2007; Cantin et al. 2006).

8 Mass Spectrometry

Anderson in 1958 first reported the use of mass spectrometry (MS) to analyze amino acids and peptides. During the process the test samples are transformed into gas and separated according to mass-to-charge ratio (*m*/*z*). Proteins are fragmented by trypsin and separated by liquid chromatography. The samples are ionized by matrix-assisted laser desorption/ionization (MALDI) and transformed into the gas. Peptides of specific mass are fragmented by collision-induced dissociation and subjected to second mass spectrometry. Results are compared with protein identification algorithms grouped as de novo search algorithms and database search algorithms.

9 Protein Microarray

Protein microarray is used to profile large quantities of complex samples, and it offers functional and classical proteome analysis (Duburcq et al. 2004; La Baer and Ramachandran 2005; Athwal et al. 2000; Ramachandran et al. 2005; Hammond-Kosack and Jones 2000). Cell lysate is allowed to pass over the surface to tie the antigen to their related antibodies. Antibody or other similar reagents such as peptides, allergens, and polysaccharides can be spotted on a flat surface such as plastic, glass, or silicon chip. Radioactively labeled proteins are used to screen the bound antigen. Generally three kinds of microarray are used in proteomic analysis including functional protein microarray, used for studying protein-protein interaction; analytical microarray, used for studying protein-DNA and protein-RNA interaction; and reversed-phase protein microarray used for differential expression profile for altered protein.

10 Conclusion

The performance of biocontrol agents is reliant upon the complex interactions that these microorganisms maintain with plants and pathogens in the soil. A superior understanding of the process and cross talk among the members will result in efficient utilization of microbial biocontrol agents for management of seed and soil-borne diseases. Current strategies including proteomics and metabolomics provide

novel insights into tripartite associations, particularly about the capacity of *Trichoderma* to sense the plant, the soil, and the microbial groups. Proteomic approaches offer easy identification of various differential proteins engaged in cross talk between biocontrol agents, plant, and pathogens. Most of the experiments conducted so far have been centered toward the two-partner interactions (Dangl and Jones 2001), thus providing a comparatively partial view of a resistance/pathogenicity processes as intervened by both pathogenic and beneficial microbes. Proteomic analysis can be very helpful to present both general and specific data on the interaction proteomes used by microbes and plants. In tripartite interactions, more than one partner is active at the same time indicating the complexity of the system, and hence a more integrated approach is needed to understand the mechanisms of biological control.

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Mycorrhizal Association and Their Role in Plant Disease Protection

Julio Alves Cardoso Filho, Sergio Florentino Pascholati, and Roberto Ramos Sabrinho

Abstract The arbuscular mycorrhizal (AM) symbiosis is a mutualistic association formed between plants and soil fungi. Members of AM-forming fungi (AMF) belong to the phylum *Glomeromycota*. They are symbiotically associated with roots of more than 80 % of terrestrial plant species. AM fungi are obligate biotrophs that require the host plant to proliferate, survive, and complete their life cycle. The AMF, during the symbiosis, establish a sink for plant photosynthate by utilizing it for biomass and metabolic energy, while the AM plants obtain nutrients and water through the AMF hyphae. The benefits of AM symbiosis on fitness plants are largely known, including a better mineral nutrition and a higher tolerance of mycorrhizal plants to abiotic stresses, such as drought, salinity, and presence of heavy metals. Additionally, recent investigations reveal that AMF can suppress pests and plant diseases through induction of systemic resistance. The knowledge about the mechanisms behind the induction of resistance by mycorrhizal symbiosis remains unknown. This chapter describes the role of mycorrhizal symbiosis in plant disease protection, with special emphasis on the mycorrhiza-induced resistance (MIR).

Keywords Plant defense • Arbuscular mycorrhizae • Induced resistance

1 Introduction

Mycorrhiza is a mutualistic association formed between plant roots and certain soil fungi (Azcon-Aguilar and Barea 1996). The symbiosis is named after the Greek words "*mycos*" and "*rhiza*" meaning "fungus root"(Smith and Read 2008). Both of them derive benefits from this interaction; mycorrhizal fungi improve the nutrient status, water absorption, and growth and enhance the host plant's resistance to

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biotic and abiotic stresses, while the host plant is necessary for fungal growth and reproduction by providing carbon in the form of photosynthates (Smith and Gianinazzi-Pearson 1988; Smith and Read 2008; Harrison 2012). Mycorrhizal association is found in 80 % of all vascular plant families (Brundrett 1991; Wang and Qiu 2006) and virtually all terrestrial ecosystems, including tropical to temperate forests, sand dunes, deserts, and grasslands as well agroecosystems (Brundrett 1991). These associations play a key role in shaping terrestrial ecosystems and are widely believed to have promoted the evolution of land plants (Zuccaro et al. 2014).

Harley and Smith (1983) recognized seven types that, for the most part, still comprise the generally accepted classification. These different groups of mycorrhizal associations have been recognized, involving different groups of fungi and host plants and distinct morphology patterns, based on the presence of various extraradical or intraradical hyphal structures (Bonfante and Perotto 2000). These include ectomycorrhizae, endomycorrhizae, ectendomycorrhizae, arbutoid mycorrhizae, monotropoid mycorrhizae, and orchid mycorrhizae (Brundrett 2004; Smith and Read 2008). Arbuscular mycorrhiza (AM) is a member of endomycorrhizae (Harley and Smith 1983). AM is the most widespread form of mycorrhizal associations and of great ecological and economic importance (Brundrett et al. 1996; Francis and Read 1994; Jeffries et al. 2003; Fitter 2005; Newman and Reddell 1987; Smith et al. 2009).

In terms of geographical distribution and phylogenetic coverage in the plant kingdom, AM is probably one of the most widespread symbioses on earth (Gutjahr and Parniske 2013). Arbuscular mycorrhizal fungus (AMF) is an obligate biotroph, which receives carbohydrates from the host plant required to complete its life cycle, in exchange of which it provides the plant with nutrients (i.e., such as nitrogen, phosphate, and sulfur) and water (Smith and Read 2008; Smith and Smith 2011). AM fungi are considered to have originated between the Ordovician and Lower Devonian, 400–500 million years ago, from a common ancestor in *Glomaceae*, based on fossil records (Pirozynski and Dalpé 1989; Simon et al. 1993) and phylogenetic evidences (Remy et al. 1994; Taylor et al. 1995; Redecker et al. 2000; Brundrett 2002; Wang and Qiu 2006).

Currently, the phylogenetic analyses of small subunit (SSU) rRNA gene sequences separated AM fungi from the polyphyletic *Zygomycota* and placed them into a new monophyletic phylum, the *Glomeromycota* (Schüßler et al. 2001). The latest classification of AM fungi (frequently updated on the webpage www.amf-phylogeny.com) contains four orders, 11 families, and 25 genera (Redecker et al. 2013; Schüßler and Walker 2010). More than 240 species have been described so far, primarily on the basis of spore morphology (Redecker and Raab 2006), but increasingly supported by DNA sequence information (Oehl et al. 2011; Krüger et al. 2012). However, molecular studies have suggested that diversity of these fungi may be much greater (Fitter 2005; Rosendahl 2008).

The development of AM symbiosis starts with a reciprocal exchange of diffusible signals between plant roots and AM fungi (Navazio et al. 2007), during preinfection stages (Harrison 2005). The host roots release signal molecules called “branching factors” (BFs) that induce extensive germination and hyphal branching in AM fungi

(Giovannetti et al. 1993; Nagahashi and Douds 2000; Akiyama and Hayashi 2006), and AM fungi have long been postulated to produce signal molecules called "Myc factors" (MFs) that induce the molecular and cellular responses leading to successful root colonization by AM fungi (Maillet et al. 2011). The root nodule symbiosis (RNS) with rhizobacteria and the actinorhizal symbiosis with cyanobacterial endosymbionts (Hocher et al. 2011; Svistoonoff et al. 2013) involve the same signaling pathway as AM, which therefore is referred to as common symbiosis signaling pathway (common SYM pathway; Favre et al. 2014).

AM colonization induced changes in the amount of phytohormones such as cytokinins, jasmonic acid (JA), certain auxins, abscisic acid (ABA), ethylene, salicylic acid (SA), and strigolactones (SL) in roots (reviewed in Hause et al. 2007; Foo et al. 2013; Carbonnel and Gutjahr 2014). In the case of mycorrhizal interactions, the plant roots release strigolactones into the rhizosphere that can stimulate hyphal branching and respiratory metabolism of AM fungi (Akiyama et al. 2005; Besserer et al. 2006). Recent studies in *Petunia* mutants defective for the strigolactone exporter PhPDR1 demonstrated that strigolactone transport is essential for the function of these signals in AM symbiosis (Kretzschmar et al. 2012). Studies showed an important role for strigolactones in the stimulation of the fungus outside the roots and possibly also in the progression of AM fungal hyphae within roots (Akiyama et al. 2005). Reciprocally, AM fungi release compounds that trigger a variety of responses in plant roots, including calcium spiking, changes in gene expression, and lateral root formation (Parniske 2008).

A good performance of this symbiosis implies remarkable changes in the physiology of the host plant (Pozo et al. 2009). The changes span from alterations in the hormonal balance and transcriptional profile to altered primary and secondary metabolism (Hause et al. 2007; Liu et al. 2007; Schliemann et al. 2008; López-Ráez et al. 2010), and many of the changes are related to defense mechanisms (Liu et al. 2007; Fiorilli et al. 2009; López-Ráez et al. 2010; Gallou et al. 2012) likely contributing to the plant maintaining control over the symbiotic partner (Fernández et al. 2014). The AM symbiotic program displayed by mycotrophic host plant roots toward AM fungi is depicted as a chronological series of events including the pre-symbiotic phase, contact, fungal entrance, and intra-radical fungal proliferation (Recorbet et al. 2013) and involving the intracellular accommodation of a microorganism by a living plant cell without causing the death of the host (Recorbet et al. 2013).

Environmental conditions can disturb the establishment of the AM symbiosis, including P (phosphorus) availability, which can inhibit the symbiotic interaction (Smith and Read 2008). Although several nutrients influence AM development (Nouri et al. 2014), most research has focused on the role of Pi (Pi, inorganic phosphate; Carbonnel and Gutjahr 2014). One explanation for suppression of AM development at high Pi could be the repression of common SYM signaling (Carbonnel and Gutjahr 2014). However, recent observations suggest that multiple layers of symbiosis control exist (Breuillin et al. 2010) and that the predominant regulatory mechanisms depend to a large extent on the plant and fungal species under study, as well as on the coculture conditions and mode of P supply.

The protection against soilborne pathogens has been widely reported in mycorrhizal plants (Linderman 1994; Azcon-Aguilar and Barea 1996; Whipps 2004; Barea et al. 2005; Pozo and Azcón-Aguilar 2007; Akhtar and Siddiqui 2008; Saldajeno et al. 2008; Pozo et al. 2009, 2010, 2012). While the exact mechanism of plant protection from the pathogen through the presence of mycorrhizal fungi is not known, it is likely that it results from a combination of better nutrition and effects of the mycorrhiza on inducing plant defense responses (Campos-Soriano et al. 2012; Vos et al. 2012).

A global change of the mycorrhizal plant functions by transcriptome reprogramming has an impact on the plant interaction with the environment, modifying its responses to biotic and abiotic stresses (Pozo et al. 2009; van Wees et al. 2008). The consequences go beyond the individual level as they may influence plant diversity and productivity in terrestrial ecosystems (van der Heijden et al. 2008). Because of this modulation, a mild but effective activation of the plant immune responses seems to occur, not only locally but also systemically (Jung et al. 2012). This modulation may result in preconditioning of the tissues for efficient activation of plant defenses upon a challenger attack.

This phenomenon called "priming" results in a faster and stronger induction of basal resistance mechanisms upon subsequent pathogen attack (reviewed in Conrath et al. 2006; Ahmad et al. 2010; Conrath 2011). This subject, "priming," will be discussed later in a specific topic, within this chapter. In this chapter, we want to discuss the role of mycorrhizal symbiosis in plant disease protection, with special emphasis on the mycorrhiza-induced resistance (MIR).

2 The Innate Immune System of Plants

Plants employ multiple layers of defense to combat pathogens (Jones and Dangl 2006). These defenses include a combination of preformed and inducible mechanisms (Jones and Dangl 2006; Spoel and Dong 2012). An innate immune perception triggers both local and systemic defense responses, allowing a plant to fight off pathogens both in a rapid and localized manner and on an extended scale of time and space (Zipfel 2014).

Harold Flor, early in the twentieth century, showed that inheritance of plant immunity to pathogens, as well as the ability of the pathogen to cause disease, is controlled by corresponding gene pairs (Flor 1971). This plant genetic factor was called the resistance (R) gene, while the pathogen genetic factor that determined the inability to cause disease was referred to as the avirulence (Avr) gene. As a result, the plant will be resistant, and the growth of the pathogen will be arrested only when both genes, R and Avr, are present (Ellis et al. 2000a, b). Therefore, for each R gene, a correspondent Avr gene coexists: this is the basis of the gene-for-gene concept, as suggested by Flor (1971). Initially it was widely thought that products of R genes act as receptors that directly interact with the products of Avr genes (Keen 1990).

This ligand–receptor model was supported by the fact that some Avr gene products are small and co-localize with R gene products, most of which encode receptor-like proteins carrying Leu-rich repeats (LRRs).

Experiments show negative results, among direct ligand–receptor interactions, which led to the formulation of the guard hypothesis, stating that R proteins monitor the state of host components that are targeted by pathogen molecules (Van der Biezen and Jones 1998). This hypothesis recognizes that pathogen molecules have intrinsic functions to promote pathogen virulence, which requires the modulation of host components that have thus become virulence targets (Van der Biezen and Jones 1998; Dangl and Jones 2001). The Guard Model was originally proposed to explain the mechanism of *Pseudomonas syringae* AvrPto perception by the tomato proteins Pto and Prf (Van der Biezen and Jones 1998) and was later generalized to perception of other effector proteins (Dangl and Jones 2001). From the guard hypothesis, the pathogen molecules that were originally referred to as avirulence factors genuinely are virulence factors.

Currently, the term "effector" is commonly used for this type of molecule (Bent and Mackey 2007; Boller and Felix 2009). Simultaneously, to research on gene-for-gene resistance, the existence of inducers of plant defense responses that were not determinants of race or cultivar specificity became evident (Ebel and Cosio 1994). These non-race-specific inducers of defense were termed elicitors and harbored a wide range of different types of molecules (Boller 1995). From the identification of the first elicitor receptor (FLS2) (Gómez-Gómez and Boller 2000), subsequent proof for its role in plant immunity (Zipfel et al. 2004), and identification of microbial effectors that suppress this type of immunity (Hauck et al. 2003), proof that both types of defense contribute to plant immunity, was eventually provided. Subsequently, the coevolution between plants and microbial pathogens has been described as a zigzag model by Jones and Dangl (2006) and can also be applied to deducing the biological activity of elicitors (Wiesel et al. 2014). According to this model, the molecules, termed elicitors or avirulence factors, have more recently been renamed to pathogen-associated molecular patterns (PAMPs) (Chisholm et al. 2006; Jones and Dangl 2006; Schwessinger and Ronald 2012; Zipfel 2014), microbe-associated molecular patterns (MAMPs) (Boller and Felix 2009; Chisholm et al. 2006; Jones and Dangl 2006; Newman et al. 2013; Schwessinger and Ronald 2012; Zipfel 2014), damage-associated molecular patterns (DAMPs) (Boller and Felix 2009), herbivore-associated molecular patterns (HAMPs) (Erb et al. 2012), and effectors (Bent and Mackey 2007). PAMPs/MAMPS are structural molecules, such as bacterial flagellin, elongation factor Tu (EF-Tu), Ax21 secreted protein, peptidoglycan (PGN), lipopolysaccharides (LPS), oomycete glucans, fungal chitin, and xylanase (Ayers et al. 1976; Felix et al. 1999; Dow et al. 2000; Gust et al. 2007; Ron and Avni 2004; Erbs et al. 2008; Miya et al. 2007; Silipo et al. 2010; Wan et al. 2008). PAMPs are widely conserved across genera, while the effectors are specific to a single or a few related species (Chisholm et al. 2006; Jones and Dangl 2006; Bent and Mackey 2007). MAMPs originate from beneficial microbes, whereas PAMPs specifically describe molecules from phytopathogens (Henry et al. 2012; Newman et al. 2013). PAMPs or MAMPs are often only present in the microbes and

not the hosts (Wu et al. 2014). Thus, PAMPS are a subgroup of MAMPs (Maffei et al. 2012). DAMPs are typically plant derived and are produced after wounding by insects or herbivores as well as degradation or perturbation of host molecules by microbes (Henry et al. 2012; Newman et al. 2013). DAMPs are endogenous molecules, such as Pep peptides (Huffaker et al. 2006; Yamaguchi et al. 2006; Krol et al. 2010; Yamaguchi and Huffaker 2014), cell wall fragment oligogalacturonides (Brutus et al. 2010), and extracellular ATP (Choi et al. 2014) released upon cell damage or pathogen recognition (Boller and Felix 2009; Newman et al. 2013; Zipfel 2014). DAMPs serve as warning signals to trigger or amplify plant defense responses (Yamaguchi and Huffaker 2014; Ma et al. 2013). HAMPs are elicitors from insect oral secretions (OS) at the site of tissue injury (Mithöfer and Boland 2008; Heil 2009). HAMP recognition and signaling, as well as into the diverse defense and tolerance strategies that plants use against herbivore attack (Bonaventure et al. 2011).

In accordance with the zigzag model, the innate immune system of plants consists of two layers of defense (Jones and Dangl 2006). At first recognition of PAMP, MAMPs (Boller and Felix 2009; Boller and He 2009), DAMPs (Boller and Felix 2009; Newman et al. 2013; Zipfel 2014), and HAMPs (Erb et al. 2012) by pattern recognition receptors (PRRs on the surface of the host cell), the pattern-triggered immunity (PTI) is activated (Tsuda and Katagiri 2010). Thus, PRRs can provide resistance to most nonadapted pathogens, as well as contribute to basal immunity during infection (Zipfel 2014). Plant PRRs are either surface-localized receptor kinases (RKs) or receptor-like proteins (RLPs) containing various ligand-binding ectodomains than PAMPs or DAMPs (Zipfel 2014). All of these molecules could universally be described as "patterns that elicit immunity" (PEIs) (Jones and Dangl 2006; Maffei et al. 2012; Newman et al. 2013). In plant the MAMP/PAMP perception by the cognate PRR elicits a cascade of early and late cellular responses and physiological changes (Boller and Felix 2009; Schwessinger and Ronald 2012). Ca^{2+} spike, extracellular alkalinization, membrane potential depolarization, ion effluxes, production of nitric oxide (NO), reactive oxygen species (ROS) and phosphatidic acid (PA), activation of an evolutionarily conserved MAP kinase (MPK) cascade, and ethylene biosynthesis are considered as early PTI responses (Boller and Felix 2009; Schwessinger and Ronald 2012), and the callose deposition and gene transcriptional reprogramming are observed later during PTI (Boller and Felix 2009; Schwessinger and Ronald 2012; Zipfel and Robatzek 2010). PTI confers low-level resistance to virulent pathogens (Vlot et al. 2009). PTI is an ancient form of plant immunity that provides sufficient protection to host-nonadapted pathogens and limited (basal) immunity to host-adapted microbes (Zipfel 2014). PTI (formerly called basal or horizontal resistance) is based on the PRR-mediated recognition of MAMPs and DAMPs, the so-called general elicitors of earlier reviews (Boller 1995; Darvill and Albersheim 1984). Conversely, PTI stimulates ethylene (ET) biosynthesis (Felix et al. 1999). The crosstalk between PTI and ET biosynthesis and signaling has recently been reviewed (Trujillo and Shirasu 2010). These first layers of defense (PTI) are believed to be sufficient to prevent the invasion of a wide range of pathogens (Huang and Zimmerli 2014). However, through

evolution, adapted pathogens have acquired effector proteins that are transferred into the plant cell where they suppress PTI (Block et al. 2008; Göhre et al. 2008; Cunnac et al. 2009), by secreting effectors in the apoplast or directly into the cytoplasm of host cells, leading to effector-triggered susceptibility (ETS) (Jones and Dangl 2006). Gram-negative bacterial pathogens acquired a type III secretion system (TTSS) through either horizontal gene transfer or adaptation of the flagellar apparatus (Chisholm et al. 2006). The evolution of the TTSS enabled bacteria to directly deliver effector proteins into plant cells, suppressing PAMP defense responses (Chisholm et al. 2006). *Pseudomonas syringae* has a type III effector, called AvrPtoB, which functions as an E3 ligase and targets the flagellin receptor FLS2 for degradation through the 26S proteasome (Göhre et al. 2008). *P. syringae* can secrete approximately 20–30 effectors during infection (Chang et al. 2005). Effectors promote pathogenicity, and the TTSS is essential for the development of disease symptoms and bacterial multiplication (Staskawicz et al. 2001). By their collective action, effectors are hypothesized to alter plant physiology in susceptible hosts to sustain pathogen growth (Chisholm et al. 2006). Both fungal and bacterial effector proteins that are delivered to plants can possess enzyme activity (Chisholm et al. 2006).

The second branch of plant immunity is a counter mechanism to ETS (Jones and Dangl 2006); this is called effector-triggered immunity (ETI) (Jones and Dangl 2006; Dodds and Rathjen 2010). In ETI formerly called R-gene-based or vertical resistance), an R gene product, usually residing inside the plant cell, recognizes the corresponding virulence-promoting effector protein(s) delivered by a pathogen (Jones and Dangl 2006). ETI is based on the highly specific, direct or indirect interaction of pathogen effectors (Fu and Dong 2013) and the products of plant R genes according to the gene-for-gene theory (Boller and Felix 2009). ETI is synonymous with pathogen race/host plant cultivar-specific plant disease (Boller and He 2009). ETI in plants is often associated with rapid, localized programmed cell death (PCD) at the infection site, a visible phenotype known as the hypersensitive response (HR) (Caplan et al. 2008; Jones and Dangl 2006), and at inoculation sites and provides effective local resistance to pathogens with a biotrophic or hemibiotrophic lifestyle (Jones and Dangl 2006). ETI employs intracellular immune receptors, which are most nucleotide binding site leucine-rich repeat (NBS-LRR) proteins, to perceive secreted virulence effectors directly or indirectly (Zipfel 2014). Based on features of the deduced domain structures and/or biochemical functions, R proteins can be divided into three classes (Dangl and Jones 2001). The two main classes of R proteins are the nucleotide binding leucine-rich repeat (NB-LRR) and the extracellular LRR (eLRR) resistance proteins (Dangl and Jones 2001).

The NB-LRR class is the most abundant, and members can possess amino-terminal coiled-coil (CC) or Toll-interleukin-1 receptor (TIR) domains (Dangl and Jones 2001; Jones and Dangl 2006). A second major class of R genes encodes extracellular LRR (eLRR) proteins. Three subclasses of eLRRs have been classified according to their domain structures (Fritz-Laylin et al. 2005). These subclasses include RLP (receptor-like proteins, extracellular LRR and transmembrane

[TM] domain), RLK (extracellular LRR, TM domain, and cytoplasmic kinase), and PGIP (polygalacturonase-inhibiting protein, cell wall LRR) (Chisholm et al. 2006). The best characterized RLPs are represented by the tomato *Cf* genes, which confer resistance to infection by the biotrophic leaf-mold pathogen *Cladosporium fulvum* (Jones et al. 1994). ETI triggers the biosynthesis and signals such as salicylic acid (SA), methyl salicylic acid (MeSA), azelaic acid (AzA), glycerol-3-phosphate (G3P), and abietane diterpenoid dehydroabietinal (DA) (Chanda et al. 2011; Chaturvedi et al. 2012; Jung et al. 2009; Malamy et al. 1990; Métraux et al. 1990; Park et al. 2007). The onset of PTI and ETI often triggers an induced resistance (IR) in tissues distal from the site of infection and involves one or more long-distance signals that propagate an enhanced defensive capacity in still undamaged plant parts (Shah and Zeier 2013). Although ETI is associated with stronger local responses than PTI, the signaling networks underlying both resistance forms partially overlap.

In symbiotic systems (e.g., mycorrhizal symbiosis), MAMPs from AM fungi are perceived and elicit a transient defense response, which later is suppressed during induction at early stages of AM (García-Garrido and Ocampo 2002). Due to their ancient origin and long coevolutionary history with plants (Bonfante and Genre 2008), it is also possible that AM associations may have predated the development of the plant innate immune system.

3 Induced Resistance in Plants

PTI and ETI, the basal immunity of the plant, contribute to the slowing down of the colonization process but are generally too weak to prevent disease (Nürnberger and Lipka 2005). The level of basal immunity of the plant can be enhanced through application of appropriate stimuli (Pastor et al. 2014). This is commonly referred to as induced resistance (IR; Kuc 1982; Hammerschmidt and Kúc 1995; Hammerschmidt 2007; Pieterse and Van Wess 2015).

The IR can lead to the direct activation of defenses, but can also lead to the priming of cells, resulting in stronger elicitation of those defenses, or other defenses, following pathogen attack (Goellner and Conrath 2008). It seems likely that most induced resistance phenomena are based on a combination of direct induction and priming (Ahmad et al. 2010). In addition to pathogen attack, resistance can also be induced by treatment with certain natural or synthetic compounds (Ryals et al. 1996; Beckers and Conrath 2007).

It is very well established that certain types of infection (pathogenic and non-pathogenic) or other treatments (e.g., abiotic stress) can induce disease resistance in plants (Cohen 2002; da Rocha and Hammerschmidt 2005; Kohler et al. 2002; Kuc 1982; Hammerschmidt and Kúc 1995; Hammerschmidt 2009; Oostendorp et al. 2001; Sticher et al. 1997; Ryals et al. 1996) and lead to horizontal resistance of the plant against a broad range of pathogenic organisms (Vallad and Goodman 2004). Induced resistance in plants can be divided into systemic acquired resistance (SAR:

reviewed in Sequeira 1983; Kessmann et al. 1994; Sticher et al. 1997; van Loon et al. 1998; Durrant and Dong 2004; Vallad and Goodman 2004; Conrath 2006; Hammerschmidt 2009; Gozzo and Faoro 2013; Fu and Dong 2013; Gruner et al. 2013), induced systemic resistance (ISR: reviewed in van Loon et al. 1998; Pieterse et al. 2001; Pieterse et al. 2014), and β-aminobutyric acid-induced resistance. Induced resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated (Van Loon et al. 1998). The IR operates in all plant parts distant from the original locus of inoculation and is therefore called systemic resistance (Durrant and Dong 2004; Hammerschmidt 2007). BABA-IR has emerged in the past decade through the discovery that BABA exogenous application can activate multiple immune responses by potentiating SA-inducible defenses and priming for pathogen-induced callose deposition, independent of salicylic acid and jasmonic acid (Gozzo and Faoro 2013), and confers resistance to biotic and abiotic stresses. The β-aminobutyric acid-induced resistance will not be addressed in this chapter.

3.1 Systemic Acquired Resistance (SAR)

A localized microbial infection of a single or a few leaves can also immunize the rest of the foliage to subsequent infection, a phenomenon known as systemic acquired resistance (SAR; Fu and Dong 2013; Shah and Zeier 2013). This type of IR requires the endogenous plant hormone SA (Métraux et al. 1990; Ryals et al. 1996; Sticher et al. 1997; Durrant and Dong 2004; Van Loon et al. 2006). SA has been implicated as an important signal for the activation of both PTI (DebRoy et al. 2004) and ETI (Dempsey et al. 1999; Durner et al. 1997; Klessig and Malamy 1994; Loake and Grant 2007). SAR is often characterized by localized necrosis, expression of pathogenesis-related (PR; van Loon et al. 2006) protein genes in the no inoculated distal tissue, to protect the rest of the plant from secondary infection and involves the SA pathway (Durrant and Dong 2004). This defense reaction aims to restrict the growth of the intruder and can lead to systemic IR leaving the plant less susceptible to subsequent pathogen attack (Henry et al. 2012). The mechanistic principles leading to SAR induction by different types of bacterial pathogens and the resulting systemic immunization patterns are highly overlapping (Mishina and Zeier 2007; Jing et al. 2011).

The SAR response is initiated by microbes eliciting PTI or ETI at inoculation sites and can also be triggered by localized leaf treatment with purified PAMPs (Gruner et al. 2013). SAR can also be induced by exogenous application of the defense hormone SA or its synthetic analogs 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole *S*-methyl ester (BTH) (Durrant and Dong 2004; Spoel and Dong 2012). Once activated, SAR provides enhanced resistance to a broad range of (hemi-) biotrophic fungal, bacterial, and viral pathogens (Sticher et al. 1997; Durrant and Dong 2004). SAR is activated by local pathogen infection that results in tissue

necrosis, also known as the hypersensitive response (HR; Ward et al. 1991). The HR is a form of PCD that is dependent on, e.g., reactive oxygen species. The resulting small necrotic lesion is involved in preventing the pathogen from spreading any further (Dangl et al. 1996). HR provides resistance to biotrophic pathogens that obtain their energy from living cells (Kumar et al. 2001). Because grafting experiments using the SA-deficient nahG rootstock have shown that SA is required only in the systemic tissue (Gaffney et al. 1993), the initial signal produced at the ETI site is a molecule other than SA (Fu and Dong 2013). These plants express the bacterial salicylate hydroxylase nahG gene, making them incapable of accumulating SA (Gaffney et al. 1993). NahG plants do not show an SAR response (Ryals et al. 1996).

3.2 Long-Distance Signal and SAR Signaling

Plant-derived substances have been proposed to participate in SAR long-distance signaling (Dempsey and Klessig 2012; Shah and Zeier 2013). These involve the putative lipid transfer protein defective in induced resistance1 (DIR1), the methyl ester of SA (MeSA), glycerol-3-phosphate (G3P), the diterpenoid dehydroabietinal, the dicarboxylic acid azelaic acid, and the Lys catabolite Pip (Maldonado et al. 2002; Park et al. 2007; Jung et al. 2009; Chanda et al. 2011; Chaturvedi et al. 2012; Návarová et al. 2012). This signal is unlikely to be effector specific, as different effector-R pairs can trigger SAR and studies have shown that virulence infection could also induce SAR (Attaran et al. 2009; Cameon et al. 1994; Jing et al. 2011). The role of several of these candidate signals for SAR induction in plants appears to depend on external parameters such as the environment light (Attaran et al. 2009; Liu et al. 2011a, b; Návarová et al. 2012).

PTI-inducing compatible *Pseudomonas syringae* pv. *maculicola* ES4326 (Psm) and ETI-inducing PsmavrRpm1 activate a similar systemic responses in *Arabidopsis thaliana*, including systemic accumulation of the SAR immune signals pipecolic acid (Pip) and SA and an increase in the systemic expression of the SAR marker genes such as *pathogenesis-related gene1 (PR1)*, *PR2*, and *PR5* (Mishina and Zeier 2006; Návarová et al. 2012). Pip enhances both its own biosynthesis and downstream signaling in SAR via intensification of ALD1 (*AGD2-like defense response protein1*) and FMO1 (*flavin-dependent monooxygenase1*) expression (Návarová et al. 2012). This suggests that ALD1 and FMO1 are involved in SA-independent signaling upstream of SA biosynthesis, processes that are required for systemic SA accumulation and SAR (Métraux 2002; Song et al. 2004a; Bartsch et al. 2006; Mishina and Zeier 2006). Pip accumulation is critical for SAR and local resistance to bacterial pathogens (Návarová et al. 2012). These authors reported that biologically induced SAR conditions plants to more effectively synthesize the phytoalexin camalexin, Pip, and salicylic acid and primes plants for early defense gene expression, and Pip has a positive regulation of salicylic acid biosynthesis and priming to guarantee effective local resistance induction and the establishment of SAR. Both

genes are not only indispensable for systemic SA accumulation and SAR but are also critical for full PTI and ETI responses at inoculation sites (Song et al. 2004a; Bartsch et al. 2006; Mishina and Zeier 2006; Koch et al. 2006).

SA is a second critical SAR metabolite that is produced in plants from chorismate by isochorismate synthase1 (ICS1; Wildermuth et al. 2001; Métraux 2002). SA induces SAR-related gene expression via the downstream regulator non-expresser of PR genes1 (NPR1; Cao et al. 1994), a transcriptional co-activator and bona fide SA receptor (Wu et al. 2012; Fu and Dong 2013). A cellular redox modulates the regulation of NPR1 (Fu and Dong 2013). It was proved that the exogenous application of SA induces a biphasic (first oxidizing and then reducing) fluctuation in cellular redox, as measured by GSH/GSSG (Mou et al. 2003). In the absence of pathogen challenge, NPR1 is retained in the cytoplasm as an oligomer through redox-sensitive intermolecular disulfide bonds (Fu et al. 2012). After the induction, these disulfide bonds are reduced, releasing NPR1 monomers into the nucleus, where NPR1 acts as a cofactor for transcription factors (TFs), such as TGAs (Dong 2004; Pieterse and Van Loon 2004), to induce defense-related genes (Fu et al. 2012; Kinkema et al. 2000; Mou et al. 2003). In the absence of a functional NPR1 protein, SA-induced transcriptional reprogramming is almost completely blocked (Fu et al. 2012). This suggests that NPR1 is important in regulating and connecting different hormone-dependent induced defense pathways (Dong 2004; Pieterse and van Loon 2004; Pieterse et al. 2009).

Spoel et al. (2009) discovered that NPR1 is constantly degraded in the nucleus by the 26S protein and revealed the underlying significance of this regulation. Degradation of NPR1 after SAR induction appeared to be required for full induction of NPR1 target genes (Fu and Dong 2013). NPR1 degradation occurs both in absence of SA and/or in the presence of high levels of this hormone, that is, during pathogen attack, and is mediated by two NPR1 paralogues, NPR3 and NPR4, that are SA receptors with different binding affinities (Fu et al. 2012). SA promotes the interaction of NPR1–NPR3 and disrupts that between NPR4 and NPR1, thus controlling NPR1 levels (Gozzo and Faoro 2013). In the early stage of infection by a pathogen triggering ETI, high SA levels facilitate NPR3-mediated degradation of NPR1 in the challenged cell, leading to HR (Gozzo and Faoro 2013). This is a mechanism to refresh the initiation complex after each round of transcription (Spoel et al. 2009). The execution of SAR involves transcriptional reprogramming regulated by a cascade of transcriptional events initiated by NPR1 (Fu and Dong 2013). The signal cascade is far from linear, as the TF controlling the NPR1-dependent ER-resident genes (TBF1) is also required for the induction of NPR1 gene expression during plant defense (Fu and Dong 2013). The list of NPR1 direct targets also includes many WRKY TFs, a plant-specific group of TFs that are highly versatile owing to their large number (74 members in *Arabidopsis*) and inducibility by various stresses (Ulker and Somssich 2004). The majority of studies on WRKY transcription factors (TFs) showed that numerous members of this multigene family are involved in the response to biotic stresses and are central components of many aspects of the innate plant immune system (Wang et al. 2014). The important role of WRKY TFs in different plant defense responses has been well established despite

the functional redundancy of this large group of proteins (Pandey and Somssich 2009). WRKY TFs are a large family of regulatory proteins forming such a network (Eulgem and Somssich 2007). They are involved in various plant processes but most notably in coping with diverse biotic and abiotic stresses (Pandey and Somssich 2009). The inducible nature of the WRKY TF encoding genes suggests that the TFs are likely auxiliary in the activation phase of a defense response and repressors in turning off defense when infection subsides (Fu and Dong 2013). Because this phenotype is similar to that of npr1 and because WRKY54 and WRKY70 are target genes of NPR1, it has been hypothesized that NPR1 feedback regulates SA biosynthesis through these WRKY TFs (Wang et al. 2006). SAR is believed to be conferred by a battery of coordinately induced antimicrobial PR proteins whose secretion requires significant enhancement of endoplasmic reticulum (ER) function (Wang and Dong 2011; Wang et al. 2005). Besides inducing SAR, SA is known to inhibit the HR during ETI (Devadas and Raina 2002; Yu et al. 1998), trigger thermogenesis in plants (Raskin et al. 1987, 1989), and inhibit plant growth, chloroplast development, and photosynthesis (Rivas-San Vicente and Plasencia 2011). It is possible that distinct SA signaling pathways control these physiological responses (Fu and Dong 2013). Several studies with transgenic plants constitutively expressing NPR1 have indicated that the NPR1-mediated mechanism is evolutionarily conserved and can be targeted for enhancing resistance against a variety of pathogens in agronomically important plants (Chern et al. 2001; Lin et al. 2004; Makandar et al. 2006; Malnoy et al. 2007; Potlakayala et al. 2007; Quilis et al. 2008; Wally et al. 2009).

SAR confers a fitness advantage under conditions of disease stress (Traw et al. 2007). A recent study indicated that the memory of SAR in *Arabidopsis* is passed on to the next generation, thus benefiting the progeny plants as well (Luna et al. 2012). However, it needs to be tightly regulated since it is an energy-driven process that diverts resources from growth and development (Heidel et al. 2004; Pajerowska-Mukhtar et al. 2012).

3.3 Induced Systemic Resistance (ISR)

Induced systemic resistance is often triggered by plant growth-promoting rhizobacteria (PGPR) (Walters et al. 2013) or plant growth-promoting fungi (PGPF; Pieterse et al. 2014), is not associated with necrosis, and involves the jasmonic acid (JA) and ethylene pathways (Henry et al. 2012; Spoel and Dong 2012; Walters and Heil 2007). PGPR and PGPF induce resistance in plants against fungal, bacterial, and viral diseases (Zamioudis and Pieterse 2012). In the early 1990s, plant growth-promoting rhizobacteria were studied for their abilities to elicit ISR, with special focus on *Pseudomonas* spp. It was discovered that when certain biocontrol pseudomonads were applied and kept spatially separated from the pathogen, they still reduced disease, suggesting that the mode of action must be plant mediated (Van Peer et al. 1991; Wei et al. 1991). The involvement of ISR, during the last two

decades, has been recognized as an effective mode of action for a range of bacterial and fungal biological control agents. In *Arabidopsis*, ISR triggered by *Pseudomonas fluorescens* WCS417r is effective against different types of pathogens (Ton et al. 2002), but it is not associated with the activation of *PR* genes (Pieterse et al. 1996).

There is a long and growing list of both biotic and abiotic agents that can protect crops against pathogens by eliciting ISR (for additional information on this topic, see these reviews: Alabouvette et al. 2009; Cameron et al. 2013; Da Rocha and Hammerschmidt 2005; Franken 2012; Jung et al. 2012; Reglinski and Walters 2009; Pozo and Azcón-Aguilar 2007; Van der Ent et al. 2009; van Loon et al. 1998; Walters et al. 2013). The cases of rhizobacteria-mediated ISR in which a role for SA had been functionally tested were reviewed by Van Loon and Bakker (2005). They concluded that the ability to activate an SA-independent ISR pathway is common for beneficial microbes and occurs in a broad range of plant species. Although the terms SAR and ISR are officially synonymous (Hammerschmidt et al. 2001), for pragmatic reasons, SAR is referred to when the induced resistance is triggered by a pathogen or demonstrated to be SA dependent and to ISR when the induced resistance is triggered by a beneficial microbe or demonstrated to be SA independent (Pieterse et al. 2014).

3.4 Early Signaling Events and ISR Signaling

The starting of ISR requires beneficial microbes to efficiently colonize the root system of host plants (Lugtenberg and Kamilova 2009). MYB72 is also induced in *Trichoderma*-colonized *Arabidopsis* roots and shown to be crucial for *Trichoderma* ISR (Alizadeh et al. 2013), suggesting that MYB72 is a node of convergence in the ISR signaling pathway triggered by different beneficial microbes (Pieterse et al. 2014). In *Arabidopsis thaliana*, the root-specific transcription factor MYB72 is required for the onset of ISR, but is also associated with plant survival under conditions of iron deficiency (De Mortel et al. 2008), pointing to a link between iron homeostasis and the onset of ISR (Zamioudis et al. 2014).

For the establishment of the mutualistic association, host plants and microbes need to respond to reciprocal signals and accordingly prioritize their responses to develop a lifestyle and the main phases of IR (for details, see the review of Pieterse et al. 2014).

In interactions between nonpathogenic rhizosphere microbes and plants, the phytohormones JA, SA, and ET regulate symbiosis and mediate ISR elicited by several groups of nonpathogenic microbes (De Vleesschauwer et al. 2009; Zamioudis and Pieterse 2012). In *Arabidopsis* mutants impaired in JA or ET signaling, it was demonstrated that JA and ET are central players in the regulation of rhizobacteria-mediated ISR (Pieterse et al. 1998). Although ISR by beneficial microbes is often regulated through SA-independent mechanisms, several PGPR have been reported to trigger an SA-dependent type of ISR that resembles pathogen-induced SAR (Pieterse et al. 2014). In plant growth-promoting rhizobacteria,

P. fluorescens P3, overexpressing the SA biosynthesis gene cluster of *P. aeruginosa* PAO1 was demonstrated to elicit SA-dependent SAR (Maurhofer et al. 1998). Many rhizobacteria have the capacity to produce SA; it is usually not the causal agent of the observed systemic resistance, because of the fact that rhizobacteria-produced SA is often not released in the rhizosphere but becomes incorporated into SA moiety-containing siderophores, which makes SA unavailable for triggering the SAR pathway (Audenaert et al. 2002; Bakker et al. 2003).

Depending on microbe species or strain, ISR can be triggered via JA/ET or SA signaling pathways, in which each pathway activates different sets of defense-associated genes (Pangesti et al. 2013). The defense regulatory protein NPR1 plays a key role in SA-dependent SAR, but has also been implicated in JA-/ET-dependent ISR (Dong 2004; Pieterse and Van Loon 2004). Many studies found an antagonistic effect of SA signaling on the JA pathway. However, in several cases JA signaling could suppress the SA pathway as well. A few molecular players have been reported to play a role in this JA–SA crosstalk, such as COI1 and MYC2 (Zheng et al. 2012). Detailed mechanistic knowledge on how the plant immune signaling network functions during multi-organism interactions is fundamental to develop novel strategies for the sustainable protection of our future crops that need to produce more with less input of pesticides and fertilizers (Pieterse and Van Wess 2015).

Crucially, this is distinct from the level of resistance induction in response to the recognition of true pathogens that are potentially capable of causing disease and where recognition would cause resistance mechanism expression that is more costly to the plant but still proportionate to potential disease cost (Walters and Heil 2007).

4 Bioprotective Effect of AM Fungi

Many studies describe particular cases of the bioprotective effect of mycorrhization against biotic stress, and hypothetical modes of action have been postulated (Garcia-Garrido 2009). Depending on the disease and the environmental conditions, any or all mechanism may be involved, such as changes in morphology of host root (Berta et al. 1993), physiological changes in host root (Smith and Gianinazzi-Pearson 1988; Dehne 1982; Dugassa et al. 1996; Dehne et al. 1978; Morandi 1996), changes in root exudates (Graham et al. 1981; Buwalda et al. 1984), enhanced nutritional status of host plants (Azcon-Aguilar and Barea 1996; Smith and Gianinazzi-Pearson 1988; Cooper and Grandison 1987), competition for space and host photosynthates (Azcon-Aguilar and Barea 1996; Linderman 1994), biological interactions in the mycorrhizosphere (Whipps 2004; Barea et al. 2002; St-Arnaud et al. 1995; Linderman 1988), enzyme production (Gianinazzi-Pearson et al. 1996), and molecular mechanisms (Harrison and Dixon 1993; Dumas-Gaudot et al. 2000; Pozo et al. 2002, 2010, 2013; Gianinazzi-Pearson et al. 1996; Zeng 2006; Pozo and

Azcón-Aguilar 2007; Vierheilig et al. 2008a, b). Although some of the suggested hypothetical modes (for review, see Azcon-Aguilar and Barea 1996; Whipps 2004; Zeng 2006; Pozo and Azcón-Aguilar 2007; Vierheilig et al. 2008a, b; Pozo et al. 2010, 2013) might play no role, they are not sufficient to explain all aspects of AM-induced bioprotection (Pozo and Azcón-Aguilar 2007; Jung et al. 2012).

The bioprotection through mycorrhization is the result of a combination of several mechanisms and not of a single mechanism (Vierheilig et al. 2008a, b). Most data about bioprotection due to mycorrhization are available from soilborne fungal pathogens (Singh et al. 2000; Azcon-Aguilar et al. 2002; Xavier and Boyetchko 2004; St-Arnaud and Vujanovic 2007), but examples of control of soil bacterial pathogens, foliar pathogens, root nematodes, and phytophagous insects are known (Garcia-Garrido 2009).

The bioprotective effect against soilborne fungal pathogens seems to depend on several biotic factors such as the host genotype, the AMF isolate, and the degree of mycorrhization (Vierheilig et al. 2008a, b; Garcia-Garrido 2009). The mycorrhizal effects on foliage diseases are contradictory and less conclusive (Vierheilig et al. 2008a, b). AM symbioses have been associated with enhanced susceptibility to biotrophic pathogens including viruses, powdery mildew, and rust fungi (*Blumeria*, *Oidium*, *Uromyces*) (Shaul et al. 1999; Gernns et al. 2001; Whipps 2004) and provides protection against the necrotroph fungus *Alternaria solani* (Fritz et al. 2006) and the shoot pathogenic bacteria *Xanthomonas campestris* (Liu et al. 2007). The bioprotection conferred by AM fungi is not effective for all plant pathogenic fungi, and the level of biological control conferred by AM fungal colonization is plant species and AM fungal isolate specific (Harrier and Watson 2004). The local (Singh et al. 2000; Azcón et al. 2002; Xavier and Boyetchko 2004; St-Arnaud and Vujanovic 2007) and the systemic (Cordier et al. 1998a, b; Pozo et al. 2002; Khaosaad et al. 2007) bioprotectional effect of mycorrhization depends on the degree of AM root colonization (Khaosaad et al. 2007). The effectiveness of AM fungi in biocontrol is dependent on the AM fungus involved, as well as the substrate and host plant (Akhtar and Siddiqui 2008).

The analysis of the published information on AM bioprotection showed that a great number of pathogen and plant species combinations and a smaller number of AM fungal species are involved in the biocontrol (Garcia-Garrido 2009). More than 80 % of the studies on the bioprotectional effect of mycorrhization have been performed with the genus *Glomus* (Vierheilig et al. 2008a, b; Garcia-Garrido 2009). The bioprotection by *Glomus* species indicates that whatever the mechanism(s) of this function, it responds to AM fungal families differently (Harrier and Watson 2004), while poor at pathogen protection, AM fungal species in the family *Gigasporaceae* most benefited the growth of the simple rooted plant species (Sikes et al. 2010). This reflects the natural occurrence of these AM fungal species or, more probably, their generalized use as model species in mycorrhizal studies (Garcia-Garrido 2009). Likewise, most of the studies on protection have been carried out in controlled environmental conditions, and field experiments have been relatively few (Garcia-Garrido 2009). Several studies showed that the mycorrhizal protection

against root fungi pathogens requires a high degree of AM root colonization, whereas intermediate and low levels of AM root colonization showed no bioprotectional effect (Vierheilig et al. 2008a, b). The root colonization by AM fungi induces biochemical changes within host tissues. These include stimulation of the phenylpropanoid pathway (Harrison and Dixon 1993; Morandi et al. 1984, 1996), changes in levels of aliphatic polyamines (El-Ghachtouli et al. 1995), activation of defense-related genes (Franken and Gnadinger 1994; Gianinazzi-Pearson et al. 1992; Harrison and Dixon 1993), and enhancement of certain hydrolase activities (Dumas-Gardot et al. 1992; Spanu et al. 1989). The colonization of root deposition of callose, activation of a distinct β-1,3 glucanase or accumulation of phytoalexins in the case of fungal attacks (Cordier et al. 1998a, b; Pozo et al. 1999; Yao et al. 2003), and the upregulation of several defense-related genes in the case of nematode infestation (Li et al. 2006; Vos et al. 2013). Mycorrhiza-induced chitinase isoforms appear to be a general phenomenon in AM symbiosis (Cordier et al. 1996; Hahn et al. 1989. These chitinases release oligosaccharide elicitors from the chitinous AM fungal cell walls which in turn stimulates the general defense responses of plants (Cordier et al. 1996; Hahn et al. 1989. Localized morphological lignification of endosperm cell walls and biochemical antifungal chitinase alterations in AM mycorrhizal roots increased resistance against wilt in tomato and cucumber (Dehne and Schonbeck 1979; Dehne et al. 1978).

Mycorrhizal roots show increased respiration than non-AM roots (Dehne 1982). The increase on the respiration of AM roots indicates higher metabolic activity which might enable mycorrhizal plants to be more resistant against root pathogens (Baas et al. 1989; Dugassa et al. 1996). AM roots show increased ethylene production and DNA demethylation. A higher demethylation can be related to gene expression for higher resistance of plants against pathogens (Dugassa et al. 1996). It has been observed that salicylic acid needs to be kept at a low level in roots for successful mycorrhization (Blilou et al. 1999), while jasmonate accumulates in mycorrhizal roots (Hause and Schaarschmidt 2009). These changes could play a role in AM-induced bioprotection by compensating root damage caused by the pathogen or by stimulating components of rhizosphere microbiota with antagonistic activity toward certain root pathogens (Azcon-Aguilar and Barea 1996; Barea et al. 2005). The activation of the plant defenses during AM formation does not only occur in the roots but also in the shoots (Liu et al. 2007; Pozo et al. 2010). Accumulation of insect antifeedant compounds (Gange 2006; Pozo et al. 2009) and transcriptional upregulation of defense-related genes (Liu et al. 2007; Pozo et al. 2009) have been described in leaves of mycorrhizal plants (Campos-Soriano et al. 2012).

Recently, Jayaraman et al. (2014) hypothesize that appropriate responses of plants to pathogenic and symbiotic microbes may require a tight integration of both chemical and mechanical stimulations exerted by these microbes, and the plants exhibit varied responses depending on the nature and intensity of these stimuli. Whether mycorrhizal plants are protected against pathogens seems to depend on the lifestyle of the pathogen (Walters et al. 2013). For example, mycorrhizal tomato plants were protected against the necrotrophic fungus *Alternaria solani* (Fritz et al.

2006; De la Noval et al. 2007), whereas pathogens with a biotrophic lifestyle such as powdery mildews appear to perform better in mycorrhizal plants (Pozo and Azcón-Aguilar 2007).

Direct and indirect modes of action have been suggested to be involved in the bioprotective effect of mycorrhization against pathogens, and recent advances regarding signaling processes in mutualistic and pathogenic associations have allowed us to define a specific mechanism of induction of resistance by arbuscular mycorrhizae (MIR; Pozo and Azcón-Aguilar 2007; Garcia-Garrido 2009).

4.1 Priming for Enhanced Defense

To compensate for their sessile life and face a broad range of biotic and abiotic stresses, plants have evolved a wide range of survival and adaptation strategies, and the higher plants are capable of inducing some stress "memory," or "stress imprinting". In plants, the IR is frequently associated with the accumulation of antimicrobial pathogenesis-related (PR) proteins (Van Loon et al. 2006) and with the so-called priming of cells (Kohler et al. 2002). Priming is the phenomenon that enables cells to respond to much lower levels of a stimulus in a more rapid and robust manner than non-primed cells (Conrath et al. 2002, 2006; Conrath 2011). Thus, plants primed by treatments that induce resistance show a faster and/or stronger activation of defense responses when subsequently challenged by pathogens or abiotic stresses (Conrath et al. 2002, 2006). Priming for enhanced defense is an important cellular process in many types of biologically and chemically in IR immunity, including SAR, ISR, and herbivore-induced resistance (Conrath 2011; Pastor et al. 2013). In the absence of such specialized cells, plants maintain immune memory through "priming" (Fu and Dong 2013).

4.1.1 Priming Induced by MIR

Priming seems to be the mechanism underlying the induced systemic resistance (ISR) observed in plants interacting with beneficial microorganisms (reviewed in Conrath et al. 2006; Goellner and Conrath 2008; Van Wees et al. 2008; Pieterse et al. 2014), including the arbuscular mycorrhizal fungi (reviewed in Pozo et al. 2009; Jung et al. 2012; Campos-Soriano et al. 2012; Cameron et al. 2013; Selosse et al. 2014), plant pathogens (Pastor et al. 2013), or insect herbivores (Rasmann et al. 2012). However, priming can be triggered also chemically, by exogenous application of low doses of SA, JA, or BABA (Conrath et al. 2002, 2006; Conrath 2011) and providing low-cost protection in relatively high stress-pressure conditions. The basal resistance by itself is too weak to protect against virulent pathogens, since it constitutes a residual level of resistance after immune suppression by the pathogen (Pastor et al. 2013). The priming-inducing stimuli can render basal

resistance more effective, particularly when the accelerated defense response precedes immune suppression by the invading pathogen (Ahmad et al. 2010).

4.2 Epigenetic Mechanisms of Molecular Priming

4.2.1 Memory of Plant Immunization

SAR-conferred immune "memory" in plants can last for weeks to months and possibly even the whole growing season (Kuć 1987). Epigenetic mechanisms, such as histone modifications or DNA methylation, have emerged as important regulatory mechanisms in plant immunity (Alvarez et al. 2010). There is evidence that post-translational modifications of histone proteins are influenced by JA-, SA-, and ABA-dependent signaling pathways (Devoto et al. 2002; Walley et al. 2008). Furthermore, exposure to disease, herbivores, and abiotic stresses can have profound impacts on patterns of symmetric and asymmetric DNA methylation (Pavet et al. 2006; Boyko et al. 2010; Verhoeven et al. 2010). It is, therefore, not surprising that priming of defense has been associated with epigenetic regulatory mechanisms (Conrath 2011).

First evidence for an epigenetic basis of defense priming came from Jaskiewicz et al. (2011), who demonstrated that infection of *Arabidopsis* by *Pseudomonas syringae* pv. *maculicola* primes stress-inducible expression of transcription factor genes via NPR1-dependent modifications of histone H3 at their promoter regions.

López et al. (2011) demonstrated that mutants blocked in RNA-directed DNA methylation are primed to activate SA-inducible defense genes, which was associated with H3 modifications marking a facilitated state of gene transcription: acetylation at lysine residue 9 (H3K9ac) and triple methylation at lysine 4. Hence, defense priming is often associated with posttranslational histone modifications at promoter regions of primed defense genes.

Recently, three independent research groups provided evidence that priming of defense can be inherited epigenetically from isogenic plants that had been treated with pathogens, herbivores, or BABA (Luna et al. 2012; Rasmann et al. 2012; Slaughter et al. 2012). These three studies demonstrated that priming for enhanced resistance also extends to next generations and that epigenetic regulatory mechanisms, such as DNA methylation, chromatin remodeling, and small interfering RNAs (siRNAs), play a central role the regulation of these transgenerational plant immune responses (Pieterse 2012).

Although these studies demonstrated an epigenetic component of defense priming, the extent by which epigenetic regulation contributes to long-lasting defense priming within one plant generation remains unknown. Some techniques are now starting to emerge as a promising alternative for sustainable modern pest management in the field, since some pesticides have been shown to actually exert their

known plant health- and yield-increasing effects through priming (Beckers and Conrath 2007).

Recent evidences suggested that priming of SA-inducible genes involves epigenetic regulatory mechanisms, such as posttranslational modifications of histone proteins and the RNA polymerase V (Jaskiewicz et al. 2011; López et al. 2011). This phenomenon is called "transgenerational SAR"; since the resistance in P1 progeny (progeny from *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000-infected *Arabidopsis*) is effective against (hemi-) biotrophic pathogens, it requires NPR1; and it is associated with priming of SA-inducible defense genes (Luna et al. 2012). This transgenerational SAR is effective against biotrophic pathogens, such as the downy mildew pathogen *Hyaloperonospora arabidopsidis* (Luna and Ton 2012). These authors conclude that transgenerational SAR is transmitted by DNA hypomethylation at CpHpG (Lindroth et al. 2001) sites.

How SAR can be sustained for so long is not clear but epigenetic modifications, such as DNA methylation and chromatin remodeling, seem critical to maintain a SAR signal (Spoel and Dong 2012). The memory associated with the inheritance of SAR is likely to be epigenetic in nature (Luna and Ton 2012; Luna et al. 2012). Hence, plants seem to have the capacity to "memorize" a stressful situation and subsequently immunize not only themselves but also their next generation against future attacks (Pastor et al. 2013). Although these studies have demonstrated an epigenetic component of defense priming, the extent by which epigenetic regulation contributes to long-lasting defense priming within one plant generation remains unknown. For plants growing in an environment with a high disease pressure, the ability to activate SAR confers a fitness advantage that can also benefit subsequent generations (Shah et al. 2014). Despite priming phenomena having been widely described, the molecular mechanisms of defense priming are still unclear (Conrath et al. 2006; Conrath 2011). The fitness benefit of priming was shown to outweigh its cost when under pathogen pressure, suggesting that priming functions as an ecological adaptation of the plant to respond faster to its hostile environment.

In agriculture, epigenetic priming of plant defenses, as has been accomplished with tomato (Rasmann et al. 2012), barley, and tobacco (Kathiria et al. 2010), has the potential to increase productivity without time-consuming breeding approaches. It is clear that, despite recent rapid advances, the importance, mechanisms, and consequences of transgenerational induction of defense for plants and their associated insects and microbes are only beginning to be unraveled. Fine-tuning defenses through priming instead of a direct activation is a cost-efficient mechanism to improve resistance (Selosse et al. 2014). From an ecological point of view, the benefits of priming are clear: rather than leading to the costly and potentially wasteful activation of defenses, a metabolic state of alert is induced after an initial infection, enabling a rapid intense resistance response to subsequent attacks. Thus, this strategy appears promising for crop protection purposes (Walters and Heil 2007).

4.3 Mycorrhiza-Induced Resistance (MIR)

Induced pathogen protection through this mechanism may either be systemic within the plant (Guillon et al. 2002) and/or through root exudation (Lioussanne et al. 2008). The effects of the AM symbiosis on plant interactions with other organisms and, in particular, the induction of resistance against deleterious organisms seem to result from the combination of multiple mechanisms that may operate simultaneously (Jung et al. 2012). A proposed hypothesis is that colonization of roots by AM fungi primes defense mechanisms leading to mycorrhiza-induced resistance (MIR; Cordier et al. 1998a, b; Pozo et al. 2002; Pozo and Azcón-Aguilar 2007; Jung et al. 2012; Cameron et al. 2013; Selosse et al. 2014). The other mechanisms discussed above might also play a role while their contribution might differ (Cameron et al. 2013). They could be operative at the same time (Pozo and Azcón-Aguilar 2007) or might act consecutively (Cameron et al. 2013). MIR includes a priming of defense-related plant genes and shares more elements with the ISR induced by rhizobacteria against necrotrophs via a jasmonate JA-mediated pathway than with salicylic acid-regulated SAR, induced by leaf pathogens via an SA-mediated pathway and directed against biotrophs (Pozo and Azcón-Aguilar 2007; Hayek et al. 2014; Jung et al. 2012; Pieterse et al. 2012).

4.3.1 Mechanisms of Mycorrhiza-Induced Resistance

Different mechanisms have been proposed to explain the role in plant bioprotection by AMF (reviewed in Azcon-Aguilar and Barea 1996; Whipps 2004; Zeng 2006; Pozo and Azcón-Aguilar 2007; Vierheilig et al. 2008a, b; Pozo et al. 2010, 2013), and these include (1) plant nutrition improvement (Berta et al. 1995; Trotta et al. 1996; Singh et al. 2000; Sharma et al. 2007), (2) competition for photosynthates and root colonization sites between an AM fungus and a pathogen (Garcia-Garrido and Ocampo 1989; St-Arnaud et al. 1995; Cordier et al. 1998a, b; Filion et al. 1999; Singh et al. 2000; Azcón-Aguilar et al. 2002; Lerat et al. 2003a, b; Pozo et al. 2002; Xavier and Boyetchko 2004), (3) changes in rhizosphere microbial populations and development of pathogen antagonism (Linderman 1994; Schreiner and Bethlenfalvy 1995; Azcón-Aguilar et al. 2002; Barea et al. 2013a, b), and (4) biochemical and molecular changes in mycorrhizal plants that induce pathogen resistance (Pozo and Azcón-Aguilar 2007; Jung et al. 2012; Cameron et al. 2013). However, Wehner et al. (2009) proposed that the existence of functionally complementary mechanisms of pathogen protection within these assemblages may be a significant driver of these phenomena and consider ways through which AM fungal diversity may influence the outcomes of these interactions. The results of these interactions may be underestimated by studies considering only single AM fungal species (Wehner et al. 2009). Species-rich AM fungal assemblages have been observed to enhance the diversity and productivity of host plants and communities (Maherali and Klironomos 2007).

4.4 Establishment of AM Symbiosis and Hormonal Modulation During MIR

4.4.1 Establishment of AM Symbiosis

The establishment of a symbiotic association usually involves mutual recognition and a high degree of coordination at the morphological and physiological level, which requires a continuous cellular and molecular dialogue between both the partners (Garg and Chandel 2010).

4.4.2 Early Symbiotic Signaling

Before physical contact, diffusible signal molecules (strigolactones and flavonoids) that are secreted by plant roots can be perceived by AM fungi (Akiyama et al. 2005) and root nodule symbiosis rhizobia–legumes (RNS; Maillet et al. 2011). The strigolactone hormones, secreted from plant roots, stimulate hyphal branching and fungal metabolism; fungal short-chain chitin oligomers as well as sulfated and nonsulfated lipochitooligosaccharides (s/nsMyc-LCOs) elicit pre-symbiosis responses in the host (Bucher et al. 2014). Two classes of such compounds were identified recently, both comprising an *N*-acetylglucosamine oligomer backbone: lipochitooligosaccharides called Myc-LCOs, able to stimulate lateral root formation and the colonization of roots by AM fungi (Maillet et al. 2011), and short-chain chitooligosaccharides (Myc-COs) that can trigger nuclear calcium spiking in host plant root cells (Genre et al. 2013).

Rhizobia–legume symbiosis produced specific symbiotic signals called Nod factors (NFs) that are lipochitooligosaccharides (LCOs; Hassan and Mathesius 2012). Nod factors are also lipochitooligosaccharides and have a similar composition (Maillet et al. 2011). It has been suggested that Nod factors developed from Myc factors and that the functions of Myc and Nod factors overlap (Bonfante and Requena 2011). Synthetic LCOs, obtained via bacterial genetic engineering, have been shown to stimulate AM colonization in plant species of diverse families (Fabaceae, Asteraceae, and Umbelliferae) (Maillet et al. 2011). The LCOs are perceived via lysin-motif (LysM; carbohydrate-binding modules found in prokaryotes and eukaryotes) receptors and activate a signaling pathway called the common symbiotic pathway (CSP), which controls both the RL (the rhizobia–legume) and the AM symbioses (Gough and Cullimore 2011). The discovery of Myc-LCO and a LysM receptor required for the AM symbiosis, therefore, not only raises questions of how plants (legume and non legume) discriminate endosymbionts from pathogenic microorganisms using structurally related LCO and CO signals and of how these perception mechanisms have evolved (Gough and Cullimore 2011).

AM and RNS are both regulated by a common set of genes that define the common SYM pathway (Bapaume and Reinhardt 2012). Symbiotic plants retain,

besides their NFRs (Nod factor receptors) and MFRs (Myc factor receptors), potent receptors for microbial cell wall constituents such as chitin and peptidoglycan oligomers, which can trigger defense responses (Shimizu et al. 2010; Willmann et al. 2011).

4.4.3 Root System Changes in Response to Pre-symbiotic Signaling in AM Symbiosis

The genes that encode the components of this signal transduction chain for both AM and RNS are called as the common *SYM* (symbiosis) genes (Kistner and Parniske 2002; Breuillin et al. 2010; Carbonnel and Gutjahr 2014). The SYM pathway is functionally conserved between monocot and dicot species (Chen et al. 2007a, 2008; Gutjahr et al. 2008), suggesting that it evolved in early land plants, and became secondarily recruited into RNS (Breuillin et al. 2010). The perception by host plants of Myc factors produced by AM fungi induces the symbiotic program through the SYM pathway (Catoira et al. 2001). This perception triggers NSP1/NSP2 (*nodulation signaling pathway* 1, 2—GRAS transcription factors) and RAM1/RAM2 (*reduced arbuscular mycorrhization* 1, 2; they also were GRAS-type transcription factors Gobbato et al. 2012). NSP1 is involved in the Myc-LCO signaling pathway (Delaux et al. 2013) and NSP2 is required for nodulation (Gobbato et al. 2013). RAM1 has a specific function in mycorrhizal signaling that regulates the expression of RAM2 (Gobbato et al. 2012), and RAM2 is a glycerol-3-phosphate acyl transferase (GPAT) that promotes cutin biosynthesis to enhance hyphopodia formation on the root surface during mycorrhizal colonization (Gobbato et al. 2012). This suggests that formation of multicomponent GRAS transcription factor complexes is a prerequisite for elicitation of nodulation or mycorrhization (Oldroyd 2013).

The perception by host plants of Myc factors produced by arbuscular mycorrhizal fungi (AMF) induces the symbiotic program through the SYM pathway (DMI "does not make infections"; Catoira et al. 2001). DMI (*DMI1*, *DMI2*, *DMI3*) genes control an NF (Nod factors) signaling pathway leading to nodulation, but are also required for the formation of mycorrhiza, indicating that the symbiotic signaling pathways activated by both the bacterial and the fungal symbionts share common steps (Olah et al. 2005). *DMI1* and *DMI2* were two genes that act upstream of Ca^{2+} spiking as part of the common SYM pathway (Olah et al. 2005). The third common SYM gene *DMI3* that acts to downstream of Ca^{2+} spiking (Maillet et al. 2011) is also required for rhizobial Nod factor-mediated lateral root induction (Olah et al. 2005). Calcium spiking is a rhythmic change in perinuclear calcium concentration, which is perceived and transmitted by calcium- and calmodulin-dependent protein kinase (CCaMK) to induce symbiotic gene expression (Oldroyd and Downie 2006; Singh and Parniske 2012). At downstream of calcium spiking, there is a nuclear calcium and calmodulin-dependent protein kinase (CCaMK) (MtDMI3/LjCCaMK) and a nuclear coiled-coil protein (MtIPD3/LjCYCLOPS) that interacts with the CCaMK to decode calcium spiking for downstream signaling (Gough and Cullimore

2011). A protein of DMI3, called IPD3 in *Medicago truncatula* and CYCLOPS in *Lotus japonicus*, was identified that was shown to be essential for infection thread formation as well as mycorrhizal arbuscule formation in *L. japonicus* (Messinese et al. 2007; Yano et al. 2008). These events lead to a signaling at the cell surface via the production of cutin monomers and the activation of cellular remodeling events in both plant (MSP1, Vapyrin D3; Pumplin et al. 2010; Expansin) and fungal (GinSTE12; Perez-Tienda et al. 2011) partners. Vapyrin (VPY) is a gene essential for intracellular progression of arbuscular mycorrhizal symbiosis and is also essential for infection by rhizobia in the nodule symbiosis of *Medicago truncatula* (Murray et al. 2011). Pumplin et al. (2010) have proposed that Vapyrin plays a cellular role to enable AM fungal development within plant cells. It has been proposed that the protein VPY could mediate Ca^{2+}-mediated membrane and cytoskeleton rearrangements during initial stages of root cell infection by rhizobia and AM fungi (Ercolin and Reinhardt 2011; Sieberer et al. 2012).

After the activation of cellular remodeling events in both plant (MSP1, Vapyrin D3; Murray et al. 2011, Expansin; Cosgrove et al. 2002; Balestrini et al. 2005) and fungal (GinSTE12; Perez-Tienda et al. 2011) partners. The elevation of VPY transcripts upon application of Nod factors, which we show to be dependent on NFP (*Nod factor perception*; Rival et al. 2012), DMI1, and DMI3, indicates that VPY acts downstream of the common signaling pathway (CSP) (Murray et al. 2011). Rival et al. (2012) suggested that a signal, produced in the epidermis under the control of NFP and DMI3, is responsible for activating DMI3 in the cortex to trigger nodule organogenesis in *Medicago truncatula*.

NFP encodes a receptor-like kinase with three extracellular lysin-motif (LysM) domains and an inactive kinase domain (Arrighi et al. 2006). These lipochitooligosaccharides (LCOs), called Nod factors, are perceived via lysin-motif (LysM) receptors and activate a signaling pathway called the CSP, which controls both the rhizobia–legume (RL) and the AM symbioses (Rival et al. 2012). The expansins are extracellular proteins involved in cell wall loosening and in the growth of plant cells (Cosgrove et al. 2002). These proteins have also been located in AM roots: they are present both in the cell walls of the host cells and in the interface, suggesting that this class of proteins, involved in cell wall loosening, may be crucial in the accommodation process of the fungus inside the cortical cells (Balestrini et al. 2005).

The host plants are able to recognize AMF as potential colonizers through pattern recognition receptors (PRRs) that perceive microbe-associated molecular patterns (MAMPs) (Zhang and Zhou 2010). As a result, a signaling cascade probably involving Rac1-mediated reactive oxygen species (ROS) production (Kiirika et al. 2012) is induced, which results in MAMP-triggered immunity (MTI) through the production of defense-related compounds. In response, to this signaling cascade, AMF developed the capacity to secrete the SP7 protein effector into the plant cytosol, which, upon targeting the nucleus, interacts with the defense-related transcription factor ERF19 to block the ERF19-mediated transcriptional program (Recorbet et al. 2013). The SYM pathway is possibly involved in the suppression of MTI (Siciliano et al. 2007a, b).

4.5 Development and Establishment of AM Symbiosis

The development of AM symbiosis can be separated into distinct steps that are characterized by the level of progression of fungal hyphae during root colonization (Gutjahr and Parniske 2013). At first in the early phase (also referred to as presymbiotic stage), a mutual recognition is characterized by hyphal-branching responses elicited by plant-derived strigolactones (SLs; Akiyama et al. 2005). Later, fungal-signaling molecules (chitooligosaccharides; Kosuta et al. 2003; Kuhn et al. 2010) induce the plant gene expression, which elicit calcium spiking in rhizodermal cells (Genre et al. 2013). After occurs the hyphopodium formation at the root surface which depends on cutin monomers produced by the plant. With the formation of the prepenetration apparatus (PPA) that guides intracellular fungal passage into deeper cell layers (Genre et al. 2008; Gutjahr and Parniske 2013), the fungal hyphae enter the plant host cell, accompanied by high-frequency calcium spiking (Sieberer et al. 2012).

Plants have receptors for chitin and peptidoglycan (Bapaume and Reinhardt 2012) to induce downstream defense reaction against pathogenic fungi and bacteria, and these receptors might also be derived from the ancestors being functional in the AM symbiosis (Parniske 2008). Symbiotic plants retain, besides their NFRs (Nod factor receptors) and MFRs (Myc factor receptors), potent receptors for microbial cell wall constituents such as chitin and peptidoglycan oligomers, which can trigger defense responses (Shimizu et al. 2010; Willmann et al. 2011). Alternatively, upon recognition of AM fungi or rhizobia, plants suppress their defense responses and actively allow symbiotic interactions (Bapaume and Reinhardt 2012). The AM fungal colonization is strictly speaking apoplastic, even if a large percent of intraradical hyphae grow through the cell lumen of epidermal and cortical cells (Genre and Bonfante 2005). The fungus leaves the plant cell and enters the apoplast, where it branches and grows laterally along the root axis (Parniske 2008). Once in the cortex, fungal hyphae progress longitudinally through the apoplast and form branches to initiate arbuscule formation in cortical cells (Genre et al. 2008; Harrison 2012). This results in the establishment of arbuscules (Breuillin et al. 2010). In this root endosymbiosis, the arbuscules serve as an exchange interface between AM fungi and host plant. Arbuscule development is accompanied by plastid proliferation and the formation of a plastidial network in close physical contact with the arbuscule (Strack and Fester 2006). The plastid is involved in numerous biosynthetic activities, including the production of apocarotenoids that specifically accumulate in AM roots (Walter et al. 2007).

Given the involvement of hormones in almost all plant developmental processes, it is thought that hormones have key roles during the development of AM (Parniske 2008). This is still a developing area of research, but abscisic and jasmonic acid have emerged as potential regulators of AM (Herrera-Medina et al. 2007; Hause et al. 2007).

4.6 Hormonal Modulation of Host Plant During MIR

The establishment of functional AM involves a series of steps which are under tight control mainly by the host plant (Breuillin et al. 2010). Plant defense responses are coordinated by small molecules that act as signal transducers (Pieterse et al. 2009). These molecules drive the coordinated expression of genes that code for defense-related proteins and compounds (Ausubel 2005; Jones and Dangl 2006).

Phytohormones also interact to regulate the establishment and functioning of the AM symbiosis (Ludwig-Müller 2010; Foo et al. 2013; Bucher et al. 2014; Gutjahr 2014). Among these molecules, the phytohormones JA, SA, abscisic acid (ABA), ET (Pieterse et al. 2009), strigolactones (SLs), auxin (Foo et al. 2013), and less well-understood gibberellins (GAs) and brassinosteriods (Foo et al. 2013) play a key role not only in the establishment but also in the functioning of the AM symbiosis (Hause et al. 2007; Herrera-Medina et al. 2007; López-Ráez et al. 2010; Ludwig-Müller 2010). Salicylic acid is a phenolic phytohormone (monohydroxybenzoic acid) required in the signal transduction cascades that regulate plant defense mechanisms against biotic and abiotic stresses (Bartoli et al. 2013). Salicylic acid is known to have a major role in plant defense against microorganisms with a biotrophic lifestyle (Pieterse et al. 2009). Jasmonic acid belongs to a group of compounds called oxylipins that are formed via oxygenation of fatty acids (Bartoli et al. 2013). JA-mediated reprogramming of gene expression is perhaps been best characterized in relation to plant–pathogen interactions. Ethylene participates in many aspects of plant biology from germination to dormancy, ripening and senescence, and the regulation of stomatal closure, as well as defenses against biotic and abiotic stresses.

In AM symbiosis, ET and salicylic acid function as negative regulators of mycorrhizal intensity (Gamalero et al. 2008; Ludwig-Müller 2010). The ET content is increased by a deficiency of ABA, which is in contrast necessary for arbuscule formation and is positively correlated to mycorrhizal establishment (Ludwig-Müller 2010; Martín-Rodríguez et al. 2011). The reduced level of ET generally found in AM plants is therefore in agreement with the increased branching of the colonized roots (Fusconi 2014).

In fact, a strong ET inhibitory effect has been observed on early symbiotic gene expression, on fungus entry into roots (Mukherjee and Ané 2011), and on intraradical fungal spread (Martín-Rodríguez et al. 2011). Ethylene and jasmonic acid synergistically activate plant defenses against necrotrophic pathogens (Thomma et al. 1998), while salicylic acid signaling triggers resistance against biotrophic and hemibiotrophic pathogens (Glazebrook 2005). Interestingly, evidence from several distantly related plant species suggests that there can be evolutionarily conserved SA and JA signaling crosstalk resulting in reciprocal antagonism between the SA and JA signaling pathways (Glazebrook 2005; Smith et al. 2009). Several authors suggested that the WRKY transcription factors may play an important role in this antagonistic interaction (Robert-Seilaniantz et al. 2011). As the AtWRKY70, the

protein encoded by this gene had been suggested to act as a positive regulator of the SA-dependent defense and a negative regulator of JA-dependent defense (Bari and Jones 2009). This is possibly adaptive since it could allow plants to fine-tune the balance between different defensive strategies (De Vos et al. 2006; Pieterse and Dicke 2007; Thaler et al. 2012). The dependence of successful mycorrhization on the control of JA and SA signaling would explain the range of protection conferred by this symbiosis (Pozo and Azcón-Aguilar 2007). The cytokinins (CKs) promote resistance against biotrophs by enhancing the SA response through NPR1 (Choi et al. 2010).

Recent studies have shown that CKs might not be involved to any great extent in the regulation of mycorrhizal development (Foo et al. 2013). The higher CK content in AM plants is in line with the reduction in the root/shoot biomass ratio, which occurs when colonization is established (Fusconi 2014). Strigolactones (SLs) are hormones constitutively exuded from higher plant roots and rhizoids of bryophytic gametophytes (Akiyama et al. 2005; Delaux et al. 2012). They induce the metabolic activity of AM fungi and provide a directional cue to guide the fungus to colonizable tissue (Parniske 2005; Besserer et al. 2006). Auxin is involved in the AM host–fungus interaction (Fusconi 2014). Auxin was shown to be required within the host roots for the early stages of AM formation, e.g., during pre-symbiotic signal exchange (Hanlon and Coenen 2011), in part through the control of the SL levels (Foo et al. 2013). The activation of auxin signaling triggers the suppression of SA biosynthesis and SA signaling (Robert-Seilaniantz et al. 2007). Activation of abscisic acid biosynthetic and signaling pathways promotes disease susceptibility to several plant pathogens (Asselbergh et al. 2007; Ton and Mauch-Mani 2004). This balance of hormonal crosstalk strongly influences the outcome of plant–pathogen interactions, including establishment of effective systemic immunity (Robert-Seilaniantz et al. 2011). For example, gibberellic acid causes degradation of the DELLA protein growth repressors, elevating accumulation of reactive oxygen species (ROS) and SA and attenuating JA signaling (Achard et al. 2003; Navarro et al. 2008). The brassinosteroid treatment enhances biotroph–hemibiotroph resistance (Nakashita et al. 2003).

Based on the literature data, we can summarize the role of phytohormones on the control of arbuscular mycorrhizal symbiosis in this way. Salicylates, ethylene, and cytokinins have negative effects at the fungal penetration or root colonization steps (Foo et al. 2013). By contrast, ABA and auxins positively regulate arbuscule development and functionality (Martín-Rodríguez et al. 2011; Etemadi et al. 2014), and positive and negative effects have been described for jasmonates (Wasternack and Hause 2013). As in other plant processes, the impact of hormones on mycorrhizae depends on pathway crosstalk (Pozo et al. 2015). The levels of these hormones seem to be altered in mycorrhizal plants (Hause et al. 2007; López-Ráez et al. 2010), probably affecting plant defense mechanisms (Pozo et al. 2010). Once established, the plant has to regulate the level of fungal proliferation within the roots to prevent excessive colonization and carbon drainage, thus maintaining the interaction at mutualistic levels (Jung et al. 2012).

4.7 Modulation of Plant Defense Responses During MIR

4.7.1 Autoregulation of Mycorrhization

Plant and AM fungi actively engage in the process of colonization, and a tight control of plant defense mechanisms is necessary (Pozo et al. 2010). The plant is able to restrict AMF colonization once plants are already mycorrhizal, a phenomenon known as autoregulation (Vierheilig et al. 2008a, b). The mechanisms operating in such autoregulation may also affect plant interactions with pathogens (Pozo et al. 2012). The bioprotective effect of mycorrhization and the autoregulation of mycorrhization are possibly two sides of the same coin (Vierheilig et al. 2008a, b). It seems plausible that an already mycorrhizal plant develops just one mechanism to repulse further colonization by fungi, not discriminating between AMF and soilborne pathogenic fungi (Vierheilig and Piché 2002; Vierheilig 2004).

Several studies of the autoregulatory mechanism of mycorrhization have excluded plant phosphorus (Vierheilig et al. 2000b) or the competition for carbon (Lerat et al. 2003a, b). Some mechanisms have been proposed for autoregulation of mycorrhization, including the long-distance transport of chemical signals (Staehelin et al. 2011), changes in phosphate levels within the plant (Breuillin et al. 2010), and the reduction in strigolactone biosynthesis (Garcıa-Garrido et al. 2009; López-Ráez et al. 2011). However, the mechanism by which AM symbiosis reduces strigolactone production is unknown (López-Ráez and Pozo 2013). Apart from that, a major transcriptional reprogramming takes place upon mycorrhizal colonization of the roots (Liu et al. 2003, 2007; Güimil et al. 2005; López-Ráez et al. 2010). This reprogramming originates alterations in the primary and secondary metabolism in mycorrhizal plants (Hause et al. 2007; Toussaint 2007; Schliemann et al. 2008). This has led to the identification of the genes, signal transduction pathways, and the chemical structures of components relevant to symbiosis; however, scientific knowledge on the physiology and function of these fungi is still limited (Garg and Chandel 2010). Transcriptional studies have also demonstrated that plant responses to AM fungi (i.e., obligate biotrophs) present similarities with plant responses to biotrophic pathogens (Sanchez et al. 2004; Güimil et al. 2005). Accordingly, all those changes may have special relevance to mycorrhizal effects on plant interactions belowground (Jung et al. 2012). A key factor in the MIR seems to be the extension of root colonization by AM fungi (Pozo et al. 2009). Studies comparing different mycorrhizal colonization levels concluded to the necessity of a well-established AM symbiosis for local and systemic induced resistance (Slezack et al. 2000; Khaosaad et al. 2007).

The presence of AM fungi within the root prior to contact with the soilborne pathogen has also been suggested as an important factor in plant protection (Azcon-Aguilar et al. 2002; Xavier and Boyetchko 2004). This aspect is probably linked with the level of mycorrhizal colonization (Vierheilig et al. 2008a, b). The fungus has to deal with the plant's immune system, contend with the defense mechanisms, and overcome them for successful colonization of the host (Kloppholz et al. 2001; Zamioudis and

Pieterse 2012). The plant responds to colonization by AMF, with a quick but transient increase of endogenous SA that occurs in the roots with accumulation, such as reactive oxygen species, and the activation of the phenylpropanoid and oxylipin pathways (Blilou et al. 1999; Dumas-Gaudot et al. 2000; Fester and Hause 2005; de Román et al. 2011). These reactions are temporally and spatially limited compared to the reaction during plant–pathogen interactions, suggesting a role in the establishment or control of the development of the fungus inside the roots (Pozo et al. 2002; Dumas-Gaudot et al. 2000; García-Garrido and Ocampo 2002).

The mechanisms, which are actually controlling the autoregulatory effect of the mycorrhizal colonization, are still unknown (Vierheilig et al. 2008a, b). Further research is needed to elucidate how the phenomenon of autoregulation during AM symbiosis is regulated and what are the chemical molecules involved (López-Ráez and Pozo 2013).

Beneficial root-inhabiting microbes, such as AM fungi, also hijack the hormone-regulated immune signaling network to establish a prolonged mutualistic association (Pieterse et al. 2012). The concept that MIR is partially determined by resistance-inducing bacteria in the mycorrhizosphere creates a novel impetus to explore the complexity of biotic interactions and chemical signals surrounding mycorrhizal roots (Cameron et al. 2013). Although the molecular basis for the regulation of plant defenses and the priming of the plant immune system during mycorrhization remains mostly unknown (Conrath 2011), a prominent role of jasmonate signaling has been confirmed (Jung et al. 2012). However, the exact contribution of jasmonates in MIR remains unclear (Hause et al. 2007), and the long-distance signals controlling MIR remain to be resolved (Cameron et al. 2013). The same authors presented, in the same article, a novel model of MIR that integrates different aspects of the induced resistance phenomenon. They propose that MIR is a cumulative effect of direct plant responses to mycorrhizal infection and indirect immune responses to ISR-eliciting rhizobacteria in the mycorrhizosphere, but this novel model requires experimental validation, because our knowledge only has demonstrated MIR under strictly axenic conditions (Gallou et al. 2011).

An open question is still why infection by AM fungi does not elicit a defense response in roots (Bapaume and Reinhardt 2012). In general, recognition of a friend versus foe is still incompletely understood in plants (Nishiguchi et al. 2008). The same authors concluded that many of these analyses regarding cultivar-dependent and culture-independent host resistance have been performed in *Arabidopsis*, which does not establish mutualistic associations with nitrogen-fixing rhizobia or with phosphate-acquiring mycorrhizal fungi. The AM fungal cell walls consist mainly of chitin; the perception of chitin fragments by plants could be expected to trigger a defense response that could block symbiosis (Bapaume and Reinhardt 2012).

It is possible that the processes of AM fungal establishment rather than resulting in the constitutive expression of defense, which is costly for the plant, enhance the plant's ability to activate defense mechanisms more efficiently when under attack (Pozo et al. 2009), such as MIR. MIR is a low-cost type of induced resistance which may be among the reasons to explain why root associations with AM fungi have been conserved during evolution and are widespread among species (Pozo et al. 2009).

5 Conclusion and Future Perspective

From the evolutionary point of view, plants developed a latent defense system that can be activated, with the goal of saving energy, contrary to the constitutive resistance that represents a real cost for the plant, since independently of the pathogen presence the plant invests its limited resources in the production of these defense factors. Thus, the induced resistance (IR) under natural conditions will represent cost only in the pathogen presence.

In this context, the mycorrhiza-induced resistance (MIR) has positive effect from the ecological point view, because MIR is a low-cost type of IR. The negative effects on plant productivity usually occur when chemical inducers are used repeatedly or in higher doses, mainly in the absence of the pathogen. We need increased efforts in research to understand the physiological, biochemical, and molecular mechanisms of MIR that will provide the "Rosetta stone" for the development of low-cost and new strategies for crop protection.

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Response of PGPR and AM Fungi Toward Growth and Secondary Metabolite Production in Medicinal and Aromatic Plants

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Abstract Plant growth-promoting rhizobacteria (PGPRs) are a group of naturally occurring beneficial soil bacteria that colonize with the plant root system and promote growth by triggering the production of growth-regulating substances and facilitate the plants in the uptake of essential nutrients from the surrounding environments. Similarly, arbuscular mycorrhizal (AM) fungi also enhanced the growth, water and nutrient uptake, and especially available phosphate through their specialized hyphae. In addition, PGPR and AM fungi are known to stimulate the accumulation of secondary metabolites in plants. For several years, they are commonly employed to increase the plant yield and productivity especially in agricultural practices. The medicinal and aromatic plants are gaining popularity worldwide due to high therapeutic properties with negligible toxic side effects. To fulfill the global demand and supply gap for medicinal and aromatic plants and their products, farmers are encouraged to cultivate these plants on a large scale. However, there is a need to understand and implement a better cultivation practices in order to improve the quality of medicinal and aromatic plants. In this regard, the utilization of PGPRs and AM fungi as biofertilizers instead of chemical fertilizers could be a promising approach to the development of medicinal and aromatic plants under the sustainable production system. The aim of this chapter is to describe the potentiality of PGPRs and AM fungi to improve growth and development of medicinal and aromatic plants and accumulation of secondary metabolites having high therapeutic worth and also pave a way in the development of new biotechnological products as biofertilizers.

Keywords Plant metabolites • Rhizobacteria • Bioinoculants • Crop productivity • Signal molecules

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1 Introduction

Medicinal and aromatic plants (MAPs) are the chief component of the flora, which provides biologically active phytochemicals used in pharmaceuticals, cosmetics, fragrance, flavor, and perfumery industry (Swamy et al. 2012; Efferth and Greten 2012; Mohanty et al. 2015). They are well utilized in various traditional medicinal practices by the indigenous peoples all over the world for treating various human ailments (Akhtar et al. 2014; Swamy and Sinniah 2015). At present synthetic drug research has much progressed toward the development of new molecules for treating various human health problems. The potential of plant metabolites are better exploited considerably using biotechnology to make new chemical drugs having greater biological activities (Gandhi et al. 2015). However, medicinal plants and their products are still considered as the major sources of medicine to treat various health problems. Throughout the world, many pharmaceutical industries are screening natural products based on plants collected mainly from developing countries which are located in tropical and subtropical regions of the globe where rich biodiversity exists. Currently, more popularity is gained by medicinal and aromatic plants because of their wide application in the preparation of various medicaments and manyfold increase in the interest toward phytotherapy research and its biodynamic studies. This is due to the fact that plant-based drugs being more efficient are very safe and cause no side effects during the treatment of various common diseases and a number of chronic illnesses (Canter 2005; Akhtar et al. 2014; Mohanty et al. 2014). The presence of various phytochemicals, referred as secondary metabolites such as phenolics, tannins, flavonoids, alkaloids, and essential oils in different compositions, is said to impart biological activities to medicinal and aromatic plants (Okigbo et al. 2009; Swamy et al. 2011; Akhtar et al. 2014; Gezahegn et al. 2015). Moreover, occurrence of few other chemical constituents can also either mediate to check the possible detrimental adverse cause of the main active ingredient or aid in the absorption of the chemical ingredient. Nowadays, many medicinal and aromatic plants are medically exploited by employing their several secondary metabolites simultaneously against single or multiple target sites which are related to physiological route (Gandhi et al. 2015).

Based on the traditional knowledge of medicinal practices, about 25 % of the modern medicines are mainly derived from MAPs (Sucher and Carles 2008; Kosalge and Fursule 2009; Sudipta et al. 2011; Pan et al. 2013; Swamy et al. 2015). Traditional system of medicine is very well practiced, in South Asia, Africa, and Europe. Traditional systems of medicine make use of several thousands of plant species to cure stomach ulcers, malaria, and many more diseases in China, India, and many other countries in South and East Asia. Ayurvedic system of medicine dates back to 5000 BC in China, India, and many other countries in South and East Asia (Swamy and Sinniah 2015). In these countries, there is a continuous support from the public to protect and promote cultural and spiritual values of traditional medical systems. About 90 % of the ingredients exploited in medicinal practices such as Ayurveda, Unani, Siddha, and homeopathy medicines are plant based, and approximately 25 %

of modern allopathic medicines constitute herbal sources. It has been also reported that about 80 % of the world's populations utilize plant-based traditional medicine to fulfill their primary healthcare needs (Swamy et al. 2011; Pan et al. 2013; Sudipta et al. 2014; Gezahegn et al. 2015; Swamy et al. 2015). More than 65 % of the rural population in India still uses traditional herbal preparation to treat various health problems. Over 1600 flowering plant species are employed in the preparation of herbal medicine in India, and the majority (90 %) of these species occur in the alpine and subalpine region of Himalaya (Uniyal et al. 2002; Raut and Karuppayil 2014). More than 9000 native plants have been identified and recorded for their curative properties, and about 1500 species are known for their aroma and flavor. In 2006, the demand for plant-based drugs in the global market was about US$ 20 billion. An approximate estimation for camptothecin (an anticancer drug) market globally was about US$ 1,000 million and predicted that plant derived drug market share to increase up to US$ 5 trillion by 2050 (Pan et al. 2013; Kaushik et al 2015).

Aromatic plants have odorous volatile essential oils, gum exudates, balsam, and oleoresins in various plant parts such as roots, woods, barks, stems, foliages, flowers, and fruits. Several spices are considered under aromatic plants as they possess a characteristic fragrance in whole, in broken, or in ground forms which adds pleasure to consume foods and beverages (Chandarana et al. 2005; Djilani and Dicko 2012). The occurrence of different chemical substances adds characteristic aroma to the essential oil. There is a rapid growth in the world demand for essential oil and natural aroma chemicals used in drug synthesis, food flavoring, fragrances, perfumes, cosmetics, and related products. Today around 3000 types of plant essential oils are traded (about 40,000–60,000 tons/annum), and their commercial market value is estimated to be US$ 700 million indicating huge global consumption of essential oils (Djilani and Dicko 2012; Raut and Karuppayil 2014). India is one of the rich countries having very rich repository of aromatic plant species with global importance. Since, engagement of science toward multipurpose utilization of MAPs is causing a threat to species abundance as well as local extinction (Aneesh et al. 2009).

As global demand is increasing, medicinal and aromatic plant population are depleting due to irrational collection and overexploitation of the flora. The rural populations of South Asian countries collect unscientifically and trade these medicinal and aromatic plants to make their income. It is observed that from a single district of Pithoragarh in Uttarakhand state of India, people collect and trade more than 1300 tons of MAPs and most of them are done illegally. These factors make these wild species to become extinct or endangered (Amujoyegbe et al. 2012). Therefore, proper utilization of the medicinal and aromatic plant plays an important role in conserving floral species and improving biodiversity in the region. The governments of many developing nations have initiated many programs and regulations to conserve the threatened plant species in their natural habitats. Farmers are also encouraged to cultivate economically important medicinal and aromatic plants to meet the global supply of raw materials. This will improve the financial stability among the rural population and indirectly maintains environmental sustainability (Kala et al. 2006; Kala 2009).

Small-scale cultivation of MAPs requires less financial inputs, but can generate income by selling at local markets. However, when its cultivation is domesticated in various farming practices, it can offer extra income to the rural family. Growing medicinal plant in home gardens is increasingly encouraged to make use of plant sources to cure very common health problems (Kumar and Nair 2004). On the other hand, large-scale cultivation of medicinal and aromatic plants depends on various factors such as selection of suitable seeds or planting materials and good cultivation practices, and management strategies such as irrigation, fertilization, and control against weeds, pests, and diseases should be adopted to obtain a better yield and good quality of the raw material. Harvesting, processing, and commercialization of processes also contribute to product yield. Therefore, large-scale cultivation of medicinal and aromatic plants requires much more attention in order to meet the global pharmaceutical demand for high-quality raw materials (Lubbe and Verpoorte 2011).

Plant-based supply of raw materials for pharmaceutical industries prefers cultivated plant materials than a wild collection because it has less chemical variation and the supply could be easily controlled. Likewise, a major problem like contamination or adulteration of materials can also be completely eliminated, and its quality remains the same as their cultivation is monitored under growing conditions. However, large-scale cultivation is hampered due to the supply of platelets, which are usually more expensive, and requires more initial investments. Moreover, most of the plant species are difficult to cultivate under different growing conditions (Schippmann et al. 2006; Lubbe and Verpoorte 2011). Even then, cultivation offers to modify some of the biologically valuable plant metabolites by manipulating the growing conditions or using conventional breeding techniques or the latest molecular biological tools and biotechnological approaches. Therefore, it is essential to improve or adopt various cultivation practices in order to produce plant-based products to meet the supply demand from the pharmaceutical industries around the world. Large-scale propagation to obtain higher yield in MAPs can be achieved by applying chemical fertilizers in farming. However, these chemicals are expensive and cause environmental pollution despite diminishing soil health and its productivity. Therefore, this necessitates adopting sustainable agricultural practices such as organic farming or makes use of PGPRs as biofertilizers to improve plant growth and development. One of the biotechnological approaches to improve the quality and yield of MAPs is by employing PGPRs or AM fungi in the cultivation practices (Malusa et al. 2012). The aim of this chapter is to describe the potentiality of PGPRs and AM fungi to improve growth and development of MAPs and accumulation of secondary metabolites having high therapeutic worth and also pave a way in the development of new biotechnological products as biofertilizers.

2 Interaction of PGPR with MAPs

PGPRs are a group of naturally occurring beneficial soil bacteria which are known to colonize with a plant root system to promote growth and development by triggering the production of growth hormones as well as facilitating an efficient uptake of

nutrients from the surrounding and release inhibitor compounds against pathogens. There are various reports on the positive synergistic effect of PGPRs and nitrogen-fixing bacteria on improvement of plant growth and yield (Jahanian et al. 2012; Ipek et al. 2014). The presence of various chemical compounds in root exudates such as proteins and polysaccharides facilitates the bacterial colonization with plant roots (Rodriguez-Navarro et al. 2007; Compant et al. 2010). PGPRs greatly influence on the plant physiology, in particular compete to colonize the roots (Barriuso et al. 2008; Akhtar and Siddiqui 2010; Akhtar et al. 2010). The secretion of root exudates by the plants directly or indirectly helps recycling of nutrients in the soil and controls the occurrence of diseases. For instance, secretions of flavonoids from leguminous plants may stimulate the growth of nitrogen-fixing bacteria and root nodule formation. However, it may also act as allelochemicals (Solaiman and Anawar 2015). Some of the PGPRs found in the rhizosphere, rhizoplane, associated with roots cortex include *Azotobacter*, *Caulobacter*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Chromobacterium*, *Agrobacterium*, *Mesorhizobium*, and *Frankia* species (Gray and Smith 2005; Solaiman and Anawar 2015).

Plants inoculated with PGPRs are benefited with increased vigor indexes such as germination rate, dry matter production, radical growth, leaf area, chlorophyll content, disease control, drought resistance, shoot weight, and other microbial activities (Lucy et al. 2004; Akhtar and Siddiqui 2008a,b; Akhtar and Panwar 2013; Akhtar and Azam 2014). Presently, PGPRs are widely utilized to improve plant growth in various fields, including agriculture, horticulture, forestry, environmental restoration, and more recently in the cultivation of MAPs. The application of many PGPRs belonging to different genera has been commercialized for agricultural usages. The growing problems of MAPs on a large scale can be solved by identifying and selecting appropriate beneficial microbes to be used as biofertilizers which improves plant growth without harming the environment (Ipek et al. 2014). Moreover, PGPRs can enhance the production of biologically active secondary metabolites in MAPs. In recent years, more research works are emphasized toward the application of PGPRs in the cultivation of MAPs to improve plant productivity (Karthikeyan et al. 2013).

3 Interaction of AM Fungi with MAPs

AM fungi have the ability to enhance the plant growth and productivity. AM fungi are known to improve the nutrient and water uptake (Gosling et al. 2006; Akhtar and Siddiqui 2008c Akhtar and Panwar 2011; Vafadar et al. 2014). Another important advantage of AM fungi is that they benefit the plants by acting as a connection between plants and rhizosphere and scavenge the available phosphate from the soil through their hyphae (Liu et al. 2000). It is reported that more than 80 % of land plant species are associated with AM fungi which helps plants to utilize phosphate from the soil (Akhtar et al. 2011; Akhtar and Abdullah 2014; Solaiman and Anawar 2015). Similarly, AM fungi when inoculated to MAPs have

shown to improve plant growth by increasing soil fertility and photosynthesis (Antunes et al. 2006; Charles et al. 2006; Vafadar et al. 2014). Likewise, AM fungi can also increase plant productivity with higher accumulation of valuable secondary metabolites (polyphenols, terpenoids, alkaloids, flavonoids, phytosterols, stilbenes, vitamins, lignans, etc.) which are beneficial for human heath (Nell et al. 2010; Zitterl-Eglseer et al. 2015). AM fungi leach compounds which play an important role in providing tolerance to plants against abiotic and biotic stresses (Gosling et al. 2006; Gianinazzi et al. 2010; Akhtar et al. 2015).

4 Impact of PGPRs and AM Fungi with MAPs: An Insight Approach

4.1 Effect of PGPRs and AM Fungi on the Growth and Development of MAPs

The plant roots provide anchorage, nutrient absorption, and water uptake and also involved in the various types of biochemical interactions taking place at belowground level. The rhizosphere is very complex in nature, and various microorganisms present in this region form with the plant root system (Mukerji et al. 2006; Badri et al. 2009; Yadav et al. 2015). This codependent benefit improved the plant growth and development through various complex phenomenons like fixing atmospheric N_2, increasing the surface area of root, and building other useful symbiotic relationships with the host plant (Vessey 2003). The direct effects of PGPR include the secretion of plant growth promoters (indoleacetic acid, cytokinins, gibberellic acid, and ethylene), fixing of atmospheric nitrogen through the symbiotic association to form root nodules, and helping in the solubilization of phosphate in the soil. In an indirect way, PGPRs reduce or completely prevent the possible harmful effect of one or more plant pathogens by competing with nutrients, produce siderophore, and induce resistance in the plant (Stefan et al. 2012). It is observed that PGPRs promote plant growth and development through high yield, enhanced chlorophyll, and other enzyme activities (Mia et al. 2010; Akhtar and Panwar 2013). The plants and microbes have adopted various strategies, for example, secreting organic acids and phosphatases into the rhizosphere soil, thereby helping in mineralization of organic phosphate. The symbiotic association of rhizobacterial strains and mycorrhizal fungi increases P uptake (Smith and Read 2008), and therefore these PGPRs can be applied in the cultivation of MAPs to increase the availability of phosphate as well as its uptake (Solaiman and Anawar 2015). Currently, many investigations on the plant improvement using rhizospheric microbes and their mechanisms involved are ever-increasing rapidly for the selection of the best bacterial strain to be used as a biofertilizer commercially (Bhattacharyya and Jha 2012; Ipek et al. 2014; Solaiman and Anawar 2015). These efforts are also observed in medicinal and aromatic plants as they are the key source for herbal medicine, and presently many research reports have confirmed the significantly increased

production of some of the industrially important secondary metabolites by rhizobacterial inoculation (Koeberl et al. 2013). The influence of PGPRs and AM fungi in increasing the growth and development of the medicinal and aromatic plants is summarized in Table 1.

The medicinal plants associate with various rhizospheric microbes and hence are required to isolate, characterize, and explore their use in formulating an eco-friendly biofertilizer or as biocontrol invent (Vasudha et al. 2013). Arora et al. (2001) isolated the soil root nodule-forming bacteria, *Rhizobium meliloti* from *Mucuna pruriens*, an important medicinal plant, and the bacteria improved the plant growth by producing siderophores. According to Giri et al. (2003), application of AM fungi (*G. fasciculatum* and *G. macrocarpum*) either alone or in combination enhanced the plant growth and nutrient uptake of *Acacia auriculiformis.* However, the study on the effect of *G. mosseae* on *Atractylodes lancea* showed a significant increase in plant height, number of leaves, leaf area and plant biomass, and NPK concentrations in AM fungi-inoculated plants compared to control treatments (Guo et al. 2006). Similarly, in the Chinese medicinal tree, *Camptotheca acuminata* (known for anticancer compound, camptothecin), it was found that seedlings inoculated with *G. diaphanum*, *G. manihot*, *G. etunicatum*, *G. versiforme*, *Acaulospora laevis*, and *A. mellea* significantly increased the growth and uptake of N and P through alteration in physiological attributes (Zhao and Yan 2006).

An experiment on the effect of PGPRs on growth parameters and the production of ajmalicine in *Catharanthus roseus* clearly indicated that *P. fluorescens* had a significant influence on the improvement of plant growth, number of leaves, and fresh and dry weights and root length under drought stress (Jaleel et al. 2007). Application of biofertilizer (*Azospirillum brasilienses*, *A. chroococcum*, *Bacillus polymyxa*, and *B. circulans*) on the plant growth, yield, and essential oil composition of *Majorana hortensis* L. revealed that the treatment of biofertilizers along with compost developed a better plant growth traits, yield, as well as essential oil composition when compared to control or chemical fertilizers (Gharib et al. 2008). Similarly, inoculation of *G. mosseae* significantly improved shoot weight and root weights up to 60 % in *Ocimum basilicum* (Toussaint et al. 2008). However, there was an existence of a close relationship between the medicinal plant, *Agathosma betulina*, and the yeast, *Cryptococcus laurentii*. This association was symbiotic and significantly improved the plant growth in nutrients deficit soil (Cloete et al. 2009; 2010).

Nevertheless, Banchio et al. (2008) observed the effect of root-colonizing PGPRs on *Origanum majorana* biomass, quality, and quantity and stated that among the tested PGPRs only *Bradyrhizobium* sp. and *P. fluorescens* significantly increased all the growth parameters such as shoot weight, shoot length, nodal number, leaf number, and dry weight of root when compared to control plants. Similarly, the promontory effects of mycorrhizal inoculation (*Acaulospora bireticulata*, *A. scrobiculata*, *Gigaspora margarita*, *G. aggregatum*, *G. mosseae*, *G. geosporum*, *Scutellospora heterogama*) were also reported in *Plectranthus amboinicus* (Rajeshkumar et al. 2008). They concluded that the shoot height and root biomass of *P. amboinicus* were improved by colonization of roots by AM fungi, which may increase nutrient availability to the plant. There was

Table 1 Effect of rhizospheric microorganisms on the growth and development of medicinal and aromatic plants

Medicinal and aromatic plants	Rhizospheric microorganisms	Growth promoting response	References
Mucuna pruriens	*Rhizobium meliloti*	Improved plant growth by producing siderophores	Arora et al. (2001)
Acacia auriculiformis	*G. fasciculatum*, *G. macrocarpum*	Increased the net photosynthesis though the production of growth promoting substances	Giri et al. (2003)
Atractylodes lancea	*G. mosseae*	Significantly improved plant length and biomass by improved nutrient uptake	Guo et al. (2006)
Camptotheca acuminata	*G. diaphanum*, *G. manihot*, *G. etunicatum*, *G. versiforme*, *Acaulospora laevis*, *A. mellea*	Significantly increased the plant growth, nitrogen, and phosphorus uptake through physiological changes	Zhao and Yan (2006)
Catharanthus roseus	*Pseudomonas fluorescens*	Significantly increased the plant growth, number of leaves, fresh and dry biomass of plant	Jaleel et al. (2007)
Origanum majorana	*Bradyrhizobium* sp., *P. fluorescens*	Significantly increased shoot and root dry weight, nodal segments, and leaf numbers	Banchio et al. (2008)
Majorana hortensis	*Azospirillum brasilienses*, *A. chroococcum*, *Bacillus polymyxa*, *B. circulans*	Enhanced plant growth and total yield	Gharib et al. (2008)
Plectranthus amboinicus	*A. bireticulata*, *A. scrobiculata*, *Gigaspora margarita*, *G. aggregatum*, *G. mosseae*, *G. geosporum*, *Scutellospora heterogama* *P. amboinicus*	Increased nutrient availability to the plant	Rajeshkumar et al. (2008)
Ocimum basilicum	*G. mosseae*	Improved the shoot and root weight up to 60 % compared to control treatment	Toussaint et al. (2008)
Agathosma betulina	*Cryptococcus laurentii*	Significantly improved the plant growth in soil with poor nutrients	Cloete et al. (2009)
Clitoria ternatea *Plumbago zeylanica* *Psoralea corylifolia*	*G. intraradices*, *G. mosseae*, *A. scrobiculata*	Enhanced the dry matter yield of plants from 0.19–422.0 % in different soils	Chandra et al. (2010)
Salvia officinalis	*G. intraradices*	Improved plant growth and dry biomass	Geneva et al. (2010)
Pelargonium graveolens	*B. subtilis*, *P. fluorescens*	Significantly enhanced the plant growth and biomass	Mishra et al. (2010)

Medicinal and aromatic plants	Rhizospheric microorganisms	Growth promoting response	References
Piper longum	*G. fasciculatum*, *G. etunicatum* *G. clarum*, *G. mosseae*, *Glomus* sp., *G. versiforme*	Treatment with various AM fungi increased he plant growth, biomass, and uptake of phosphorus and potassium contents	Gogoi and Singh (2011)
Ocimum basilicum	*Pseudomonas* sp., *B. lentus*	Significantly increased proline, total carbohydrate, chlorophyll contents under water stress	Heidari et al. (2011)
Solanum viarum	*Bacillus coagulans*, *G. aggregatum*, *Trichoderma harzianum*	Greatly improved plant height and dry weight	Hemashenpagam and Selvaraj (2011)
Mentha viridis, *Origanum onites*	*Glomus etunicatum*, *G. lamellosum*	Increased plant growth due to improved nutrient absorption from the soil	Karagiannidisa et al. (2011)
Angelica dahurica	*G. claroideum*, *G. intraradices*	Promoted plant growth and biomass	Zhao and He (2011)
Stevia rebaudiana	*Burkholderia gladioli*, *B. gladioli*, *Enterobacter aerogenes*, *Serratia marcescens*	Improved the plant growth	Gupta et al. (2011)
Coleus forskohlii	*G. fasciculatum*, *Achromobacter xylosoxidans*, *A. lipoferum*	Increased the plant growth	Sakthivel and Karthikeyan (2012)
Pogostemon cablin	*Azotobacter chroococcum*, *G. intraradices*, *P. fluorescens*	Improved shoot and root biomass	Singh et al. (2012)
Plectranthus tenuiflorus	*B. megaterium*, *B. pumilus*, *B. licheniformis*, *Paenibacillus* sp., *Pseudomonas* sp.,	Improved the plant growth	El-Deeb et al. (2013)
Hyoscyamus niger	*P. putida*, *P. fluorescens*	Increase the plant growth and vigor index	Ghorbanpour et al. (2013)
Acorus calamus, *Aloe vera*, *Andrographis paniculata* *Aquilegia vulgaris*, *Mimosa pudica*, *Ocimum sanctum*, *Tagetes erecta*, *Withania somnifera*	*Pantoea* sp., *Bacillus* sp., *Pseudomonas* sp.	Enhanced germination rate, plant growth, and vigor index of all the tested medicinal and aromatic plants	Malleswari and Bagyanarayana (2013)

(continued)

Table 1 (continued)

Medicinal and aromatic plants	Rhizospheric microorganisms	Growth promoting response	References
Bacopa monnieri	*B. pumilus*, *Exiguobacterium oxidotolerans*	The plants performed well in terms of growth as well as yield attributes	Murugappan et al. (2013)
Coleus forskohlii	*G. fasciculatum* *P. monteilii*	Considerably increased plant height	Singh et al. (2013)
Panax ginseng	*Streptomyces pactum*	Improved the plant yield	Zhang et al. (2013)
Stevia rebaudiana	*Glomus*, *Bacillus*, *Azotobacte*r, and *Pseudomonas* sp.	Improved the plant growth	Vafadar et al. (2014)

an increased dry matter of the medicinal plants such as *Abelmoschus moschatus*, *Clitoria ternatea*, *Plumbago zeylanica*, *Psoralea corylifolia*, and *Withania somnifera* when they were inoculated with mycorrhizal fungi (Chandra et al. 2010). They found that inoculation of *G. intraradices*, *G. mosseae*, and *Acaulospora scrobiculata* produced higher dry matter in all the plant species investigated. The dependency of plants on the AM fungi was in the following order: *W. somnifera* > *P. corylifolia* > *P. cerneatea* > *P. zeylanica* > *A. moschatus*. Similarly, Nisha and Rajeshkumar (2010) have reported that inoculation of *A. delicata*, *G. aggregatum*, *G. feugianum*, *G. fasciculatum*, *G. rubiforme*, *G. margarita*, and *Scutellospora heterogama* significantly stimulated the growth of *Wedelia chinensis*. There was an increased plant growth, biomass, and nutrition uptake of plants inoculated with fungal strains compared to non-inoculated plants. Among all the tested mycorrhizal fungi, *G. fasciculatum* was found best in improving the total seedling biomass and nutrition uptake. Karagiannidisa et al. (2011) have also reported that when medicinal plants (*Mentha viridis* and *Origanum onites*) are inoculated with *G. etunicatum* and *G. lamellosum*, increase in plant growth due to improved nutrient was observed in mycorrhizal plants compared to non-mycorrhizal plants.

In an interesting experiment, Mishra et al. (2010) have reported that the production of ammonia by the rhizobacterial strains (*B. subtilis* and *P. fluorescens*) isolated from an aromatic herb *Pelargonium graveolens* L. Herit. significantly increased the plant growth and biomass. Gogoi and Singh (2011) observed that inoculation of all the tested AM fungi, namely, *G. fasciculatum*, *G. versiforme*, *G. clarum*, *Glomus* sp., *G. mosseae*, and *G. etunicatum* appreciably improved the biomass and nutrient uptake of *Piper longum* L. compared to control. However, the results of Zhao and He (2011) showed that inoculation of *G. claroideum* (BEG 180), *G. intraradices* (BEG 193), and native AM fungi to *Angelica dahurica* plants promoted the plant growth and biomass. A field experiment was conducted by Heidari et al. (2011) to study the effect of PGPRs on physiology and uptake of minerals (proline, carbohydrates, chlorophyll, and mineral contents) in *Ocimum basilicum* L. under water stress. They concluded that *Pseudomonas* sp. and *B. lentus* significantly increased proline, total carbohydrate, chlorophyll, and nutrient uptake under water stress. Gupta et al. (2011) evaluated the prospective uses of phosphate-solubilizing bacterial strains such as *Burkholderia gladioli*, *B. gladioli*, *Enterobacter aerogenes*, and *Serratia marcescens* to utilize Mussoorie rock phosphate and to improve the growth of *Stevia rebaudiana* and secondary metabolites (stevioside and rebaudioside A). They concluded that a significant increase in plant growth and metabolite production (stevioside and rebaudioside A) was recorded in the plants treated with combination of bacterial strains grown in Mussoorie rock phosphate amended soil compared to unamended soil.

Hemashenpagam and Selvaraj (2011) studied the effect of *G. aggregatum*, *B. coagulans*, and *Trichoderma harzianum* on growth, nutrition, and secondary metabolite production in the seedlings of *Solanum viarum*. The results showed that inoculation of these microbes significantly improved the plant fresh and dry biomass. Likewise, maximum root colonization and higher P, Zn, K, Mn, Cu, and Fe contents of a leaf were observed in the plants treated with *G. aggregatum*, *B. coagulans*, and *T. harzianum*. However, Arun et al. (2012) stated that the application of PGPRs significantly

augmented the growth of *Cassia occidentalis* and could also be used as an alternative to chemical fertilization to obtain a better agricultural crop yield. Similarly, Sakthivel and Karthikeyan (2012) have reported the increased plant height due to inoculation of *G. fasciculatum* and rhizobacterial strains in *Coleus forskohlii*. Moreover, Singh et al. (2012) revealed the improvement in shoot and root biomass of nursery cuttings of *Pogostemon cablin* Benth., treated with bioinoculants (*A. chroococcum*, *G. intraradices*, and *P. fluorescens*). In another study, Singh et al. (2013) have also found that the inoculation with *G. fasciculatum* and *P. monteilii* significantly increased tuber yields by enhancing nutrient uptake in the inoculated plant compared to control under field conditions. Malleswari and Bagyanarayana (2013) isolated a total of 219 isolates from various MAPs (*Coleus forskohlii*, *Withania somnifera*, *Ocimum sanctum*, *Andrographis paniculata*, *Mentha spicata*, *Aloe vera*, *Tagetes erecta*, *Artemisia vulgaris*, *Acorus calamus*, and *Mimosa pudica*) and screened all these isolates for their plant growth-promoting traits, production of ammonia, indoleacetic acid, hydrogen cyanide, phosphate solubilization, and antifungal properties. They concluded that out of 219 isolates, 201 isolates produced ammonia, 186 isolates produced indoleacetic acid, 43 isolates solubilized phosphate, 58 isolates produced hydrogen cyanide, and 43 isolates showed antifungal activity against the pathogen, *Macrophomina phaseolina*. This clearly indicated the multiple rhizobacterial significance on the seed germination under in vitro environment. This allowed the selection of efficient PGPRs such as *Pantoea* sp., *Bacillus* sp., and *Pseudomonas* sp. on the basis of their exhibited traits and enhancement of germination rate, growth of the plant, and its vigor index. Similarly, El-Deeb et al. (2013) have isolated 28 different rhizobacterial strains from different parts of the medicinal herb, *Plectranthus tenuiflorus*. All the isolated rhizobacterial strains, *Bacillus* sp. (*B. megaterium*, *B. pumilus*, *B. licheniformis*), *Paenibacillus* sp., and *Pseudomonas* sp., improved the plant growth and production of a secondary metabolites. However, Murugappan et al. (2013) reported that the application of *B. pumilus* enhanced the growth of *Ocimum sanctum*, while the inoculation of *B. pumilus* and *Exiguobacterium oxidotolerans* to Brahmi (*Bacopa monnieri*) increased the plant growth 26 % and 50 %, respectively, compared to control plants (Bharti et al. 2013). Vafadar et al. (2014) reported that the use of *Glomus* and *Bacillus* or *Azotobacter* and *Pseudomonas* consortium significantly improved the shoot and root biomass, chlorophyll content, and NPK content in *Stevia rebaudiana* Bertoni, which is commonly used as antidiabetic plant.

4.2 Effect of PGPRs on the Production of Secondary Metabolites from MAPs

In the present world, herbal products are gaining more importance as they are the major source of pharmacological and therapeutical uses. The essential oils produced from the aromatic plants are widely used in flavor and fragrance industries. Plants are used in the ancient medicinal practices, and currently, about 80 % of modern medicines are plant based (Swamy et al. 2011; Akhtar et al. 2014). Hence, the

ever-increased demand for plant-based phytochemicals in the modern world has forced the cultivation of MAPs on a large scale by adopting better agricultural practices. There are many reports on the advantageous effect of PGPRs and AM fungi when applied to MAPs. These PGPRs influenced the plant growth, nutrient utilization, and production of various medicinally useful plant chemical metabolites like flavonoids, phenols, saponins, alkaloids, and tannins (Egamberdieva and da Silva 2015). Similarly, AM fungi symbioses with various medicinally important plants enhanced the production of several secondary metabolites useful for treating various human ailments (Zeng et al. 2013; Lingua et al. 2013) (Table 2).

Gupta et al. (2002) reported that the inoculation of *G. fasciculatum* to *Mentha arvensis* increased the plant yield and oil content. Ponce et al. (2004) compared the effect of inoculation of *G. intraradices* on the root and shoot flavonoid contents in *Trifolium repens*. They observed the different flavonoid content from roots and shoots of *T. repens* inoculated with mycorrhizal fungus increased the root colonization of AM fungi, which may alter the plant metabolism to secrete different flavone derivatives such as 4′,5,6,7,8-pentahydroxy-3-methoxyflavone and 5,6,7,8-tetrahyd roxy-3-methoxyflavone and 3,7-dihydroxy-4′-metho-xyflavone. Moreover, the AM fungi-inoculated roots also produced quercetin, rhamnetin, and acacetin. Sailo and Bagyaraj (2005) have reported that inoculation of *G. bagyarajii* and *Scutellospora calospora* to *Coleus forskohlii* increased the accumulation of forskolin content which is widely used in treating glaucoma, heart diseases, asthma, and cancer. According to Khaosaad et al. (2006), root colonization by *G. mosseae* with *Origanum vulgare* has shown no significant effect on the composition of essential oil. Copetta et al. (2006) studied the effects of *G. mosseae*, *Gigaspora margarita*, and *G. rosea* on the yield of essential oil in *Ocimum basilicum* L. var. Genovese. They concluded that among the tested AM fungi, *G. rosea* cause a significant increase in root and shoot biomass, root branching, root length, and the essential oil mainly α-terpineol content compared to other treatments. This improved yield of oil is said to be associated with increased number of glandular trichomes present in the zones of basal and central leaves. Catford et al. (2006) studied the suppression of isoflavonoids and their effects on stimulating nodule formation and mycorrhizal association in split root systems in *Medicago sativa*. They found that inoculation with *Sinorhizobium meliloti* or *G. mosseae* or nod factor altered the secretion of flavonoids (formononetin and ononin). According to Liu et al. (2007), triterpenoid saponin (glycyrrhizic acid) content increased with the mycorrhizal symbiosis in *Glycyrrhiza uralensis*. However, the inoculation of *G. mosseae* in *O. basilicum* had no effect on the phytoconstituents, rosmarinic acids, and caffeic acids or essential oil yield (Toussaint et al. 2008).

In the medicinal plant, *Catharanthus roseus*, the inoculation of *P. fluorescens* has improved the synthesis of alkaloids such as ajmalicine, catharanthine, tabersonine, serpentine, and vindoline and also increased the accumulation of tabersonine, serpentine, and vindoline contents in roots (Jaleel et al. 2007). Morone-Fortunato and Avato (2008) reported that the essential oil content increased when the *G. viscosum* was inoculated with micropropagated plants of *Origanum vulgare* L. However, the colonization of PGPRs improved the qualitative and quantitative composition of

Table 2 Influence of rhizospheric microorganisms in the production of metabolites in medicinal and aromatic plants

Medicinal and aromatic plants	Rhizosphere microbes	Plant metabolites/essential oil components	References
Mentha arvensis	*Glomus fasciculatum*	Increased essential oil contents	Gupta et al. (2002)
Trifolium repens	*G. intraradices*	Increased the flavonoid contents and produced 4,5,6,7,8-pentahydroxy-3-methoxy-flavone and 5,6,7,8-tetrahydroxy-3-methoxyflavone, 3,7-dihydroxy-4′-methoxy-flavone, quercetin, rhamnetin and acacetin	Ponce et al. (2004)
Coleus forskohlii	*G. bagyarajii Scutellospora calospora*	Increased the accumulation of the forskolin in roots	Sailo and Bagyaraj (2005)
Medicago sativa	*Sinorhizobium meliloti* *G. mosseae*	Suppressed the production of formononetin and ononin	Catford et al. (2006)
Ocimum basilicum	*G. mosseae*, *Gigaspora rosea*, *G. margarita*	Significantly increased the essential oil with higher α-terpineol and eugenol contents	Copetta et al. (2006)
Catharanthus roseus	*Pseudomonas fluorescens*	Significantly increased the biosynthesis of alkaloids such as ajmalicine, catharanthine, tabersonine, serpentine, and vindoline	Jaleel et al. (2007)
Origanum vulgare	*G. mosseae*	Significantly increased essential oil production	Khaosaad et al. (2006)
Glycyrrhiza uralensis	*G. mosseae* *G. veriforme*	Increased glycyrrhizic acid accumulation	Liu et al. (2007)
Origanum majorana	*P. fluorescens* *Bradyrhizobium* sp.	Showed increased essential oil yield up to 24-fold and significantly increased terpinene- 4-ol, cis-sabinene hydrate, trans-sabinene hydrate, and a-terpineol	Banchio et al. (2008)
Origanum vulgare	*G. viscosum*	Increased essential oil contents	Morone-Fortunato and Avato (2008)
Ocimum basilicum	*G. mosseae*	No significant effect on the phytoconstituents, rosmarinic acids, and caffeic acids or essential oil yield	Toussaint et al. (2008)
Echinacea purpurea	*G. intraradices*	Significantly increased phenolic concentrations mainly cynarine and cichoric, caftaric, and chlorogenic acids	Araim et al. (2009)
Allium sativum	*G. fasciculatum*	Increased the alliin contents	Borde et al. (2009)
Salvia officinalis	*G. mosseae*, *G.intraradices*	Significantly increased the accumulation of rosmarinic acids	Nell et al. (2009)
Cynara cardunculus	*G.intraradices*, *G. mosseae*	Markedly increased the total phenolic contents	Ceccarelli et al. (2010)

Medicinal and aromatic plants	Rhizosphere microbes	Plant metabolites/essential oil components	References
Salvia officinalis	*G. intraradices*	Significantly accumulated higher 1,8-cineole, bornyl acetate, camphor, α-thujone, and β-thujones contents in the oil	Geneva et al. (2010)
Arnica montana	*G. intraradices*	Increased the concentrations of phenolic acids and sesquiterpene lactone	Jurkiewicz et al. (2010)
Catharanthus roseus	*Bacillus* sp., *Azotobacter* sp., *Pseudomonas* sp.	Significantly increased alkaloid content compared, significantly enhanced the production of antineoplastic alkaloid	Karthikeyan et al. (2010)
Valeriana officinalis	*G. mosseae*, *G. intraradices*	Promoted the secretion of sesquiterpenic acids	Nell et al. (2010)
Inula ensifolia	*G. intraradices*, *G. clarum*	Increased the production of all the thymol derivatives	Zubek et al. (2010)
Solanum viarum	*G. aggregatum*, *B. coagulans*, *T. harzianum*	Improved the accumulation of total phenols, ortho dihydroxy phenols, alkaloids, saponins, flavonoids, and tannins	Hemashenpagam and Selvaraj (2011)
Andrographis paniculata	*Gigaspora albida*	Significantly increased the accumulation of andrographolide	Radhika and Rodrigues (2011)
Catharanthus roseus	*Glomus sp.*	Significantly increased total phenolic compounds and vinblastine contents	Rosa-Mera et al. (2011)
Mentha piperita	*P. fluorescens*, *B. subtilis* *Azospirillum brasilense*	Increased the production of pulegone and menthone	Santoro et al. (2011)
Angelica dahurica	*G. claroideum*, *G. intraradices*	Significant increased total coumarin and imperatorin contents	Zhao and He (2011)
Hypericum perforatum	*Rhizophagus intraradices*, *Funneliformis mosseae*, *F. constrictum* *F. geosporum*	Significantly increased the hypericin and pseudohypericin contents	Zubek et al. (2012)
Bacopa monnieri	*B. pumilus Exiguobacterium oxidotolerans*	Increased the accumulation of the bacoside A content	Bharti et al. (2013)
Hyoscyamus niger	*P. putida* *P. fluorescens*	Produced higher hyoscyamine and scopolamine contents	Ghorbanpour et al. (2013)
Stevia rebaudiana	*P. putida*, *B. polymyxa* *Azotobacter chroococcum*, *G. intraradices*	Significantly increased the production of the stevioside contents	Vafadar et al. (2014)
Angelica archangelica	*G. mosseae*, *G. intraradices* AMF mixture Symbivit^{R}	Markedly increased the monoterpenoids and coumarins	Zitterl-Eglseer et al. (2015)

essential oils in *O. majorana*. Moreover, inoculation of *P. fluorescens* and *Bradyrhizobium* sp. has showed an increase (>1000-fold) in the essential chemical constituents compared to control plants (Banchio et al. 2008). The alliin content of *Allium sativum* increased after inoculation of *G. fasciculatum* under field condition (Borde et al. 2009). According to Nell et al. (2009), there was significant difference in the accumulation of rosmarinic acid in *Salvia officinalis* plant roots. Inoculation of *G. intraradices* has shown higher secretion of phenolics in *Echinacea purpurea* (Araim et al. 2009) and *Arnica montana* (Jurkiewicz et al. 2010). Similarly, Nell et al. (2010) reported that the *G. mosseae* and *G. intraradices* have promoted the secretion of sesquiterpenic acid in *Valeriana officinalis*. Likewise, inoculation of AM fungi (*G. intraradices* and *G. clarum*) increased the production of thymol derivative in *Inula ensifolia* (Zubek et al. 2010). PGPRs such as *Bacillus*, *Azotobacter*, and *Pseudomonas* when inoculated to *Catharanthus roseus* either individually or in combination had significantly increased the plant height, root length, as well as secondary metabolite compared to the non-inoculated control plants and also increased the nutrient (N, P, K, Ca, and Mg) concentration (Karthikeyan et al. 2010). *Salvia officinalis* when inoculated with *G. intraradices* produced higher yield of the essential oil as well as its quality. There was an increased antioxidant metabolite content and significant accumulation of 1,8-cineole, bornyl acetate, camphor, α-thujone, and β-thujone chemical components in the essential oil (Geneva et al. 2010).

From the field and greenhouse experiments, it was observed that the association of mycorrhizal symbiosis with the *Cynara cardunculus* L. alone or in combination markedly increased the total phenolic content and antioxidant property compared to control plants (Ceccarelli et al. 2010). In another study, Meng and He (2011) found the mycorrhizal plant significantly increased the soluble sugar, soluble protein, proline, total nitrogen, and flavonoid contents in both shoots and roots of *Salvia miltiorrhiza* under drought condition. Likewise, Zhao and He (2011) found a significant increase of total coumarin as well as imperatorin content in different plant parts of *Angelica dahurica* inoculated with the AM fungus. The results showed that the plant growth, chlorophyll, carotenoids, soluble sugar, soluble protein, SOD, and POD activity were higher in AM fungus-inoculated plants compared to control treatments. However, the contents of MDA and proline decreased, but inoculation of AM fungi significantly increased the total coumarin and imperatorin content. Radhika and Rodrigues (2011) investigated the influence of different AM fungi on the accumulation of andrographolide (secondary metabolite) in *Andrographis paniculata* and reported that among the tested AM fungi, *Gigaspora albida* caused a significant increase in the concentrations of andrographolide. However, in an interesting experiment, Rosa-Mera et al. (2011) evaluated the effect of AM fungi on the accumulation of antineoplastic alkaloids such as vinblastine in *C. roseus* and confirmed the positive effect on production of total phenolic compounds, total antioxidant activity, and vinblastine contents in the leaves. The production of secondary metabolites such as total phenols, ortho dihydroxy phenols, alkaloids, saponins, flavonoids, and tannins in the leaves and fruits of *Solanum viarum* was found maximum when plants were inoculated with *G. aggregatum*, *B. coagulans*, and *T. harzianum* (Hemashenpagam and Selvaraj 2011).

PGPRs such as *P. fluorescens*, *B. subtilis*, and *Azospirillum brasilense* increased essential oil composition in *Mentha piperita* with the twofold increase of monoterpene production {(+)pulegone and (−)menthone} in *P. fluorescens*-inoculated plants (Santoro et al. 2011). Zubek et al. (2012) stated that the AM fungi has increased the hypericin and pseudohypericin contents significantly in *Hypericum perforatum* used for the treatment of HIV. Tropane alkaloids such as hyoscyamine and scopolamine production in Black henbane (*Hyoscyamus niger*) under water-deficit stress conditions were increased by the application of *P. putida* and *P. fluorescens* (Ghorbanpour et al. 2013). The plants treated with *P. putida* under water stress condition had an increased root area and thus resulted in higher hyoscyamine and scopolamine production. Their results suggest that the plants are stimulated by the inoculation of *P. putida* and *P. fluorescens* to produce more antioxidant enzymes and proline accumulation. However, *B. monnieri* (Brahmi) inoculated with *B. pumilus* and *E. oxidotolerans* increased the yield up to 138 % and bacoside A content up to 376 % higher under saline condition. Under salinity regime, the inoculated plants showed an increased level of proline content and lipid peroxidation. Increased catalase and ascorbate peroxidase activity was also observed in the plants inoculated with PGPRs in comparison to the control plants (Bharti et al. 2013). Recently, Vafadar et al. (2014) found that inoculation of PGPRs (*P. putida*, *B. polymyxa*, and *Azotobacter chroococcum*) and AM fungus (*G. intraradices*) caused a significant increase in chlorophyll contents, nutrient uptake, and production of stevioside contents in tissue culture-raised plantlets of *Stevia rebaudiana* Bertoni. However, Zitterl-Eglseer et al. (2015) reported that monoterpenoid and coumarin contents were increased in two genotypes of *Angelica archangelica*, when inoculated with *G. mosseae* and *G. intraradices* of the AMF mixture Symbivit^{R}.

5 Conclusions and Future Prospects

Exploitation of PGPRs and AM fungi for the cultivation of MAPs is a promising approach. The use of these bioinoculants improved the host physiology, stimulated the accumulation of secondary metabolites and nutrient acquisition, and overall improved the plant health desired for sustainable agricultural practices. Nowadays, more efforts should be made toward maximizing the potential of these bioinoculants for the commercial cultivation of MAPs all over the world. Moreover, the research should focus on identification of novel and useful isolates for understanding the mechanisms of their association with the host plant and biochemical and molecular changes that regulate and stimulate the signaling pathways of molecules to enhance plant secondary metabolites. However, still interaction of these bioinoculants with MAPs is not well understood under different agroecological conditions. Therefore, more attention should be focused on the future studies related to gene expression and their regulatory mechanisms involved in controlling the secretion of these chemical exudates by these microorganisms through genomic as well as proteomic approaches. The mechanisms could reveal the molecular mechanisms of MAPs

responding to various environmental conditions. However, application of biotechnological tools to develop transgenic MAPs by incorporating useful genes from rhizospheric microbes is a trusted area of future research. This approach would enable the commercialization of MAPs under stressed environmental conditions and also improved the production of biologically active metabolites for their therapeutic purposes toward the development of new drugs.

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Interaction Among Rhizospheric Microbes, Soil, and Plant Roots: Influence on Micronutrient Uptake and Bioavailability

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Abstract Soils resulting in micronutrient deficiency in agricultural land and pastureland are increasing globally. Such micronutrient deficiency is due to lower nutrient availability, lower nutrient mobility, and lower capacity of plants to take up nutrients from the rhizosphere. The rhizosphere extends up to a few millimeters from the root surface into the surrounding soil and is rich in microbial activity and diversity. The activity and types of microbes and the soil characteristics influence the uptake and transport of micronutrients in the roots. From the root zone, mobilization of micronutrients in the edible part of plants and their bioavailability is another question. The availability and uptake of various micronutrients in the rhizosphere is again influenced by soil properties and plant root exudates, and depends on microbial interactions with plant roots. The micronutrient transfer dynamics from the microbial cell to the plant cell is also influenced by the physiology of plant–microbe interactions. For diffusion-supplied micronutrients, if a large diffusion gradient exists between the root surfaces and the soil, a large amount could be shipped toward the roots. Conversely, when the capacity of root cells to take up micronutrients exceeds the rate of nutrient replenishment in the root zone, the uptake rate is regulated by nutrient availability rather than the capacity of plant roots to absorb nutrients. Plants exude a wide range of organic compounds and inorganic ions into the rhizosphere, changing the micro-chemical and biological zone of the rhizosphere and enhancing acclimatization or modification toward a

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particular biotic and abiotic environment. Absolute understanding of the multifaceted and intricate interactions dominating the relationship among plants, microbes, and soil that influence the composition of root exudates is still far off. Understanding of the plant–microbe–soil interaction mechanism for the uptake and mobilization of micronutrients and their bioavailability in the edible part of plants will open an avenue in biological science which could help solve the problem of micronutrient deficiency in consumers.

Keywords Bioavailability • Micronutrients • Microbes • Rhizosphere

1 Introduction

The rhizosphere is the region of soil that surrounds plant roots. The root zone acts as a source of excretion of various organic compounds into the sink that is the rhizosphere; therefore, the rhizosphere contains solution-phase, volatile, and gas-phase compounds (Belnap et al. 2003). From the microbial point of view, the rhizosphere is a rich zone of nutrients. The real and active zone of the rhizosphere is not defined, but it ranges up to few millimeters and depends on the type of soil and roots. The study of microbes in the rhizosphere and their interaction with plant roots is not a simple task compared with the study of microbes and microbial communication in bulk soil. A multifaceted microbial community could be present on soil mass, in the form of a biofilm, or on a segment of the exterior or interior of a root hair, where border is difficult or complicated to define (Belnap et al. 2003).

On the other hand, physically removing microbes from soil aggregate is not easy, and it is extremely difficult, in particular, to remove them from intact rhizosphere. Modern improved and sophisticated molecular biology tools to characterize and categorize soil microbes have resulted in our jumping from beyond merely culturing soil microorganisms to the study of their community interaction and diversity. Plant types may also play an important role in determining the bacterial and fungal community structure in the rhizosphere and in bulk soil (Yang et al. 2001). The microbial community of a plant is influenced by the plant age, genotype, and phenotypic characters, abiotic and biotic stress (Smith et al. 1999), the nutrient status of the plant (Yang and Crowley 2000), physiological abnormalities due to pathogen infection (Yang et al. 2001), and colonization by arbuscular mycorrhizal (AM) fungi (Gadkar et al. 2001). In the root systems, the microorganism population can vary in the various zones (Yang and Crowley 2000), and the microbial community varies as the root elongates and enters the bulk soil (Marilley and Aragno 1999). The region of root elongation has been reported to harbor the largest number of microbes (Jaeger et al. 1999).

The rhizosphere is very complex and it also supports a varied microbial community. The effect of microorganisms in the rhizosphere is habitually mutualistic. Therefore, it will be more ecologically significant and important to understand and

study microbes at the community level. The characterization of the microbial community and its structure is generally related to those microbes which we know best, such as plant-growth-promoting microbes (PGPMs) such as members of *Azotobacter*, pseudomonads (Noori and Saud 2012), phosphate solubilizers (Kumar and Narula 1999; Yazdani et al. 2009), or AM fungi (Sabannavar and Lakshman 2009). The microbial community diversity and beneficial aspects have been studied mainly in economically important agricultural crops; namely, wheat, rice, maize, oilseeds, vegetables, and medicinal and aromatic plants (Solanki et al. 2011; Kumar et al. 2013).

2 Micronutrient Uptake

Micronutrients are essential for plant growth and production; they also play a vital and significant role in balanced crop nutrition and physiological functions. The common micronutrients which are important for overall plant growth and metabolic activities are iron, copper, zinc, boron, nickel, manganese, molybdenum, and chloride. Although plants do not require as much micronutrients, or trace nutrients, they are as necessary for plant nutrition as primary and secondary nutrients are. Deficiency of any one of the micronutrients in the soil could check plant growth and production, even if all other macronutrients or micronutrients are present in sufficient quantity (Yu and Renegal 1999). Most of the soils in world are deficient in micronutrients for many reasons. Some reasons limiting the incidental addition of micronutrients include harvesting of micronutrients from the soil by the growing of high-yield crops, increased use of NPK fertilizers containing lesser amounts of micronutrients, developments in fertilizer technology decreasing the residual addition of micronutrients, less use of organic matter, and the desire to achieve bumper production.

On the other hand, deficiency of important micronutrients such as iron and zinc leads to brain development impairment and wound healing and the person becomes immune compromised to common infectious diseases such as pneumonia, diarrhea, and malaria (Prasad 2013). Moreover, iron deficiency causes poor physical development, poor learning ability, and poor intellectual maturity among children, and leads to anemia and poor reproductivity (Black 2003). Mostly, the zinc and iron deficiencies are caused by a diet deficient in micronutrients or their nonbioavailability (Welch and Graham 2004).

3 Micronutrient Uptake Mechanism

The active root exudates of plants are able to decide and establish the microbial organization on the root by repressing or stimulating a particular type of microbial community (Doornbos et al. 2012). Kamilova et al. (2006) studied the root exudates

of seed, seedlings, and roots of tomato (*Lycopersicon esculentum*), cucumber (*Cucumis sativus*), and sweet pepper (*Capsicum annuum*), and reported that the major organic acids were citric acid, succinic acid, and malic acid. In vitro growth of rhizobacteria on citric acid as the sole carbon source seems to be associated with the root-establishing capability. This suggests that a particular plant species can choose a particular microbe by producing explicit and particular root exudates. Berg and Smalla (2009) found using stable isotope probing techniques that for plants grown under $^{13}CO_2$, bacteria could assimilate the root exudates. Low molecular weight phytochemicals such as organic acids, phenolics, amino acids, sugars, and other secondary metabolites form the bulk of root exudates and can protect the plant from invasion by pathogens. Generally, root exudates such as phenolics and terpenoids have strong external antimicrobial qualities (Bais et al. 2006). Phenolic metabolites produced by root exudates also function proficiently in attracting some rhizospheric and bulk soil microbes and can successfully manipulate the resident soil microbial population (Brimecombe et al. 2001).

Therefore, these micronutrients are imperative for usual and natural functioning of plant metabolism, productivity, seed viability, chlorophyll synthesis, fruit development, and nutrient and water uptake and transportation. They are essential for amino acid structure and enzymatic activities. Deficiency of any one micronutrient affects the plant normal growth and function, which gives an opportunity for pathogenic fungi, viruses, and bacteria to attack the plant. For many reasons (dry or flooded soil, lack or low availability, soil texture, trend of fertilizer application) micronutrients are not sufficient in soil or their availability is low. Plant roots are not as efficient in taking up an adequate amount of all essential trace elements as they are in taking up water and macroelements. Because of the low efficiency of plant roots for micronutrient uptake, microbes can help in the uptake and transport of vital nutrients using many uptake mechanisms.

These micronutrients are used in metalloproteins or cofactors to protect the organism from toxic effects of excess metals. These metals are present in ionic forms with variable oxidation states and take part in most cellular redox reactions according to biological needs. Thus, for healthy plant growth and development, a range of transition metals must be acquired from the soil, transported around the plant, and distributed and compartmentalized within different tissues and cells. Clearly membrane transport systems likely play a key role in these events (Guerinot 2000; Hall and Williams 2003; Grotz and Guerinot 2006; Krämer et al. 2007; Smith and Smith 2011; Barberon et al. 2011). Symbiotic mutualistic interactions of AM fungi with plants resulted in increased nutrient uptake, especially supply of low-mobility mineral nutrients; for example, phosphorus and other micronutrients such as zinc and copper. The hyphal networks AM fungi develop in the soil enhance the surface area of soil contact, movement of nutrients into the environment, and storage capacity, and thus increase nutrient availability to plants by up to four times. By reducing the distance required for diffusion, mycorrhizal hyphae more efficiently take up phosphorus (up to four times higher) as compared with the plant root system, and in return the plant provides carbon compounds to the fungus (Smith and Smith 2011).

4 Role of Transporters in Nutrient Acquisition

The selectively permeable nature of the plasma membrane ensures the entry of crucial metals and metabolites into the cell and also maintains intracellular homeostasis of the cytoplasm. The structural components of membranes are primarily phospholipid bilayers with transmembrane proteins; they helps in the trafficking of metals, ions, water, and metabolites across the membrane and maintenance of a cytosolic pH. Generally, O_2 and CO_2 can permeate phospholipid bilayers, but inorganic ions and other hydrophilic solutes, such as sucrose and amino acids, cannot permeate them. Therefore, certain specific proteins facilitate the entry of protons, inorganic ions, and organic solutes the through plasma membrane, and these are called "transporters." The recent cloning of the genes for a large number of transport proteins and the availability of knockout mutants make it possible to dissect transport processes in greater detail and to begin to understand the interactions between ion uptake processes that have often been observed at the organ level (Grotz and Guerinot 2006; Krämer et al. 2007). Plants associated with mycorrhizal fungi follow two pathways for nutrient uptake. The plant root epidermis and hairs possess high-affinity phosphorus and nitrogen transporters, which are responsible for uptake of nutrients; this is the direct pathway. These transporters are downregulated in a mycorrhizal symbiotic root association. In the mycorrhizal pathway, mycorrhiza-inducible phosphorus and nitrogen transporters present in the periarbuscular membrane are involved in uptake of nutrients. This is main pathway for nutrient uptake in a symbiotic or mutualistic association (Guerinot 2000; Hall and Williams 2003; Smith and Smith 2011).

Metal uptake, metal distribution, and metal delivery to various plant cell types and organs, conjugation with the required protein, and storage and remobilization require the operation of efficient metal transporters. Higher accumulation can lead to toxicity in plants, thus, high precision and specificity is required in metal transport and its regulation (Krämer et al. 2007; Smith et al. 2004; Smith and Smith 2011; Barberon et al. 2011). Transport of micronutrients and other metabolite metals in plants is mediated by some specific protein families: for example, the iron-regulated transporter protein (ZIP) family, zinc-regulated transporter, the subfamily of P-type ATPases, the natural-resistance-associated macrophage protein (NRAMP) family, the yellow stripe like 1 (YSL1) subfamily of the oligopeptide transporter (OPT) superfamily, the copper transporter (COPT) family, the Ca^{2+}-sensitive cross complementer 1 (CCC1) family, the iron-regulated protein (IREG) family, ATP-binding cassette (ABC) transporters, ABC transporters of the mitochondria (ATM), and pleiotropic drug resistance (PDR) transporters (Barberon et al. 2011; Montanini et al. 2007; Hall and Williams 2003).

5 ZIP Transporters

The ZIP family of transporters has been characterized universally, including in bacteria, fungi, plants, insects, and mammals, and plays important roles in metal uptake and transport. ZIP proteins consist of 309–476 amino acid residues with

eight potential transmembrane domains, and generally contribute to metal ion homeostasis by transporting divalent cations, including Fe^{2+}, Zn^{2+}, Mn^{2+}, and Cd^{2+}, from microorganisms such as yeast into the cytoplasm. Transmembrane domains 3 and 4 contain N and C terminals on the plasma membrane surface, with variable regions between both domains. These transmembrane domains possess a potential metal-binding domain rich in histidine residues frequently located in the variable region between the domains 3 and 4. Transmembrane domain 4 of the ZIP family proteins is the most conserved region, and forms an amphipathic helix rich in histidine residues. The adjacent polar residue and this histidine-rich region may form part of an intramembranous heavy metal binding site that is part of the transport pathway (Curie et al. 2000; Guerinot 2000; Eide 2011). ZIP transporter activity is regulated by both transcriptional and posttranscriptional control mechanisms.

6 Cation Diffusion Facilitator Family

The cation diffusion facilitator (CDF) family is responsible for cation homeostasis in bacteria, fungi, plants, and animals. Members of this family facilitate the transportation of micronutrients such as zinc, cobalt, nickel, and copper. The protein structure reveals six transmembrane domains with two significant regions: an N-terminal signature sequence and a C-terminal cation-binding domain. They are also rich in histidine, between the N and/or C termini. Such histidine-rich regions are predicted to function as potential metal-binding domains. Some of the CDFs play important role in homooligomeric complexes or heterooligomeric complexes, and this may lead to regulation of the activity of other proteins bound to metals. CDF family members transport principal metals: Zn^{2+}, Mn^{2+}, or Fe^{2+}/Zn^{2+}. These proteins are responsible for the transport of more than one metal. CDFs create an electrochemical gradient by using H^+ or K^+ ions and transport the metals against a concentration gradient (Podar et al. 2012; Grotz and Guerinot 2006; Montanini et al. 2007; Gustin et al. 2011; Desbrosses-Fonrouge et al. 2005; Kobae et al. 2004)

The proteins range in size from 280 and 740 residues. In *Arabidopsis thaliana*, 12 CDF transporter genes were reported, and one of them was named Z*INC TRANSPORTER OF ARABIDOPSIS THALIANA (ZAT*), or *METAL TOLERANCE PROTEIN 1* (*MTP1*) *MPT1* is expressed constitutively throughout the plant and was induced by increased zinc concentrations. MTP1 is present in all tissues, and the uptake of zinc is limited by the expression of *MPT1*. Overexpression of the gene leads to resistance of toxic levels of zinc. Increased sequestration rather than efflux of zinc is the main reason for such resistance. Since MTP1 has been localized to the vacuolar membrane of plants, it also supports zinc transportation in vacuoles, which are highly sensitive to zinc concentrations in plants.

7 ABC Transporters

The ABC transporters are the largest protein family, present in microbes to specialized human cells, and are actively engaged in active transport process by gaining energy from ATP hydrolysis. The energy released is used by the phosphorylated intermediates of ABC family members and pumps metals or large organic molecules across the plasma membrane of the cell. ABC transporters act as proton cotransporters and proton antiporters involved in, similarly to the P-type ATPases, and associated with metal deposition in vacuoles, where detoxification of heavy metals follows (Morel et al. 2009). An ABC transporter from tonoplasts has been identified and named "HMT1." It is a heavy metal phytochelatin complex (Garmory and Titball 2004; Vert et al. 2002). Song et al. (2010) identified the plant vacuolar phytochelatin transporters. The reason why these transporters remained undiscovered for such a long time is that two ABCC proteins, AtABCC1 and AtABCC2, exhibit a redundant function, rendering reverse genetic approaches unsuitable for their identification. Two other ABC transporters, AtABCC1 and AtABCC2, contribute to Cd^{2+} and Hg^{2+} tolerance. AtABCC1 plays a significant role in tolerance to the divalent heavy metals in the absence of AtABCC2. In plants, final detoxification processes occur in vacuoles with the help of ABC proteins, which are responsible for metal/metalloid deposition in vacuoles, where detoxification occurs (Kang et al. 2011; Garmory and Titball 2004).

8 NRAMP Family

The NRAMP family is a ubiquitous metal transport family from bacteria to humans which participates in metal ion homeostasis. Members of the NRAMP family help in Zn^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+}, Ni^{2+}, Mn^{2+}, and Co^{2+} transport. They also have highly conserved protein sequences and show 28 % (yeast), 40 % (plant), and 55 % (fly) sequence identity with the mammalian proteins (46 %, 58 %, and 73 % similarity respectively). The NRAMP/SLC11 transmembrane proteins constitute a ubiquitous family of metal transporters important for host–microbe interactions and responsible for competitive acquisition for divalent cations (Grotz and Guerinot 2006). One member, divalent-metal transporter 1, is responsible for dietary iron absorption. Three (AtNRAMP1, AtNRAMP3, and AtNRAMP4) of seven members of the NRAMP family identified from the model organism *Arabidopsis thaliana* mediate the transport of iron. When iron deficiency occurs in soil, the messenger RNA of the corresponding genes accumulated in roots (*ATNRAMP1*, *ATNRAMP3*, and *ATNRAMP4*) and shoots (*ATNRAMP4*). At toxic iron concentrations, overexpression of *ATNRAMP1* increases the resistance of plants by excess transportation of iron into the plastids to prevent toxicity. Overexpression of *ATNRAMP3* increases the iron concentration in the cytosol, and in response *IRT1* and *FRO2* messenger RNA (accumulated during iron deficiency) is downregulated and localizes to the vascular system of the roots and shoots of *Arabidopsis* (Lin et al. 2009, Cellier 2012; Nevo and Nelson 2006).

9 P-Type ATPases

A superfamily of transporters, P-type ATPases posses unique signature motifs and have been identified in prokaryotes and eukaryotes, including yeasts, insects, plants, and mammals. As the name suggests, formation of a phosphoenzyme intermediate, in which one phosphate is transferred to highly conserved DKTGT motif, is a unique character of this superfamily. P-type ATPases catalyze heavy metal transport, and this transport activity is characterized by a Cys-Pro-Xaa (or Xaa-Pro-Cys) motif, where Xaa is Cys, Ser, or His, in the sixth transmembrane helix (Okkeri and Haltia 2006; Hussain et al. 2006). This superfamily is divided into subgroups with distinct substrate specificities involved in heavy metal transport across cellular membranes. For example, some subfamilies include H^+-ATPases (type 3A) in plants and fungi, Na^+/K^+-ATPases (types 2C and 2D) in animals, Ca^{2+}-ATPases (types 2A and AB), and heavy-metal-transporting ATPases (type 1B). Two types of coppers transporters, RAN1 (also called "HMA7") and PAA1 (P-type ATPase 1 of *Arabidopsis*) have also been identified in plants and are responsible for the delivery of copper to the plastid. Members of HLA alleles also play essential roles in the homeostasis of zinc in *Arabidopsis* (Argüello 2003; Argüello et al. 2007; Wu 2006; Lewinson et al. 2009)

10 Rhizospheric Microbial Interaction

Rhizospheric communication is essential and critical for almost all sorts of interactions and led to evolution of plant–microbe interactions. Molecular signaling systems, especially in bacteria, have been well identified. Bacteria can sense the biomolecules excreted by the plant roots. Many rhizospheric microbes, especially bacteria, also secrete phytohormones (Kumar et al. 2000, 2001), which influences plant growth. Additionally, there is also competition and communication among various types of microbes in the rhizosphere, which also modulates the rhizosphere, influencing plant growth and the biome (Faure et al. 2009). The best known plant–microbe interaction is symbiotic association of a *Rhizobium* and legume plant, where legume roots secrete betaines and flavonoids (Franche et al. 2009). On the other hand, other signaling biomolecules play an important role in the legume–*Rhizobium* communication that leads to a mutualism (Faure et al. 2009). AM fungi also excrete strigolactones, which are a group of sesquiterpene lactones which act as signaling molecules between roots and AM fungi (Akiyama and Hayashi 2006; Paszkowski 2006). The low concentrations of sesquiterpene strigolactones encourage and persuade the widespread hyphal branching in the germinating spores of the AM fungus *Gigaspora margarita* (Bouwmeester et al. 2007). Phosphate deficiency enhances strigolactone release in plants infected with AM fungi (Yoneyama et al. 2008), whereas excess of phosphate leads to nongermination of fungal spores. The identification of novel plant microbe communicating signals leads to an understanding of the precise basis of AM fungus–plant interactions.

Various biotic interactions also occur in the rhizosphere that may influence the type and organization of the microbial population on the root surface. These include interactions of roots with mycorrhizal fungi and root pathogens, root–bacteria interactions, interactions within the microbial community, and interactions of mesofauna with the microbial community. Biotic interactions are typically commoner in the rhizosphere than in bulk soil and in many cases may equal or exceed the importance of abiotic factors in determining microbial community composition. During the course of plant root generation and growth, the root passes through soil particles and activates the indigenous microbial community there, through its exudates, which leads to competition among microbes for food and space, which results in the final determination of the rhizospheric community. During this course of development, microbes may intermingle with each other through detection and excretion of organic molecules. In the process of chemical interactions, bacteria sense a quorum, which leads to a change in gene expression and therefore bacterial activities (Loh et al. 2002). The kind of chemical indicator or signal is precise for a particular microorganism; closely related microbes share a common type of indicator or signal (Loh et al. 2002). The Gram-negative bacteria signals are commonly acyl homoserine lactones, and for Gram-positive bacteria, they are modified polypeptides. One receptor has been reported in most soil bacteria that sense a particular signal and a synthase that produces a signal or indicator, but there are reports that some rhizospheric bacteria are known to produce many types of signal molecules.

Additionally, plant hormones, such as jasmonic acid and salicylic acid (defense-related plant hormones), can intercede in or modulate the composition of root exudates (Carvalhais et al. 2013). Pieterse et al. (2009) proposed that alterations in the protection- or resistance-related hormones, as observed for the period of insect and pathogen damage of leaf tissues, could potentially manipulate the chemical composition of the root exudates and also the microbial community in the rhizosphere. On the other hand, chemical initiation of plant resistance by foliar application of salicylic acid and jasmonic acid did not considerably influence the rhizosphere microbiome (López et al. 2008). Therefore, use of precise and responsive microbial profiling methods is essential to notice active alterations or changes in the plant microbial community.

11 Micronutrient Uptake and Mobilization

A normal human requires 49 nutrients to meet his/her metabolic demands. Insufficient intake of even one of these nutrients will lead to metabolic disorders. This will lead to poor and underdeveloped health, especially in children. The health of an adult person is also affected by micronutrient deficiency, which ultimately negatively affects the efficiency and economic costs of a society (Calton 2010). Prominently, the chief resource of all micronutrients comes from agricultural produce. If agricultural produce does not supply sufficient quantities of all micronutrients, then the population will suffer from deficiency diseases and people will not

have healthy lives. Regrettably, this is the case for most of the agricultural systems in many developing countries (Govindaraj and Kannan 2011). Micronutrient deficiencies in the soil influence crop production, and ultimately reduce the quality of the produce. It has been estimated that more than three billion people experience micronutrient deficiency and about two billion of these have iron deficiency, and the numbers are increasing (Hennessy et al. 2014). To augment the accumulation of various micronutrients in crop produce, biofortification is a viable and feasible choice. On the other hand, the quality of crop produce biofortification depends on the chemical properties of the soli, crop genotypes, agricultural management practices, and climatic factors (Schulin et al. 2009). Biofortification is the process of augmenting the bioavailable concentrations of necessary and vital elements in edible portions of crop plants through agronomic practices or genetic methods.

Many research organizations worldwide are investing hugely and investigating the genetic potential of crop plants to enhance micronutrient bioavailability in common staple food crops such as wheat, rice, beans, and oilseeds (Cakmak et al. 2010). For micronutrient biofortification, plant breeding approaches facilitate enhancement of the amount of a number of minerals concurrently in edible tissues of food without any consequences for growth and yield parameters, whereas transgenic approaches depend on improving nutrient mobilization from the soil, uptake from the rhizosphere, transport to the shoot and leaf, and buildup of mineral elements in bioavailable forms in edible tissues (Borrill et al. 2014). The plant breeding approach to increase micronutrient uptake by plant roots is tedious, and results take a long time; on the other hand, the transgenic approach is costly. The use of potential PGPMs could be a better choice to increase micronutrient uptake by roots.

This underlines the promise of the use of PGPMs as a simple, effortless, and economically encouraging method for biofortification. The PGPM attributes could facilitate in crop plants the growth of deep root systems in nutrient-deficient soils and the excretion of ligands/siderophores or acids/alkalis to mobilize micronutrients. Many researchers have reported the uptake of micronutrients by a variety of crops using microorganisms. Ardakani et al. (2011) reported the higher uptake of iron, manganese, zinc, and copper in wheat using *Azospirillum* and mycorrhizae compared with nonmicrobial inoculation. Inoculation of roots with AM fungi increased zinc uptake and mobilization and growth of rice (Purakayastha and Chhonkar 2001). AM fungi inoculation has been shown to increase wheat and maize growth along with higher zinc uptake in zinc-deficient soils where zinc was applied as a fertilizer (Kothari et al. 1990). Yildirim et al. (2011) investigated the effects of root inoculations with *Bacillus cereus* (N_2 fixing), *Brevibacillus reuszeri* (phosphorus solubilizing), and *Rhizobium rubi* (both N_2 fixing and phosphorus solubilizing) on plant growth, nutrient uptake, and yield of broccoli in comparison with manure (control) and mineral fertilizer application under field conditions. Bacterial inoculations with manure compared with the control significantly increased yield, plant weight, head diameter, chlorophyll content, and nitrogen, potassium, calcium, sulfur, phosphorus, magnesium, iron, manganese, zinc, and copper content of broccoli. Senthilkumar et al. (2014) observed that the combination of fertigation and a consortium of biofertilizers in banana significantly enhanced accumulation of secondary nutrients and micronutrients (Fe, Zn, and

Mn) in the leaves, pseudostem, and fruits at harvest. Goteti et al. (2013) screened ten strains for zinc solubilization, and tested them on a maize crop in a short-term pot culture experiment and observed that seed bacterization with zinc solubilizing *Pseudomonas* sp. (strain P29) the P29 strain significantly enhanced the concentrations of macronutrients and micronutrients such as manganese (60 ppm) and zinc (278.8 ppm) compared with the uninoculated control.

Li et al. (2007) investigated in a hydroponic experiment with different concentrations of cadmium and zinc the effects of bacteria (*Burkholderia cepacia*) on metal uptake by the hyperaccumulating plant *Sedum alfredii*. It was observed that inoculation with bacteria significantly enhanced plant growth (up to 110 % with zinc treatment), phosphorus uptake (up to 56.1 % with cadmium treatment), and metal uptake (up to 243 % and 96.3 % with cadmium and zinc treatment respectively) in shoots, the tolerance index (up to 134 % with zinc treatment), and translocation of metals (up to 296 % and 135 % with cadmium and zinc treatment respectively) from root to shoot. Kuffner et al. (2008) studied ten rhizospheric isolates (*Pseudomonas*, *Janthinobacterium*, *Serratia*, *Flavobacterium*, *Streptomyces*, and *Agromyces*) from heavy-metal-accumulating willows. These isolates were analyzed for plant growth promotion and zinc and cadmium uptake in *Salix caprea* plantlets grown in sterilized, zinc–cadmium–lead-contaminated soil. *Agromyces* AR33 increased plant growth and also enhanced the total amount of zinc and cadmium extracted from soil. In an additional study, three bacterial strains–namely, BC, AX, and AB—isolated from a zinc-deficient rice–wheat field belonging to the genera *Burkholderia* and *Acinetobacter* were investigated for growth promotion and zinc uptake in rice plants. Bacterial inoculations also significantly enhanced the total zinc uptake per pot (52.5 %) as well as grain methionine concentration (38.8 %). The effect of bacterial treatments on the bioavailability of zinc was assessed by estimation of the levels of phytic acid in grains. A reduction of nearly 38.4 % in the phytate-to-zinc ratio in grains was observed under bacterial inoculations (Vaid et al. 2014). Zaidi et al. (2006) reported that inoculation with *Bacillus subtilis* (SJ-101) not only prevented the plant from experiencing nickel toxicity but also enhanced the growth of *Brassica juncea* in metal rich soil. Tariq et al. (2007) reported that mobilization of aboriginal soil zinc in rice (*Oryza sativa* L.) rhizosphere was observed with plant-growth-promoting rhizobacteria and compared the effect with that obtained with an available form of a chemical zinc source as Zn-EDTA. Application of plant-growth-promoting rhizobacteria mitigated the zinc deficiency symptoms and enhanced the total biomass (23 %), grain yield (65 %), and zinc concentration in the grain. Microbial inoculation had a positive impact on zinc uptake and mobilization efficiency compared with no inoculation, and microbially inoculated rice plants were more efficient in taking up zinc compared with noncolonized plants. Sharma et al. (2003) reported that the plant-growth-promoting bacterium *Pseudomonas* sp. strain GRP3 influences iron acquisition in mung bean (*Vigna radiata* L. Wilzeck). Root colonization by AM fungi resulted in increased uptake of comparatively stationary metal micronutrients, such as copper in white clover (Li et al. 1991), copper, zinc, manganese, and iron in *Zea mays* (Liu et al. 2000), and zinc in field pea crops without influencing yield or phosphorus uptake (Ryan and Angus 2003).

12 Micronutrient Bioavailability

Nutritional value is a vital and significant limiting aspect to sufficient diet in many resource-limited regions. One of the important features of food value is the bioavailability of nutrients present in a given food; this availability could be enhanced by biofortification. Biofortification is a method in which plants take up the macroelements and microelements (e.g., Fe and Zn) from the soil and transport them in the grains so that these elements become bioavailable to humans or to produce nutritionally rich grains that maintain the nutritional requirement of the consumer. This approach has been proved to be viable, comparatively cost-effective, and effectual (Hotz and Gibson 2007). Regarding bioavailability of micronutrients in the edible part of a crop, most research has been done on iron and zinc because these two elements are very essential and significant for normal metabolism and enzymatic activities and influence the overall health of a human. Because of this, the following discussion on bioavailability of micronutrients will focus on iron and zinc. Ryan et al. (2008) reported that the zinc concentration of wheat grain decreased by 33–39 % in response to phosphorus fertilizer. Phosphorus fertilizer also increased the concentrations of grain phosphorus by 17 % and grain phytic acid by 19 % but had little effect on the concentrations of calcium, iron, and polyphenols. The bioavailability of grain zinc, as shown by the phytic acid to zinc and calcium times phytic acid to zinc molar ratios, mostly reflected zinc concentration and was low in all treatments. After milling, the phytic acid to zinc molar ratio suggested low zinc bioavailability for flour from wheat grown with phosphorus fertilizer after canola or fallow. In a greenhouse and field experiment it has been demonstrated that mycorrhizal symbiosis facilitates the availability of both iron and zinc in maize. The relation between these two micronutrients may aid in increased mobilization of iron and zinc, which are eventually translocated into developing grains. AM fungi inoculation helped in biofortification of grains with trace minerals besides mitigating the effect of antinutritional factors present in grains (Balakrishnan and Subramanian 2012). Imran et al. (2014) reported that soil microbial communities play a vital role in enhancing bioavailability of zinc to plants in soil matrix. Similarly, application of a consortium of *Azospirillum lipoferum* JCM-1270, *Azospirillum lipoferum* ER-20, *Pseudomonas* sp. K-1, *Pseudomonas* sp. 96-51, and *Agrobacterium* sp. Ca-18 has been reported by Tariq et al. (2007) to promote zinc availability to rice plants (*Oriza sativa*) and substantially increase root weight, length, and volume and zinc uptake in straw and grain. Similarly, Biari et al. (2008) reported on the bioavailability of iron, copper, manganese, and zinc in *Zea mays* promoted by *Azotobacter chroococcum* and *Azospirillum brasilense* under field conditions. Nyoki and Ndakidemi (2014) reported on zinc, iron, manganese, and copper uptake in cowpea pods promoted by *Bradirhizobium japonicum*.

13 Future Aspects and Concluding Remarks

This is an exciting research field in rhizospheric microbial ecology. The advancement of innovative approaches for studying the integral rhizosphere is just like entering another world. In this chapter we have discussed current progress in rhizospheric microbial interaction, the role of rhizospheric microbial communities in uptake of micronutrients and their bioavailability. The rhizosphere is very different from bulk soil in mineral composition, pH, organic matter content, redox potential, and gaseous exchange. These parameters control and regulate the uptake and mobility of micronutrients, which results in different biogeochemistry of these nutrients in the rhizosphere compared with bulk soil. The future research direction should be to enhance the many aspects of plant root and rhizospheric systems and also to augment our concept of scavenging of microbial microelements from the rhizosphere and their transport into plant parts. One also has to keep the following things in mind; the root morphology, its biomass, physiology, and exact mode of adsorption, microbe–root association and interaction, micronutrient uptake and mobilization through plant root exudates, production of chelating agents by roots and microbes, and consumption and degradation of root exudates by microbes for trace element uptake and mobilization enhancement. Better understanding of rhizospheric microbial interaction will help us to comprehend the uptake of micronutrients for better plant growth. Development of molecular biology and genetic engineering technology for better understanding of microbe–plant–root association and inoculation with suitable potential microbes will help to remove more microelements.

Employment of a range of bacterial and fungal inoculants to plant roots, seeds, and soil had produced promising results in terms of improving the levels of micronutrients in soil, plant tissues, and the sink, and thereby improving overall yield. Regardless of the reality that research related to the influence of these microbial inoculants on increasing uptake of trace elements from soil to the plant edible parts is incomplete and restricted, the promise of such potential bioinoculants has not been greatly investigated, and only a few microbes have been tested under field conditions, there are still some realistically and convincingly excellent reports about the significance of these microbial inoculants in improving the micronutrient bioavailability fraction from soil and reducing micronutrient deficiency in edible crop plants and also decreasing the need for costly fertilizers. For that reason, potential microbes with greater micronutrient scavenging ability and greater ability to transport these micronutrients into the edible part of the plant should be developed. Additionally, focus on the genes responsible for or involved in micronutrient uptake and mobilization is very important so that more competent microbial strains can be developed and formulated. There could be a further possibility of transferring the genes responsible for micronutrient mobilization into a bioavailable form from microbes into the plant. This will be highly useful and advantageous to solve the dependency of plant roots on any external agency for micronutrient mobilization into a bioavailable form.

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Bacterial Determinants and Plant Defense Induction: Their Role as Biocontrol Agents in Sustainable Agriculture

Stuti Patel, Riyaz Z. Sayyed, and Meenu Saraf

Abstract In an environment consisting of harmful microorganisms, survival of plants mainly depends on efficient microbial recognition and rapid defense mechanisms. After infection with a necrotizing pathogen, many plants develop resistance against attack by phytopathogens. This resistance is regarded as systemic acquired resistance, which is a key portion of plant defense against pathogen infection. Induction of acquired resistance in plants occurs mainly by enhancement of the levels of pathogenesis-related proteins and salicylic acid. Some groups of plant-growth-promoting rhizobacteria are involved in an indirect mechanism, either by their antagonistic effect against phytopathogens or by induced systemic resistance (ISR) mechanisms in plants. ISR has been studied with respect to the underlying signaling pathways and to its application in crop protection. The signaling pathway regulating ISR functions independently of salicylic acid, and is mainly dependent on the plant hormones jasmonic acid and ethylene. Apart from these, NPR1, a defensive regulatory protein, is also involved in both systemic and acquired resistance in plants. In this chapter, the molecular and genetic relationship between basal resistance and induced resistance is highlighted.

Keywords Biocontrol • Induced systemic resistance • Systemic acquired resistance

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1 Introduction

Plant diseases have always been major problems. Various abiotic and biotic factors affect plant productivity worldwide. Biotic factors such as viroids and higher organisms are plant pathogen parasites that cause diseases. Plant disease resistance is a prerequisite for modern agriculture; the dynamics of plant–microbe interaction has an immense and positive effect worldwide for the management and control of plant diseases. Since the twentieth century, it has been reported that plants have an innate ability to combat infection, recover from diseases, and evade future infections (Chester 1933). Colonization on host plants and thereby utilization of the reserves of plant stores are the features of phytopathogens. Detection of pathogens and the defensive response of the plant is in the form of secretion of antimicrobial compounds and other stress responses. The abilities of a pathogen to induce a disease in a host plant is usually the exception as plants are capable of recognizing the invading pathogens and establishing a successful defense mechanism. In contrast, some pathogens can successfully produce diseases in plants as they are able to evade suppression of the host defense mechanism (Borrás-Hidalgo 2004). Plant–pathogen interaction results either in a disease condition in the host plant or in a resistance mechanism which prevents the spread and multiplication of the nonhost pathogen. Plants depend on an innate immunity to defend themselves. Innate immunity includes two interrelated branches: pathogen-associated molecular pattern (PAMP)/microbe-associated molecular pattern (MAMP)-triggered immunity and effector-triggered immunity (Euglem and Somssich 2007). PAMPs/MAMPs are identified by host cell-surface-localized pattern recognition receptors and activate plant immunity. PAMP-triggered immunity restricts pathogen proliferation. Since the last decade, plant–pathogen association has been known to lead to the development of multiple mechanisms of surveillance in plants (Zhang and Zhou 2010). By delivering virulence effector proteins into host cells, pathogens adapt themselves into the host plant on inhibiting PAMP-triggered immunity (Abramovitch et al. 2006). To overcome this, plants developed immune receptors known as resistance proteins, which either directly or indirectly detect pathogen-specific effector protein activities inside the plant cell and trigger disease resistance, which results in effector-triggered immunity, which is very specific and mostly leads to hypersensitive responses (Tsuda et al. 2009).

There are mainly two possible kinds of plant resistance mechanisms: active and passive. The active resistance mechanism of the plant depends on the defense mechanism induced only after an attack by a pathogen, whereas the passive resistance mechanism relies only on constitutively expressed defenses. Active defense against an incompatible pathogen is in the form of induced resistance that is categorized by a highly localized defense expression such as the hypersensitive response and phytoalexins (Hammerschmidt and Nicholson 1999). Induced resistance, depending on the mode of expression, can be of two types: local and systemic. Local induced resistance means resistance is induced only in the specific tissue where the attack by the pathogen occurs, whereas systemic induced resistance occurs in a part of a plant that is spatially separated by an induction point (Hammerschmidt 1999). Local systemic

resistance involves induction of certain pathogenesis-related (PR) proteins to stop the proliferation of the challenging pathogen; in the case of systemic resistance, the induction of cells away from the induction site occurs, which permits the cells to defend themselves against the challenge by what is called "priming" (Conrath et al. 2002). On the basis of the type of inducing agent and the host signaling pathways, induced resistance is characterized into two forms: systemic acquired resistance (SAR) and induced systemic resistance (ISR) (van Loon et al. 1998). SAR develops subsequent to a localized necrosis, and is dependent on salicylic acid (SA) signaling and on the expression of PR proteins. ISR develops systemically because of plant root colonization by plant-growth-promoting rhizobacteria (PGPR) and plant-growth-promoting fungi. Moreover, besides induced resistance in plants, rhizobacteria are also known to be involved in an indirect mechanism by acting as biocontrol agents (Akhtar and Siddiqui 2010; Glick 2012). Biocontrol activity include nutrient competition, exclusion of niches, and production of antifungal metabolites (lytic enzymes) as chief modes of the mechanism (Lugtenberg and Kamilova 2009) against phytopathogens.

2 Indirect Mechanisms

2.1 Role of PGPR in Suppression of Disease Caused by Phytopathogens

There are several biocontrol methods for the control of soilborne phytopathogens and plant diseases. These methods involve an attempt either to increase soil antagonist activity with pathogens (Gamliel et al. 2000) or to protect plants by the use of bioinoculants (Compant et al. 2005; Akhtar and Siddiqui 2009; Akhtar et al. 2010). Increasing the soil fertility may also reduce the efficacy of bioinoculants, whose niches may be reduced (Hoitink and Boehm 1999). The mechanisms by which PGPR control the damage to plants resulting from pathogen invasion include siderophore secretion, physical displacement, and production of antibiotics, enzymes, and a variety of molecules that inhibit phytopathogen growth (Niranjan Raj et al. 2006). One of the major mechanisms that can control the proliferation of phytopathogens is the production of siderophores with a very much higher affinity for iron than fungal pathogens (Siddiqui et al. 2007; Sayyed and Chincholkar 2009; Sayyed and Patel 2011; Glick 2012; Sayyed et al. 2013; Shaikh et al. 2014; Shaikh and Sayyed 2015). Another effective mechanism is the production of antibiotics, which are deleterious to the metabolism or growth of other pathogens (Doornbos et al. 2012). A large number of antibiotics have been identified, produced by members of *Pseudomonads*, *Bacillus*, *Stenotrophomonas*, and *Streptomyces* (Compant et al. 2005).

Soilborne microorganisms are capable of producing extracellular enzymes such as cellulases chitinases, lipases, β-1-3-glucanases, and proteases, thereby hydrolyzing a wide variety of polymeric compounds, including proteins, chitin, hemicelluloses, and cellulose, which hinders the growth of pathogens (Markovich and Kononova 2003). These enzymes together with antibiotics play an important role in

the defense against phytopathogenic fungi as a antagonistic effect (Fogliano et al. 2002). PGPR that produce such enzymes have been shown to have a biocontrol effect against fungi, including *Sclerotium rolfsii*, *Botrytis cinerea*, *Phytophthora* spp., *Fusarium oxysporum*, *Pythium ultimum*, and *Rhizoctonia solani* (Glick 2012).

2.2 Systemic Acquired Resistance

On primary invasion by pathogens in plants, tissue necrosis at the site of infection by pathogens is activated by SAR (Ryals et al. 1996). SAR is known to be associated with a PR gene expression which results in accumulation of PR proteins involved in antimicrobial action and thereby induces resistance. Expression of the PR-1 protein is a molecular marker for SAR induction. The PR-1 proteins are usually appear as a result of SA accumulation (van Loon et al. 2006). It was demonstrated that a transgenic plant expressing the bacterial salicylate hydroxylase gene (*nahG*) was incapable of accumulating SA. Plants expressing *nahG* do not show an SAR response as they convert SA to inactive catechol (Lawton et al. 1996). Likewise, the SA-production-deficient mutants sid1 and sid2 do not show SAR after infection with a necrotizing pathogen, which indicates that SA is necessary and sufficient for the induction of SAR (Verhagen et al. 2006). However, SA action requires the protein NPR1, also known as "NIM1," an ankyrin repeat family protein structurally (Cao et al. 1997). In the presence of SA, oligomers of NPR1 in the cytoplasm are reduced to monomers by redox reactions and interact with specific TGA transcription factors for the expression of gene codings for PRs (Dong 2004). NPR1 is a master regulatory protein identified through genetic screens for SAR-compromised mutants in *Arabidopsis thaliana* (Dong 2004; Pieterse and van Loon 2004). It significantly increases the binding of TGA2 to SA promoter elements in the *Arabidopsis* PR-1 gene (Despres et al. 2000). Subramaniam et al. (2001) showed interactions between NPR1 and TGA2 by using a protein fragment complementation assay in vivo, and demonstrated that the SA-induced interaction is strictly localized in the nucleus. The steps involved in SAR signaling are shown in Fig. 1.

2.3 Induced Systemic Resistance

In the last three decades, various reports have confirmed a beneficial effect of root-colonizing bacteria such as PGPR on plant development and disease resistance (Kloepper et al. 1980). A specific recognition factor between the plant and the systemic-resistance-inducing rhizobacteria is needed for the induction of resistance. For instance, *Pseudomonas fluorescens* and *Pseudomonas putida* perform differently on different host species, as *Arabidopsis* responds to *P. putida*, whereas carnation and radish do not (van Wees et al. 1997). Conversely, radish is responsive to *P. fluorescens*, whereas *Arabidopsis* is not (Leeman et al. 1995a). Plant-growth-promoting activities

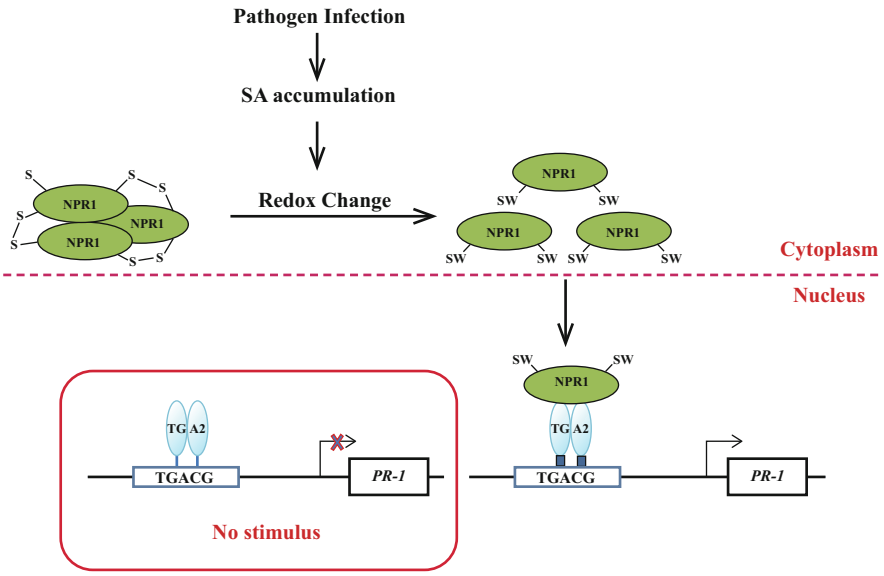

Fig. 1 SAR signaling induced by Phytopathogens in plants (modified from Pieterse and Van Loon 2004)

of PGPR can directly affect plant growth but are mostly related to a biocontrol activity of soil microorganisms which can be depend on a few mechanisms, including nutrient competition, siderophore-mediated competition for iron, and antibiotic production (Patel et al. 2012). Van Peer et al. (1991) demonstrated that PGPR also reduce pathogen infections on aboveground parts of plants such as stems and leaves. Some biochemical compounds of PGPR affect the complementary receptors on the plant surface for the successful elicitation of systemic resistance. Root colonization of ISR-triggering bacteria results in a discriminating level of resistance against a wide range of pathogens, and no defense mechanisms are frequently triggered in aboveground plant tissues on the recognition of the resistance-inducing signal. The phenomenon of expressing the basal defense responses faster on pathogen attack on the tissues is known as "priming" (Conrath et al. 2002). Priming shows efficient resistance strategies that assist the plant to efficiently react to any invader by enhancing infection-induced cellular defense responses (Beckers and Conrath 2007). Pieterse et al. (2000) demonstrated that in *Arabidopsis*, *P. fluorescens* WCS417r-mediated ISR functions require components of the jasmonic acid (JA) and ethylene response pathways but not SA. Like SAR, *P. fluorescens* WCS417r-mediated ISR relies on NPR1.

It is well known that only a few PGPR strains trigger ISR by ethylene-, JA-, and NPR1-dependent pathways. Rhizobacteria-mediated systemic resistance is actively efficient against a vast range of fungal phytopathogens in many plant species (van Loon et al. 1998). Certain bacterial-derived compounds have been implicated in elicitation of ISR (van Loon and Bakker 2006). Bacterial cell wall determinants such as flagella and lipopolysaccharides (LPS), secondary metabolites such as siderophores and antibiotics (Bakker et al. 2003; Iavicoli et al. 2003), and a bacterial

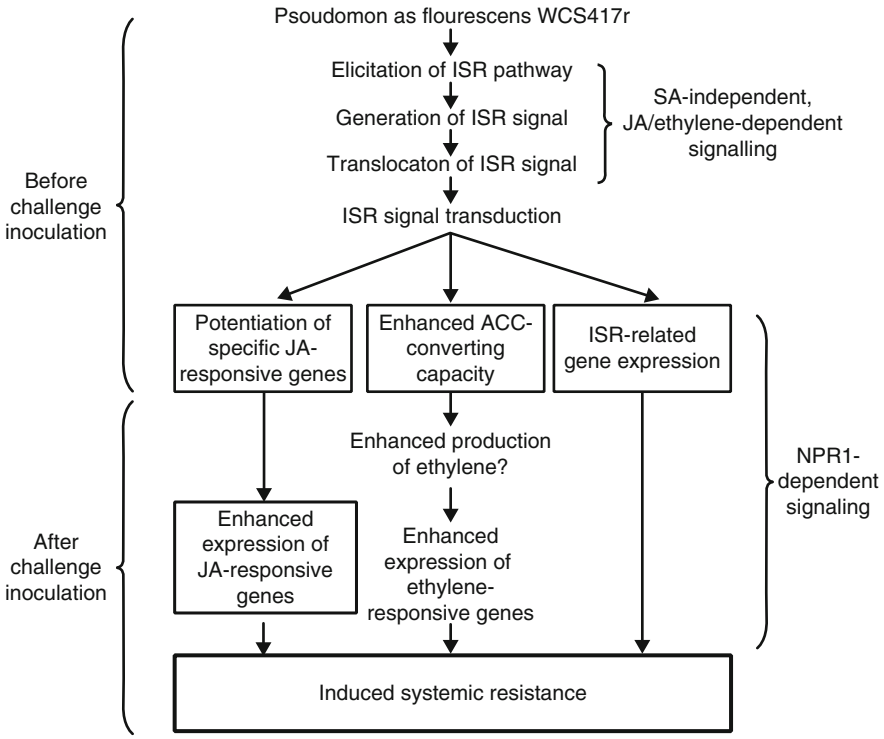

Fig. 2 ISR signaling mediated by Rhizobacteria in plants (adapted from Pieterse et al. 2001)

flagellin receptor have been recognized as bacterial elicitors (Gómez-Gómez and Boller 2000). The prominent homologies with recognition mechanisms for PAMPs in the innate immune response of plants demonstrated that rhizobacteria are recognized as general immunity mechanisms (Nurnberger et al. 2004). The important steps involved in the ISR mechanisms are summarized in Fig. 2.

3 Signaling in Rhizobacteria-Mediated ISR

3.1 SA-Independent Signaling

It has been found that there is no connection between resistance induced by *P. fluorescens* WCS417r (the Dutch reference strain) and accumulation of messenger RNA of certain PR genes ensures SAR and SA action (van Wees et al. 1997). It is known that ISR is not related to changes in endogenous SA content (Pieterse et al. 2000). ISR mediated by *P. fluorescens* WCS417 in SA-nonaccumulating *Arabidopsis nahG* transformants (van Wees et al. 1997) is an independent mechanism and is regulated by signaling pathways different from those for pathogen-induced SAR. The

Arabidopsis ethylene mutant *etr1* and JA mutant *jar1* were studied for their capacity to mount ISR. Both root-colonizing mutant strains of *P. fluorescens* WCS417r failed to induce systemic resistance against *P. syringae* pv. *tomato* (Pieterse et al. 1998), showing ISR signaling is dependent on these phytohormones. Certain well-identified ethylene-signaling mutants do not show enhanced resistance on root treatment with *P. fluorescens* WCS417r against *P. syringae* pv. *tomato* (Knoester et al. 1999), indicating that an ethylene signaling response is necessary for the development of ISR, whereas on leaf infiltration by *P. fluorescens* WCS417r, systemic resistance was observed, indicating ethylene is required for rhizobacterial induction (Knoester et al. 1999). Moreover, it is known that the activation of ISR in the host by *P. fluorescens* WCS417r depends on the responsiveness to ethylene and JA but not an increased level of these defense regulators. The sensitivity to ethylene and JA is enhanced as a result of ISR elicitation. This has been supported by two hypotheses. The first is that in plants showing ISR the ability to convert 1-aminocyclopropane-1-carboxylate (ACC) to ethylene is significantly enhanced, providing an ability to produce ethylene on pathogen invasion (Hase et al. 2003). The expression of ethylene and JA responsive genes is greater in induced plants than in noninduced plants after pathogen attack (Pozo et al. 2008). Thus, it seems that induced plants are more sensitive to the recognition of pathogen-induced ethylene and JA, thus resulting in an enhanced and potent response to subsequent pathogen invasion (Conrath et al. 2006). However, elucidation of the ISR signal-transduction pathway resulted in the conclusion that NPR1 acts downstream of the JA- and ethylene-dependent steps (Pieterse et al. 1998).

3.2 SA-Dependent Signaling

It has become clear that only a few rhizobacteria triggering ISR are facilitated by ethylene/JA, although it has been found that rhizobacteria-facilitated systemic resistance is not regulated by SA. The role of SA was first reported in *Pseudomonas aeruginosa* 7NSK2 and its mutant producing SA. Induction of SA-dependent resistance by *Pseudomonas* strains is similar to the resistance response of *Tobacco mosaic virus*, which is not expressed in *nahG* tobacco (De Meyer et al. 1999), whereas resistance to *B. cinerea* cannot be triggered in *nahG* tomato (Audenaert et al. 2002). Systemic resistance induced by some *Bacillus* strains requires SA but not JA and NPR1, although some strains of *Bacillus* sp. operate through an ethylene/JA-dependent mechanism and require NPR1 similarly to *P. fluorescens* WCS417r (Barriuso et al. 2008). ISR in *Arabidopsis* against *Verticillium dahliae* in response to root treatment with *Paenibacillus alvei* K165 requires an SA-dependent mechanism of resistance (Tjamos et al. 2005). Similarly, Domenech et al. (2007) reported an SA- and ethylene-dependent pathway in *Bacillus* strain N1137 induces systemic resistance to *Xanthomonas campestris* in *Arabidopsis*. However, Djavaheri (2007) also reported that ISR against *Turnip crinkle virus* in *Arabidopsis* is SA and NPR1 dependent in *P. fluorescens*.

4 Expression of ISR in Plants

After challenge introduction of a pathogen, expression of ISR is almost similar to that of SAR, in which the severity of disease is decreased with reduced pathogen growth and colonization of induced tissues, confirming that the plant is able to fight the pathogen (van Loon 2000). However, a decrease in disease incidence may protect the plant and increase the yield of the crop. Induced proteins on induction of systemic resistance can be taken as reliable markers for the induced state (van Wees et al. 1999).

There was an increase in the activities of stress-related enzymes such as peroxidase, glucanase, polyphenol oxidase, chitinase, and phenylalanine ammonia-lyase (PAL) as well as total phenolic compounds in PGPR-treated plants (van Loon and van Strien 1999). PAL is an important phenolic biosynthesis enzyme, and oxidative enzymes such as polyphenoloxidase and peroxidase have a vital role in lignification of tissue (Barcelo 1997). The activities of PAL, peroxidase, and phenolic content are responsive to changes in the environment and stresses; these changes often occur as a result of rhizobacterial treatments. Root colonization of cucumber by ISR-mediating *Pseudomonas chlororaphis* O6 against *Corynespora cassiicola* causes leaf spot, and effective accumulation of transcripts of six distinct genes on challenge inoculation was found (Kim et al. 2004). Induction was not by *P. chlororaphis* O6 colonization alone but became evident only after pathogen inoculation.

5 PGPR-Mediated ISR for Disease Suppression Under Field Conditions

Besides laboratory and greenhouse evidence, some experimental evidence indicates that systemic resistance by PGPRs can also be useful for plant protection under field conditions (Nandakumar 1998). *Serratia marcescens* strain 90-166, *P. putida* 89B-27, and *Flavimonas oryzihabitans* strain INR-5 showed ISR against angular leaf spot disease and bacterial wilt in field trials (Kloepper et al. 1993). Several PGPRs on application as bacterization of seed, alone, or as seed treatment plus soil drenching at the time of transplantation have protected cucumber plants against anthracnose, angular leaf spot, and bacterial wilt (Zehnder et al. 2001). In rice, treatment with PGPR strain mixtures of *P. fluorescens* strains Pf1 and PB2 reduces rice sheath blight disease penetration and increases yield under field trials (Nandakumar 1998). Thus, mixture of strains would be more effective than a single strain against a broad range of pathogens and pests (Raupach and Kloepper 2000; Akhtar and Siddiqui 2010).

6 Rhizobacterial Determinant Help in Indirect Mechanisms

A number of bacterial determinants are involved in the ISR by PGPR as summarized in the following sections.

6.1 Siderophores

The presence of iron is the limiting condition for both plant and microorganism growth. Disease suppression is performed by rhizobacteria against soilborne pathogens by the release of iron chelators known as "siderophores" in the rhizosphere for competition. Besides competition for ferric iron, siderophore production also triggers ISR and plays a dual role in disease suppression (Hofte and Bakker 2007). Leeman et al. (1996) reported that pseudobactin, a siderophore, produced by *P. fluorescens* strain WCS374 was responsible for ISR in radish against *Fusarium* wilt and not the LPS. Application of purified pseudobactin from *P. fluorescens* strain WCS374 to the roots of radish induces systemic resistance. Pseudobactins from *P. putida* strain WCS358 were tested for *Ralstonia solanacearum* suppression in *Eucalyptus urophylla*, *Erwinia carotovora* suppression in tobacco, and *B. cinerea* suppression in tomato. In all three cases, the purified pseudobactin 358 was as effective as the wild type (van Loon et al. 2008). Some bacteria also produce SA-containing siderophores, which means their SA secretion is the precursor for SA-containing siderophores such as pseudomonine and pyochelin produced by *P. fluorescens* WCS374r and *P. aeruginosa* 7NSK2 respectively. SA is not excreted by bacteria under iron-limiting conditions, but is channeled into the production of SA-containing siderophores. Audenaert et al. (2002) described that induction of systemic resistance does not depend on SA produced by *P. aeruginosa* 7NSK2; rather it depends on the synergistic interaction between the siderophore and pyochelin derived from SA (De Vleesschauwer and Hofte 2009). It has been observed that all the siderophores are not involved in induction of systemic resistance as all siderophores possess different chemical structures produced from various bacterial sources (Höfte 1993).

6.2 Lipopolysaccharides

LPS are made up of three different components: lipid A, core oligopolysaccharide, and an O-linked polysaccharide. LPS have an important function in stabilizing the outer membrane structure of gram-negative bacteria and also play another role of interacting with the outer membrane of the eukaryotic hosts. LPS are important to plants in preventing the hypersensitive response induced in plants by a virulent or nonhost bacterial adhesion to the plasma membrane receptors of plants such as tobacco, pepper, turnips, and *Arabidopsis* (Dow et al. 2000) referred to as a "localized induced response." Plant pathogenic LPS have also been proven to induce the rapid burst of NO which is responsible for innate immunity in plants. Besides, LPS present in the outer membranes of cells are the major component of ISR in certain PGPRs. LPS from *Burkholderia cepacia* with which tobacco leaves were pretreated was associated with the accumulation of PR proteins (Coventry and Dubery 2001) and phosphorylation of ERK-like mitogen-activated protein kinase (Piater et al. 2004) against *Phytophthora nicotianae*, whereas, in tobacco cell suspensions, it has been observed that LPS enhances a rapid flux of Ca^{2+} ion in aequorin-transformed cells, which is correlated with the production of reactive

oxygen and nitrogen species, alkalinization of the extracellular culture medium, and fast phosphorylation of certain proteins (Gerber et al. 2004) due to signaling and regulatory defense mechanism (Gerber et al. 2006) and changes in the expression of several genes (Sanabria and Dubery 2006). LPS from *P. fluorescens* strains WCS374 and WCS417 induces resistance in radish against *F. oxysporum* f. sp. *raphani* (Leeman et al. 1995b). Whereas the mutant of *P. fluorescens* strain WCS417 lacking the O-antigen side chain of LPS does not induce resistance in radish, the O-antigen side chain triggers a defense in radish plants. LPS from *P. putida* WCS358, which has the O-antigen side chain, does not show ISR in radish. Van Wees et al. (1997) showed the O-antigen side chain of *P. fluorescens* WCS417r elicits a defense mechanism in *Arabidopsis*. This shows that LPS from rhizobacteria differs with different host plants and it is not the only trait defining the ISR.

6.3 Exopolysaccharides

Exopolysaccharides (EPS) are high molecular weight polysaccharides that are secreted by most bacteria. EPS help in colonization of the bacteria within the host tissue as well as on the plant surface (Denny 1995). Jones et al. (2008) suggested that EPS can be used as signaling molecules for the developmental response in plants or to suppress host defense response for, for example, EPS secreted by the alfalfa-symbiotic bacterium *Sinorhizobium meliloti* (Mendrygal and González 2000). EPS produced by *Pantoea agglomerans* YAS34 was associated with plant growth promotion of sunflower (Alami et al. 2000). EPS from plant pathogenic *Pantoea agglomerans* elicited a quick flux of active oxygen species in tobacco, parsley, wheat, and rice cell culture (Conrath et al. 2006). However, elicitation of ISR by EPS from PGPR has not been reported. EPS from *Burkholderia gladioli* IN-26, a strain of PGPR, can induce systemic resistance to *Colletotrichum orbiculare* in cucumber when it infiltrates leaves or is applied via seed soaking at a concentration of 200 ppm (Park et al. 2008). However, Ipper et al. (2008) reported that EPS from *Serratia* strain Gsm01 at a concentration of 200 ppm on tobacco leaves affected with *Cucumber mosaic virus* results in accumulation of peroxidase, PAL, and phenols, and an increased level of PR-1b protein expression.

6.4 Antibiotics

Antibiotics are low molecular weight compounds produced by a few microorganisms, and are harmful to the growth and metabolism of other microorganisms. A large diversity of antibiotics exists (e.g., 2,4-diacetylophloroglucinol, pyrrolnitrin, pyoluteorin, and phenazines), and their involvement in biocontrol has been well studied (Haas and Défago 2005). 2,4-Diacetylophloroglucinol is an elicitor of systemic resistance against *Hyaloperonaspora parasitica* induced by *P. fluorescens*

CHA0, whereas its mutant strain does not show any ISR effect (Iavicoli et al. 2003). Moreover, Siddiqui and Shaukat (2003) have demonstrated that *P. fluorescens* CHA0 producing 2,4-diacetylophloroglucinol induces resistance in tomato against root-knot nematode *Meloidogyne javanica*, whereas its mutant strain does not have the capacity to induce resistance. Resistance induced by 2,4-diacetylophloroglucinol follows a signaling route that induces ethylene signaling (Iavicoli et al. 2003). Another antibiotic, pyocyanin (an N-containing heterocyclic blue phenanzine pigment), is also considered a bacterial determinant eliciting ISR (Britigan et al. 1997). Pyocyanin production by *P. aeruginosa* 7NSK2 along with SA-derivative pyochelin triggered ISR in beans and tomato against *B. cinerea* (Audenaert et al. 2002). De Vleesschauwer et al. (2006) reported the dual role of pyocyanin in *P. aeruginosa* 7NSK2-triggered ISR, acting as a positive regulator of resistance to *Magnaporthe oryzae* while also rendering ornamental plants hypersusceptibile to *Rhizoctonia solani*.

6.5 Flagella

The protein flagellin is the building block of the flagellum, a motility organ. It is recognized by most plants, an indication that detection of flagellin is evolutionarily ancient (Boller and Felix 2009), and is required for root colonization by rhizobacteria (De Weger et al. 1987). Conserved peptides of flagellin are observed in Toll-like receptor-like kinase FLS2 in *Arabidopsis* (Gómez-Gómez et al. 2001) and in tomato (Robatzek et al. 2007). Flagella from *P. putida* WCS358 have been widely studied in *Arabidopsis*, bean, and tomato plants (Meziane et al. 2005) but it was shown that its mutant strain lacking flagella also induced resistance; hence, it was concluded that flagella do not play a role in induction of systemic resistance by *P. putida* WCS358. Therefore, there must be other bacterial determinants involved in induction of systemic resistance by *P. putida* WCS358. Plant cells have some receptors which recognize the stretching of 15–22 amino acids of flg22 in a conserved domain, which is a potent elicitor in cell culture of certain plant species such as *Arabidopsis*, tobacco, potato, and tomato. In tomato, flg22 receptor is active at a concentration of 1 pM and has half-maximal resistance at a concentration of 30 pM (Felix et al. 1999). Chinchilla et al. (2006) reported that flagellin is identified through its interaction with FLS2 in *Arabidopsis*. Thereafter, it was identified in *Nicotiana benthamiana*, tomato, *Brassica* sp., and rice (Takai et al. 2008). FLS2 present on the plasma membrane was internalized on flg22 stimulation (Robatzek et al. 2006).

6.6 Volatile Metabolites

Chemically diverse, volatile metabolites are produced by plants and microorganisms, such as terpenes, indoles, fatty acid derivatives, and molecules from other chemical families (Pare and Tumlinson 1999). Ryu et al. (2004) reported that

volatile organic compounds (VOCs) released from PGPR trigger ISR in *Arabidopsis* using an in vitro Petri plate method against the necrotrophic pathogen *Pectobacterium carotovorum* subsp. *carotovorum* using the PGPR strains GB03 and IN937a. The VOCs which were involved were analyzed by gas chromatography–mass spectrometry and were found to be 2,3-butanediol and its precursor 3-hydroxy-2-butanone (Farag et al. 2013). However, it is yet to be investigated whether recognition of VOCs is by aboveground or belowground parts and how plants recognize VOC signals (Pare et al. 2005). Heil and Ton (2008) postulated that in the presence VOCs, changes in transmembrane channels lead to enhanced gene activity.

6.7 Other Compounds

Biosurfactants, most specifically cyclic lipopeptides, act as ISR signaling molecules in plants. Cyclic lipopeptides such as members of the fengycin, iturin, and surfactin families from *Bacillus* sp. are known to induce resistance mechanisms in plants (Ongena and Jacques 2008). Pure fengycin and surfactins provided ISR-mediated protection in beans against *B. cinerea* similarly to that induced by *B. subtilis* S499 (Ongena et al. 2007). Massitolide A cyclic lipopeptide, a member of the viscoin group from *P. fluorescens* strain SS101, shows direct antagonisms and not ISR, but massitolide A when applied alone reduces lesion areas in tomato but not disease incidence, whereas its mutant strain does not show any such effect (Tran et al. 2007).

Certain compounds, such as *N*-acyl homoserine lactones (AHL), a class of bacterial quorum-sensing signals from *Pseudomonas*, assist bacterial cells to regulate gene expression in shoots and roots, and modulate defense and cell growth responses, depending on the population density (Jha and Saraf 2012). *P. putida* strain IsoF producing four different 3-oxo-AHL molecules with acyl side chains showed marked reduction in tomato damage when plants were challenged with *Alternaria*, whereas the mutant strain was 50 % as effective as the wild-type strain. A microarray analysis of defense gene expression of tomato leaves on application of *N*-hexanoyl and *N*-butanoyl homoserine lactones to the roots showed enhanced production of PR proteins and acidic chitinase (Schuhegger et al. 2006). Pure benzylamine at a concentration of 1 μM induced systemic resistance in plants such as beans and cucumber, indicating that the amino group is involved in the resistance (De Vleesschauwer et al. 2008). *N*-Dimethyl-*N*-tetradecyl-*N*-benzylammonium released by *P. putida* BTP1 seems to be the bacterial determinant of systemic resistance in cucumber (Ongena et al. 2008).

7 Conclusions and Future Prospects

Induction of resistance in plants has opened a new horizon in disease maintenance and plant protection. It is a promising tool for ecofriendly disease control and sustainable agricultural practices. PGPR help the plant by plant growth promotion mechanisms, biocontrol, and inducing systemic resistance in host plants. SA-dependent and SA-independent pathways are both involved in systemic signaling for defense responses. The variety of rhizobacterial determinants shows their vital role in ISR and their regulation in the rhizosphere against multiple pathogens attacking crops. The various bacterial determinants and their regulation in the rhizosphere to explore the fundamentals of plant–microbe interactions will a hot topic of future research because it may offer an opportunity to use the above-mentioned attributes of PGPR in crop management strategies.Acknowledgments We thank the Department of Microbiology, Gujarat University, for encouraging us and helping us with the required facilities and British Petroleum International for financial support.

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Occurrence, Distribution, and Molecular Identification of Phytoplasma-associated Diseases in Ornamental Plants

Akil Ahmad Khan, Shoeb Ahmad, and Mohd Sayeed Akhtar

Abstract Phytoplasma is recognized as the serious constraints for the many economically ornamental plants all around the world. It may reduce the quality and yield of ornamental plants and is recognized internationally because of its unspecific symptoms, severe losses, and diverse epidemiology. The epidemics of these diseases have compelled the withdrawal of many ornamental plant species such as gladiolus, lily, chrysanthemum, and rose from cultivation. So far, more than 42 ornamental plant species were reported as infected by phytoplasma. The general symptom includes flower malformation, growth abnormalities, yellowing or decline of leaves, elongation and etiolation of internodes, witches' broom, stunting, little leaf, and virescence. The knowledge on the diversity and identification of phytoplasma has been explored with the molecular tools and techniques showing that phytoplasma infecting the ornamental plant *Candidatus* Phytoplasma asteris belongs to a major 16Srl group. The other known groups of phytoplasmas are 16Srll, 16Srlll, 16SrV, 16SrVl, 16SrVll, 16SrlX, 16SrX, 16SrXll, 16SrXlll, and 16SrXV. For the detection of phytoplasma in the infected plant parts or tissues, the 16S rRNA gene fragments were amplified using phytoplasma universal primer pairs P1/P7 in a polymerase chain reaction (PCR) followed by primer pairs R16F2n/R16R2 in the nested PCR. Nevertheless, for the finer detection of phytoplasma-related *Candidatus* Phytoplasma asteris, DNA samples were used to extend the *RP* and *Tuf* gene fragments by PCR using aster yellows group-specific primer pairs RP(l)F1A/RP(l)R1A and fTufAy/rTufAy, respectively. However, the restriction fragment length polymorphism (RFLP) analysis of *RP* gene fragments digested with AluI, MseI, and Tsp5091 restriction enzymes indicates the presence of aster yellows group. The aim of the present chapter is to provide an overview of the phytoplasma-associated diseases in ornamental plants, their mode of transmission, and the molecular techniques employed to detect the phytoplasma in the infected plant parts or tissues.

Keywords *Candidatus* • PCR • RFLP • Transmission • Aster yellows disease

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1 Introduction

Phytoplasmas are strangely small obligate parasites found in the phloem tissues of the plants. They are filamentous in shape with a very small genome. Phytoplasmas vary in size ranging from 200 to 800 nm and usually characterized by a lack of a cell wall. Doi et al. (1967) were the first to report that the aster yellows symptoms in mulberry are caused by cell wall-less prokaryotes not the plant viruses. They named these etiological agents as "mycoplasma-like organisms" (MLOs). Later, in 1994, these microorganisms were renamed as phytoplasma by the International Organization for Mycoplasmology (Hogenhout et al. 2008). Phytoplamas are the group of plant-pathogenic wall-less, non-helical bacteria associated with diseases in several plant species worldwide (Harrison et al. 2009; Marcone 2014). These disorders are characterized by flower malformation, growth abnormalities, yellowing or decline of leaves, elongation and etiolation of internodes, witches' broom, stunting, little leaf, and virescence (Bertaccini 2007; Bertaccini and Duduk 2009). These disorders affect directly the plant health and reduced the quality and yield in the infected plants. Mostly phytoplasmas are host dependent, but sometimes also occur through transovarian transmission (Hanboonsong et al. 2002; Tedeschi et al. 2006).

Phytoplasma-associated diseases are transmitted in a persistent manner by insects belonging to the families Cicadellidae, Cixiidae, Psyllidae, Delphacidae, and Derbidae (Weintraub and Beanland 2006). Molecular data on phytoplasma has provided considerable insights into their diversity and genetic interrelationships that are the basis for several comprehensive studies on phytoplasma phylogeny and taxonomy (Hogenhout et al. 2008). Some investigations, particularly sequence analysis of 16S rDNA, have shown that phytoplasmas constitute a coherent, genus-level taxon. In the monophyletic phytoplasma clade, groups and subgroups were delineated, many of which are now considered as species under the provisional status '*Candidatus*' for incompletely described prokaryotes (Murray and Stackebrandt 1995). Several provisional species have been described to date and rules for future putative species delineation have been defined (IRPCM 2004). The first comprehensive phytoplasma classification scheme based on restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rRNA was proposed by Lee et al. (1998). It is a reliable technique for the differentiation of a wide array of sensitive phytoplasma. The accurate detection of these microorganisms is a prerequisite for the management of phytoplasma-associated diseases. Phytoplasma-associated diseases are associated with more than 600 plant species, including many important food, vegetable, and fruit crops, ornamental plants, timbers, and shade trees. The list of diseases caused by phytoplasma continues to grow with many newly emerging diseases of uncertain etiology and with diverse disease distribution being identified. The aim of the present chapter is to provide an overview of the phytoplasma-associated diseases in ornamental plants, their mode of transmission, and the molecular techniques employed to detect the phytoplasma in the infected plant parts or tissues.

2 Phytoplasma as Plant Pathogens

Phytoplasmas or mollicutes are associated with diseases in several plant species and cause serious economic losses in ornamental plants (Chaturvedi et al. 2010a; Singh et al. 2011). The epidemics of phytoplasmas have been compelled the withdrawal of many ornamental varieties from cultivation. General yellowing and stunted growth of plants, proliferation of shoots, phyllody, virescence, and reduced size of flowers and reddening of leaves are the most common symptoms observed in ornamental plants. The common symptoms in ornamental plants associated with phytoplasma are yellowing of *Aster* leaves; virescence in *Hydrangea*, *Gladiolus*, and *Lilium*; and stunted growth in *Lilium*. Plant diseases associated with the presence of phytoplasma typically exhibit a number of symptoms that are suggestive of disturbances in the normal balance of plant hormones (Schneider et al. 2005). These symptoms include virescence, phyllody, proliferation of auxiliary shoots resulting in witches' broom, sterility of flowers, compact growth at the end of stems, yellowing, phloem necrosis, and dieback of branches in woody plants (Mc Coy et al. 1989; Bertaccini 2007). Phytoplasmas are known to cause considerable losses in ornamentals including *Asclepias curassavica* (Griffiths et al. 1994), *Tanacetum parthenium* (Barros et al. 1998), *Limonium sinuatum* (Kaminska et al. 1999; Shiomi et al. 1999), *Tagetes patula* and *T. erecta* (Wang and Hiruki 2001), and *Gomphocarpus physocarpus* (d'Aquillo et al. 2002).

Natural occurrences of little-leaf disease in several *C. morifolium* plants were observed in gardens and nurseries at Lucknow, India (Raj et al. 2007a, b). The symptoms were excessive proliferation, tiny narrow leaves, and shortening of internodes, which altogether gives rise to witches' broom appearance. Since, *Chrysanthemum* are propagated through suckers or cuttings and the phytoplasma is known to be transmitted by vegetative propagation through cuttings, the identification of the causal agent of the little-leaf disease of *C. morifolium* was attempted (Raj et al. 2007a). However, Singh et al. (2011) have identified phytoplasma infection in five ornamental species grown in the gardens of Uttar Pradesh and Uttarakhand, India, showing suspected phytoplasma symptoms through nested-PCR assays. Based on sequence identities and phylogenetic relationships, the new phytoplasma strains identified have been classified as related to *Candidatus* Phytoplasma asteris (16SrI group). Recently, Sichani et al. (2014) detected phytoplasma-related *Candidatus* Phytoplasma asteris in several annual field crops, vegetables, ornamentals, oilseed crops, and weeds.

3 Phytoplasma-associated Diseases on Ornamental Plants

Phytoplasmas cause diseases in several ornamentals and resulted in serious threats to alternative natural hosts belonging to various economically important plants (Chaturvedi et al. 2010a, b). Phytoplasmas causes premature germination of corn kernels in *Gladiolus* (Bertaccini and Duduk 2009); chlorosis, stunted growth, and

virescence in tulip (Bertaccini and Duduk 2009); phyllody and virescence in *Ranunculus* plants (Parrella et al. 2008); virescence in *Hydrangea macrophylla* (Hiruki et al. 1994) and *Cyclamen* (Bertaccini 1990); and dieback, rose rosette, witches' broom, and bud proliferation in rose (Kaminska and Sliwa 2003, 2004). Table 1 summarizes the various phytoplasma-associated diseases and the symptoms appearing in different plant families.

Table 1 Summary of phytoplasma diseases and their symptoms associated within various plant families

Diseases	Symptoms	Plant families	References
Periwinkle little leaf	Little leaf	Apocynaceae	Davis et al. (1990)
Aster yellows	Yellowing	Asteraceae	Lee et al. (1992)
Spiraea stunt	Stunting	Rosaceae	Griffiths et al. (1994))
Alstroemeria dieback	Deformation, absence of pigmentation in leaves, and dieback of floral stems	Amaryllidaceae	Bertaccini et al. (1996) and Cervantes-Diaz et al. (2004)
European aster yellows	Yellowing	Asteraceae	Vibio et al. (1994, 1996)
Gladiolus aster yellows	Yellowing	Iridaceae	
Chrysanthemum witches' broom	Downward curling, yellowing, and witches' broom	Asteraceae	Okuda et al. (1997)
Dahlia cultorum	Stunted growth and shoot proliferation	Asteraceae	Marzachi et al. (1999)
Abnormal proliferation of cladodes	Severe proliferation of cladodes with lack of flower, fruit, and spine production	Cactaceae	Tessitori et al. (2006)
Chrysanthemum indicum hybridum	Virescence and abnormal flower proliferation	Asteraceae	Duduk et al. (2006)
Little leaf	Reduced leaf size	Portulacaceae	Ajayakumar et al. (2007)
Jasminum witches' broom	Curling of leaves, stunting, yellowing, and witches' broom	Oleaceae	Al-Zadjali et al. (2007)
Chrysanthemum little leaf	Little leaf	Asteraceae	Raj et al. (2007a)
Little leaf of rose	Little leaf	Rosaceae	Raj et al. (2007b)
Purple coneflower	Phyllody	Echinacea	Lee et al. (2008)
Little leaf	Bud proliferation, downward curling, stunting and yellowing, rosettes and proliferation, witches' broom	Portulacaceae	Samad et al. (2008)
Rose little leaf	Little leaf and chlorosis	Rosaceae	Chaturvedi et al. (2009a)
Little leaf of *Catharanthus*	Little-leaf phyllody	Apocynaceae	Chaturvedi et al. (2009b)

(continued)

Table 1 (continued)

Diseases	Symptoms	Plant families	References
Lethal decline	Lethal decline, large number of reddish brown leaves in the mid-crown, wilting, necrosis of the youngest leaf	Arecaceae	Harrison et al. (2009)
Malformation of floral spikes	Flower yellowing, malformation, stunting, small corms, poor root	Iridaceae	Raj et al. (2009)
Leaf roll	Leaf roll, little-leaf symptoms	Sapindaceae	Zhang et al. (2009)
Hibiscus little leaf	Leaf roll, little-leaf symptoms	Malvaceae	Chaturvedi et al. (2010b)

The symptoms include general chlorosis, leaf bronzing or reddening, dieback, abnormal production of secondary shoots, upright growth habit, and flower malformation. The flowers are sterile, reduced in size with necrotic petals (Kaminska and Malinowski 1996). However, free-branching production, reported in *Euphorbia pulcherrima*, was also associated with phytoplasma (Lee et al. 1997). In *Silene nicaeensis*-phytoplasma infected plants showed narrow leaves, shortened internodes, reddish brown discoloration of leaves and stems, proliferation of auxiliary shoots, and phyllody (Cozza et al. 2008). Moreover, *Alstroemeria*, *Calendula*, and *Digitalis lanata* Ehrh with virescence in flowers and *Asclepias physocarpa* with plant malformation and growth stunting were also linked with phytoplasma (Bellardi et al. 2007; Bertaccini and Duduk 2009; Chaturvedi et al. 2010a).

3.1 Diseases in Dicentra

Dicentra spectabilis produces fleshy tuberous roots, but is frequently listed in perennial plants than bulbous crops. It is propagated by cutting tubers into pieces, by shoot and leaf cuttings, or from seeds. Kaminska et al. (2004) have reported the phytoplasma disease on this plant in Poland.

3.2 Diseases in Lily

It is not clear when aster yellows-type disease was described for the first time in lilies. Most probably the earliest description of aster yellows-type disease in lilies in the United States was by Ogilvie and Guterman (1929). They have described a disease on *Lilium longiflorum*, characterized by stunted growth, leaf chlorosis, and malformation and flower distortion. Later on, the PCR amplification of 16S rDNA and RFLP analysis proved that stunted growth and flower bud deficiency symptoms in hybrids Casablanca were associated with infection with aster yellows phytoplasma and viruses (Bertaccini et al. 2006).

3.3 *Diseases in Magnolia*

The genus *Magnolia* comprises about 80 species of trees and shrubs, naturally distributed throughout Eastern North America and Southeastern Asia. Magnolias are relatively free of pest and diseases. A new severe phytoplasma disease, designated as Magnolia stunt and yellows, was observed in magnolia grown in some gardens and nurseries in Poland (Kaminska et al. 2001a, b).

3.4 *Diseases in Rose*

Rose is the most common garden plant and important commercial-cut flower cultivated under cover several viruses like diseases worldwide. The first record of the rose wilt was found in Australia in 1908, but the symptoms were lately described by Grieve (1931) and Fry and Hammett (1971) in New Zealand. It is also known as a rose witches' broom. This disease is endemic in Southeast, South Central, and North Central United States. In 1976 rose leaf curl (Slack et al. 1976), which resembles that of rose wilt disease, and rose spring dwarf were described in the United States. Due to the similarity in the mentioned diseases, Thomas (1981) named them rose degeneration syndrome.

3.5 *Diseases in* Catharanthus

Detection of phytoplasma in *C. roseus* has been reported throughout the world (Jimenez and Montano 2010). *C. roseus* is the best host for phytoplasma; thus, an extensive research has been carried out to understand their interaction with hosts on this particular plant. Molecular cloning and detection of DNA of clover proliferation and little-leaf disease in *C. roseus* were also carried out (Deng and Hiruki 1990). However, Musetti et al. (2007) observed the effects of fungal endophytes in *C. roseus* tissues infected with phytoplasma disease.

3.6 *Diseases in* Chrysanthemum

Phytoplasma disease on *Chrysanthemum* sp. was reported by Pettersson and Tomenius (1979)in Sweden, by Verhoyen et al. (1979) in Belgium, by Shiomi and Sugiura (1983) in Japan, by Israel et al. (1988) in the United States, and by Duduk et al. (2006) in Serbia.

3.7 Diseases in Chinese Aster

Chinese aster is one of the species where phytoplasmas were first detected (Doi et al. 1967). Haggis and Sinha (1978) observed MLOs in the petiole tissues of affected Chinese aster plants with the help of scanning electron microscopy (SEM). However, Hemmati and Mc Lean (1980) observed ovoid polymorphic and pleomorphic bodies in Chinese aster infected with aster yellows. Similarly, Wang and Hiruki (2001) detected and estimated the genetic diversity of phytoplasma associated with the Chinese aster yellows with heteroduplex mobility assay (HMA) in Canada.

3.8 Diseases in Phlox

Zajak (1979) observed MLOs in sieve tubes of *Phlox paniculata* with symptoms of phyllody. Similarly, Misra et al. (1985) detected these prokaryotes in phloem sieve tubes of *Phlox drummondii* in Rajasthan, India.

3.9 Diseases in Syringa

Hibben et al. (1986) and Griffiths et al. (1999) detected the presence of MLOs in phloem sieve tubes of *Syringa* plant infected with lilac witches' broom. However, Hibben and Franzen (1989) reported susceptibility of *Syringa* to these prokaryotes in the United States.

3.10 Diseases in Portulaca

Ajayakumar et al. (2007) and Samad et al. (2008) described a phytoplasma disease associated with little leaf of *Portulaca grandiflora* in Lucknow, India.

3.11 Diseases in Other Ornamental Plants

Lange et al. (1978) described four diseases of unknown etiology of *Anemone nemorosa* in Denmark. Similarly, Ulrychova et al. (1983) observed yellows-type disease in Czech Republic. However, Marwitz et al. (1984) observed virescence and phyllody in *Primula denticulata* and *P. vulgaris* in Germany, while Behncken (1984) reported a new vector of a little-leaf disease in *Ipomoea* in Australia. Sharma et al. (1985) described phyllody of marigold in India. Hiruki and Rocha (1986) reported little-leaf disease on *Brugmansia candida* in Australia. Bellardi and

Bertaccini (1990) observed spheroid or elongated MLOs in the phloem of virescent *Sinningia speciosa* in Italy. However, Rojas-Martinez et al. (2003) identified phytoplasma associated with *Cosmos bipinnatus* plants by RFLP analysis of 16S rDNA in Mexico. Similarly, Cervantes-Diaz et al. (2004) reported the molecular detection of phytoplasma associated with *Alstroemeria* in Mexico. However, Hong et al. (2005) detected and identified phytoplasmas associated with witches' broom disease in *Cassia surattensis* in China. A new phytoplasma was identified as infecting *Cassia italica* in Oman (Al-Saady et al. 2008). Siddique (2006) reported phyllody on *Gerbera jamesonii* in Australia, while Ribeiro et al. (2006) reported shoot proliferation in *Begonia* in Brazil. Jones and Arocha (2006) reported yellowing and little-leaf disease on *Veronica scutellata* in the United Kingdom.

Al-Zadjali et al. (2007) identified and molecularly characterized the phytoplasma associated with *Jasminum sambac* witches' broom in Oman. Sobolev et al. (2007) described phytoplasma symptoms in *Mirabilis jalapa* plant in Israel. Nicolaisen and Christensen (2007) reported changes in gene expression in *E. pulcherrima* due to phytoplasma infection. Habili et al. (2007) detected for the first time *Candidatus* Phytoplasma australiense in *Liquidambar styraciflua* in Australia. Davino et al. (2007) detected phytoplasma presence in *Matthiola incana* by applying PCR/RFLP techniques. Mafia et al. (2007) reported witches' broom disease on *Tabebuia pentaphylla* in Brazil. Kaminska and Sliwa (2008) reported mixed infection of apple proliferation and aster yellows phytoplasmas in dahlia plants. Lee et al. (2008) reported purple coneflower phyllody associated with aster yellows phytoplasmas (16SrIB) in Maryland. Harrison and Helmick (2008) reported for the first time *Candidatus* Phytoplasma asteris-related strain associated with the little-leaf disease of *Helianthus debilis* in Florida. However, Harju et al. (2008) identified an X-disease (16SrIII) group phytoplasma infecting *Delphinium* sp. in the United Kingdom, while Zhang et al. (2009) reported aster yellows group phytoplasma associated with a leaf roll disease in shiny leaf yellowhorn in China. Similarly, Chaturvedi et al. (2010b) reported the little-leaf disease in *Hibiscus rosa-sinensis* in Gorakhpur. However, Singh et al. (2011) observed the presence of phytoplasmas in five ornamental plants, namely, *Alstroemeria*, *Duranta*, *Streblus*, *Petunia*, and *Zenia*, from the gardens of Uttar Pradesh and Uttarakhand.

4 Mode of Transmission of Phytoplasma

Phytoplasma transmission occurs through sap-sucking insect vectors belonging to families Cicadellidae (leafhoppers) and Fulgoridae (plant hoppers). It could also be spread through vegetative propagation techniques such as grafting of infected plant onto healthy plant, cutting, micropropagation, or other techniques. A number of insect vectors were responsible for the transmission of phytoplasma in ornamental plants (Kaminska 2008; Favali et al. 2008; Chung 2008). For instance, the causal agent of aster yellows disease in *Gladiolus* is transmitted by the leafhopper *Macrosteles sexnotatus* (Fraitag and Tompkins 1963). However, aster yellows phytoplasmas in diseased lilies and roses were experimentally transmitted by grafting

the *Cuscuta* to *C. roseus* and *Alstroemeria* seedlings (Kaminska and Korbin 2000; Kaminska et al. 2001a). The agent of *Chrysanthemum* yellows phytoplasma was inoculated by multiplying with diverse leafhopper species (Bosco et al. 2007). Similarly, Bertaccini et al. (1993) detected the virescence symptom in *Hydrangea* sp. by grafting it onto healthy plants, while the grafting of different phytoplasma exhibited diverse branch-inducing abilities in poinsettia (Pondrelli et al. 2002).

Aster yellows and apple proliferation phytoplasmas were successfully transmitted by grafting from diseased magnolias to *C. roseus* (Sliwa and Kaminska 2004). However, Epstein and Hill (1995) reported that the pathogen of rose rosette disease was transmitted by *Phyllocoptes fructiphilus*. Artificial transmission of phytoplasma from host plants to *C. roseus* and vice versa was successfully done by Carraro et al. (2004) by grafting with *Cuscuta*. However, it has been also demonstrated that *Cuscuta* transmits different phytoplasma to periwinkle with diverse efficiency due to their pathogenic effects on the vectors (Carraro et al. 1991; Musetti et al. 1992). Bressan et al. (2005) reported that the flavescence dorée phytoplasma greatly reduced the longevity and fecundity of leafhoppers. However, studies on the reproduction of *M. quadrilineatus* increased considerably when this leafhopper was reared on either AY phytoplasma-infected *Arabidopsis thaliana* (Sugio et al. 2011). They concluded that when plants are infected with phytoplasmas, adults live longer and lay eggs and the nymphs are hatched after 15 days from eggs.

5 Phytoplasma Detection and Identification

Plants infected by the phytoplasma exhibited various degrees of symptoms on their hosts, which may be correlated with imbalance in quantities in the presence of growth hormones (Lee and Davis 1992). The most characteristic symptoms caused by phytoplasma in ornamental plants are virescence or phyllody, flower sterility, witches' broom, and abnormal internode elongation. The generalized symptoms include stunted growth, yellowing, little leaf, phloem necrosis, chlorosis, crinkling, shoot proliferation, leaf burn, and death of the plant (Mc Coy et al. 1989). The symptom of phytoplasma disease in lily appears as fasciation such as flattening of the stem, bunchy appearance of apical of stems, and shortening of internodes resulting in multiple flowers (Chung and Jeong 2003). Similarly, Chung (2008) reported the stunting, yellowing, leaf cupping, and vein clearing symptoms in *Chrysanthemum* infected by phytoplasma. However, Kaminska (2008) observed the rose dieback symptoms at the end of winter or early in the spring. Favali et al. (2008) reported the most characteristic symptoms on *C. roseus* plants are yellowing of the leaves, virescence, phyllody and proliferation, witches' broom induced by the premature development of axillary buds, and inhibition of root growth. Most of the phytoplasmas infecting ornamental plants were detected on the basis of symptoms, DAPI staining, electron microscopy, PCR/RFLP analysis, and phylogenetic relatedness. However, the recent studies on the phylogeny based on the mentioned less-conserved genes were nearly matching with that inferred by 16S rDNA sequence analysis, indicating similar interrelatedness among phytoplasma taxa (Fabre et al. 2011; Durante et al. 2012; Valiunas et al. 2013).

5.1 Microscopy and Staining Technique

In the past the phytoplasmas were detected on the basis of microscopic observations and DNA-specific 6-diamidino-2-phenylindole (DAPI) staining techniques. The microscopic methods include transmission electron microscopy (TEM) and light microscopy (LM). Compared to TEM and LM, DAPI is the most sensitive technique. Shin and La (1984) have used Dienes' stains, while, Hiruki and Rocha (1986) have used DAPI stain to detect the MLOs in *C. roseus*. However, electron microscopy was used to detect the phytoplasma in *Aster*, *Gladiolus*, *Ranunculus*, *Cyclamen*, and *Lilium* (Doi et al. 1967; Hemmati and Mc Lean 1980; Bertaccini and Marani 1982; Bertaccini et al. 1988, 1990a, b), while Conti et al. (1988) have detected the presence of this disease-causing microorganism in *Chrysanthemum frutescens*. Moreover, bio-imaging methods require sensitive, specific, and nontoxic fluorescent dyes and the use of confocal or multiphoton microscopy for the identification of phytoplasma in the living host tissues (Christensen et al. 2004). These microscopic methods have also limitations and they didn't identify the pathogen if the infection is in low titer host tissues (Rao et al. 2012).

5.2 PCR/RFLP Assays and Phylogenetic Relationships

The identification of phytoplasma in diseased plants relied on symptoms that already appeared, but sometimes occurrence of peculiar symptoms in infected plants makes it difficult to identify the diseases associated with phytoplasma. In this regard, molecular identification has provided significant acumen on their genetic diversity and interrelationships and could serve as the basis for the comprehensive phylogenic and taxonomic studies (Hogenhout et al. 2008). The investigations on the sequence analysis of 16S rDNA have shown that phytoplasma constitute a coherent, genus-level taxon. The first comprehensive phytoplasma classification scheme was based on restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified 16S rDNA (Lee et al. 2000). This approach provided a reliable tool for broad differentiation among phytoplasmas. This system has classified phytoplasma into 19 groups and more than 40 subgroups and recognized as the most comprehensive and widely accepted phytoplasma classification system (Arocha et al. 2005; Al-Saady et al. 2008). Sensitive and accurate detection of these microorganisms is a prerequisite for the study and management of phytoplasma-associated diseases. After their discovery, phytoplasmas were initially difficult to detect due to their low concentration, especially in woody hosts, and their erratic distribution in the sieve tubes of infected plants (Berges et al. 2000).

Dot and Southern blot hybridization assays were used as the molecular tools for phytoplasma detection in the last few years, but nowadays, they have been replaced by the PCR assays using universal primers. Several universal and group-specific

primers have been designed for the detection of phytoplasma by the past researchers (Lee et al. 1993; Lorenz et al. 1995; Smart et al. 1996; Gundersen and Lee 1996; Khadhair et al. 1998; Berges et al. 2000). The nested-PCR assay using the group-specific primers was the most sensitive and specific technique for the detection of the phytoplasma. Application of the nested-PCR assay to detect the phytoplasma-associated disease in ornamental plant has greatly assisted in the identification of a varied range of phytoplasma in ornamental plants (Chung 2008; Favali et al. 2008; Mertelik et al. 2008). This approach is adept to sense the manifestation of dual or multiple phytoplasma in the infected plant tissues or mixed infection (Lee et al. 1995). Therefore, this technique is more valuable in the amplification of the phytoplasma from samples having low titers or inhibitors (Heinrich et al. 2001). For amplification rDNA of phytoplasma, the universal primer pairs P1/P7, P4/P7, and P1/P6 are being used to amplify 16S ribosomal region, plus spacer region (Deng and Hiruki 1991; Schneider et al. 1995). However, Gundersen and Lee (1996) used the 16S rRNA primer pairs R16F2n/R16R2 to detect the phytoplasma using RFLP. Since then, this primer pair has been more popular for the detection of phytoplasma. The major benefit to this approach is that the PCR products could be sequenced directly from amplicons. These sequences were used to determine the genetic relatedness of the phytoplasma. The taxonomic and molecular classification of phytoplasma detected in ornamental plants has been summarized in Table 2.

Table 2 Molecular characterization of phytoplasma stains based on RFLP analysis detected in ornamental plants

Phytoplasma strains	16Sr group to subgroup	Accession number	References
CY	16SrI-B	EF634457	Bertaccini et al. (1990b)
AY1	16SrI-B	L33767	Lee et al. (1992)
GLY	16SrI-B	–	Vibio et al. (1994, 1996)
AUSGU	16SrXII-B	L76865	Davis et al. (1997)
PpYC	16SrII-D	Y10097	White et al. (1998)
JHP	16SrXII-D	AB010425	Sawayanagi et al. (1999)
HibWB26	16SrXV-A	AF147708	Montano et al. (2001)
CnWB	16SrXIX-A	AB054986	Jung et al. (2002)
PD1	16SrX-C	AJ542543	Seemüller and Schneider (2004)
PAY	16SrXVII-A	AY725234	Arocha et al. (2005)
APPTW12-NE	16SrXVIII-A	DQ174122	Lee et al. (2006)
THP	nd	EF199549	Arocha et al. (2007)
IM-1	16SrXXIX-A	EF666051	Al-Saady et al. (2008)
SCWB1	16SrXXX-A	FJ432664	Zhao et al. (2009)
SoySTc1	16SrXXXI-A	HQ225630	Lee et al. (2011)
16SrVI-I	PassWB-Br3	GU292081	Davis et al. (2012)
MaPV	16SrXXXII-A	EU371934	Nejat et al. (2013)
LYDM-178	16SrXXII-A	KF751387	Harrison et al. (2014)

6 Conclusions and Future Prospects

In this modern era, attention has been paid for the development of floriculture, particularly for the benefit of small farming businesses to produce seedlings of ornamental plants for the domestic market as well as foreign export. Likewise, in other sections of agricultural economics, this trade is also susceptible by plant diseases particularly phytoplasma. The infestation of ornamental plants by phytoplasma is too much severe and is the burning topic of research worldwide because of unspecific symptoms, severe losses, and diverse epidemiology. Epidemics of these diseases have compelled the withdrawal of many ornamental plant species such as gladiolus, lily, chrysanthemum, and rose from cultivation. Nowadays, the diversity of phytoplasma has been explored with the help of the sophisticated molecular tools and techniques. Several factors have been identified in the past few decades throughout the world for the transmission of this disease at a grave extent, but still this unique etiological agent required more attention for the exploration of new possibilities. However, detection of various alternative hosts, which may carry the identical phytoplasma and their mode of transmission, will be addressed in the near future by planned and strict management strategy.

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Isolation and Identification of Allelochemicals from Ascocarp of *Tuber* Species

Paola Angelini, Emma Bricchi, Mohd. Sayeed Akhtar, Alessandro Properzi, Jeri-Lynn Elizabeth Fleming, Bruno Tirillini, and Roberto Venanzoni

Abstract Truffles (*Tuber* spp.) belong to the fruiting bodies of certain hypogeous ascomycetes, which may grow in ectomycorrhizal symbioses with specified shrub and tree species. Some truffles, notably *Tuber melanosporum* and *T. aestivum,* form 'burnt' area, also known as 'burn' or 'brûlé' around their symbiotic hosts. Increasingly focused interest has been centred on an in-depth research and study of truffle methanolic extracts and their fatty acid allelochemicals. These metabolites have been recognised as biochemical and have great influence in the burnt formation. This present chapter contributes the knowledge of truffle methanolic extracts and fatty acids regarding allelopathic activity to understand the applicability and sustainability of truffles in agricultural practices for the management of weed and plant pathogens. However, it will also be helpful to the companies specialising in the processing of truffle and the recovery and reinsertion of waste truffles through the production process for the isolation of important allelopathic compounds.

Keywords Bioassay • Fatty acids • LC/MS analysis • *Tuber aestivum* • *T. borchii* • *T. magnatum* • *T. melanosporum*

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1 Introduction

Fungi are microorganisms known to occupy every ecological niche of the earth. Their environmental requirements are different, from those species that are capable of utilising nutrients in various substrates to species with very specific habitat needs (Angelini et al. 2008, 2014a, 2016; Pagiotti et al. 2011; Picco et al. 2011; Perotto et al. 2013). Fungi are a rich source of secondary metabolites and natural products. These compounds recovered from the fungi are low molecular weight molecules, are highly dispensable and are advantageous in various environmental conditions (Gerke and Braus 2014). However, the interest in secondary metabolites of symbiotic fungi found in close association with land plants, lichens, marine organisms and insects has recently intensified due to the belief that natural products synthesised by these fungi during ecological interactions exhibited strong biological activities (Keller and Turner 2012; Streiblova et al. 2012; Angelini et al. 2015b).

True truffles are subterranean fruiting bodies produced by members of the ascomycete genus *Tuber*, comprising of over 200 species (Bonito et al. 2012). Some of these species produce edible fruiting bodies (ascocarps) of high nutritional values commonly used in both traditional European cooking and haute cuisine worldwide. These fungi form as ectomycorrhizal symbiosis with the roots of trees or shrub to complete their life cycle. The production points of some species of truffles, such as *T. melanosporum* and *T. aestivum*, are known as burns or brûlés because of their activity to clear the vegetation around host plants through the secretion of phytotoxic allelochemicals from the mycelia and ascocarps into their environment as exudates or leachates (Angelini et al. 1998, 2010a, b, 2015; Zacchi et al. 2003; Splivallo et al. 2007a, b, 2011; Splivallo 2008; Streiblova et al. 2012; Azul et al. 2014). They are also able to effectively 'kill off' the competing plants by creating a seemingly burnt area at their vicinity. Truffles are naturally occurring in the Northern Hemisphere (Payen et al. 2014). Black truffle (*T. melanosporum*), the black summer truffle (*T. aestivum*) and white truffle (*T. magnatum*) are edible in nature and are consumed throughout the world (Otsing and Tedersoo 2015). However, there are some other economically important truffle species such as pecan truffles (*T. lyonii*) found in North America and Chinese truffles (*T. indicum* and *T. sinoaestivum*) found in Asia (Bonito et al. 2012; Reyna and Garcia-Barreda 2014).

The natural production of truffle in the past century has been drastically declining due to many factors, including deforestation of the natural habitat of the *Tuber* spp., poor forest management, unselective harvesting of fruiting bodies as well as the introduction of new or exotic species which is unable to form a symbiotic relationship with edible mushrooms (Rubini et al. 2005). Other factors include acid rain, global climate change and pollution (Büntgen et al. 2012; Olivier et al. 2012). This decline and the flourishing truffle market have encouraged researchers and entrepreneurs to develop methods for cultivating truffles in varying climatic conditions and on different soils (Otsing and Tedersoo 2015). Despite the fact that truffle orchards have been and are

being established in various countries around the world, including New Zealand and Israel, many basic aspects of truffle biology are scarcely understood and the ecological requirements of some species remain unknown to this day (Murat et al. 2013; Martin et al. 2014). Thus, innovative tools are now required to make truffle cultivation in a sustainable manner and profitable in the truffle industry.

2 Truffle Plantations: Weed and Herbaceous Plant Competition and Necessity of Allelopathy

The ecological requisites of truffle species are relatively well known, and cultivation methods have been developed based on these, thus allowing many once-endemic species to be sown in truffle plantations worldwide especially in New Zealand, Finland and Israel. It is not possible to cultivate successfully all the species in truffle beds (Iotti et al. 2012; Reyna and Garcia-Barreda 2014). Aside from the importance of site suitability and seedling quality, one critical factor in the precocity and regularity of truffle production has proven to be correct management of the truffle beds. All ectomycorrhizal fungi demand very specific conditions to be able to develop in a given soil, pH, moisture, fertility, temperature, aeration, texture, organic matter and content found in and the canopy cover of a given site all contribute to the possible development or inhibition of fungi, such as truffles (Olivier et al. 2012; Salerni et al. 2013). These factors and more are modifiable with certain cultural practices and may contribute to creating a more favourable environment for the production of truffles, i.e. weed control, irrigation and foliar fertilisation treatments applied to young truffle plantations have resulted in modifications in plant growth in general as well as in the root system and the status of the ectomycorrhizal colonisation itself (Olivera et al. 2011).

Competition from weeds and herbaceous plants has been shown to reduce the colonisation (Mamoun and Olivier 1997) and production (Ricard et al. 2003) of truffles citing the competition with these plants for water, nutrients and light (Cahill 1999; Olivera et al. 2014). Black truffles act as a natural herbicide as they are known to exert a phytotoxic effect on the root systems of many herbaceous species. This results in a 'burn' pattern, an area void of competing vegetation which forms at the base of colonised trees even years before the first truffle appears. This means before the formation of the truffle bun, the development of the young seedlings (host plant) may be constrained due to competition from weeds which could eventually lead to plantation failure (Olivera et al. 2014). Clearly, the impact herbaceous competition must be reduced so as to create productive plantations. Traditional methods of weed control in truffle orchards are based essentially on labour-intensive tillage and hand hoeing for the first few years. Unfortunately, these may also adversely affect soil structure itself and excessively break down the aggregates (Reicosky et al. 2003).

During a plantation establishment phase, both tillage and herbicides are recognised methods as stated by Ricard et al. (2003); indeed Bonnet et al. (2007) noted

that using glyphosate furnished not only greatly reduced mortality rates in first-year seedlings after out planting but also a non-reduction of truffle mycorrhizae. However, Busse et al. (2004) found that though mycorrhizal formation itself may not be inhibited by commercial herbicides, root tip growth may be subjected to serious damage. Seeing the increasing cause for concern with risks involving human and environmental health for the use of the generally unsafe chemical herbicides (Duke et al. 2001), it becomes clear that an adequate and sustainable modern agricultural practice requires new strategies in the improvement of weed and pathogen management. Allelopathy offers just this new approach towards the discovery, isolation and utilisation of new lead compounds as organic herbicides and pesticides driving directly from the natural biological phenomena of negative allelopathy in plants, fungi and microorganisms (Bhadoria 2011).

3 Allelopathy and Allelochemicals

The term allelopathy (Molisch 1937) refers to the direct or indirect harmful or beneficial effects of one plant (or microbes) on other plants through the release of chemical compounds in the environment, known as allelochemicals (Rice 1984). Allelopathy plays an important role in the pattern of vegetation, i.e. as plant communities, climax vegetation and crop productivity (Chou 1999; Cruz-Ortega et al. 2007).

According to the International Allelopathy Society, allelopathy can be regarded as 'any process involving secondary metabolites produced by plants, algae, bacteria, and fungi that influences the growth and development of agriculture and biological systems' (Muller 1966). Which is to say, allelopathic compounds may influence the germination and the growth and development of plants, affecting their photosynthetic, respiratory, transpiratory and biochemical metabolic processes reaching as far as to affect the molecular basis of their protein and nucleic acid synthesis (Reigosa et al. 2006; Angelini et al. 2009).

Allelochemicals or allelochemics are secondary metabolites which may institute allelopathic influences (Chandra et al. 2012). Taking into consideration the structures and properties of allelochemical compounds, it is possible to descry essentially ten categories, as follows: (1) water-soluble organic acids, straight-chain alcohols, aliphatic aldehydes and ketones; (2) simple unsaturated lactones; (3) long-chain fatty acids and polyacetylenes; (4) quinines (benzoquinone, anthraquinone and complex quinines); (5) phenolics; (6) cinnamic acid and its derivatives; (7) coumarins; (8) flavonoids; (9) tannins; and (10) steroids and terpenoids (sesquiterpene lactones, diterpenes and triterpenoids) (Li et al. 2010). Despite the fact that tens of thousands of secondary substances have been identified to date, the number of these which have been revealed as utilisable allelochemicals is very limited (An 2005).

3.1 Competition or Allelopathic Interference

A vast gamma of scientific studies regarding the biology and genetics of pathological, symbiotic and associative interactions on a molecular level is defined by plant-microbe interactions; competition and allelopathy are paramount in these. As it is reliant upon the effective release of chemical compounds (particularly secondary metabolites) into the surrounding environment, allelopathy, a form of chemical competition, is distinct from the competition, which instead removes or reduces a necessary factor for other individuals in a shared habitat (Bertholdsson 2012). Competition is used by microbes and plants to assure its place in nature. In the field, both allelopathy and competition usually act simultaneously (Bertholdsson 2012).

4 Allelopathic Activities of Methanolic Extract and Allelochemicals from *Tuber* spp.

The experiment conducted at the Mycology Laboratory, Department of Chemistry, Biology and Biotechnology, University of Perugia, Italy, studies and observes the allelopathic effects of methanolic extracts of truffles and fatty acid allelochemicals on germination and growth of *Arabidopsis thaliana*, *Lotus corniculatus*, *Melica ciliata* and *Silene vulgaris* seeds and seedlings (Angelini et al. 2015). Here below are offered the descriptions of the tested truffles, methods of experimentation and the results of this experiment.

4.1 Truffle Fruiting Bodies

In the truffle allelopathy studies (Angelini et al. 2010a, b, 2015), fruiting bodies from *T. melanosporum* Vittad., *T. aestivum* Vittad., *T. magnatum* Pico and *T. borchii* Vittad. were purchased from a truffle company and identified according to macro- and micromorphological characteristics paying particular attention to the size, the shape and the ornamentation of spores (Granetti et al. 2005; Angelini et al. 2014b).

4.1.1 Description of Truffles

Tuber aestivum Vittad.

(Great Britain: Summer white truffle; Italy: Scorzone; France: Truffe de la Sainte Jean, Truffe d'été)

Globose ascoma, irregular, from (1)3 to 10(14) cm, often with a, not very pronounced, concavity at the base, firm and compact, black-brown, intense black, with

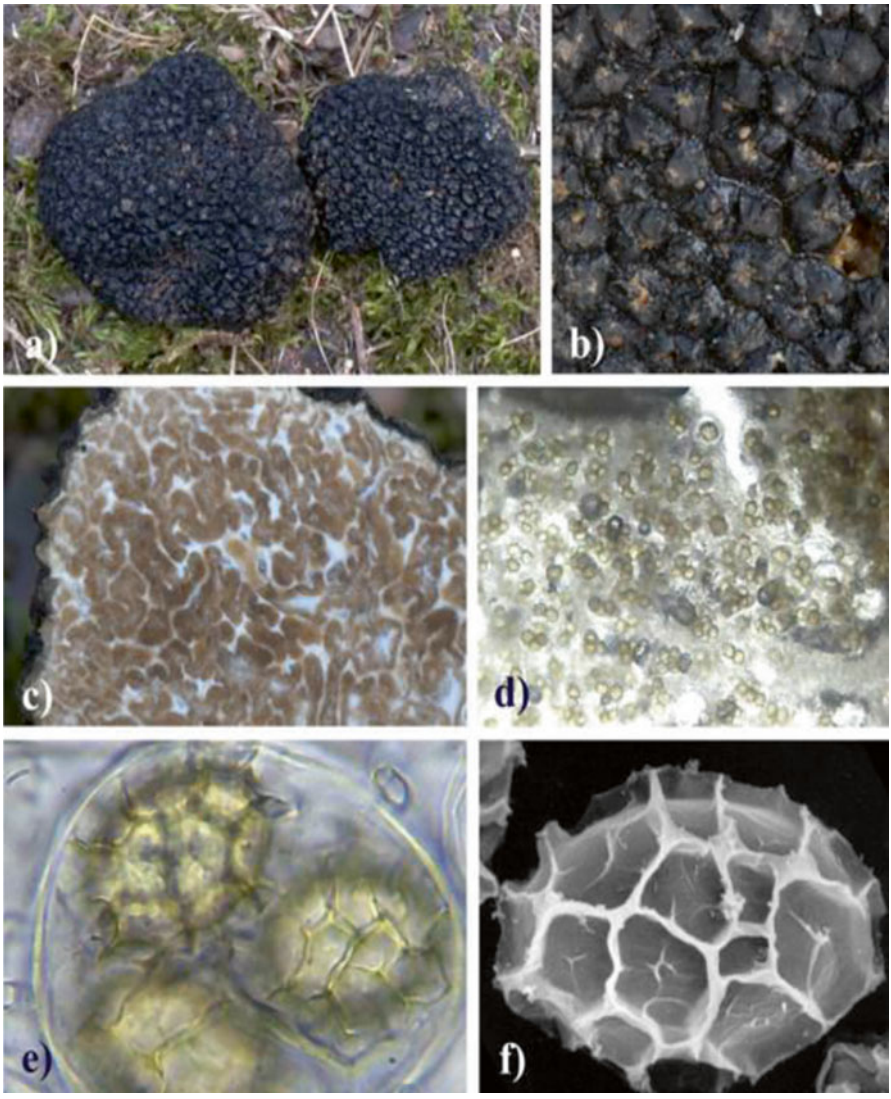

Fig. 1 *Tuber aestivum*: (**a**) Two truffle samples (photo: Giancarlo Bistocchi); (**b**) truffle surface detail with marked verrucas (photo: Giancarlo Bistocchi); (**c** and **d**) gleba section (photo: Giancarlo Bistocchi); (**e**) ascus with three spores (photo: Giancarlo Bistocchi); (**f**) SEM spore with medium-sized alveoles (photo: Emma Bricchi)

4–6(7)-sided pyramidal warts also very large at the base (from 2 up to 12 mm) and (0.5–)1–2(5) mm high, often seen with a depressed or concave apex and with risen radical crests and small parallel transversal striations; warts also with longitudinal cracks (Fig. 1a).

Peridium thickness 200–480 µm comprising a layer of 100 µm corresponding to the warts, essentially pseudoparenchymatic with polygonal cells of 8–18(20) µm,

the external, with a thick wall, coloured brown-black, the internal coloured a pale ochre and an internal layer with a pseudoparenchymatic structure in the proximity of the gleba (Fig. 1b). Global firm and pulpy, at first white, the ochre-like, brunette, often spotted with reddish here and there with plectenchimatic zones; numerous veins, white, very ramified, thin (Fig. 1c, d). Globular asci or subglobular, short stalked or sessile, 80–100 × 55–75 µm, (1)3–5(6) spores (Fig. 1e). Alveolate-reticulum spores, subglobular or largely ellipsoidal, coloured ochre or brownish-red, (18–)24–35(–40) × (15–)18–27(–36) µm excluding the ornamentation; with large irregular alveoli 4–10 µm and 2–5(–7) µm deep, often with internal crests and with walls often hooked at the apex (Fig. 1e, f and Table 1). Odour at first weak, then strong, pleasant, fruity and pleasant flavour.

Habitat

In calcareous terrain, drained and rocky of diverse geological origins with pH 7–8, in mixed and broad-leaved woods, and in conifer plantations, but also under isolated plants, with not preferred exposition, at variable altitudes above sea level up to 1400–1600 m. Fructification also on the surface. Solitary or aggregate.

Maturation Period

From late spring to winter.

Symbiotic Nature

It forms the symbiosis with *Quercus pubescens* Willd., *Q. ilex* L., *Q. ilex* L. var. *ballota* (Desf.) Samp., *Q. robur* L., *Q. petraea* (Mattushka) Liebl., *Q. cerris* L., *Corylus avellana* L., *Ostrya carpinifolia* Scop., *Carpinus betulus* Willd., *Tilia platyphyllos* Scop., *Fagus sylvatica* L., *Betula verrucosa* Ehrh., *Salix* spp., *Populus* spp., *Pinus nigra* Arnold, *P. pinea* L., *P. sylvestris* L., *P. halepensis* Mill., *P. brutia* Ten., *Picea abies* (L.) Karst. and *Cedrus* spp. (Chevalier 1979; Stobbe et al. 2013; Hilszczanska et al. 2014; Garcia-Montero et al. 2014).

Geographic Distribution

Distributed in almost all Europe (between 37° and 57° N), North Africa, Korea and China (Weden et al. 2004; Granetti et al. 2005; Hall et al. 2007; Jeandroz et al. 2008; Milenkovic et al. 2009; Yun and Liu 2009; Chevalier 2010; Streiblova et al. 2010; Gryndler et al. 2011; Gogan et al. 2012; Salerni et al. 2013; Azul et al. 2014; Gezer et al. 2014).

Tuber borchii Vittad.

(Great Britain: Whitish truffle, whitebait; Italy: Tartufo bianchetto, Marzuolo; France: Blanche du Piemont)

Table 1 Morphological features of *Tuber* spp. spores

Tuber spp.	Spore colour	Spore length (µm)	Spore width (µm)	Spore shape	Episporium ornamentation	Ornamentation width	Ornamentation height
Tuber magnatum	Light yellow	35–50	32–42	Subglobose, spheroidal, ellipsoidal	Alveolae 5–6 sided; 2–3 alveolae along spore length	Alveolae 10–20 µm	Walls 4–5 µm
T. aestivum	Yellow-brown	25–36	18–28	Ellipsoidal	Polygonal alveolae sometimes incomplete with inner ridges; 3–4–(5) alveolae along spore length	Alveolae 8–9 µm	Walls 1.5–2.5 µm
T. borchii	Yellow-brown	30–45	24–32	Ellipsoidal, subglobose	Small, orderly alveolate-reticulum; 7–8–(9) alveolae along spore length	Alveolae 4–7 µm	Walls 1–2.5 µm
T. melanosporum	Light brown to mid brown	23–45	19–28	Ellipsoidal	Broad base spines, longitudinally furrowed, sometimes with curved tips	Strong spines, stronger at poles	Spine length 2.5–3 µm

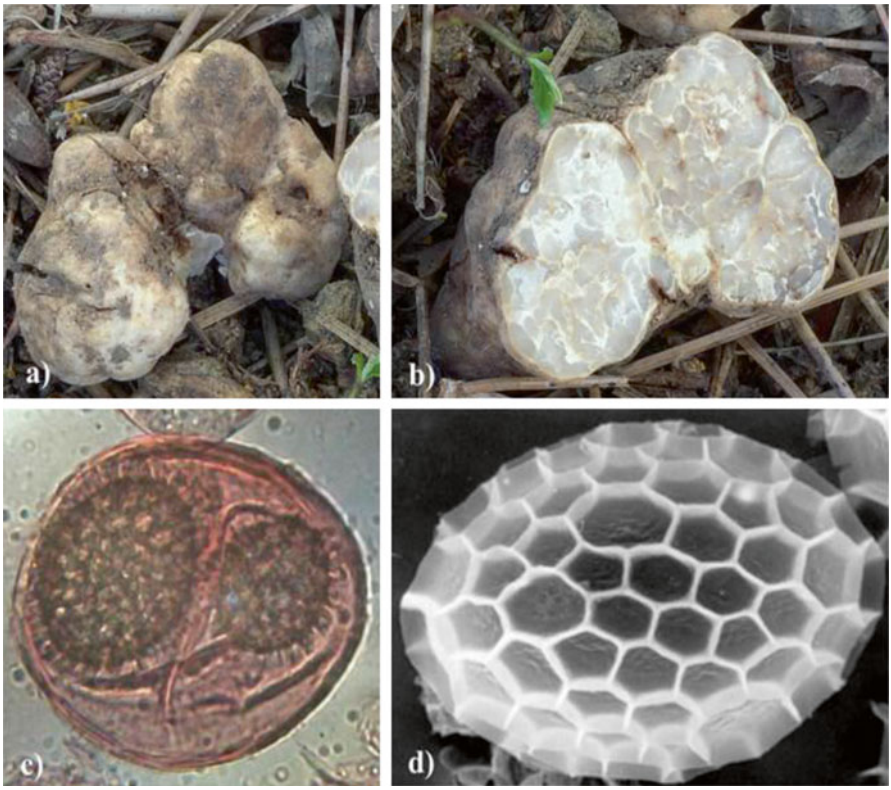

Fig. 2 *Tuber borchii*: (**a**) Truffle sample (photo: Giancarlo Bistocchi); (**b**) truffle section (photo: Giancarlo Bistocchi); (**c**) ascus with two spores (photo: Giancarlo Bistocchi); (**d**) SEM spore showing small regular alveoles (photo: Emma Bricchi)

Globose ascoma, often lobbed or irregular, often with a depressed base, but without cavities, 2–3(–7) cm in diameter (Fig. 2a). Peridium on the surface at first whitish, then greyish-yellow and finally brown ochre, when immature quite pubescent and glabrous at maturity, humid, smooth, often with darker or lighter reddish spots; thickness (100–)200–500 μm, when immature with sparse superficial, short hyphae, up to 50–80(–120) × 3–5 μm in height, generally with thin walls, occasionally thickened and yellowish near the surface, in proximity of the gleba plectenchimatic with meshed hyphae up to 5–9 μm in width (Fig. 2a, b). Pale ochre-coloured gleba, often a bit reddish then yellowish and brownish, smooth plectenchimatic structure, not unlike that of the more internal layer of the peridium, with more or less numerous veins, white and tending towards ochre-like or brownish-red, ramified from multiple points of the peridium and anastomosed (Fig. 2b).

Asci mostly globose, sessile or barely stalked, 73–80 × 52–67 μm, with 1–3(4) spores (Fig. 2c). Alveolate-reticulum spores, ellipsoidal, mostly ellipsoidal,

rarely subspherical, reddish-brown, (20–)30–45(–55) × (18–)24–32(–40) µm excluding ornamentation, with more or less regular polygonal alveoli, numbering (4)5–7 along the major dimension of the spore, 4–8(–10) µm wide and 3–5 µm deep (Fig. 2d and Table 1). Pleasant in odour, then strong and garlic-like; strong flavour, intense, not very pleasant.

Habitat

In calcareous terrain, clayish (hilly), but also sandy (coastal pinewoods) and in humus not very acidic, in broad-leaved, conifer or mixed woods, at a shallow depth in the soil, from sea level to 1000 m in altitude. Solitary and aggregated.

Maturation Period

From autumn to spring.

Symbiotic Nature

It forms the symbiosis with *Q. pubescens* Willd., *Q. ilex* L., *Q. cerris* L., *Q. petraea* (Mattuschka) Liebl., *Fagus sylvatica* L., *Corylus avellana* L., *Carpinus betulus* L., *Ostrya* sp., *Tilia* sp., *Populus alba* L., *P. nigra* L., *P. tremula* L., *Cistus* spp., *Salix alba* L., *S. caprea* L., *Pinus nigra* Arnold, *P. pinea* L., *P. halepensis* Miller, *Larix decidua* Miller, *Cedrus* sp., *Abies* sp. and *Pseudotsuga menziesii* (Mirbel) Franco for *T. levissimum* Gilkey (Angelini and Granetti 2001; Granetti et al. 2005; Iotti et al. 2010).

Geographic Distribution

Commonly distributed in almost all Europe (between 37° and 55° N) (Hall et al. 2007; Jeandroz et al. 2008; Lawrynowicz et al. 2008; Gezer et al. 2014), south-west China and Taiwan for *T. sphaerospermum* (Malençon) (≡ *Tuber borchii* var. *sphaerosperma* Malençon) (Yun and Liu 2009).

Tuber magnatum Pico

(Great Britain: Piedmont white truffle; Italy: Tartufo bianco del Piemonte, Trifola bianca; France: Truffe des Magnats, Truffe Gris)

Globose ascoma more or less regular, often with lobes, gibbous, often flattened with variable dimensions from 1–2 cm up to 10–15 cm and beyond, yellowish, pale ochre, more or less greyish, often with greenish or reddish tones (Fig. 3a, b). Peridium smooth on the surface, under a dense minutely papillose, adherent, 50–350(–500) µm thick, pale grey ochre, essentially pseudoparenchymatic with few plectenchimatic zones, internally paler (Fig. 3a, b). Whitish gleba, pale yellow, pale ochre, brownish-grey or reddish-brown, often with reddish spots, soapy to the touch, essentially pseudoparenchymatic. Thin veins, whitish, ramified, anastomosed, also in the white

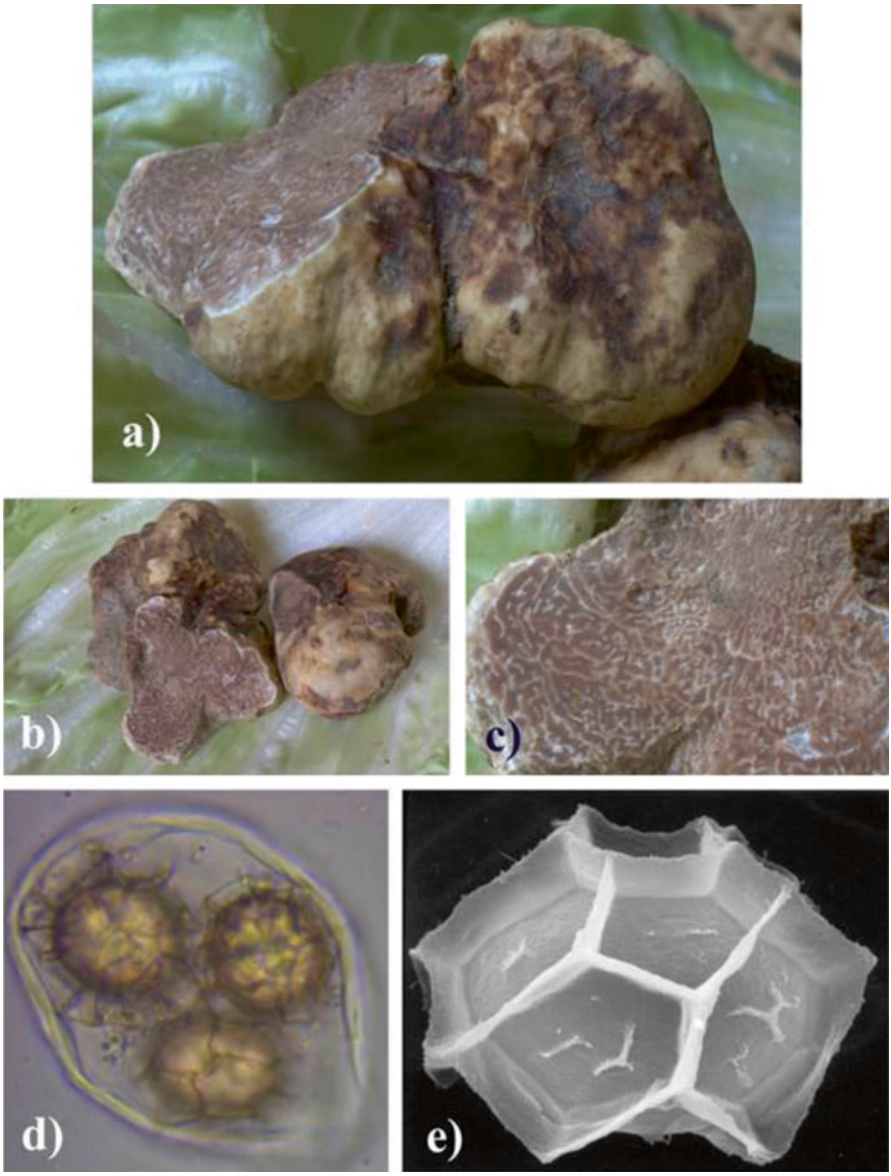

Fig. 3 *Tuber magnatum*: (**a**) Mature truffle sample (photo: Giancarlo Bistocchi); (**b**) three truffle samples (photo: Giancarlo Bistocchi); (**c**) truffle section with sterile white veins and fertile light brown veins (photo: Giancarlo Bistocchi); (**d**) ascus with three spores (photo: Giancarlo Bistocchi); (**e**) SEM spore with wide alveoles (photo: Emma Bricchi)

ganglia(Fig.3c).Ascimostlyglobose,sessileorshortstalked65–80(90)×42–65(70)µm with 1–3(4) spores (Fig. 3d).

Alveolate-reticulum spores, from yellowish, pale ochre to brownish, globose or ovoid-globose, (20–)35–50×(15–)32–42 µm excluding ornamentation, with more or less regular alveole, by number 2–3 according to the diameter of the spore and depth 4–6, 5(–8) µm with internal crests (Fig. 3d, e and Table 1). Pleasant odour, penetrating, garlic-like and strongly cheesy. Pleasant flavour, intense and garlic-like.

Habitat

In calcareous marl terrain, constituted of sandstone, marl, marly limestone and marl clay; mostly at the depth of 10–30 cm (and beyond up to 80 cm), but also more superficially (2 cm), solitary or rarely aggregated. Grows below 700(–800) m in altitude, in the valley bottoms, along ditches, in tilled terrain, slightly humid, but drained, poor in P and N, at pH 7–8(8.5), poor in humus, not very inclined and not very sunny, especially alluvial, sedimentary, of landslides, and of escarpment; does not grow in sandy or siliceous terrain.

Maturation Period

Late summer, autumn and early winter.

Symbiotic Nature

It forms the symbiosis with *Q. robur* L., *Q. petrea* Liebl., *Q. pubescens* Willd., *Q. cerris* L., *Q. ilex* L., *Populus alba* L., *P. nigra* L., *P. tremula* L., *P. pyramidalis* Roz., *Salix alba* L., *S. viminalis* L., *S. caprea* L., *S. appennina* Skvortsov, *Tilia cordata* Miller, *T. platyphyllos* Scop., *Corylus avellana* L., *Ostrya carpinifolia* Scop., *Alnus cordata* (Loisel.) Desf., *Carpinus betulus* L., *Pinus pinea* L. and *Abies alba* Miller (Granetti et al. 2005; Figliuolo et al. 2013; Iotti et al. 2014).

Geographic Distribution

Distributed in Central and Southern Europe (between 40° and 46° N) (Rubini et al. 2005; Piltaver and Ratosa 2006; Jeandroz et al. 2008; Milenkovic et al. 2009; Christopolous et al. 2013).

Tuber melanosporum Vittad.

(Great Britain: Black truffle, Black diamond; Italy: Tartufo nero pregiato; France: Truffe du Périgord; Spain: Trufa negra)

Globose ascoma, regular or irregular, occasionally lobed, compact, with a diameter from (2)5 to 8(10) cm, at first reddish-brown then reddish-black,

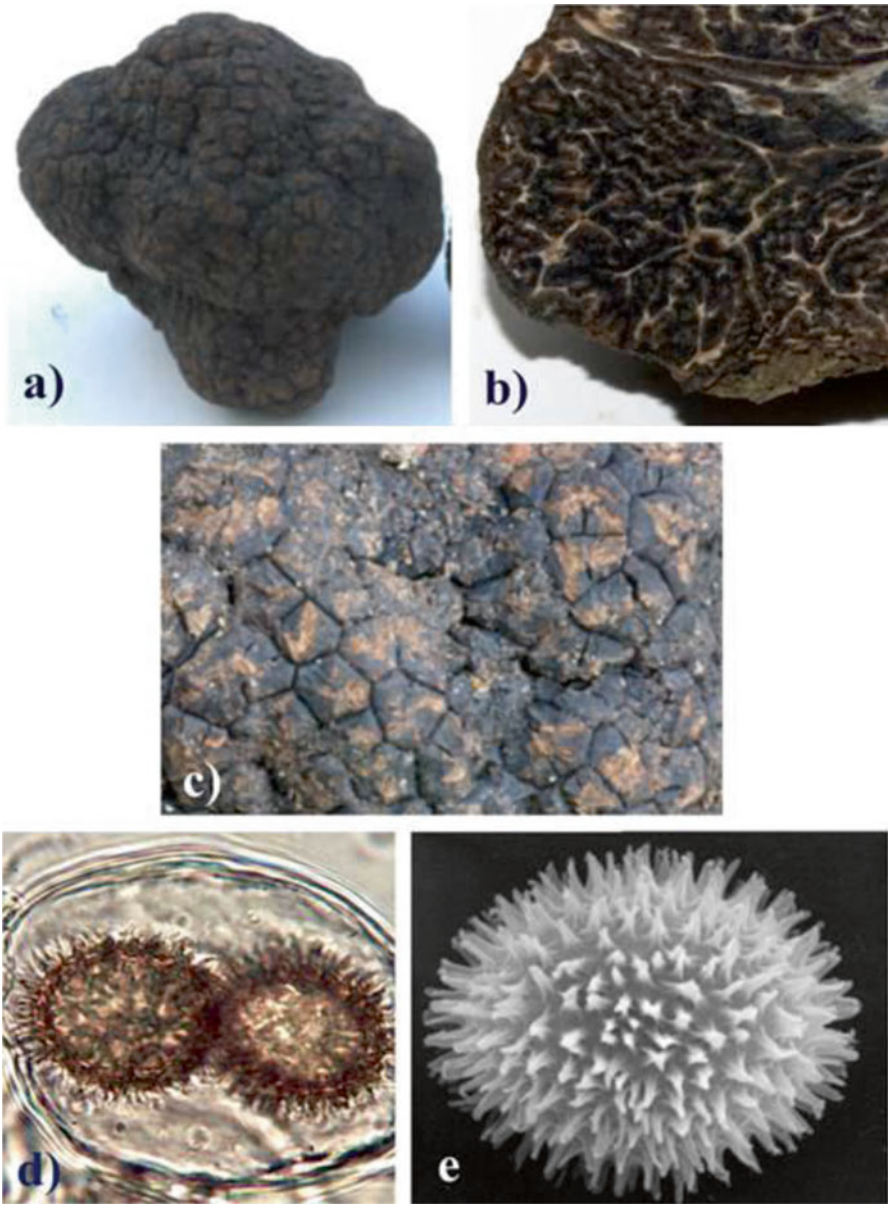

Fig. 4 *Tuber melanosporum*: (**a**) Truffle sample (photo: Giancarlo Bistocchi); (**b**) truffle section with sterile clear veins and fertile blackish veins (photo: Giancarlo Bistocchi); (**c**) surface detail with verrucas (photo: Giancarlo Bistocchi); (**d**) ascus with two ellipsoidal spores (photo: Giancarlo Bistocchi); (**e**) SEM spore with broad base spines (photo: Emma Bricchi)

brownish-black, blackish sometimes with dark red or rusty zones, with pyramidal warts, depressed at the apex and 3–6 mm wide at the base with four to six lateral faces with radial walls (Fig. 4a).

Peridium strongly adherent to the gleba, up to 700 µm thick, pseudoparenchymatic, blackish-brown with large polyhedral cells with thickened walls up to 2 µm, especially in the peripheral part, clearer towards the internal, with a pseudoparenchymatic structure with thin-walled cells and plectenchimatic structure in the proximity of the gleba (Fig. 4c). Plectenchimatic gleba, at first white, then greyish, reddish-grey, tending towards a purplish-black or a brownish-black with violet highlights, with numerous remified, thin, sterile veins, at first white which become reddish in the air at maturation (Fig. 4b).

Globose asci, sessile or short stalked, pale ochre colour, numerous, 85–145×78–127 µm with (1)3–4(6) spores (Fig. 4d). Aculeate spores, non-transparent at maturation, intense brownish colour, elongated ellipsoidal, (20–)23–45×(14–)19–28 µm excluding short and rigid spines, sometimes curved in the mature spores, 2.5–3 µm long (Fig. 4d, e and Table 1). Odour intense, musky and very pleasant. Flavour intense, spicy and very pleasant.

Habitat

In calcareous or limestone and clay terrain, with 40 % or less clay, presence of Fe, microelements and scarce N, P and K, a permeable surface, but not necessarily, well-aerated, porous, sandy or rocky, sunny, rarely towards the north, on a gradient, at pH 7.5–8.5, with 1.5–8 % humus, mostly at a depth of 5–15(–30) cm. Grows mostly aggregated from 100 to 1100 m above sea level and requires a well-distributed rainfall throughout the year. Rain in spring and in June, storms in summer, rain in autumn and moderate rain in winter.

Maturation Period

From autumn to winter.

Symbiotic Nature

It form the symbiosis with *Q. pubescens* Willd., *Q. robur* L., *Q. petraea* (Mattuschka) Liebl., *Q. ilex* L., *Q. coccifera* L., *Q. faginea* Lam., *Q. cerris* L., *Q. trojana* Webb, *Q. suber* L., *Fagus sylvatica* L., *Corylus avellana* L., *C. colurna* L., *Salix caprea* L., *S. viminalis* L., *S. alba* L., *Populus nigra* L., *P. tremula* L., *P. alba* L., *P. carolinensis*, *Tilia cordata* Miller, *T. platyphyllos* Scop., *Ostrya carpinifolia* Scop., *Carpinus betulus* L., *Alnus cordata* (Loisel) Desf., *A. glutinosa* (L.) Gaertner, *Betula verrucosa* Ehrh., *Castanea sativa* Miller, *Eucalyptus* sp., *Cistus albidus* L., *C. incanus* L., *C. salvifolius* L., *C. laurifolius* L., *C. crispus* L., *C. monspeliensis* L., *Fumana procumbens* (Dunal) Gren. et Godron, *Pinus halepensis* Miller, *P. nigra* Arnold, *P. sylvestris* L., *P. pinaster* Aiton, *P. pinea* L., *Picea abies* (L.) Karsten, *Abies alba* Miller, *Cedrus atlantica* (Endl.) Carrièrè, *C. deodara* (D. Don) G. Don. and *Arbutus unedo* L. (Granetti et al. 2005; Comandini et al. 2006; Rubini et al. 2011; Belfiori et al. 2012; Taschen et al. 2015).

Geographic Distribution

Southern Europe (between 40° and 48° N) (Piltaver and Ratosa 2006; Jeandroz et al. 2008; Rubini et al. 2011; Parlade et al. 2013; Le Tacon et al. 2013).

4.2 Experimental Methods

4.2.1 Preparation of the Methanolic Extracts

Fresh fruiting bodies of *T. melanosporum*, *T. aestivum*, *T. magnatum* and *T. borchii* (200 g of each truffle species) are extracted with methanol (1:10 ml) at room temperature for 7 days to obtain an extract after evaporation under a vacuum (40 °C). The samples are held at −20 °C until analysed (Angelini et al. 2010a, b, 2015).

4.2.2 Separation and Characterisation of Chemicals

The fruiting bodies of *Tuber* spp. are poor in lipids, ranging from 5 to 9 % by dry weight according to the species and age. Normal extraction protocols don't extract spore contents which is a considerable weight of the fruiting bodies; on the other hand, spores are not digestible and their contents currently are not well studied. The lipids have a pattern of total FAs that consist mainly of linoleic, oleic and palmitic acids even if many other FAs were identified by Tang et al. (2011); 28 kinds of FAs were found within the fruiting bodies of *T. aestivum*, *T. indicum*, *T. himalayense* and *T. borchii*. The total FA fraction is the result of free FAs and esterified FAs being these two fraction distinguishable with particular protocols. The classical total FA determination consists of an extraction of lipid fraction (e.g. chloroform/methanol 2:1 in a Soxhlet apparatus). The residue was redissolved in methanol and treated with KOH methanolic solution. With this extraction protocol, esterified FAs and free FAs were derivatised all together to their methyl ester forms. The use of methyl derivatives is necessary because the quali-quantitative analysis of the FA mixture was due to gas-chromatographic methods that required the volatilisation of the sample (methyl derivatives have a lower boiling point referring to the corresponding non-methylated acid). The quantitative analysis requires the use of an FID detector that has about the same responsive factor even for FAs of relative different C-chain number (calibration curves are necessary at least for groups of similar FAs). The qualitative analysis was performed using an MS detector set at 70 eV. The standard use of such a detector has produced, during the last 60 years, a very big mass spectra database of every sort of chemical compound so it is quite easy to label a GC peak (automatic softwares of research in the mass database were furnished with the GC/MS instruments).

Another approach in the separation and identification of complex matrices was the use of HPLC with an MS detector, the ESI interface being the most useful (with the promising liquid chromatography-electron ionisation-mass spectrometry (LC-EI-MS) which is able to separate a complex matrix and give a

mass spectra of the peak at 70 eV, therefore allowing the use of GC mass spectra database, but lacking in sensitivity). The modern instruments HPLC-ESI with various interfaces for the fragmentation of the compounds (i.e. laser desorption/ionisation) and MS/MS/TOF detectors are highly sensitive but give a mass fingerprint of first and second fragmentation that aren't useful with the GC mass spectra library. Currently, the HPLC mass spectra libraries are continuously refurnished by mass spectra of new compounds, but a correct use of the report of such instruments needs the use of the original standards. Other problems are in the quantitative analysis and are due to the different fragmentation patterns of each compound even concentration dependent that require a calibration curve for each compound.

HPLS/ESI/MS/TOF is widely used because each analysis furnishes a lot of information on the analysed matrix and gives a large survey on the compounds of many kinds. A possibility to analyse a large pool of compounds simultaneously has greatly improved the knowledge of many aspects, and the scientific papers on-omic studies are in exponential rise. The use of less invasive extraction and analysis protocols is well accepted and produces less chemical artefacts (Li et al. 2011; Tang et al. 2011; Han et al. 2012; Bhattacharya 2013; Brenna 2013; Fouillen et al. 2013; Hartler et al. 2013; Lam and Shui 2013; Schone et al. 2013).

A lipidomic approach for the determination of the free fatty acids (FFAs) in some *Tuber* species was carried out by Angelini et al. (2015). Methanolic extracts from fresh fruiting bodies from *T. melanosporum* Vittad., *T. aestivum* Vittad., *T. magnatum* Pico and *T. borchii* Vittad. were analysed on an LC system interfaced with a Q-TOF/MS/MS detector equipped with an ESI source, operating in negative ion mode. The identification of FFAs was based on commercially available standards. The components were identified by comparing the MS/MS spectra and the retention times (RT) of the chromatographic peaks with those of authentic compounds run under the same conditions. The relative amounts of FFAs were obtained calculating the response factor for the linoleic acid. Among the more than 100 separated compounds, only the FFAs were considered and the mainly FAs were in accordance with the GC results (Angelini et al. 2015).

4.2.3 Bioassays

A suitable bioassay is a necessity in the investigation of allelopathy and in finding out the role of truffle methanolic extract and fatty acid compounds. According to Selim et al. (2012), the most frequently used bioassay tests are the influence on seed germination seeing as its results are fairly direct and reputable. Though many techniques are applied, all share some common ground; seeds are placed on a substrate saturated with the test solution. Germination is defined as the emergence of the radicle, often 2 mm beyond the seed coat, and is scored over a period of time (Angelini et al. 2010b). Properly conducted bioassays are invaluable. They are straightforward and require a little test solution (Khaliq et al. 2012). When the quantity of test solution is problematic, agar cultures may be utilised, placing the

pre-germinated seed on the surface of the agar containing allelochemicals. The elongation of the hypocotyl or coleoptile could be considered in conjunction with germination percentage. The measure of the elongation itself or dry mass can be used to mark the growth (Mardani et al. 2014). It should be taken into consideration that growth bioassays are often more sensitive than germination bioassays.

4.3 Truffle Allelopathy

4.3.1 Allelopathic Activities of *Tuber* spp. Methanolic Extract

Metabolites of many fungi may have adverse or stimulatory effects on plants (Heisey et al. 1985; Rice 1995) such as suppression of seed germination, malformation and retardation of seedling growth (Lynch and Clark 1984; Eaton and Ayres 2002). To evaluate possible allelopathic effects of the *Tuber* spp. methanolic extracts, extract concentrations from 0.03 to 0.3 g/ml were used to test the in vitro effects on germination, root length and plant height of *A. thaliana*, *L. corniculatus*, *M. ciliata* and *S. vulgaris* expressed as inhibition ratio {IR = [1 − (obtained value/control value)] × 100} (Angelini et al. 2015).

The *Tuber* spp. methanolic extracts significantly affected the germination of seeds as well as the development of seedlings (Fig. 5a). The extracts were able to inhibit root length as well as seedling height in the tested plant species. The magnitude of the extract's effect was dependent on the concentration. The tested plants demonstrated varying responses to each methanolic extract (Fig. 5b, c). Differences in root length and plant height (IR values) in the morphological indices of the tested subjects were observed among *Tuber* spp. extracts. Phytotoxic activity in the *T. melanosporum* and *T. aestivum* extracts was nearly consistently greater than that in *T. magnatum* and *T. borchii* (Fig. 5b, c) (Angelini et al. 2015). The extracts also induced leaching in the cotyledon leaves and tissue darkening in the roots. This preliminary in vitro study has demonstrated the allelopathic potency of the methanolic extract of *Tuber* spp. (Angelini et al. 2010a, b). Active methanolic extract compounds of *Tuber* spp. are particularly interesting, because they can act as allelochemicals to inhibit some plant species or be a signal to specific microbes.

4.3.2 *Tuber* spp. Allelochemicals

The FFA compositions in the extracts with methanol from *T. melanosporum*, *T. aestivum*, *T. magnatum* and *T. borchii* were shown in Table 2 (Angelini et al. 2015). There were a total of 12 FFAs identified from the *Tuber* spp. extract, but the extracts could be differentiated from each other on the basis of relative amounts of FFAs (Table 2). The main saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) were palmitic acid (ca. 38.53–77.38 μg/g) and linoleic acid (63.88–185.89 μg/g), respectively; they were more abundant in *T. aestivum* than in the other

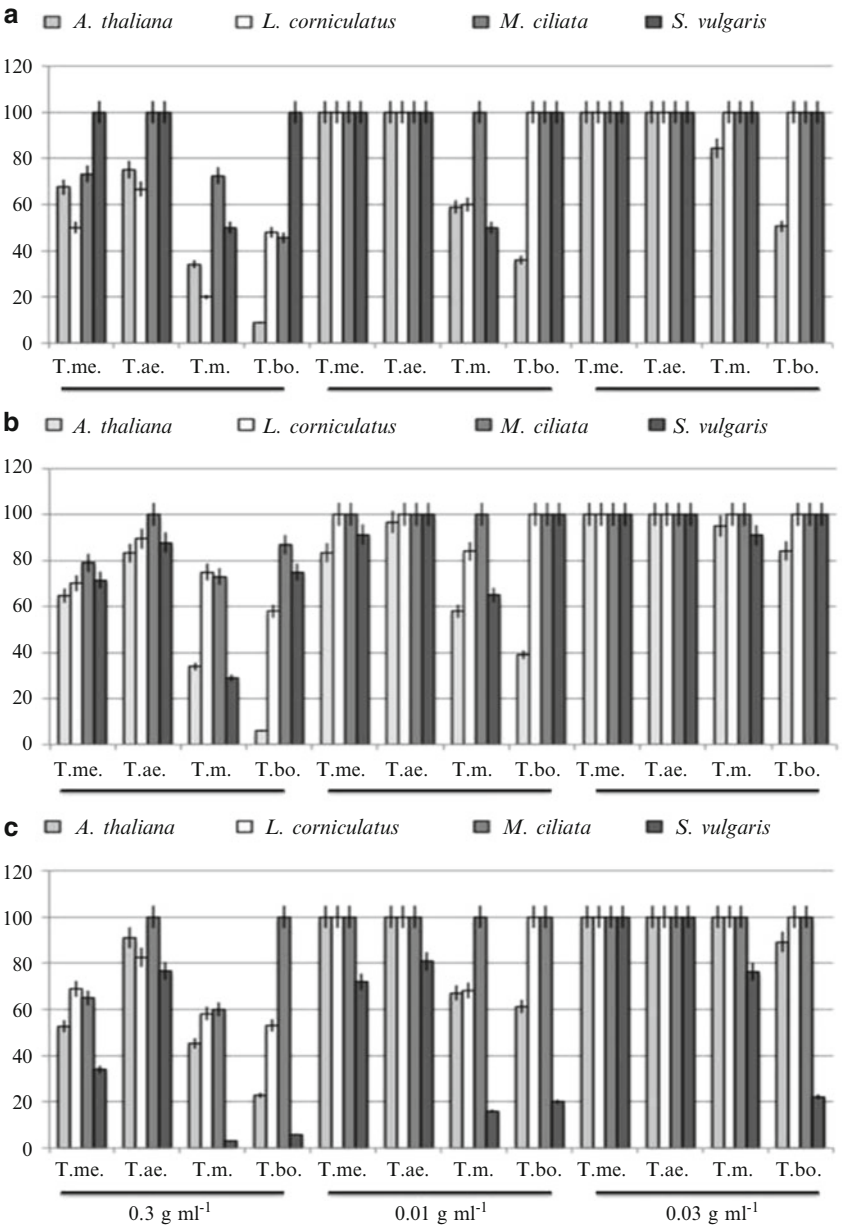

Fig. 5 IR (%) of *Tuber* spp. methanolic extracts on seed germination (**a**), root length (**b**) and hypocotyl length (**c**), 12 days after sowing. The values are the means of three repetitions. Error bars represent the 95 % confidence interval of a mean

Table 2 Free fatty acids (FFAs) composition of *Tuber* spp. methanolic extracts (Angelini et al. 2015)

FFAs		*Tuber* spp.			
Systematic name (shorthand notation)	Trivial name	*T. melanosporum*	*T. aestivum*	*T. magnatum*	*T. borchii*
Decanoic (C10:0)	Capric	0.11 ± 0.02^{a}	0.69 ± 0.21^{b}	0.43 ± 0.16^{ab}	0.32 ± 0.11^{ab}
Tetradecanoic (C14:0)	Myristic acid	5.91 ± 1.90^{ab}	6.71 ± 1.37^{b}	4.19 ± 1.37^{ab}	2.49 ± 1.00^{a}
Pentadecanoic (C15:0)	Pentadecylic	18.03 ± 4.26^{b}	18.6 ± 3.36^{b}	10.04 ± 1.85^{a}	6.08 ± 1.28^{a}
Hexadecanoic (C16:0)	Palmitic acid	63.25 ± 7.46^{ab}	77.38 ± 20.01^{b}	40.44 ± 8.34^{a}	38.53 ± 9.00^{a}
cis-10-heptadecenoic (C17:1)	–	1.05 ± 0.45^{ab}	1.40 ± 0.27^{b}	0.98 ± 0.41^{ab}	0.20 ± 0.14^{a}
Heptadecanoic (C17:0)	Margaric	0.11 ± 0.04^{ab}	0.17 ± 0.05^{b}	0.07 ± 0.01^{a}	0.09 ± 0.02^{ab}
cis-9,12-octadecadienoic (C18:2n6)	Linoleic acid	136.98 ± 34.97^{bc}	185.89 ± 32.4^{c}	90 ± 22.97^{ab}	63.88 ± 15.54^{a}
cis-9-octadecenoic (C18:1n9)	Oleic acid	62.42 ± 15.2^{b}	62.93 ± 17.53^{b}	26.88 ± 5.43^{a}	24.88 ± 6.06^{a}
cis-11-14 eicosadienoic (C20:0)	Dihomo-gamma-linoleic	14.00 ± 3.23^{ab}	18.41 ± 5.79^{b}	6.75 ± 2.46^{a}	6.05 ± 1.90^{a}
cis-11-eicosenoic (C20:2)	Gadoleic	12.45 ± 3.5^{ab}	20.52 ± 5.54^{b}	10.31 ± 2.37^{a}	9.96 ± 1.06^{a}
Eicosanoic (C20:0)	Arachidic	28.88 ± 9.69^{ab}	40.9 ± 6.51^{b}	21.31 ± 3.9^{a}	18.43 ± 4.95^{a}
Heneicosanoic (C21:0)	Heneicosylic	21.24 ± 4.38^{bc}	31.67 ± 5.58^{c}	17.27 ± 4.5^{ab}	8.6 ± 1.58^{a}
Yield of the compounds		364.43 ± 1.73^{c}	465.27 ± 2.88^{d}	228.67 ± 5.77^{b}	179.51 ± 3.54^{a}

Means of three analyses ± standard error; each value is expressed as mg/g of free fatty acids in dry matter. Values in the same row bearing different letters were significantly different ($p < 0.05$)

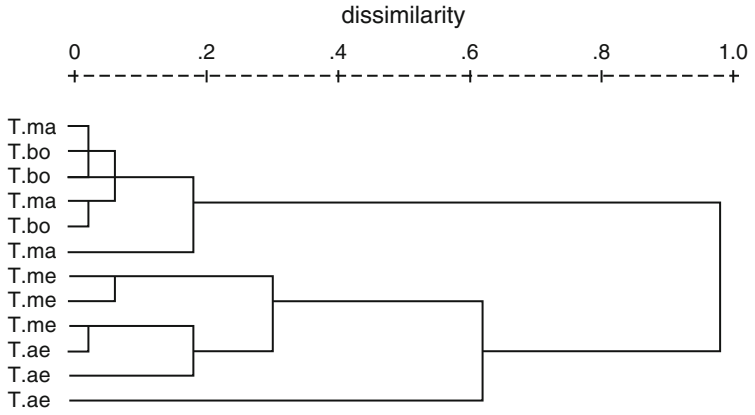

Fig. 6 Dendrogram of *Tuber* spp. methanolic extract with fatty acids contents (Angelini et al. 2015). T.me: *Tuber melanosporum*, T. ae: *T. aestivum*, T.ma: *T. magnatum*, T.bo: *T. borchii*

species (Table 2). In general, unsaturated FFA levels were higher than saturated. This is in agreement with previous published data that showed high proportions of unsaturated FFAs, especially linoleic acid (Ribeiro et al. 2009; Tang et al. 2011). It is known that linoleic acid is the precursor of 1-octen-3-ol, known as the alcohol of fungi, which is the principal aromatic compound in most fungi, and might contribute to mushroom flavour (Ullrich and Grosch 1987; Cullere et al. 2010). In terms of total production, *T. melanosporum* and *T. aestivum* synthesised significantly higher amounts of all identified FFAs than *T. magnatum* and *T. borchii* (Table 2).

The HCA (hierarchical cluster analysis) of the *Tuber* spp. methanolic extract and FFA variables was used to examine the distance among the samples of truffle extracts in two-dimensional plot (dendrogram) and cluster samples with similarity (Fig. 6). The results of this computation showed that the four investigated truffle species were separated into two groups, the group I containing the samples from *T. borchii* and *T. magnatum* extracts, while the group II is constituted of *T. melanosporum* and *T. aestivum* extracts. These results show that the FFA compositions of the group I samples were similar, whereas they were significantly different from those of the group II samples. These marked dissimilarities were principally the result of the distinctions among species and various complex factors such as habitat and relative harvest times (Sancholle et al. 1988; Tang et al. 2011).

4.3.3 Growth Inhibitory Effects (IR) of Isolated Allelochemicals

The toxic effects of the examined FFA (linoleic, oleic and palmitic acids) on seed germination and root and hypocotyl growth of the tested plants varied (Fig. 7a–c). Among the three FFAs studied, higher toxicity was detected for linoleic in all tested plants, followed by oleic acid and finally palmitic (Fig. 7b, c). *M. ciliata* was observed to be the most sensitive plant. Also oleic acid acted as a growth inhibitor

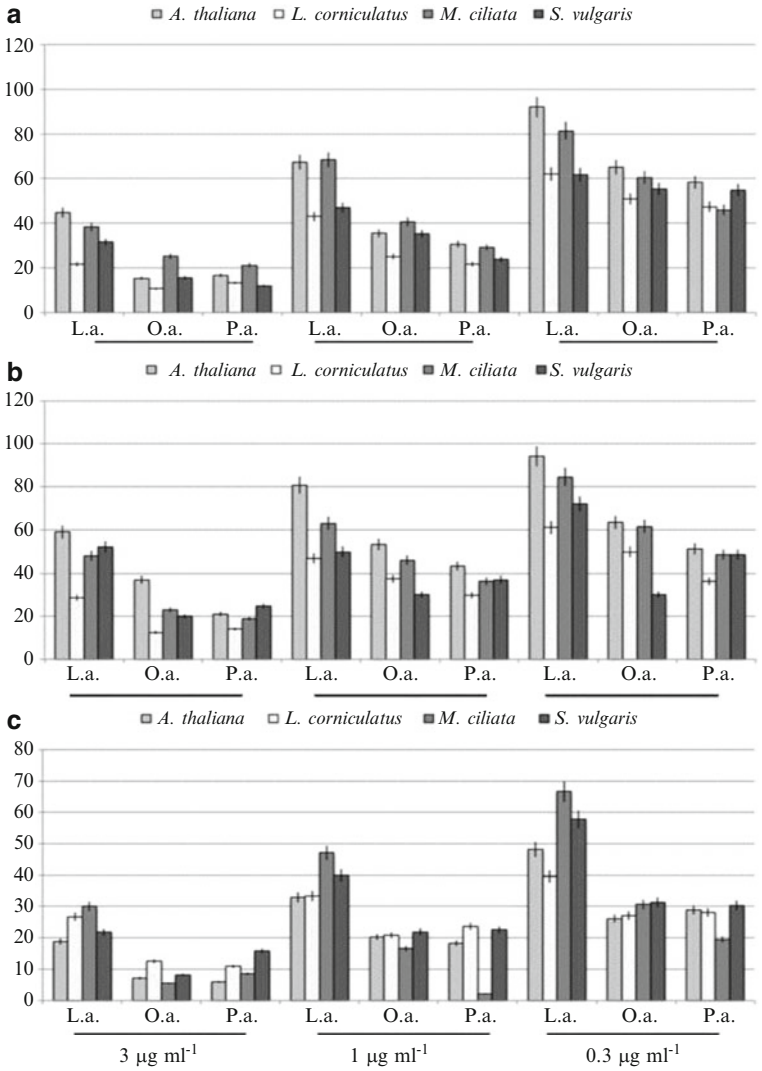

Fig. 7 IR (%) of allelochemicals on seed germination (**a**), root length (**b**) and hypocotyl length (**c**), 12 days after sowing. The values are the means of three repetitions. Error bars represent the 95 % confidence interval of a mean

on all of the tested plants (Fig. 7), with diverse respective IR values, according to the section of and species tested. *A. thaliana* was revealed as the most sensitive; on the other hand, *S. vulgaris* was the most resistant (Fig. 7). However, the effect of growth inhibition of oleic acid on the tested plants was overall less apparent than the other tested compounds. Palmitic acid demonstrated itself as a greater influence in the roots than in the hypocotyl with *A. thaliana* being the most sensitive to stearic acid and *L. corniculatus* being the most resistant. Angelini et al. (2015) reported

that the allelochemicals such as linoleic, oleic and palmitic acids may offer a plausible explanation for the aggressive characteristics of the *T. melanosporum* and *T. aestivum* along with the strong cytotoxic effects observed in their brûlé (Streiblova et al. 2012). Phytotoxic properties found within fatty acids have become part of the general knowledge in their respective fields of study (Duke et al. 2013). It is believed that the mode of action in long-chain fatty acids is observable in the disruption of the cell wall building function as these can be utilised by the proteins that construct the cell walls as a sort of building block (Kim and Kim 2000).

A number of studies have also indicated that the toxic activity of polyunsaturated fatty acids (PUFAs) may be due to oxidation products derived through photo-oxidation or metabolic processes (Duke et al. 2013). Linoleic, oleic and palmitic acids are well-known allelochemicals (Macias et al. 2006; Noguchi 2008; Quintana et al. 2009). Regardlessly, the study of Angelini et al. (2015) is the first report in which their effects on the weeds of truffle plants (*L. corniculatus*, *M. ciliate* and *S. vulgaris*) were exposed. It was found that unsaturated FFAs such as linoleic acid were the most inhibitory and that allelopathic efficacy increased with an increase in double bonds (Kakisawa et al. 1988).

FFAs are abundant in natural sources, relatively nontoxic and 'generally regarded as safe' (Khanh et al. 2007). It is also important to note that generally, the possible induced evolution of FFA-resistant phenotypes would prove to be a minor problem compared to the evolution of synthetic herbicide-resistant phenotypes (Desbois and Smith 2010; Busi et al. 2013). Disregarding the glaring potential of the phytotoxic and allelopathic properties of FFAs, they are still vastly unexploited. Possibly due to their structurally unstable nature, particularly found in the long-chain polyunsaturated FFAs, which are easily denaturalised when heat or pressure is applied (Storch and McDermott 2009). Otherwise, the usefulness of FFAs as allelochemicals could be seen as not patentable owing to their ubiquity. These complications are minor and surmountable; therefore, studies regarding the allelopathic applications of FFAs should proceed in the future (Desbois and Smith 2010).

4.3.4 Species Specificity of Truffle Allelopathic Activity

Investigations of truffle methanolic extracts suggest that some active compounds (FFAs) that are selective against specific weed species might also be responsible for the observed selective allelopathic activity of truffle methanolic extracts (Angelini et al. 2010a, b, 2015). These species specific responses of weed species to truffle allelochemicals has also been witnessed in other allelopathic fungal and plant species (Berestetskiy 2008). The test species' seedlings of these experiments were germinated and grown in an isolated Petri dish free from any form of intra-species resource competition as early seedlings extract their nutrients directly from the seed itself without the necessity of sunlight at this stage (Ashrafi et al. 2008). It is then deducible that the revealed growth inhibition of the tested species was likely due to an allelopathic reaction and not competitive

interference. Additionally, methanolic extracts and compounds proved to more greatly inhibit root growth than hypocotyl growth in the test subjects. Mominul Islam and Kato-Noguchi (2013) reported that root growth is more sensitive to the extracts of allelopathic plants than the hypocotyl/coleoptile growth as the root directly absorbs the undiluted allelochemicals from the soil. Furthermore, there is more intensive contact between roots and fungal or plant extracts (Salam and Kato-Noguchi 2010). These results suggest a possibility to develop biological herbicides for weed management from these compounds.

4.3.5 Comparative Analysis of Allelopathic Activities of Isolated FFAs and Methanolic Extracts

The comparative analysis between the identified FFAs and the methanolic extracts (linoleic, oleic and palmitic acids) from *Tuber* spp. has shown a vital role in the allelopathic activity. The greater phytotoxicity was found in the black truffles (*T. melanosporum* and *T. aestivum*) extracts compared to white truffles (*T. magnatum* and *T. borchii*). It may be justified with the release of huge relative quantities of the principle FFAs in the black truffles. On the contrary, multiple potential allelopathic compounds are likely to be responsible for the allelopathy of methanolic extracts of *Tuber* spp. It could also be hypothesised that synergistic reactions between some or all of the extract's present compounds may create its allelopathic potential (Angelini et al. 2015).

5 Conclusions and Future Prospects

Recently used innovative technologies are obliging in quantification and identification of minute amount of allelochemicals isolated from the various *Tuber* spp. It may grant the truffle processing companies to generate the revenue from the development of bio-herbicides based on truffle-extracted allelochemicals known as waste truffle. The isolation of natural, economically sustainable and ecologically sound herbicide from *Tuber* spp., through modern tool and techniques, has open the horizon of the future for equally beneficial, organic and/or integrated plant cultivations, but to understand the ability of allelopathic mechanisms of FFAs, a more in-depth research regarding the productivity, function and stability of FFAs is desired. The simulation of the innumerable field conditions is affected by a lot of biological interactions, which differ completely from laboratory experiments. The experimental setting under natural condition to explore the processes involved in the retention, transformation and transferring of chemicals in the environment could be a promising prospective in the future. It may explore our understanding towards the *Tuber* spp., their influence with surrounding environments and the health of the soil flora and fauna. These factors may also answer why some truffle species apparently do not create a brûlé' characteristic of giving black truffles despite the constant release and accumulation of phytotoxins in the soil. Moreover, it may also reconnoiter the indulgences of the chemical reactions with soil

microorganisms to the allelochemicals found in *Tuber* spp. and could be possible to set off a growing chain of implicated and applicable potentiality of *Tuber* ecosystems.

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Mycorrhizal Association: A Safeguard for Plant Pathogen

Madhumati Bora and Ami Lokhandwala

Abstract Biological control refers to the potential application of introducing or inhabitant microorganisms to reduce the damage caused by one or more plant pathogens. A continuous interplay between ecosystem and its rhizospheric organisms brings the control of soilborne pathogens which are more difficult to manage. Since last two decades, biocontrol has been gaining considerable interest to maintain sustainable agriculture system. Mycorrhizae are among the most primeval, intimate, and vital association which colonize symbiotically through the roots of various vascular, nonvascular, and crop plants. The arbuscular mycorrhizal (AM) fungi develops a complex mutualistic association with host plant roots via various mechanisms. From the initiation of symbiosis till the formation of arbuscules and/or vesicles, a number of changes occur in plant metabolism, plant nutritional status, and plant resistance. The key indicators of AM fungi for an effective defense response are reduction in the infectivity of soilborne pathogens or suppression of pathogen metabolism or increase in plant's tolerance toward pathogen. Proposed grounds for a healthier state of plants associated with AM fungi are attributed to (1) improvement in nutritional status of plants, (2) competition between mycorrhiza and pathogen for nutrition and infection sites, (3) modifications in root anatomy and morphology, (4) release of plant root exudates, (5) harboring microbial flora antagonistic to root pathogens, and (6) improved localized as well as systemic resistance in plants. The effective and prolonged mycorrhiza-induced resistance involves various phytohormones, secondary metabolites, pathogen-related proteins, and defense enzymes. Such activation leads to a primed state in plant, which allows quicker and mightier defense response against pathogens. This chapter provides an overview of the underlying mechanisms involved in mycorrhiza-induced disease resistance and priming in plants.

Keywords Biological control • Arbuscular mycorrhizal fungi • Mechanisms involved

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1 Introduction

The term "rhizosphere" was first coined by Lorenz Hiltner in 1904 (Hartmann et al. 2008) to describe the plant-root interface, a word originating in part from the Greek word "rhiza," meaning root. Rhizosphere is that section of soil which is in direct proximity of plant roots harboring a unique population of microorganisms (Azcón-Aguilar and Barea 1992). Rhizosphere develops at soil-root interface and so it communicates both with soil and plant roots via physical interaction and/or chemical or biochemical signals. It is metabolically busier and more competitive environment than the surrounding soil. This tripartite cross talk is affected by inherent soil characteristics and ecosystem conditions (Lynch 1990). The "dialogue" between these three partners can be either plant-plant communication caused by two overlapping rhizospheres, plant-microbe communication, or synergistic/antagonist microbe-microbe communication. Two factors determine plant-microbe interaction: first, the rhizospheric effect, i.e., plant activities that stimulate growth of microorganisms around the roots, and second, microbial activities that either benefit or detriment the plant (Lynch 1990). The beneficial action of biological organisms also named natural enemies against pathogens is termed as "biological control." A key component of ecosystem for biocontrol of pathogens is microbial diversity (Kennedy and Smith 1995). Under natural conditions, the majority of plant species form mycorrhizal associations to enable AMF survival. Arbuscular mycorrhizal fungi (AMF), being the most widespread type in regular crops of all ecosystems (Akhtar and Siddiqui 2008a, b; Smith and Read 2008; Akhtar and Panwar 2011), are the most common association prevalent in almost all climatic conditions. AM fungi had the ability to modify the rhizospheric microbial activity through the alteration in the quality and abundance of rhizosphere microflora (Akhtar and Siddiqui 2008a, b). These fungi induce changes in the host root exudation pattern following host colonization which alters the microbial equilibrium in the mycorrhizosphere (Akhtar et al. 2011). AM fungi reduced the severity of plant diseases, increasing the yield of various crops through their biocontrol potential against the wide range of pathogens in sustainable agricultural practices. This chapter provides an overview of the underlying mechanisms involved in mycorrhiza-induced disease resistance and priming in plants.

2 Understanding of Arbuscular Mycorrhizal Fungi as Bioprotectant

AMF belongs to order Glomales of Zygomycetes family (Rosendahl et al. 1994). Since AMF are totally dependent on their host plant to multiply and survive, they are best known as "obligate biotrophs." The joint venture thus develops between plant and fungus is based on the mutual profits attained by both. AMF colonizes

the plant root cortex and forms bush or little treelike structures known as arbuscules meant for nutrient exchange between the partners (Jung et al. 2012). Extraradical hyphae formed by AMF in soil increase the absorption efficiency of plant roots by crossing nutrient depletion areas and thereby improve the reach for inorganic nutrients like phosphate and nitrate (Smith et al. 2011). Plants reciprocate this help by supplying photosynthates to fungal partner (Smith and Smith 2011). This kind of association ensures a tight bidirectional regulation of mutualism (Kiers et al. 2011). Plants manage this barter practice by bringing essential modifications in its primary as well as secondary metabolism and also amending its defense mechanisms (Fester and Hause 2005). These amendments build up the plants potential to muddle through stresses.

The fact that mycorrhiza provides nutritional benefit to plant is as old as human civilization. The enhanced growth and/or yield in mycorrhizal host plants are solely credited to improved nutritional status (Smith and Read 2008). Moreover, advancement in this field rendered another potential of mycorrhiza alleviating the damage under stress conditions (Pozo and Azcón-Aguilar 2007; Koricheva et al. 2009; Smith et al. 2010; Campos-Soriano et al. 2012). Upon mycorrhizal association, noteworthy changes develop in host plant and its surrounding environment. Plants release root exudates in soil upon which alters soil structure, carbon deposition in soil, and its microbial diversity, thus influencing rhizospheric milieu (Jung et al. 2012). Changes associated with defense mechanism are noticed not only in rhizosphere but in non-colonized root parts and aboveground parts of mycorrhizal-colonized plant (Pozo et al. 2002; Pozo and Azcón-Aguilar 2007). The obvious changes observed upon efficient colonization are alteration in root design, metabolic modifications, and accumulation of certain defense-related compounds (Strack et al. 2003; Hause et al. 2007; Schliemann et al. 2008; Péret et al. 2009; Lopez-Raez et al. 2010a, b; Akhtar et al. 2011) and accumulation of apocarotenoids (Strack and Fester 2006; Floss et al. 2008; Schliemann et al. 2008), flavonoid contents (Akiyama et al. 2002; Vierheilig and Piché 2002), phenolic compounds, defense-related phytohormones, and reactive oxygen species (Fester and Hause 2005; Lopez-Raez et al. 2010a, b). This implies that plants too exhibit defense mechanisms, coordinated by their immune system to enable the plant in recognizing nonself alien organisms through molecules like flagellins, lipopolysaccharides, or peptidoglycans, collectively known as pathogen-assisted molecular patterns (PAMPs) (Jung et al. 2012). Detection of these PAMPs by host's transmembrane receptors leads to activation of necessary reactions in host and ultimately PAMP-triggered immunity (Jones and Dangl 2006; Boller and He 2009; Thomma et al. 2011). Pathogen can suppress this immunity by secreting effector proteins for successful infection; sometimes plants may also recognize these effector proteins and initiate rapid, long-lasting, and robust responses known as effector-triggered immunity (Boller and He 2009; Thomma et al. 2011). These activated responses in plant are synchronized by signal transducers which control the expression of certain defense-related molecules (Jones and Dangl 2006; Thomma et al. 2011). Therefore, for successful establishment, mycorrhiza has to cope up effectively with these changes and regulate the responses of host. Such

modulations may result in priming and preconditioning of the tissues for competent activation of plant defense response (Pozo and Azcón-Aguilar 2007). Priming is believed to be the basic strategy behind induced systemic resistance (ISR) reported in plants associated with beneficial organisms (Conrath et al. 2006; Goellner and Conrath 2008; van Wees et al. 2008) (Table 1).

The molecular mechanisms of priming and its biological relevance in plants immunity has been studied thoroughly, and now further attention is on to decipher its trans-generational effects. Biocontrol and biofertilizer properties associated with mycorrhizal symbiosis has become an important alternative to chemical fertilizers and pesticides for sustainable agriculture research (Harrier and Watson 2004; Mukerji and Ciancio 2007; Fester and Sawers 2011).

3 Underlying Mechanisms of Mycorrhiza-Induced Resistance (MIR)

3.1 Enhanced Nutritional Status in Host Plant

Plants having AMF association shows better nutrition uptake ability (Smith and Read 2008). Phosphorous and several other nutrients are made available in larger amounts to plants in exchange of photosynthates (Pearson and Jakobsen 1993). Whether this improved nutritional status of plant can increase the strength to cope with pathogens or not is still debatable. There are reports where plants colonized with AMF symbiont like *Glomus intraradices* and *G. mosseae* have shown a concurrent raise in mineral uptake and tolerance (Bodker et al. 1998; Karagiannidis et al. 2002; Akhtar and Siddiqui 2008a, b); on the contrary, some reports indicate improved nutritional status did not lead to any change in tolerance (Shaul et al. 1999; Fritz et al. 2006). Interestingly, it has also been reported that AMF provides protection toward pathogen to plants without affecting host phosphorous level (Newsham et al. 1995). Though the results obtained are not unanimous in ascribing the role of enhanced nutrition by AMF-colonized plants, it can be suggested that tolerance is indirectly contributed either by profused root growth or by efficient phosphorous uptake (Jansa et al. 2005) or by maintaining root cell activity through arbuscule formation (Cordier et al. 1996; Akhtar and Siddiqui 2008a, b; Smith and Read 2008; Akhtar and Panwar 2011).

3.2 Role of Phosphorus and Photosynthates

The most important aspect of mycorrhizal symbiosis is nutritional exchange of phosphorous and photosynthates which occurs mainly in arbusculated cells (Helber et al. 2011). Roots colonized with AMF are suppressed by higher P levels (Balzergue

Table 1 Few examples of defense mechanisms underlying mycorrhiza-induced resistance machinery

Host	Mycorrhiza	Pathogen/parasitic plants stresses	Mechanism involved	Results	References
Tomato	*G. mosseae*	*Erwinia carotovora* pv. *carotovora*	Competition for nutrients	Mycorrhiza inoculated plant exhibited normal growth in presence of pathogen	Garcia-Garrido et al (1991)
Marigold	*G. intraradices*	*Pythium ultimum*	Modulation of defense responses	Lower pathogen infection in mycorrhized plants	St-Arnaud et al. (1994)
Pea	*G. intraradices*	*Aphanomyces euteiches*	Increased P uptake	Reduced the disease severity	Bodker et al. (1998)
Tomato	*G. mosseae*, *G. intraradices*	*Phytophthora parasitica*	Induction of hydrolytic and antioxidant enzymes	Induction of mycorrhiza-related and new isoforms of the hydrolytic enzymes chitinase, chitosanase, and β-1,3-glucanase, superoxide dismutase	Cordier et al. (1998)
Lotus, Medicago	*G. margarita*	–	Modulation of superoxide dismutase	SOD was differentially expressed during the fungal life cycle; highest transcript levels were found in fungal structures inside the roots as observed on two host plants	Lanfranco et al. (2005)

(continued)

Table 1 (continued)

Host	Mycorrhiza	Pathogen/parasitic plants stresses	Mechanism involved	Results	References
Cucumber	*G. intraradices*	*Colletotrichum orbiculare*	Changes in host physiology at pathogen penetration sites	The pathogenicity declined on the leaves of cucumber plant colonized with *G. intraradices* Callose formation was notably high on the leaves of *G. intraradices* colonized plants	Lee et al. (2005)
Cucumber	*G. mosseae*	*C. orbiculare*	Association with other plant growth-promoting microbiota in soil	Anthracnose disease reduction in mycorrhiza colonized plants when combined with other bioprotective isolates	Chandanie et al. (2006)
Tomato	*AMF*	*Alternaria solani*	Induced systemic resistance	Reduced the *A. solani* symptoms	Fritz et al. (2006)
Mays, *Sorghum*	*G. clarum*, *G. margarita*	*Striga hermonthica*	Hormonal changes	Reduction in the number of *S. hermonthica* shoot	Lendzemo et al. (2007)
Barley	*G. mosseae*	*Gaeumannomyces graminis* var. *tritici*	Increase in SA content	Concentration of salicylic acid increased with colonization rate in the mycorrhizal fungus	Khaosaad et al. (2007)

Medicago	*G. intraradices*, *G. gigantea*, *G. versiforme*	*Xanthomonas campesteris*	Local and systemic defense responses in root and shoot	Differential expression of 68 genes in shoots and 41 in roots of host plant. Moreover, 21 genes found in both colonized and non-colonized host plant reduced the development of the pathogen	Liu et al. (2007)
Tomato	*G. intraradices*	*Rhizoctonia solani*	Association with other plant growth-promoting microbiota in soil	Soil application of *T. harzianum* or/and *G. intraradices* radically abridged tomato seedlings damping-off driven by *Rhizoctonia solani*	Amer and Abou-El-Seoud (2008)
Banana	*G. intraradices*	*Radopholus similis* and *P. coffeae*	Induced systemic resistance	The AMF reduced both nematode species by more than 50 %	Elsen et al. (2008)
Date palm	*AMF*	*F. oxysporum* f. sp. *albedinis*	Increase in phenolics	Accretion of hydroxycinnamic acid derivatives—synaptic derivative a I2, play a vital role in resistance of date palm to Foa in mycorrhized seedlings	Jaiti et al. (2008)

(continued)

Table 1 (continued)

Host	Mycorrhiza	Pathogen/parasitic plants stresses	Mechanism involved	Results	References
Tomato	*G. macrocarpum*, *G. fasciculatum*	*F. oxysporum* f. sp. *lycopersici*	Increase in JA	Increased the growth, phenyl-alanine ammonia lyase activity, phenol and foliar trichome density	Kapoor (2008)
Cotton	*G. etunicatum*, *G. intraradices*, *G. versiforme*	*Verticillium wilt*	Competition between AMF and pathogen	Mycorrhizal symbiosis and growth of AMF-related structures reduced when AMF and pathogen infected the same host root	Kobra et al. (2009)
Tomato	*G. mosseae* and *G. intraradices*	*P. parasitica*	Acidic and basic β-1,3-glucanase isoforms	Differential expression of glucanase isoforms in mycorrhiza colonized plants	Pozo et al. (1999)
Pea	*G. mosseae*, *G. intraradices*	*Orobanche crenata*, *O. foetida*, *Phelipanche aegyptiaca*	Nutritional and hormonal changes	Inhibition of parasitic plants germination	Fernandez-Aparicio et al. (2010)

et al. 2011), while higher level of photosynthates promotes density of AM fungal vesicles (Bago et al. 2000) and lateral root development (Fusconi 2014). The negative role of P may be due to signaling pathway which senses the P level in roots and thus upregulates defense gene expression in plants for enzymes like chitinase, glucanase (Lambais and Mehdy 1993), and catalase (Lambais 2000). Phosphorous acts by systemic suppression of essential symbiotic genes, especially the genes encoding enzymes of carotenoid and strigolactone biosynthesis, and symbiosis associated phosphate transporters (Breuillin et al. 2010). Arbuscules are the most active structures in this association where trading of nutrient occurs (Garcia-Garrido and Ocampo 2002); thus, they are greater sucrose sinks for more energy requirements (Vierheilig et al. 2001). Blee and Anderson (2000) discussed the relationship between sugar catabolism, activation of defense-related genes, and in turn activation of systemic resistance (Herbers et al. 1996). Since interchange between phosphorous and carbon is the major movement in AMF association, it has a pivotal role in the activation of defense responses in host plants.

3.3 Hormonal Changes in Plants

Mycorrhizal association is well known for acclimatizing host plant to various stresses. Plant hormones, key controller of plant development and immunity, not only coordinate plant responses to the fluctuating environment but also regulate mycorrhizal symbioses either by regulating initial steps of colonization or by bringing morphological changes in plants to accommodate AMF, its expansion, and functionality (Pozo et al. 2015). These phytohormones interact positive or negative to regulate a large number of plant activities.

Hormones are having defined roles at onset of colonization and after colonization. Strigolactones are one of the positive hormones secreted by plant inducing fungal chitin oligomer, mandatory for fungal incursion and activating symbiosis with plant roots (Gutjahr and Parniske 2013; Ruyter-Spira et al. 2013). However, salicylates, ethylene, and cytokinins behave antagonistically for the same (Foo et al. 2013). Development of arbuscule and its survival are also controlled by plant hormones (Gutjahr and Parniske 2013) where ABA and auxins are indispensable, while gibberellins exhibit inhibition (Floss et al. 2013; Foo et al. 2013; Etemadi et al. 2014; Martín-Rodríguez et al. 2015).

Imbalance in plant hormone levels in mycorrhizal plants makes plants more tolerant (Fernandez et al. 2014; Selosse et al. 2014). Enhanced plant nutrition, changes in root structure, and priming plant defense responses regulated by plant hormones (Jung et al. 2012); while changes in root architecture alter the level of cytokinins, auxins, and strigolactones (Fusconi 2014); and ABA levels in colonized plants that increase their water absorption capacity (Ruiz-Lozano et al. 2012) are few examples of it. Though levels of ABA remain unaffected under normal conditions, ABA increases in osmotic stress, probably leading to primed responses (Aroca et al. 2013). ABA deficiency suppresses AMF formation (Martín-Rodríguez et al. 2015)

by inducing defense cell wall-related genes (Garcia-Garrido et al. 2010). Hormonal changes affect the interaction of plant with various airborne and soilborne pathogens. Production of strigolactones in mycorrhizal plants is able to reduce *Striga hermonthica* infection by root pathogen (Lendzemo et al. 2007). Similar, results were seen in peas and tomatoes (Fernandez-Aparicio et al. 2010; Lopez-Raez et al. 2012). Thus, it explains the role of plant hormone to alleviate plants' response to abiotic and biotic stresses in AMF plants.

3.4 Struggle for Host Photosynthates and Colonization or Infection Sites

AMF competes with pathogenic microorganisms in soil or root (Filion et al. 2003; St-Arnaud et al. 1994) for their establishment in the same host. This may be because both of them utilize common sources to survive which are host photosynthates such as glucose and fructose and a space to infect/colonize within the root (Whipps 2004). Arbuscules and intercellular hyphae are the structures which are actively involved in uptake of photosynthetic products (Douds et al. 2000; Kaiser et al. 2015). Photosynthates are transferred to the fungus as sucrose or hexose via mycorrhizal intraradical structures (hyphae or arbuscules in the root cortex) and transferred as glycogen or triacylglycerol through the large extraradical hyphae network (Bago et al. 2002, 2003; Parniske 2008). Increased inflow of sucrose, fructose, and glucose leads to active catabolism in arbuscules which in turn activates defense-related genes (Ernst and Siri-Prieto 2009; Vierheilig et al. 2001). Resistance mechanism depends upon type of pathogen in colonized plants which can be localized (Jalali and Jalali 1991) or systemic (Dehne 1982; Smith 1987). Utilization of plant resources and space by AMF consequently decreases the survival rate of pathogen.

3.5 Changes in Plant Root Architecture

AMF promotes profusely branched root system (Paszkowski et al. 2002; Olah et al. 2005; Gutjahr et al. 2009). Eggplant colonized with *Glomus* spp. showed higher lignin concentrations in first- and second-order root on attack of a root pathogen *Verticillium dahliae* (Matsubara et al. 1995). Moreover, AMF also caused the plant to produced thickened third-order roots. However, in tomato with AMF, root branching was not affected, but there was a decrease in number of infection loci of *Phytophthora parasitica* (Vigo et al. 2000). It is noteworthy here that increase in lateral root tips and developing meristems due to AMF colonization results in higher susceptibility of plants toward pathogen, which in turn necessitates AMF to protect plants by other mechanisms (Newsham et al. 1995; Norman et al. 1996). Thus, changes in root architecture such as the root physical force, rhizodeposition, root

entanglement of soil particles, and also the soil water regime (Rillig and Mummey 2006) on colonization by AMF can directly or indirectly affect the infectivity of pathogen to host plant (Dighton 2014).

3.6 Microbial Community and Its Associations in Mycorrhizosphere

Colonization by AMF in plants roots changes the quality and quantity of root exudates released in soil, which affects the microbial populations in soil (Azcón-Aguilar and Bago 1994; Akhtar et al. 2011). Changes in these root exudates in soil rhizosphere may be inhibitory to root pathogens and nematodes (Pozo et al. 2013). AMF reduces the number of sporangia and zoospores formed in soil by *Phytophthora cinnamomi* (Meyer and Linderman 1986); also the reduction is seen in *Fusarium* populations in mycorrhizospheric soil of tomato plants (Caron 1989). This prophylactic activity of AMF is also endorsed to some root pathogen antagonists such as *Trichoderma*, *Gliocladium*, and PGPR like *Pseudomonas* and *Bacillus* (Kloepper et al. 1991; Barea et al. 1996). Growth-promoting bacteria and fungi work in collaboration with AMF by improvement in plant rooting, enhancement of plant growth and nutrition (Gopal et al. 2012; Vafadar et al. 2014), abiotic stress tolerance (Xun et al. 2015), and biological control of pathogens; they also improve as well as facilitate symbiosis (Barea et al. 1996). That means the presence of microbial community in soil rhizosphere or changes in rhizosphere enhances AMF-assisted antagonism to root pathogens.

3.7 Production of Endogenous and Exogenous Elicitors

Development of resistance by AMF association can also be attributed to preventing the release of endogenous elicitor molecules from plant cell wall or degradation of the exogenous elicitor molecules produced by AMF. This crosstalk between both the partners can be one plausible reason for boosting plant defense response. Hydrolases are core enzymes of both the partners, wherein plant hydrolase can degrade fungal elicitors, while fungal hydrolase can degrade plant cell wall (Garcia-Garrido and Ocampo 2002). Potential hydrolases like chitinases, chitosanases, and β-1,3-glucanases produced constitutively by mycorrhiza regulate differentially in process of AMF colonization, revealing their key role in it (Dassi et al. 1996; Pozo et al. 1998; Salzer et al. 2000). Stimulation of plant defense reaction by an elicitor produced from extraradical mycelium of *G. intraradices* (Lambais 2000) further supports this theory. For elicitor breakdown, plant chitinase expresses constitutively in the early phase of mycorrhization,

whereas mycorrhiza-specific isozymes are expressed at later stage (Salzer and Boller 2000; Salzer et al. 2000). Besides, defense regulation through degradation of exogenous elicitor molecules and prevention of endogenous elicitor formation plays an equal role. These endogenous elicitors formed on degradation of cell wall; if prevented, then effective plant defense mechanism can be induced. This hypothesis is supported by the fact that *Glomus mosseae* produces very little plant cell wall-degrading pectolytic enzymes like pectin esterase, endo-polymethylgalacturonase, pectin lyase, and pectate lyase (Garcia-Romera et al. 1991). Production of hydrolytic enzymes is minimal by mycorrhiza which is required specifically for their incursion in plants only. There is no obvious role of these fungal enzymes in endogenous elicitor production. Apart from hydrolytic enzyme regulation by endo/exo elicitor molecules, arbuscules also play a role wherein localized defense response is generated when plant/fungal lytic enzymes generate fragments/elicitors from molecules like cellulose, pectin, xyloglucan, and HRGP in new interface compartment created by AMF shared by membranes from both partners (Bonfante 2001).

3.8 Interplay Between Hydrogen Peroxide and Salicylic Acid (SA)

A rapid and transient ROS production had been observed during early plant-pathogen interactions at the site of infection. Following this oxidative burst by ROS production, SA accumulates at the site of infection (Draper 1997). SA is potential inhibitor of antioxidant enzymes such as catalase and peroxidase, thus stimulating ROS response after pathogen attack. This same type of interplay has been observed between ROS and SA in mycorrhizal plants as a possible mechanism to attenuate plant defense response. Both ROS and SA have been used as secondary messengers in AMF associations (Garcia-Garrido and Ocampo 2002). Amount of H_2O_2 and other ROS have not been measured directly in AMF-colonized roots, but evidences suggest that ROS increase upon mycorrhizal colonization (Salzer et al. 1999). During AMF colonization, a transient increase in anti-oxidative enzymes like catalase, peroxidase, and superoxide dismutase is observed in mycorrhizal roots (Falahian et al. 2007; Younesi et al. 2013; Chen et al. 2014). Along with production of such enzymes, changes in their isoenzyme pattern in colonized roots have also been observed at initial stages of fungal penetration, indicating involvement of H_2O_2 and other ROS in signal transduction processes. To address the experimental evidence of the above results, we have tested the production and expression of peroxidase, polyphenol oxidase, and catalase in tomato when colonized with *G. geosporum* MBAL upon attack of *Fusarium oxysporum* f. sp. *lycopersici*. Peroxidase (Fig. 1), polyphenol oxidase, and catalase were produced in higher amounts and expressed different isoenzyme pattern in mycorrhizal plants irrespective of pathogen attack (Fig. 2a–c). Tomato plants

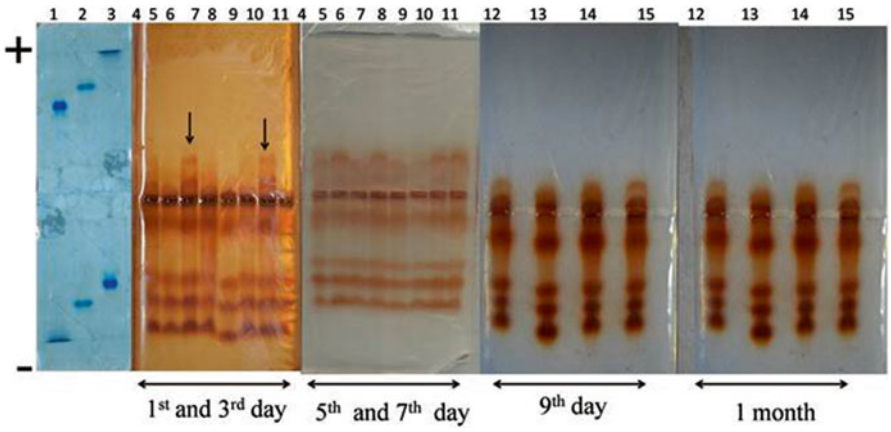

Fig. 1 Agarose gel electrophoresis. *Lane 1*: trypsin soya bean inhibitor (18.40 kDa); *Lane 2*: lactoglobulin (20.10); *Lane 3*: ovalbumin (43 kDa); *Lanes 4*, *5*, *12*: control; *Lanes 6*, *7*, *13*: plant + mycorrhiza; *Lanes 8*, *9*, *14*: plant + pathogen; *Lanes 10*, *11*, *15*: plant + mycorrhiza + pathogen

associated with *G. clarum* expressed all the isoforms of both peroxidase and polyphenol oxidase more strongly than that associated with *G. fasciculatum* (Rodríguez et al. 2001). In bean roots, catalase and ACC oxidase activity is synchronized in accordance with infection potential of AMF and P availability (Lambais 2000). Increase in anti-oxidative burst has been also ascribed to fungal penetration and appresoria formation (Spanu and Bonfante-Fasolo 1988). Accumulation and expression of defense enzymes in the arbuscules may be a localized regulation of defense mechanism (Garcia-Garrido and Ocampo 2002). Temporary increase in catalase and peroxidase activity coincides with that of free SA in AM-colonized tobacco roots and rice roots (Blilou et al. 2000a, b); even external application of SA did not concur with appresoria formation, rather it delayed the mycorrhization of roots. SA level increases only after successful completion of mycorrhization (Pozo and Azcón-Aguilar 2007). This implies that there must be a correlation between SA accumulation and mycorrhizal fungi infectivity. However, precise role of SA in successful mycorrhization is still hazy, but it appears that there is an explicit relationship between antioxidant enzymes and SA accumulation, which in turn regulates mycorrhiza-induced plant defense mechanism in plants.

3.9 Jasmonic Acid as an Endogenous Signal

Jasmonic acid (JA) and its derivatives play a major role in AM symbiosis (Morcillo et al. 2012). JA acts differently at different stages of mycorrhizal colonization (Foo et al. 2013). They are thought to be involved in establishment of AM fungus in host plant (Hause et al. 2007). Arbuscules containing cells actively express genes which is evident by an increase

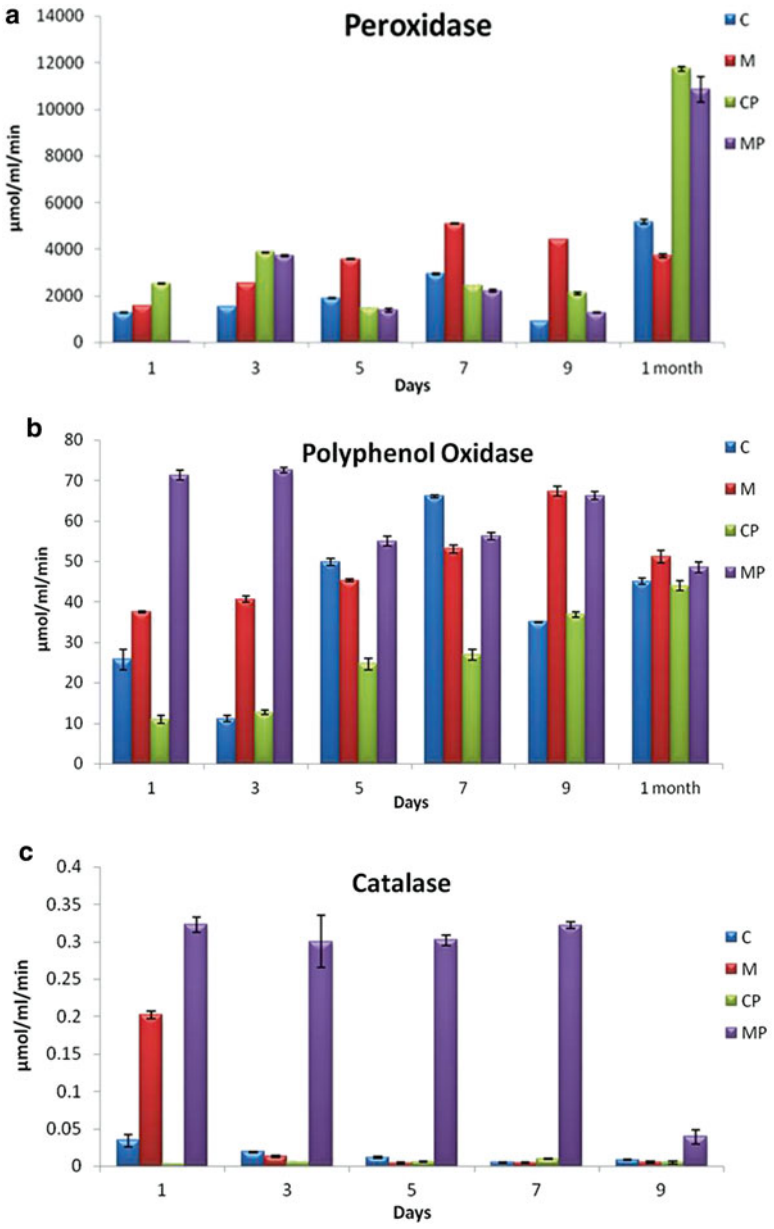

Fig. 2 (**a–c**) Analysis of enzyme (peroxidase, polyphenol oxidase, and catalase). *C* control, *M* mycorrhizal plant, *CP* control plant inoculated with *Fusarium oxysporum* f. sp. *lycopersici*, *MP* mycorrhizal plant inoculated with *Fusarium oxysporum* f. sp. *lycopersici*

in level of endogenous JA in mycorrhizal roots (Hause et al. 2002). This increase in JA may be a reason for prominent resistance shown by mycorrhizal plants. In tomato plants, the JA pathway is involved in defense priming by AMF upon herbivore attack (Song et al. 2012) Further, exogenous supply of JA increases mycorrhizal colonization through modifying the regulation of carbohydrate partitioning in tomato plants (Tejeda-Sartorius et al. 2008). Thus, for successful establishment of AMF, an increased JA is necessary requirement (Hause and Fester 2005). JA-regulated defense responses in mycorrhizal plants can be observed in aboveground tissues, particularly those colonized by *G. mosseae* (Pozo et al. 2009). Another interesting feature is eavesdropping of JA-regulated defense-related genes from colonized plants to their neighboring plants via common mycorrhizal networks (Song et al. 2010). So, it concludes the successful role of JA in colonization and activation of defense responses in mycorrhizal plants against pathogens.

3.10 Activation of Phenylpropanoid Pathway

Another widespread response of plant toward pathogen attack is production of phenylpropanoid derivatives. Flavonoids obtained from phenylpropanoid pathway are responsible for an increase in hyphal growth, spore germination, and more profused hyphal branching (Abdel-Lateif et al. 2012). Isoflavonoid phytoalexins are among the end products of phenylpropanoid pathway whose role is involved in resistance mechanism. These phytoalexins are found only at early stages of mycorrhization (Morandi et al. 1984), for example, glyceollin and medicarpin were found during early stages of mycorrhiza colonization in soya bean and *M. truncatula*, respectively (Wyss et al. 1991; Harrison and Dixon 1994). Similarly, phenylpropanoid-related enzymes, i.e., phenylalanine-lyase (PAL), first enzyme in phenylpropanoid pathway, and chalcone isomerase, the second enzyme, show their increased level in early stages of *G. intraradices* colonization (Lambais and Mehdy 1993; Volpin et al. 1994). Some enzymes that metabolize important reactions in phenylpropanoid pathway like PAL and CHS could be observed in arbuscules, while other enzymes of same pathway like CHI of IFR were not found in mycorrhiza (Harrison and Dixon 1994). However, accumulation of flavonoid/isoflavonoids depends on genotype of photobiont and mycobiont (Harrison and Dixon 1994; Volpin et al. 1995). It can be concluded here that mycorrhizal symbiosis leads to activation of weak, localized, and uncoordinated induction of phenylpropanoid pathway and accumulation of its products (Morandi 1989).

4 Conclusions and Future Prospects

Wide distribution of AM fungi throughout the world and its association with roots of most agricultural and horticultural plants is accepted unanimously. Moreover, it is an eco-friendly approach to combat the toxic effects of chemical fertilizers and pesticides. A thorough understanding of plant interaction with AM fungi, chemical

cross talk, and protection pathways induced after successful colonization will develop a feasible technique to use AM fungi as a potential safeguard. Further research on specific cues on mechanism regulated and products generated may result in an AMF-based green technology for sustainable agriculture.

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Potential of *Bacillus thuringiensis* in the Management of Pernicious Lepidopteran Pests

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Abstract Microbial products have a long history of safe use and most of the microbial agents are compatible with other methods of pest control. A number of microbial biopesticides have been registered for field application on various vegetables, fruits, and other crops of agricultural, horticultural, and forest importance. During sporulation phase, *Bacillus thuringiensis* accumulates certain insecticidal crystal proteins which are pathogenic to a number of insect orders. Thousands of toxicogenic strains of *B. thuringiensis* exist and each strain produces its own unique well-known insecticidal crystal protein. *B. thuringiensis* is biodegradable and safe to nontarget organisms as the conditions required for complex steps in the mode of action do not exist in mammals or most of invertebrates. Development of agricultural crop varieties that contain *B. thuringiensis* proteins provides a safe alternative to the use of chemical insecticides. Tobacco and tomato were the first transgenic plants encoding for *B. thuringiensis* insecticidal crystal protein. The development of resistance to *B. thuringiensis* toxins is, however, particularly unfortunate. Thousands of *B. thuringiensis* isolates are available around the world, and fortunately almost all the major insect pests are susceptible to these strains. Moreover synthetic insecticides in combination with biopesticides are economic,

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effective, and eco-friendly. The aim of this chapter is to focus on the potentiality of *B. thuringiensis* in the management of pernicious lepidopteran pests and their mode of their interactions to develop the cost-effective medium for the formulation of biopesticides.

Keywords *B. thuringiensis* • Insecticidal crystal proteins • Lepidoptera

1 Introduction

It is a well-established fact that weather, insects, and plant diseases affect agricultural products. The integration between the two out of the three and sometimes among all the three is so complicated that it becomes difficult to determine the actual origin of the trouble. In India alone, 30 % of the crop yield potential is lost as a result of insects, disease, and weeds, corresponding to 30 million tons of food grain (Koul 2011). Insects are one of the main natural hazards to any agricultural products and comprise a remarkable group in animal kingdom, accounting 70 % of all the animals present in this universe. Damage caused by them in one or the other way is incompatible, and to estimate, it is really a hard nut to crack. It may be safely stated that next to the vagaries of climate, insects aggravate the farmer's problem the world over. The human desire to control insects has existed about as long as humans themselves have. As the world's population increases, the need to keep away insects from destroying food crops becomes even more urgent. An estimated one third of global agricultural production valued at several billion dollars is destroyed annually by over 20,000 species of insect pests in field and storage (Mariapackiam and Ignacimuthu 2008).

During ancient time, insect pest control has been practiced in various manners. Greek philosopher Homer reported the use of sulfur for fumigation and other pest control uses (1000 B.C.). Pliny the Elder reported pest control practices from Greek literature (70 A.D.) including the use of pepper, tobacco extracts, soapy water, vinegar, turpentine, fish oil, brine, etc. After World War II, the Green Revolution provided great agricultural advantages via the use of agrochemicals, chemical fertilizers, highly productive cultivars, and mechanization. The result was a considerable decrease in a great variety of insect populations, and as a consequence, synthetic insecticidal compounds became popular due to the long residual action and the wide toxicity spectrum. However, synthetic chemical insecticides appeared fully in 1940, when organochlorinated and organophosphate insecticides were discovered. These insecticides were applied during all growing seasons to attack all the developmental stages of insect pests.

One of the most spectacular episodes of insect control began in 1945–1946 with the commercial introduction of the synthetic insecticides like DDT. With the discovery of organochlorines, organophosphorus, organocarbamates, and synthetic pyrethroids, chemical insecticides have been the backbone of insect control. Human

attempts at insect control have changed over time from synthetic chemical control to natural methods, to overcome the demerits associated with synthetic chemical insecticides. Serious environmental and health issues began to be recognized by the presence of chemical residues in food, water, and air. Long-term exposure to these synthetic insecticides, like DDT, has been associated with cancer, liver damage, immunotoxicity, birth defects, and reproductive problems in humans and other animals (Kegley and Wise 1998). Moreover when an insecticide is repeatedly applied against a population, unaffected individuals survive to pass their genes on to following generations. Over time, a greater and greater proportion of the insect population is unaffected by that insecticide. Unfortunately insects have developed resistance to most of the synthetic chemical insecticides that are used widely. In 1979, the United Nations Environmental Programme declared pesticide resistance "one of the world's most serious environmental problem." Its seriousness to the environment has posed a burning and alarming situation from documented adverse effects on the beneficial insects, wildlife, spread of disease by resistant insects, and addition to the environment of new and potentially dangerous insecticides to which pests have already gained resistance (Pimentel and Burgess 1985).

With the growing realization of hazards and side effects associated with the extensive and indiscriminate use of pesticides, entomologists have adopted a new concept of pest control, termed as integrated pest management (IPM) which refers to a system that utilizes all suitable techniques and methods in as compatible manner as possible and maintain the pest population at levels below those causing economic threshold (Mahtur and Kishor 1987). In this context, the role of biocontrol agents, viz., predators, parasitoids, and microbes, needs no emphasis due to their specificity, effectiveness, and safety to nontargeted organisms besides the other components in relation to man and biosphere.

Microbial products have a long history of safe use, and most of the microbial agents are compatible with other methods of pest control. In recent years, entomologists are leaning their attention on the exploration of microbial agents including bacteria, virus, fungi, nematode, and protozoa for pest suppression. Facultative pathogens of some insect species are commonly used as a biopesticide or microbial control agent. Interestingly, some of them have been widely tested and proved very effective against pernicious insect pests of agricultural crops. In certain developing and developed countries, a number of microbial biopesticides have been registered for field application on various vegetables, fruits, and other crops of agricultural, horticultural, and forest importance.

Of the large number of microbial species belonging to all the major groups like bacteria, virus, fungi, and nematode, the bacterial pathogens have been exploited the most and are recommended as potential biocontrol agent for the control of major insect pests. Bacteria are prokaryotic, unicellular organisms varying in size from less than 1 μm to several μm in length along with spherical, spiral, and rod shaped. Most of the insect pathogenic bacteria occur under the families *Bacillaceae*, *Pseudomonadaceae*, *Enterobacteriaceae*, and *Streptococcaceae* (Kalha et al. 2014). Members of *Bacillaceae*, particularly *Bacillus* spp., have received maximum attention as microbial control agents. *Bacillus thuringiensis*

Berliner, which occupied 90 % of the world biopesticide market, is pathogenic to more than 525 insect species belonging to various orders, but mainly to Lepidoptera, Diptera, Coleoptera, and Hymenoptera (Jayaraj 1986). The aim of this chapter is to focus on the potentiality of *B. thuringiensis* in the management of pernicious lepidopteran pests and their mode of their interactions to develop the cost-effective medium for the formulation of biopesticides.

2 *Bacillus thuringiensis*

B. thuringiensis is a rod-shaped, gram-positive, spore-forming, aerobic common soil bacterium and was first discovered in Japan in 1901 by Ishiwata and then in 1911 in Germany by Berliner (Baum et al. 1999). It is distributed worldwide in soil, stored products, insects, insect-breeding environments, and the phylloplane (Hofte and Whiteley 1989). Vegetative cells of *B. thuringiensis* are 0.2–5 μm in size with peritrichous flagella. They divide by binary fission and frequently occur in chains. During sporulation phase of growth, this bacterium accumulates certain insecticidal crystal proteins (ICPs)/δ-endotoxin. These proteins accumulate as inclusion bodies in the mother cell compartment and are finally released to the environment along with the spore at the end of sporulation. Some ICPs have toxicity at par with that of widely used organophosphate pesticides. Unlike organophosphates, which are quite general in their effects, *B. thuringiensis* ICPs are very specific to certain harmful insects and are therefore safe to most beneficial insects and other animals. Additionally, *B. thuringiensis* toxins are biodegradable and do not persist in the environment (Van Frankenhuyzen 1993). Owing to its greater effectiveness to defoliating lepidopterans, a worldwide recognition has been given to this bacterium.

The Environmental Protection Agency (EPA) has exempted from tolerance several *B. thuringiensis* ICPs for introduction into agricultural crops to control insect pests. These tolerance exemptions were based on the extensive safety data on history of safe use of microbial pesticide formulations containing *B. thuringiensis* proteins. ICPs were also subjected to safety testing to confirm that they posed no meaningful risks to mammals, especially human being. None of the incidents are attributable to exposure to the proteins produced by *B. thuringiensis* (EPA 1988).

The first record of its application to control insects was in Hungary at the end of 1920, and in Yugoslavia at the beginning of the 1930s, it was applied to control the European corn borer. During the following two decades, several field tests were conducted to evaluate its effectiveness against lepidopterans, both in Europe and in the United States (Ceron 2001), and results favored the development of formulations against on this pathogen. Subsequently, the first commercial product was produced in 1938 by Libec in France (Aronson et al. 1986) and in the 1950s in the United States. The first product of *B. thuringiensis* was registered in 1961; since then bacterium has been applied continuously for an expending number of uses in agriculture, forestry, and control of insect vector. Now there are many countries like the United States, France, Germany, Russia, etc. where *B. thuringiensis* has

been registered under the different trade names for the control of insect pests. Thousands of toxicogenic strains of *B. thuringiensis* exist (Lereclus et al. 1993), and each strain produces its own unique insecticidal crystal protein which is encoded by a single gene on a plasmid in the bacterium. The insecticidal activity of the toxins from each strain is also known to vary widely. In addition to that, there is often a considerable diversity of such activity within a single strain. The medium on which a bacterial strain is grown also affects the toxicity of the pathogen (Srivastava and Ramakrishnan 1980). There are several other factors, viz., pH of the gut, stage, age, feeding habit of the insect, method of application, exposure period, and environmental conditions, which determine the pathogenic effects of the bacterium against the particular insect species.

There are 34 recognized subspecies of *B. thuringiensis* with two distinct groups of toxin proteins, *Cry* (crystal delta-endotoxins) and *Cyt* (cytolysins), pathogenic to insect pests (Schnepf et al. 1998). *Cry* proteins are named according to their amino acids similarity to established holotype proteins (Crickmore et al. 1995). In general, commercially available *B. thuringiensis* products are of two types. The predominant type is *B. thuringiensis* subsp. *kurstaki*. Since early 1960, these have been sold under several trade names like Dipel, Biobit, Bactospeine, Thuricide, Delfin, etc. for the control of a number of lepidopteran pests of important field crops, forest trees, vegetable crops, and ornamentals. In the late 1970s, another *B. thuringiensis* product based on *B. thuringiensis* subsp. *israelensis* was introduced for the control of a number of dipteran, including the vectors of human diseases like mosquito and flies. These products are sold under various trade names like Tekhnar, VectoBac, etc. (Lee et al. 1998).

In the 1980s, commercial interest in *B. thuringiensis* grew very rapidly as many popular synthetic insecticides became ineffective due to insect resistance or became unusable due to environmental restrictions and also as the field of genetic engineering emerged. The first report of genes insertion, encoding for *B. thuringiensis* δ-endotoxins into plants, came in 1987 with a tag on tobacco and tomato to be the first transgenic plants (Van Frankenhuyzen 1993). Development of agricultural crop varieties that contain *B. thuringiensis* proteins provides a safe alternative to the use of chemical insecticides. Many lepidopterans bore into the stalk of the plants and destroy its structural integrity. In the stalk, the pest is relatively safe from pesticide application. The engineering of plants to express *B. thuringiensis* δ-endotoxins has been especially helpful against pests that attack parts of the plant that are usually not well protected by conventional insecticide applications. Using *B. thuringiensis* in the form of transgenic crops is now very common. Such crops have also been commercialized and are in wide use. Most of the transgenic are in major crop plants such as cotton, corn, rice, potato, soybean, tobacco, and tomato (Perlak et al. 1990; Jenkins et al. 1993). The discovery of *Bt.* with new insecticidal spectra and the advent of recombinant DNA technology have led to a substantial increase in the number of *B. thuringiensis* products available for pest control. At present more than 100 *B. thuringiensis* insecticidal formulations (based on naturally occurring isolates) are available. We have summarized some of them in tabular form (Table 1).

Table 1 Classification of insecticidal crystal proteins (ICPs) from *B. thuringiensis* (Source: Lee et al. 1998)

Old name	New name	Host range	Mass (kDa)
CryIA (a)	*Cry1Aa*	Lepidopteran	133.5
CryIA (b)	*Cry1Ab*	Lepidopteran	131.0
CryIA (c)	*Cry1Ac*	Lepidopteran	133.3
CryIB	*Cry1Ba*	Lepidopteran	138.0
CryIC (a)	*Cry1ca*	Lepidopteran	134.8
CryIC (b)	*Cry1Cb*	Lepidopteran	134.0
CryID	*Cry1Da*	Lepidopteran	132.5
CryIF	*Cry1Fa*	Lepidopteran	133.6
CryIG	*Cry9Aa*	Lepidopteran	129.7
CryIIA	*Cry2Aa*	Lepidopteran/dipteran	70.9
CryIIB	*Cry2Ab*	Lepidopteran	70.8
CryIIIA	*Cry3Aa*	Coleopteran	73.1
CryIIIB	*Cry3Ba*	Coleopteran	74.2
CryIIIC	*Cry7Aa*	Coleopteran	74.4
CryIIID	*Cry3Ca*	Coleopteran	73.8
CryIVA	*Cry4Aa*	Dipteran	134.4
CryIVB	*Cry4Ba*	Dipteran	127.8
CryIVC	*Cry10Aa*	Dipteran	77.8
CryIVD	*Cry11Aa*	Dipteran	72.4
CryV	*Cry11b*	Coleopteran/lepidopteran	81.2
CytA	*Cyt1Aa*	Dipteran/cytolytic	27.4
CytB	*Cyt2aA*	Dipteran/cytolytic	29.2

The development of resistance to *B. thuringiensis* toxins is, however, particularly unfortunate. If *B. thuringiensis*-based products become ineffective due to resistance, organic farmers would lose this irreplaceable resource (McGaughey et al. 1998). In 1985, the first evidence of resistance developing in the field against *B. thuringiensis* δ-endotoxin was published. Low levels of resistance was found in *Plodia interpunctella* (Hubner), the Indian meal moth, in storage bins of *B. thuringiensis*-treated grain. Prior to this, *B. thuringiensis* δ-endotoxin resistance had been seen in neither the field nor the lab, though attempts were made to select for resistance in laboratory populations (McGaughey 1985). In 1990, *Plutella xylostella* (L.) was found to be losing susceptibility to *B. thuringiensis* toxin in Hawaii, Florida, and New York. In *P. xylostella* the resistance was detected after intensive use of these insecticides in several other countries, including Japan, China, the Philippines, and Thailand (Liu and Tabashnik 1997).

About 20 years after *B. thuringiensis* resistance was discovered in *P. interpunctella*, *B. thuringiensis* resistance has been reported in laboratory populations of a total of 13 insect species. Eleven of these species have developed resistance to various strains of *B. thuringiensis* toxin in the laboratory but not in the field (Tabashnik et al. 1994; Gould et al. 1997; Wirth et al. 1997; Huang et al. 1999; Liu et al. 1999).

These laboratory studies show that the potential to develop resistance is real. To overcome the resistance problem in *B. thuringiensis*-based biopesticides, it is necessary to minimize the uses of similar *B. thuringiensis* strains or even different strains having the same mode of actions against a particular insect pest.

2.1 *History of* B. thuringiensis

B. thuringiensis was isolated as early as 1911 by Berliner from the diseased larvae of Mediterranean flour moth *Anagasta kuehniella* Zeller (Faust 1974). The events of pioneering research of Steinhaus (1951) on *B. thuringiensis* and a growing realization that the organic insecticides were deleterious to the environment and human health spurred a renewed interest in *B. thuringiensis* in the early 1960s. Hannay (1953) reported a crystalline body formed at sporulation in the cells of *B. thuringiensis* and suggested it might be associated with the insecticidal activity of this bacterium. Angus (1954) demonstrated that the crystal contained an alkaline soluble toxin for insects. In 1962, Edouard Kurstak isolated another subspecies of *B. thuringiensis* from diseased *A. kuehniella* larva from a floor mill at Bures-sur-Yvette near Paris in France (Kurstak 1962). Heimpel (1967) named this agent the δ-endotoxin and reported that the effectiveness of *B. thuringiensis* depends on the presence of this toxin, and bacterial spore count is not a reliable index of potency. In 1970, Dulmage isolated a new strain of *B. thuringiensis* var. *alesti* from diseased pink bollworm, *Pectinophora gossypiella* (Saunders) larvae, at the Southwestern Cotton Insects Investigations Laboratory at Brownsville, Texas, and he designated this strain as HD-1 (Dulmage 1970). It has a high insecticidal activity. Later on, different types of biopesticide based on *B. thuringiensis* were introduced into the market. Simultaneously, intensive screening programs were undertaken in western countries to find out new strains of *B. thuringiensis* that could kill wider spectrum of insects. Cabbage looper, *Trichoplusia ni*, is used in the standardization of preparations of *B. thuringiensis*, and the potency of the pathogen is expressed in international unit (IU)/mg. Earlier in the use of conventional *B. thuringiensis* insecticides, there were some limitations like narrow specificity, short shelf life, low potency, lack of systematic activity, and the presence of viable spores. To some extent these problems are now being tackled by various molecular and genetic engineering approaches along with the conventional microbiological methods (Dov-Ben et al. 1995). Now, thousands of *B. thuringiensis* isolates are available around the world, and fortunately almost all the major insect pests are susceptible to these strains.

2.2 *Role of* B. thuringiensis *in Agriculture*

B. thuringiensis strains have attracted worldwide interest in various pest management applications because of their specific pesticide activities. It is the most widely used commercially successful biological control agents in different forms (Table 2).

Table 2 Types of *Bt.*-based microbial insecticides formulation

Formulation	Application
Briquettes	Aquatic systems
Emulsions	Agriculture and forestry
Encapsulations	Agriculture and forestry
Granules	Agriculture and forestry
Powders	Forestry
Wettable powders	Agriculture and gardens

δ-endotoxins produced by *B. thuringiensis* have been successfully and safely used against several agricultural and forest pests, as well as in vector control, for decades (Federici et al. 2006). Depending upon the subsp. and strains of the bacterium, it is pathogenic to a number of major insect orders. In India, *B. thuringiensis*-based products are being marketed by various agrochemical industries, whereas new strains are being investigated for development as potential microbial biopesticide (Arora et al. 2000a). Annual worldwide production of *B. thuringiensis* represents about 2 % of the total global insecticide market with worth of approximately $90 million clearly indicating that *B. thuringiensis* is the widely used bacterial pest control agents.

Different *B. thuringiensis* subsp. are known to vary in their efficacy to different insect pests, and even different isolates of the same strain may show variable toxicity against the same species of insect pests (Khan et al. 1995; Adams et al. 1996; Barker 1998). Dulmage (1970) reported an isolate of *B. thuringiensis* subsp. *kurstaki* strain HD-1 that was later produced by Abbott Laboratories as the first major commercial product called as Dipel. Presently subsp. *kurstaki* HD-1-based products are registered for nearly 30 crops against over 100 insect pest species worldwide.

2.3 Toxins Produced by B. thuringiensis

Although *B. thuringiensis* contain four major toxins (α-, β-, and γ-exotoxin and δ-endotoxin), more attention has been paid only to crystalline δ-endotoxin, because of its high toxicity to insect pests of agricultural importance. The α-exotoxin is a proteinaceous, thermolabile toxin that is highly toxic to some insects by oral and intra-hemocoelic inoculations. It is also toxic to mice and other vertebrates. The β-exotoxin or thuringiensin is a thermostable toxin, which kills the insect by per os and parental inoculations. It is formed during vegetative phase of the bacteria and secreted into the medium. Thuringiensin affects a broad spectrum of insect orders like Lepidoptera, Diptera, Coleoptera, Hymenoptera, Isoptera, Orthoptera, Hemiptera, and Neuroptera (Kalha et al. 2014). On the basis of molecular mass, δ-endotoxins may be grouped into two classes: one containing both high- (125–144 kDa, P1) and medium-sized

(60–144 kDa, P2) proteins and a second class consisting of only the high-molecular-weight polypeptides. Structure of δ-endotoxin from *B. thuringiensis* subsp. *tenebrionis* which is especially toxic to coleopterans has been determined by Li et al. (1991) at 2.5 Å resolution.

2.4 *New Strains of* B. thuringiensis

Isolation and characterization of new *B. thuringiensis* strains are needed to discover strains with novel or high insecticidal activities. Intensive screening programs are leading to a broader activity spectrum of toxins as the result of isolation and characterization of new strains with different combinations of crystal proteins, as well as the discovery of new toxins (Silva-Werneck and Ellar 2008). Shojaaddini et al. (2012) isolated a new *B. thuringiensis* subsp. *aizawai* strain EF495116 from a dead *P. interpunctella* larva in Tabriz University, Iran. The strain produced a major parasporal protein band of about 135 kDa and shows toxicity against *P. interpunctella* and *P. xylostella* larvae, with LC_{50} values of 7.13 and 3.1 mg/ml, respectively. Yilmaz et al. (2012) isolated a highly pathogenic *B. thuringiensis* strain SY49.1 from a soil sample in Turkey. This strain harbors several cry genes producing crystalline inclusions known to have toxicity on lepidopteran, dipteran, and coleopteran pests. Ecological distribution of Cry proteins and their genotypes of *B. thuringiensis* isolates from warehouses in China has also been reported by Hongyu et al. (2000). More than 3000 isolates of *B. thuringiensis* from 20 countries have been collected (Stotzky 2002).

2.5 *Classification of* B. thuringiensis *Subspecies*

Different workers have classified *B. thuringiensis* into a large number of subsp., varieties, strains, serotypes, serovars, biovars, pathovars, or cristovars. The subsp. have been differentiated by various methods such as biochemical tests, flagella serology, parasporal body (crystal) antigen, plasmid profiling, and molecular techniques (Adams et al. 1996). DeBarjac and Bonnefoi (1962) developed a key for taxonomic division of *B. thuringiensis*, based on the antigenic properties of the flagella. In 1990, DeBarjac and Franchon revised the classification based on H antigen. The insecticidal proteins of *B. thuringiensis* are grouped in two big families named Cry and Cyt toxins. Lee et al. (1998) classified *B. thuringiensis* Cry proteins as cryl (lepidopteran specific), cryll (lepidopteran and dipteran specific), crylll (coleopteran specific), crylV (dipteran specific), cryV (coleopteran and lepidopteran specific), and cytl and cytll (dipteran and cytolytic). A number of techniques including serotyping, crystal serology, crystal morphology, protein profiles, peptide mapping, DNA probes, and insecticidal activity can be used to characterize a *B. thuringiensis* strain.

2.6 Crystal Protein Genes and Their Classification

Several genes code for various ICPs, produced by *B. thuringiensis*. These genes are usually plasmid borne but are also chromosomally located (Carlson and Kolsto 1993). They are normally present on large plasmids some of which are self-transmissible and often in low copy number. Many of the plasmid borne genes are bordered by transposons and/or insertional sequences. Several *B. thuringiensis* toxin genes have homology in sequence and show similar insecticidal spectrum. However, there are certain genes which are highly homologous but have different insecticidal spectrum. These genes have been sequenced and analyzed. Since the insecticidal activity of *B. thuringiensis* is determined by the cry genes within the host bacterium, cloning and sequencing of cry genes is an obvious key to understanding this relationship.

Based on comparison of the insecticidal spectra, Hofte and Whiteley (1989) organized a classification system for the 42 sequenced genes. Fourteen cry genes were designated holotypes and placed into four major classes: *CryI*, *CryII*, *CryIII*, and *CryIV* (Table 3). Feitselson et al. (1992) analyzed toxin domains of 29 distinct ICPs and added two new major classes, cryV and cryVI. Koni and Ellar (1994) reported a new 27.3 kDa protein, isolated from *B. thuringiensis* subsp. *kyushuensis*, and it was named as *cytB*. For classifying the *cry* genes and their protein products, Crickmore et al. (1998) have introduced a systematic nomenclature, based on amino acid sequence of full-length gene products. This shifting from function-based to amino acid sequence-based nomenclature allows closely related toxins to be ranked together. Over the past few years, however, the discovery of new *Cry* proteins with new insecticidal properties shows that the screening programs continue to hold promise for identifying proteins that could prove useful in pest control.

2.7 Structure of Insecticidal Crystal Proteins

Insecticidal crystal proteins, produced during sporulation phase of the bacterium, are bipyramidal with eight similar faces, on which rows of protein molecules are arranged. These crystal proteins comprise three domains, which are from N- to C-terminal, a seven-helix bundle, a three-sheet domain, and a β-sandwich. The bundle of long, hydrophobic, and amphipathic helices is equipped for pore formation in the insect midgut membrane, and the regions of three-sheet domain are probably responsible for receptor binding. Biochemically crystal proteins are insoluble in water, dilute nitric acid, and several common organic solvents, whereas soluble in weak alkaline solutions like sodium carbonate, ammonium carbonate, and sodium hydroxide. Insecticidal crystal proteins contain only protein and silicon, while other constituents like carbohydrate, phosphorus, and fats are altogether absent. Federici et al. (1990) extensively reviewed the biochemical properties along with the toxicity of different crystal protein components.

Table 3 Commercial *B. thuringiensis*-based bioinsecticides (Source: Ninfa and Garcia 2009)

Company	Commercial name	Active ingredient	Target pest
Certis	Agree WG	*B. thuringiensis* v. *aizawai*	Lepidopterans
Certis	Condor	*B. thuringiensis* v. *kurstaki*	Lepidopterans
Certis	CoStar	*B. thuringiensis* v. *kurstaki*	Lepidopterans
Certis	Crymax	Genetically engineered *B. thuringiensis* v. *kurstaki* and *B. thuringiensis* v. *aizawai*	Lepidopterans
Certis	Deliver	*B. thuringiensis* v. *kurstaki*	Lepidopterans
Certis	Jackpot WP	*B. thuringiensis* v. *kurstaki*	Lepidopterans
Certis	Javelin/Delfin	*B. thuringiensis* v. *kurstaki*	Lepidopterans
Certis	Lepinox WDG	*B. thuringiensis* v. *kurstaki*	Lepidopterans
Certis	Turix WP/Agree WP	*B. thuringiensis* v. *kurstaki*	Lepidopterans
AFA Environment Inc.	Agribac	*B. thuringiensis* v. *kurstaki*	More than 30 insect species
Valent Biosciences Corp.	Dipel	*B. thuringiensis* v. *kurstaki*	Lepidopterans
Valent Biosciences Corp.	XenTari	*B. thuringiensis* v. *kurstaki*	*Spodoptera* sp. and *P. xylostella*
Valent Biosciences Corp.	Biobit	*B. thuringiensis* v. *kurstaki*	Lepidopterans
Valent Biosciences Corp.	Novodor	*B. thuringiensis* v. *tenebrionis*	Coleopterans
Valent Biosciences Corp.	VectoBac	*B. thuringiensis* v. *israelensis*	Mosquito and fly larvae
Valent Biosciences Corp.	Teknar	*B. thuringiensis* v. *israelensis*	Mosquito and black fly larvae
Valent Biosciences Corp.	Gnatrol DG	*B. thuringiensis* v. *israelensis*	Larval stage of sciarid mushroom flies
Valent Biosciences Corp.	Foray	*B. thuringiensis* v. *kurstaki*	Lepidopterans
Som Phytopharma	Lipel	*B. thuringiensis* v. *kurstaki*	Lepidopterans
Biotech International	Biolep	*B. thuringiensis* v. *kurstaki*	Lepidopterans
Valent Biosciences Corp.[a]	Thuricide	*B. thuringiensis* v. *kurstaki*	Lepidopterans and certain leaf-eating worms

[a]Valent BioSciences has also acquired exclusive marketing rights to Thuricide biological insecticide. Thuricide is a registered trademark of Certis, USA

2.8 Mode of Action

The pathogenic effect of *B. thuringiensis* generally occurs in insects only after ingestion of toxin with food, and target organ is the insect midgut. Upon ingestion by insect crystalline, inclusions are solubilized at high pH in the midgut, releasing δ-endotoxins (protoxins). These toxins are activated by resident protease and bind to receptor proteins localizing on brush border membrane of the larval midgut and form pore on the membrane that lead to insect death (Chang et al. 2001). The main toxicity symptom appears as cessation of feeding followed by gut paralysis. In certain cases, as a result of inhibition of feeding, the insect dies within 2–4 days. However, Contreras et al. (2015) reported that differences in susceptibility to *B. thuringiensis* infection might not only rely on toxin–receptor interaction but also on host defense mechanisms. The toxicity caused by δ-endotoxins is also known to affect the biology of insect pests. Conditions required for complex steps in the mode of action do not exist in mammals or most of invertebrates. Only a relatively small subset of insects has been identified that support this complex series of events that leads to cell death.

3 Preparation of Bacterial Concentration

For seed flasks, prepare 72-h-old nutrient agar slants of *B. thuringiensis* and store them at 4 °C until use. Take loopsful of the bacterial growth from these slants to inoculate 500-ml Erlenmeyer seed flasks containing 100 ml of tryptose phosphate broth that had been autoclaved at 121 °C for 35 min. Incubate these flasks on a rotary shaker at 280 rpm at 32 °C for 24 h. Use 3 % (by volume) of the first-passage seed to inoculate second similar seed flasks and incubate these flasks for 18–24 h at the same conditions. For fermentation flasks, use 2 % of the second-passage seed to inoculate fermentation flasks, and incubate at same conditions on rotary shaker for 3 days (Dulmage 1970) which can be harvested when sporulation and cell lysis are essentially complete, usually at about 72 h. The spore–crystal complex can be harvested by the acetone coprecipitation procedure described by Dulmage et al. (1970) (Fig. 1). The number of viable spores can be determined by first pasteurizing samples for 10 min at 65 °C and then plating them in nutrient agar (Dulmage 1970). However, the required concentrations of the pathogen can be calculated using Pearson's square method. For example, to prepare 0.05 % of spray solution, 0.05 ml of 10 % microbial stock solution and 9.95 ml of DDW is required.

3.1 Cost-Effective Medium for Large-Scale Production

Large-scale production of *B. thuringiensis* is expensive because of the high cost of the raw materials used in the medium. Several workers made attempts to develop a cost-effective medium, based on a locally available raw material. Salama et al.

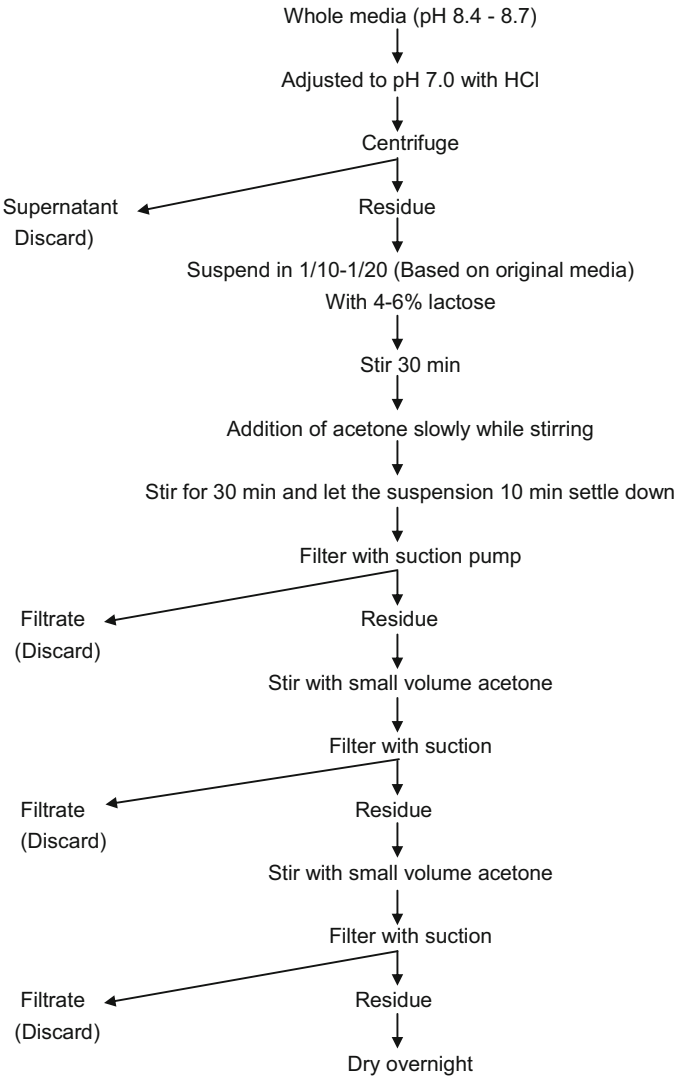

Fig. 1 Flow sheet representation of recovery process for spore–crystal complex (Dulmage et al. 1970)

(1983) used fodder yeast, beef blood, and slaughterhouse residue by-products for yielding good sporulation titers and potent spore–δ-endotoxin preparations. Formulations of subsp. *kurstaki* produced from media containing these nutrients killed 80–100 % of larvae of *H. armigera* when tested at 500 pg/ml diet. Most of the formulations derived from fermentations using leguminous seeds as sole sources of protein also contained high levels of spores and endotoxin. Prabakaran et al. (2008)

reported that coconut water-based culture medium is economical for the production of *B. thuringiensis* var. *israelensis*, widely used in mosquito control programs. Paul et al. (2011) develop an economical medium, based on inexpensive, locally available raw materials. They reported that *Parthenium hysterophorus* L. leaf extract-based culture medium resulted in highest toxicity (LC_{50} 14.628 µg/ml) against 7-day-old *Spodoptera litura* (Fab) larvae. Cost-effective media using locally available raw materials, wheat bran (Vimala Devi et al. 2005), and spent mushroom substrate (Wu et al. 2014) were also reported.

4 Effect of *B. thuringiensis* Against Lepidopteran Pests

Many lepidopteran insects can effectively be controlled by *B. thuringiensis kurstaki* (Nethravathi et al. 2010). *Bt*. spore–crystal mixtures have been successfully used as bioinsecticides against lepidopteran pests. Several workers have reported efficacy of this pathogen against different stages, egg, larva, pupa, and adult (Fig. 2), of lepidopteran pests. By restricting the amount of damage caused by lepidopteran pests to the infested crop, *B. thuringiensis*-based biopesticides have contributed to yield increases in different crops like rice (Kandibane et al. 2010), cauliflower (Justin et al. 2003), corn (Tamez-Guerra et al. 1998), etc. Mortality symptoms among different stages of the pest are shown in Fig. 3.

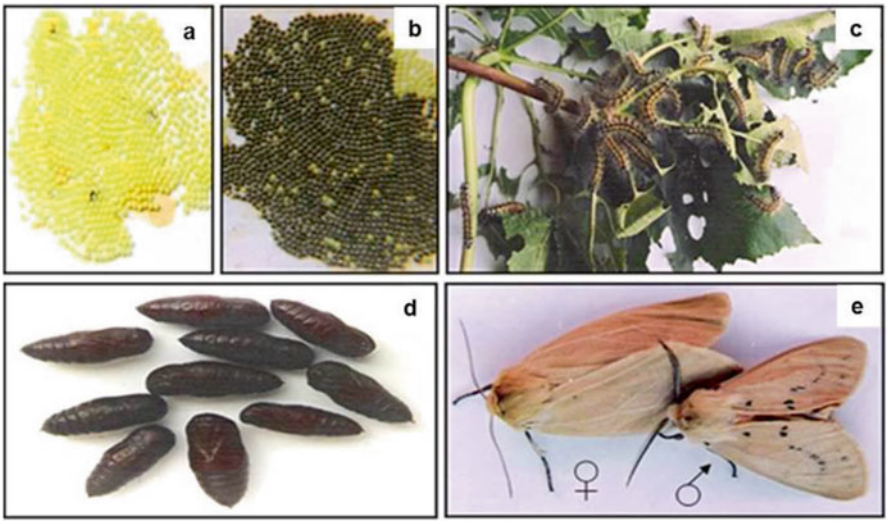

Fig. 2 Various life cycle stages of *Spilarctia obliqua* (Walker) (Lepidoptera: Arctiidae): (**a**) fresh eggs; (**b**) eggs near to hatch; (**c**) 7-day-old larva; (**d**) pupa; (**e**) adult

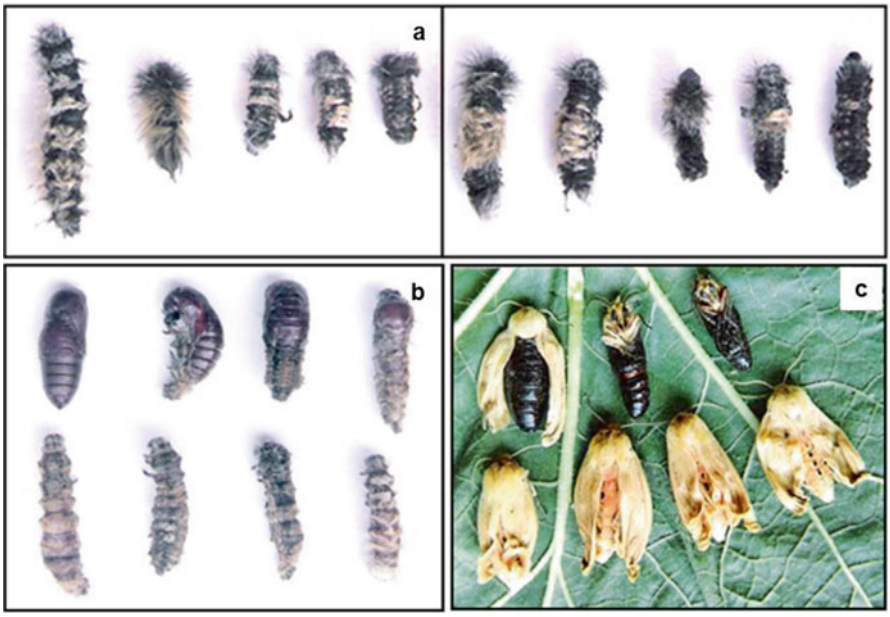

Fig. 3 Mortality symptoms at different stages of *Spilarctia obliqua* (Walker) (Lepidoptera: Arctiidae)

4.1 Larval Mortality

Traditionally the insecticidal activity of *B. thuringiensis* crystal protein has been investigated by using a crude preparation of spore–crystal mixtures. Kulkarni and Amonkar (1988) studied toxicity effects of purified spore–crystal formation from three isolates of *B. thuringiensis* subsp. *kurstaki*. They revealed that the mixture of spore–crystal formation pronounces 100 % mortality against second instar larvae of pod borer *Helicoverpa armigera* (Hubner). However, spores alone registered 10–20 % larval mortality. Larvae of *Chilo partellus* (Swinhoe) showed >80 % mortality when treated with spore–crystal preparations from *B. thuringiensis* strains (Khanna et al. 1995). Citrus butterfly (*Papilio demoleus* L.) was found to be completely controlled 5 days after application of different concentrations of *B. thuringiensis* (Narayanamma and Savithri 2003).

Cornu et al. (1996) demonstrated that *B. thuringiensis* subsp. *kurstaki* HD-1 and HD-73 strains when tested against *P. xylostella* showed that the cryIA(a) protein of HD-73 is about three times better in toxicity than cry1A(a) from HD-1. *B. thuringiensis* subsp. *kurstaki* strain as S93 was analyzed by Silva-Werneck et al. (1999) for its cry gene and their efficacy against third instar larvae of *Spodoptera frugiperda*. They noticed 12.3-fold lower LC_{50} for the S93 strain when compared with the standard HD-1 strain. Gujar et al. (2004) studied the

susceptibility of *H. armigera* neonates, collected from different crops and locations in north India, against *B. thuringiensis* subsp. *kurstaki* HD-73. They reported that there was a relative influence of host crops on insect susceptibility. *B. thuringiensis* strains K9903 and K9805, from subsp. *kurstaki* and *aizawai*, respectively, were reported as highly biological active against *P. xylostella* and *S. exigua* (Li et al. 2000). Comparative efficacy for two *B. thuringiensis* strains, Toarrow-Ct and Bacilex, was carried out by Adachi and Grey (1996) against *P. xylostella* and reported that the LC_{50} for Toarrow-Ct was 96.0 ppm, whereas, for Bacilex, it was 78.8 ppm. Kat et al. (2005) isolate a *B. thuringiensis* strain (MnD) from lepidopteran insect *Malacosoma neustria* L. They performed the toxicity tests against seven insect species from Lepidoptera, Coleoptera, and Diptera groups and noticed that crystal–spore suspensions show toxicity only against Lepidoptera species, *M. neustria*, *Lymantria dispar* (L.), and *Hyphantria cunea* (Drury). Hernandez-Martınez et al. (2008) evaluated the susceptibility of *S. exigua* larvae to nine toxins from *B. thuringiensis*. They observed that Cry1Ca, Cry1Da, and Cry1Fa were the most effective toxins with all strains.

B. thuringiensis subsp. *kurstaki*-based commercial formulations, viz., Bioasp, Biobit, Biolep, Dipel WP, and Dipel 8L, were evaluated against *P. xylostella* in field trial (Battu et al. 1997). They reported that 7 days after the first spray, Bioasp at 0.075 kg/ha recorded maximum larval mortality (61.1 %). However, 3 days after the second spray, Dipel 8L and Biobit at the same concentration recorded the highest mortality, 90 % and 89.6 %, respectively. Further they mentioned that in comparison chemical insecticides endosulfan and fenvalerate recorded 27.5 % and 32.7 % larval mortality, respectively, at 833 and 250 ml/ha. In another experiment Arora et al. (2000b) observed that *P. xylostella* was effectively controlled by two applications of Dipel 8L, Biolep, and Biobit at 0.75 kg/ha in cauliflower field in Punjab, India, and 5 days after *B. thuringiensis* treatment, the larval mortality was more than 80 % as compared to 53 % in standard fenvalerate. Considerable mortality in *P. xylostella* larvae was also noticed by Chandle and Mane (1994) using another *B. thuringiensis* subsp. *kurstaki*-based product Agree 50 WP.

Under environmentally controlled conditions, Ajanta et al. (1999) evaluated the effectiveness of commercial formulations from *B. thuringiensis* subsp. *kurstaki*, viz., Biolep, Biobit, and Dipel, against third instar larvae of *H. armigera* and reported LC_{50} 0.114, 0.211, and 0.213 %, respectively, with an exposure period of 48 h. These all concentration of biopesticides had adverse effect on growth and development of *H. armigera*. Biswas et al. (1994) noticed that Dipel is the most toxic (LC_{50} = 0.08) and acted more rapidly followed by Thuricide and Bactospeine, respectively, against third instar larvae of *S. obliqua*. Gujar et al. (2000) also noticed that HD-1 caused highest mortality to 5-day-old larvae of *H. armigera* (LC_{50} 1.71 a.i. ppm), followed by Biobit and Biolep. Dhawan (1999) suggested that the spray of Thuricide/Biotrol effectively controlled *H. armigera* at 1.25 l/ha. Moreover Biswas et al. (1996) mentioned that Dipel was compatible with synthetic chemical insecticides against *S. obliqua* larvae. Other commercial formulations of subsp. *kurstaki*, viz., Delfin, Halt, Dipel DF, and Biobit, were tested by Gopalakrishnan and Gangavisalakshy (2005) for their field efficacy

against *P. demoleus* on citrus. They reported that five applications of bacterial formulations at 1 kg/ha effectively controlled the larval population of *P. demoleus* on citrus compared with the untreated control. An optimal mixture of molasses and henna minimizes the degradation of *B. thuringiensis* formulations caused by sunlight, and the stability of formulations can also be improved by adding a sugar solution to them (Zareie et al. 2003). Results suggest that *B. thuringiensis* is a potential biopesticide for controlling lepidopteran larvae.

4.2 Larval Growth and Development

Toxins from *B. thuringiensis* are known to effect larval growth and development. Several workers have reported various effects of *B. thuringiensis* toxins on larval development. Larvae of *Achaea janata* (Tiwari and Mehrotra 1980), *S. litura* (Sareen et al. 1983), and *H. armigera* (Gujar et al. 2000) when fed on leaves treated with different concentration of *B. thuringiensis* lost their weight along with prolonged larval period. Retardation in larval growth, prolonged pupal period, and low rate of pupation among *H. armigera* were also reported by Wang et al. (1994) when larvae were fed on diet treated with *B. thuringiensis* at LC_{50} (1.2 µg protein crystal/g). Ma et al. (2008) evaluated the mortality and survival of moth *Ostrinia furnacalis* using crystal protein, Cry1Ac, produced by *B. thuringiensis*. They reported that not only were larval growth and development delayed, but pupation, pupal weight, and adult emergency also decreased when larvae were fed on artificial diet containing purified Cry1Ac toxin. Barker (1998) noticed that larval feeding of banded sunflower moth *Cochylis hospes* Walsingham stopped almost at a sudden when *B. thuringiensis* toxin was present in the diet. Total developmental period from hatching of eggs to pupation was 21.4 ± 0.1 and 33.8 ± 1.1 days, respectively, for control and treated larvae. Weight loss in tobacco budworm *H. virescens* larvae has also been reported by Navon et al. (1992), when larvae were fed upon diet treated with lethal dose, 20 µg/g of *B. thuringiensis* subsp. *kurstaki* strain HD-73, for 24 h. However, the larvae feeding on fresh food attained normal weight. Janmaat et al. (2014) reported that susceptible larvae of *Trichoplusia ni* exhibited reductions in growth and frass production at all tested *B. thuringiensis* concentrations.

Commercial formulations of *B. thuringiensis* were also noticed to effect larval growth. Morris (1973) reported that larvae of forest pests *Lymantria dispar* (L.) when fed at Dipel-treated diet gained weight at a considerably slower rate as compared to the untreated ones. Reduction in larval weight intensified as dosages of microbe were increased. Weight loss caused by *B. thuringiensis* commercial formulations, viz., E-16, Bactospeine, and Dipel, in the case of *A. janata* larvae was also reported by Srivastava (1991). Other formulations, Biobit, Biolep, and Dipel, were evaluated by Chandra et al. (1998) against *H. armigera* at 0.114, 0.211, and 0.212 %, respectively. They noticed an increase in larvae mortality, larval period, and growth inhibition along with decrease in total numbers of pupae, pupal weight, and adult emergence as the doses of *B. thuringiensis* were increased.

4.3 Larval Age/Stage

A critical growth phase, responsible for deformities in the larva, was noticed. Dulmage et al. (1978) assigned this stage between hatch and the third instar in *H. virescens*. Once this stage is over, little difference occurs between treated and control larvae. Different larval instars of *P. xylostella* were tested against *B. thuringiensis* subsp. *kurstaki* at 0.01 % concentration by Singh et al. (2002). They observed the mean percent mortality of 16.67, 53.33, 86.67, and 66.67 % for the first, second, third, and fourth instar larvae, respectively, indicating that the third instar larvae were the most susceptible, whereas first instar larvae were the least. They suggested that the difference could be because of the leaf-mining habit of neonates, greater physiological tolerance of neonates to *B. thuringiensis* subsp. *kurstaki*, or both. The third instar larvae followed by fourth instar larvae were most susceptible because of voracious feeding habit and consuming more leaf area treated with the insecticide. Highest larval mortality at third instar stage was also reported by Singh et al. (2003) using commercial formulation of *B. thuringiensis*, Biobit and Biolep, respectively.

Valicente and Fonseca (2004) noticed that larval mortality was inversely proportional to the age of *S. frugiperda* larvae treated with *B. thuringiensis* subsp. *tolworthi*. Li and Bouwer (2012) also reported that second instar larvae of *H. armigera* were consistently less susceptible to the evaluated Cry proteins than neonate larvae. Pramanik and Somchoudhury (2002), however, reported that larval mortality was directly proportional to concentration of microbial insecticide and duration of treatment in *S. obliqua*. These differences among observations of various workers may be because of different species of insects or subsp. of microbe used in studies.

4.4 Exposure Period/Time

Dulmage and Martinez (1973) noticed that the constant exposure of *H. virescens* larvae even to sublethal concentration results in heavy mortality at prepupal and pupal stage. Dulmage et al. (1978) observed that the larvae of *H. virescens* exposed to *B. thuringiensis* for a short period could recover completely, although the capacity for recovery decreased as the exposure time or dosage rate (Abdul Sattar and Watson 1982) increased. Fast and Regniere (1984) also reported that the extension of exposure period from 1 day to continuous 6 days resulted in 500-fold reduction in LC_{50} and equivalent reduction in LT_{50} of spruce budworm larvae. Valicente and Fonseca (2004) evaluated different concentrations of *B. thuringiensis* subsp. *tolworthi* against 2-day-old larvae of *S. frugiperda* for 24, 48, and 72 h of exposure. The highest mortality was observed at 72-h exposure. Gupta et al. (2000) stated that in the case of laboratory-prepared *B. thuringiensis* strains, the toxicity symptoms in the larvae of *H. armigera* were noticed only within 24 h, but complete larval mortality, in infected larvae, was observed 120 h after exposure.

A proportional relationship between larval mortality and concentration of *B. thuringiensis* along with time exposure and anti-proportional relationship to the growth of insect was reported by Pramanik and Somchoudhury (2002) in *S. obliqua*. Salama et al. (1981), however, reported that at higher concentration larval mortality was cent per cent irrespective of the exposure period in *S. littoralis* and *H. armigera*. On the contrary Chatterjee and Choudhury (2003) reported a decline in toxicity of *B. thuringiensis* subsp. *kurstaki* and other biopesticides, *Beauveria bassiana* and avermectin, with increase in time against third instar larvae of European cabbageworm *Pieris brassicae*.

4.5 Compatibility of B. thuringiensis *with Chemical Insecticides*

Chemical pesticides are compatible with the bacterium *B. thuringiensis* having little or no effect on spore germination or cell multiplication (Benz 1971). In combination, some additive effects of chemical pesticides have also been reported. Tan et al. (1999) mentioned that sensitivity to fenvalerate in *H. armigera* larvae increases when the larvae are treated with *B. thuringiensis* for 24 h. It shows that *B. thuringiensis* in combination with chemical insecticides in an integrated pest management system is feasible. Rao and Singh (2003) also mentioned that synthetic insecticides in combination with biopesticides exhibited moderate effects on pest damage as well as on predator's populations, which were at par with alone biopesticidal treatment indicating that synthetic insecticides in combination with biopesticides were economic, effective, and eco-friendly. Combination of insecticides and *B. thuringiensis* to limit the population of lepidopteran pests has also been reported by (Khan et al. 2010).

5 Conclusions and Future Prospects

Annual worldwide production of *B. thuringiensis* represents about 2 % of the total global insecticide market indicating that *B. thuringiensis* is now the most widely used bacterial pest control agents. Earlier in the use of conventional *B. thuringiensis* insecticides, there were some limitations. To some extent these problems are now being tackled using various molecular and genetic engineering approaches. *B. thuringiensis*-based products are being marketed by various agrochemical industries. Thousands of toxicogenic strains of *B. thuringiensis* exist, and each strain produces its own unique insecticidal crystal protein. The insecticidal activity of the toxins from each strain is also known to vary widely. Therefore, isolation and characterization of new *B. thuringiensis* strains are needed to discover strains with novel or high insecticidal activities. Large-scale production of *B. thuringiensis* is expensive because of the high cost of raw materials used in the medium. Locally available

raw materials are required to develope cost-effective medium. To overcome the resistance problem in *B. thuringiensis*-based biopesticides, it is necessary to minimize the uses of similar *B. thuringiensis* strains or even different strains having the same mode of actions against a particular insect pest. As synthetic insecticides in combination with biopesticides were economic, effective, and eco-friendly, various combinations must be standardized.

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Genomics of Plant, Soil, and Microbe Interaction

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Abstract Plants are exposed to a wide variety of microbes in the environment. Owing to the diverse range of microbes, a complex set of molecular mechanisms mediate the plant–microbe interactions. These interactions, consequently, may be presented in the form of beneficial or harmful effects on plants. On receiving a specific stimulation from the host, certain plant-associated microbes improve plant growth and development. They colonize the host plant and either contribute to nitrogen fixation process or suppress the invasion of pathogenic microbes. Moreover, they also enhance the acquisition of nutrients by the plant based on the mycorrhizal association. Plants also release a variety of secondary metabolites that disturb the microbial growth and availability of nutrition to these pathogenic microbes. The interaction between plants and microbes is mediated by very specific signaling molecules that allow only the compatible bacteria to colonize the plant. Albeit the specificity, a number of pathogens have evolved mechanisms to overtake plant defense resulting in plant diseases. The study of genomics of plant–microbe interaction and the signaling pathways involved in the process offer an interesting avenue for the improvement of biological and agricultural outcomes. Research in this field can help in improving the background knowledge on plant tolerance to various biotic stress factors. Moreover, understanding the genomics of plant interaction may benefit crop productivity by exploiting the signaling pathways and designing suitable interventional strategies. The current chapter provides an account of the essential microbial genes and pathways involved in plant–microbe interaction.

Keywords Plant–microbe interaction • Symbiosis • Pathogenesis • Genomics • Signaling pathways • Phytohormones

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1 Introduction

Rhizosphere is a dynamic platform for the interaction of plant roots with microbes, insects, and soil. The plant battles a wide range of pathogens including fungi, bacteria, and insects within the rhizosphere (Hirsch et al. 2003; Hirsch 2004). Here, the plant–microbe signaling occurs via a complex chemical interaction in which both the partners actively participate. The plants release a diverse set of compounds, mainly comprising of root exudates, mucilage, border cells, volatile organic carbon, root cap, and the metabolites of dead tissue, collectively referred to as rhizodeposition (Jones et al. 2009). Plant, soil, and microbe interactions are generally characterized on the basis of location and relationship with fungi and bacteria (Hirsch 2004).

Plant actively produces and releases small-molecular-weight compounds—root exudates—into the rhizosphere. These comprise of carbon-containing primary and secondary metabolites, enzyme, mucilage, and ions (Uren 2000; Bertin et al. 2003). Plant root interaction is quite complex as multiple chemical, physical, and biological entities are involved in this relationship between the plants, microbes, and roots of neighboring plants (Evangelisti et al. 2014; Badri et al. 2009). Consequently, the recipient microbes show a diverse range of responses depending upon the composition of root exudates that may be deleterious for one organism while attracting another organism (Morris et al. 1998). Microbes, generally, are attracted toward plants owing to the exudation of nutrients that increase the activity and population of microorganisms in plant root vicinity (Hiltner 1904). Plant roots provide a carbon-rich environment to these microbes and aid the initiation of molecular cross talk to initiate colonization (Bais et al. 2006). The type of colonizing bacteria, hence, depends upon the composition of rhizodeposits around plant roots. A wide range of prokaryotes and eukaryotes are attracted toward the plants (Turner et al. 2013). In a rhizosphere, plant roots release amino acid and carbohydrates that act as chemoattractant for bacteria, thereby increasing their number in the vicinity (Bacilio-Jimenez et al. 2003). *Pseudomonas aeruginosa* strain PAO1, for instance, showed a differential gene expression contributing to bacterial colonization and rhizosphere competition in response to various root exudates of sugar beet varieties (Mark et al. 2005). Isoflavones are mainly involved in chemical signaling to attract microbes like *Phytophthora sojae* and *Bradyrhizobium japonicum* on soybean roots (Morris et al. 1998).

Microbes interact with plants in harmony as root exudates either accelerate plant resistance against pathogenic infection or initiate production of plant volatiles that attracts predators (Wardle et al. 1998; Inderjit and Weiner 2001). Root exudates also alter biological processes and soil chemistry by regulating the availability of soil nutrients that stimulates competition among plants (Bais et al. 2006). However, certain root exudates inhibit the competition with neighboring plants, insects, and microbes via signaling due to their antibiotic potential, for instance, the generation of “defensive zone” by root tip mucilage to protect the meristematic and root elongating cells from other biological agents (Walker et al. 2003). Therefore, root exudates are perceived as positive or negative signal

depending on the type of organism and the chemical moiety released by the plant. Figure 1 represents the complexity of plant–microbe interactions and the myriad presentations observed as a result of this relationship.

2 Role of Flavonoids in Plant–Soil Interaction

Plants release different types and amounts of metabolites through active root exudation (Cesco et al. 2010). Chief among the ones released as a result of root decomposition and injury are flavonols, isoflavonoids, flavanones, flavones, and chalcones (Shaw et al. 2006). Flavonoids play a vital role in soil interaction. They are polyphenolic compounds that are produced by phenylpropanoid or shikimic acid pathways. The type and amount of flavonoid released depend on plant species, types of stress factors, plant development, and nutrient availability in soil (Cesco et al. 2010). However, the potency of flavonoids in soil is very low in comparison to in planta mainly because of its degradation and rapid absorption by surface reactive soil. Therefore, the interaction mechanism is regulated by a number of factors including the presence of exact amount and time of release of flavonoids, soil

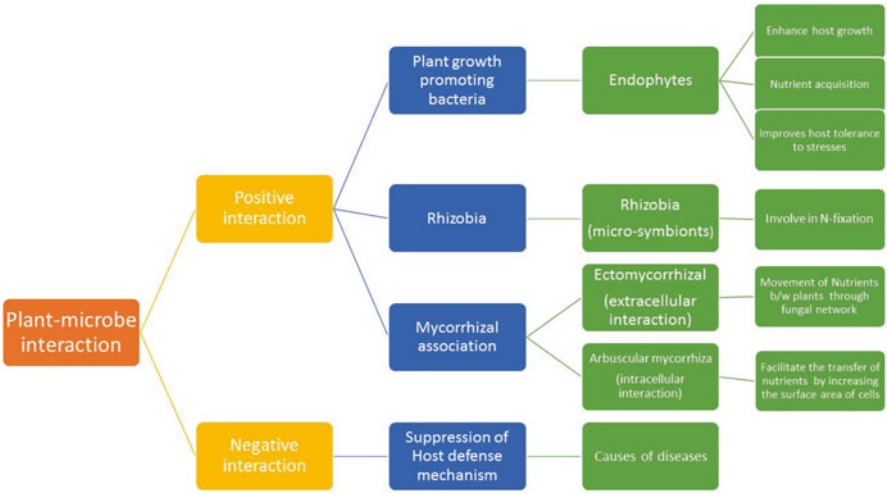

Fig. 1 Plant–microbe interactions: The interactions involve positive or negative interaction between the participants. Here, positive interactions are divided into plant growth-promoting bacteria that either act as free-living bacteria or endophytes, enhance host growth, help in nutrition acquisition, or develop host tolerance to diseases (Han et al. 2011). Rhizobia are involved in nitrogen fixation by forming nodules in host mycorrhizal association that also enhances nutrient uptake in plants depending on the area of colonization (Oldroyd and Downie 2004). However, negative association also exists, where plant responses are suppressed and result in infection and disease (Bonfante and Genre 2010)

heterogeneity (variable pH, surface reactive solid phase), and properties of flavonoids (reactivity of flavonoid molecules and their chemical structures) (Alford et al. 2007; Barto and Cipollini 2009; Chaves et al. 2001; Perry et al. 2007a, b; Inderjit and Dakshini 1992a). A number of studies have reported the multifunctionality of flavonoids. The most significant of these include the plant protection from diseases (Dakora and Phillips 2002; Dakora 2003), nutrition regulation (Rao 1990; Kuiters 1990; Garg and Geetanjali 2007), regulating root development and function (Buer et al. 2010; Rao 1990), allelopathic effect on neighboring plants (Chaves et al. 2001), and development of mechanisms to resist the microbial degradation (Hättenschwiler and Vitousek 2000).

Once flavonoids are released into soil, their concentration is reduced by flavonoid-degrading bacteria that stimulate the biodegradation of xenobiotics (Cooper 2004a; Shaw et al. 2006). Thereafter, there is an increase in the free flavonoid concentration due to the microbial activity leading to the hydrolysis of flavonol glycosides and release of unconjugated aglycones. Further degradation of free flavonoids is carried out due to their high affinity for organo-mineral matrix (Hartwig and Phillips 1991). Phenols in soil undergo biotic and abiotic metabolism as they are used as carbon source by microorganisms (Hättenschwiler and Vitousek 2000), absorbed by clay minerals (Huang et al. 1999) or soil matter (Makino et al. 1996). They are also polymerized to recalcitrant humic substances (Hättenschwiler and Vitousek 2000). They are also transformed chemically into a variety of other compounds depending upon the inherent properties of phenols and the properties of soil (Okumura et al. 1999; Blum et al. 1999). Similarly, aglycone flavonoids from *Cistus ladanifer* become inaccessible to soil microbes due to the entrapped micropores of soil aggregates (Pignatello and Xing 1996; Sosa et al. 2010). Sosa and colleagues (2010) demonstrated that root exudates of *C. ladanifer* L. in soils showed five types of aglycone flavonoids including apigenin, 7(*O*)-methyl apigenin, 4′(*O*)-methyl apigenin, 3(*O*)-methyl kaempferol, and 3,7-di(*O*)-methyl kaempferol. The persistence of these compounds in soil, however, varies according to the biological and pedological properties of the compounds (Cesco et al. 2012). Flavonoids, hence, act as the initiator molecules for many plant–microbe interactions.

3 Positive Plant–Microbe Interactions

The study of plant–microbe interactions aids the characterization of pathways involved in the association of two diverse biological communities. The outcomes of these interactions may be beneficial or harmful to either one or both the members. The symbiotic outcomes of the interactions are observed between mycorrhizae and plants (Smith and Smith 2011) or rhizobia that fix nitrogen in legume root nodules (Oldroyd et al. 2011) and endophytic bacteria and plant growth-promoting bacteria in plant development. The negative effects are generated in case of pathogenic relations with host owing to the suppressed immune

system and disease in plant (Kachroo and Robin 2013; Dodds and Rathjen. 2010; Wirthmueller et al. 2013). The positive and negative aspects of the plant–microbe interactions have been discussed in the following sections.

3.1 Plant Growth-Promoting Bacteria

Plant growth-promoting bacteria or PGPBs are a group of bacteria that live in symbiosis with plants and contribute to its growth. PGPBs exist either as rhizobacteria—residents of soil—or later colonize the plant roots to attain their endophytic status (Compant et al. 2010). They are involved in performing a variety of activities such as nitrogen acquisition, production of phytohormones, protecting plants by controlling pathogens, and enhancing or mobilizing mineral uptake (Compant et al. 2010). In response, these rhizobia thrive on abundant nutrients and attain shelter from plants (Gray and Smith 2005).

3.1.1 Genomics of Bacterial Endophytes

A large and diverse population of soil bacteria not only occupy soil environment of plants but also inhabit the plant parts without causing any disease symptoms. These are referred to as bacterial endophytes. Successful colonization of endophytes requires compatible host and specific plant parts to obtain nutrients and multiply. Endophytes gain access into lateral roots via cracks and reside in specific tissues, intracellular fluids, or cells (Rosenblueth and Martınez-Romero 2006). These plant–microbe interactions mediate a variety of outcomes in plants such as plant growth (Moulin et al. 2001) and tolerance to biotic or abiotic stress factors (Schardl et al. 2004). Majority of endophytes exist in free-living state in soil. However, a few of them become a part of plant microbiome by circumventing the host immunity and defense response. Studies have reported the transition of endophytes from soil to plant body especially for the competent microbes that are well established in rhizosphere and actively colonize the host roots (Compant et al. 2010). After gaining access to the plant body, they may colonize intracellular or extracellular regions within the plant body. *Herbaspirillum seropedicae*, for instance, colonizes intercellular spaces, cortical cells of roots, and xylem vessels of the shoots (James et al. 2002). *Azoarcus* sp. strain BH72—a strict nitrogen-fixing endophyte—encodes nitrogenase genes (*nif*) in rice roots (Miché et al. 2006). On residing inside plant cells, necessary transition occurs in motility, with secretion of pectinase and cellulase to aid its transformation to the endophytic form (Reinhold-Hurek and Hurek 2011). In rice roots, jasmonic acid-mediated defense response is activated following the bacterial colonization. Endophytic bacteria are, generally, harmless and do not cause any cellular damage in plants. To explain the phenomenon, Zinniel et al. (2002) demonstrated that endophytes, in comparison to pathogens, evade plant defenses and exist in low density in plant tissues.

The genomic analysis of *Variovorax paradoxus* S110, a PGPR, revealed 6,754,997 bp genome in two separate chromosomes having 6,279 ORFs. Around 91.4 % of the genome comprises of coding regions with 4557 genes that have known biological role. The rest of the coding region has unknown function, and studies to decipher their roles are still undergoing. The genomic studies have shown that there are genes that are involved in the symbiotic interaction and contribute to the adaptation under diverse environmental conditions of rhizosphere and endosphere (Han et al. 2011). *V. paradoxus* under both conditions—living as free microbe or in plant cells—competes for iron acquisition (Neilands 1995) via active ferric siderophore complex that controls iron uptake by TonB-dependent proteins. In genome of this rhizobacteria, 24 genes and 12 ORFs function in iron transport and for the maintenance of homeostasis. Another 16 genes are involved in the synthesis of siderophore. Nevertheless, plant–microbe association depends on metabolic activity of the microbe. As observed in the case of strain S110, a superfamily of ATP-binding cassette (ABC) transporter of 143 members is involved in the transportation of elemental agents. Strain S110 successfully colonizes and acts as endophyte by using polar flagellum and pili (Dörr et al. 1998). Its genome analysis established the role of 32 genes in the assembly and regulation of IV pilus (tfp). The pilin performs the signal peptidase activity along with the assembly and translocation of pilus. Pilus retraction is performed by NTP-binding protein. The genome also contains five genes that encode the filamentous hemagglutinin involved in the attachment to the plant (Gottig et al. 2009). Rhizobacteria are attracted to the plants by means of a chemosensory protein system that is encoded by 45 genes. Similar to this, *P. aeruginosa* PAO1 also includes cluster of chemotaxis: cheYZABW and cheR (Shitashiro et al. 2005). *V. paradoxus* requires carbon and nitrogen sources to produce ethylene and utilize 1-aminocyclopropane-1-carboxylate (ACC) as instant precursor for synthesis of phytohormone (Belimov et al. 2009). S110 contains an ACC deaminase gene that fragments ACC into ammonia and ketobutyrate for further utilization as carbon and nitrogen source. Similarly, spermidine synthase gene and its transporter gene synthesize spermidine, a polyamine phytohormone, responsible for plant growth during cell division (Han et al. 2011).

Plants protect themselves from pathogen attack by producing reactive oxygen species. Rhizobacteria strain S110 comprises of 21 glutathione S-transferases (GST), five catalase genes, eight hydroperoxide reductase genes, and nine peroxidase genes, all of which are collectively involved in the production of free radicals and, hence, protection of the plants (Han et al. 2011). Although, PGPR lacks hydrolytic enzymes (cellulases and pectinases) and toxins due to absence of toxin genes, yet they possess β-glycosidase genes that allow colonization in *Azoarcus*. The genome also encodes feruloylesterases that hydrolyze feruloyl polysaccharides. These sugars control the cross-linking of plant cell wall, prevent microbial degradation, and help in improving host compatibility (Krause et al. 2006). Genome sequencing of a number of endophytes has been reported including *Serratia proteamaculans* 568, *Methylobacterium populi* BJ001, *Pseudomonas putida* W619, *Stenotrophomonas maltophilia* R551-3, and *Enterobacter* sp.638 (www.jgi.doe.gov). Genome sequence analysis of *Azoarcus* sp. strain BH72, a nitrogen-fixing

endophyte, exhibited that it lacked genes for toxins, cell wall-hydrolyzing enzyme, T3SS, T4SS, *N*-acyl homoserine lactone-based QSS (quorum sensing system), and Nod factors. These have been reported in *Azoarcus* sp. strain EbN1 and other plant-associated strains (Krause et al. 2006; Hurek and Reinhold-Hurek 2003). Most plant-related microbes and pathogens contain these essential genes involved in colonization of plant body (Büttner and Bonas 2006; Preston et al. 2001). Detailed analysis of BH72 genome identified various factors responsible for host interaction including proteins of type I and II secretion systems, for iron–siderophore uptake, type IV pili proteins for chemotaxis and flagella, and surface polysaccharides (Krause et al. 2006) (Fig. 2).

Endophytic bacteria prevent pathogenic infections by triggering induced systemic resistance (ISR) in plants (Kloepper and Ryu 2006). The essential components of microbes identified by plant system are called microbe-associated molecular patterns (MAMPs) that trigger plant immunity called MAMP-triggered

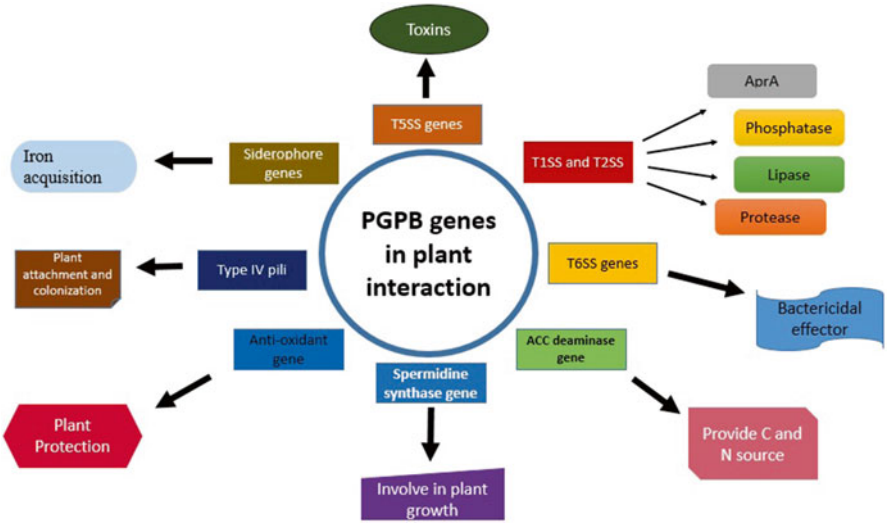

Fig. 2 Plant growth-promoting bacterial genes in plant interaction: Genomic analysis of PGPB reveals unique properties of genes involved in symbiotic interaction in environmental conditions of rhizosphere and as endophyte. In genome of WCS strains, type II, III, V, and VI secretion systems are already determined, whereas T1SS and T2SS are involved in secretion of AprA, phosphatase, lipase, and extracellular protease to facilitate nutrient acquisition. T5SS-related gene encodes for toxins in contact-dependent growth inhibition of surrounding bacteria (Ruhe et al. 2013), whereas T6SS competes in a cell contact-dependent manner with microbes by delivering bactericidal effector. ACC deaminase gene fragments help in the conversion to ammonia and ketobutyrate to be utilized as carbon and nitrogen as energy source, while spermidine synthase gene is in charge of plant growth during cell division. PGPB also comprises of antioxidant genes that protect plants by producing antioxidant enzymes, whereas type IV pili allow colonization and attachment in plants (Han et al. 2011)

immunity (MTI). As a result, nitrogen species and reactive oxygen species (ROS) are produced that are involved in exhibiting antimicrobial effects. In terms of pathogenicity, MAMPs are highly conserved in all types of bacteria including beneficial bacteria, pathogens, and endophytes (Newman et al. 2013). In plants, ISR triggered by endophytes is independent of pathogen-related protein induction and salicylic acid accumulation. This factor helps to mediate the response of systemic acquired resistance (SAR) triggered under pathogenic attack (van Loon et al. 1998; Pieterse et al. 1998). Conn and colleagues (2008) confirmed the protective role of two strains of *Streptomyces* species (endophyte) when pre-inoculated in *Arabidopsis* seedlings and later with pathogenic *Erwinia carotovora* increased duration of resistance. Although both the strains were similar, yet they evoked a completely different response in *Arabidopsis*. One of the strains elicited ISR induction while other showed SAR induction. Plant responses are fine-tuned for every bacterial signal as bacterial exudates confirmed induction of ISR on complex medium. On the other hand, SAR induction was confirmed by some strains that exudate on minimal medium (Conn et al. 2008).

3.1.2 PGPB Interacts via Phytohormones and Biofilm

Some PGPB strains are phyto-stimulators, i.e., they increase the plant growth; for example, *Azospirillum* spp. secrete phytohormones like cytokinins, auxins, and gibberellins for that purpose (Steenhoudt and Vanderleyden 2000). Phytohormones produced by microbes are essential to initiate biosynthesis cross talk and communication of indoleacetic acid (IAA) and ethylene with plants (Yuan et al. 2008; Spaepen et al. 2007; Tsavkelova et al. 2006; Bottini et al. 2004). Rothballer and coworkers (2008) demonstrated that *Herbaspirillum frisingense* GSF30T produces IAA in culture. Similarly, wheat seedlings inoculated with *B. subtilis* increased seedling growth by producing auxins (Egorshina et al. 2011). *Azospirillum* spp. are also known for fixing atmospheric nitrogen and stimulating plant growth by synthesizing auxins and other phytohormones (Steenhoudt and Vanderleyden 2000). Some bacterial species protect plants by acting as biocontrol agents against potential pathogens by either forming a biofilm or producing antibiotic agents (Bais et al. 2004a, b). However, recent studies have shown that a few toxic or allelopathic compounds are degraded by the pathogenic microbes (Gray and Smith 2005). Molecular characterization has shown that the members of the genus *Pseudomonas* contribute most actively to the biocontrol mechanisms. They produce antifungal metabolites like pyoluteorin, phenazines, and pyrrolnitrin 2,4-diacetylphloroglucinol (DAPG) (Ryu et al. 2004). These compounds create suppressive soil that prevents fungal and bacterial attacks on plants. Biocontrol activity of microbes is controlled by niche exclusion, antifungal metabolite production, nutrient competition, and siderophore secretion. Siderophores are small iron-chelating compounds that enhance ferric iron uptake from surrounding environment under iron-stress conditions (Lemanceau et al. 2009). Biocontrol activity of certain *Pseudomonas* WCS strains is linked with iron competition via siderophore (Bakker et al. 1986; Duijff et al. 1999). These

microbes recognize and uptake cognate iron–siderophore complexes via specific siderophore receptors (Cornelis 2013; Loper and Henkels 1999). A number of rhizobacteria possess siderophore receptors to compete for iron uptake produced by soil microbes (Hartney et al. 2013; Bakker et al. 1990). Plants under pathogenic attack signal to recruit surrounding soil microbe; for example, when *P. syringae* attacks *Arabidopsis*, it induces malic acid (MA) transporters. The increased MA in rhizosphere helps in the recruitment of *Bacillus subtilis*, beneficial rhizobacteria that stimulate ISR in plants to restrict pathogenic infection from spreading to the plant body (Lakshmanan et al. 2012).

Ryu and coworkers (2004) discovered a reduction in the symptoms of *Cucumber mosaic virus (CMV)* infection in wild-type *Arabidopsis thaliana* when pre-inoculated with strains of PGPBs: *Bacillus pumilus* strain SE34 and *Serratia marcescens* strain 90–166 (Ryu et al. 2004). Interestingly, *B. pumilus* strain 90–166 induces acquired resistance in *Arabidopsis* that helped in its protection from *CMV* (Ryu et al. 2004). On the other hand, gram-positive *B. subtilis* 6051 strain protected the plant from gram-negative pathogen invasion. Certain *Pseudomonas syringae* pv. DC3000 have genes that code for protective biofilm layer formation on the roots of *A. thaliana* (Bais et al. 2004a). This helped in the prevention of colonization of pathogenic bacteria on the plants (Raaijmakers et al. 2010). Though the *Pseudomonas* spp. are plant pathogens, they are found to increase the plant immunity and serve as PGPR model organisms to study their role in plant protection (Lugtenberg and Kamilova 2011; Pieterse et al. 2014).

3.1.3 Genomics of PGPB Interaction with Plants

In rhizosphere, population and density of WCS strains depend on effective competition for space and nutrients with other microbes. Moreover, the secretions from plant roots help in the provision of nutrients and shelter to the microbial community around the roots (Berendsen et al. 2012). As a result, rhizobacteria return the favor by modulating immune responses and regulating the composition of root excretion (Zamioudis et al. 2014; Millet et al. 2010). Three strains of *Pseudomonas* WCS have been shown to be capable of eliciting the ISR on the basis of host specificity (Berendsen et al. 2015). The strain WCS417 failed to elicit response in radish. However, WCS374 and WCS358 displayed a potent ISR in radish (Leeman et al. 1996). WCS417 strain demonstrated ISR mediated by specific transcription factor, MYB72, present in the roots of the plant (Zamioudis et al. 2014; Van der Ent et al. 2008). The study showed that in these plants, ISR coordinates well with phytohormones like ethylene (ET) and jasmonic acid (JA) yet works without salicylic acid (SA) (Pieterse et al. 1998; Pieterse et al. 1996). As a result, WCS417-ISR elicits a different resistance pattern in comparison to the pathogen-induced systemic acquired resistance (SAR) (Fu and Dong 2013). The ISR redundancy has been investigated in different plant species, and it has been found that tomato WCS358 strain works effectively to elicit ISR against *Botrytis cinerea* (Meziane et al. 2005). Several bacterial determinates such as LPS, flagella, and siderophores induce

systemic resistance (Weller et al. 2012; Iavicoli et al. 2003; Tran et al. 2007). Additionally, pyoverdine production plays a key role in biocontrol activity of WCS strains (Berendsen et al. 2015). WCS strains have been demonstrated to induce resistance in a variety of crops including bean, carnation, eucalypt, flax, radish potato, and tomato (Bakker et al. 1986; Duijff et al. 1999; Ran et al. 2005). The protective role of these strains depends on siderophore-mediated iron competition as pyoverdines initiate onset of ISR; e.g., PVD358 is known to cause ISR in *Arabidopsis*, eucalypt bean, and tomato (Ran et al. 2005; Meziane et al. 2005). It is, hence, established that siderophores are involved in host specificity to elicit ISR in rhizobacteria. Genomic analysis of PVD374 and PVD417 showed that isomeric configuration of single amino acid can cause high specificity to differentiate the pyoverdines (Berendsen et al. 2015). *Pseudomonas* WCS374 strain, for instance, produces siderophore pseudomonine and pyoverdine (Berendsen et al. 2015). SA moiety is present in the compound pseudomonine generated by isochorismate-pyruvate lyase gene pmsB and isochorismate synthase gene pmsC. These genes are a part of pseudomonine biosynthesis gene cluster pmsCEAB (Mercado-Blanco et al. 2001). Previous studies showed that SA is a potent inducer of systemic acquired resistance (SAR) as exogenous application of SA induces systemic resistance in *Arabidopsis* (Van Wees et al. 1997). Meanwhile, as strain WC374 colonizes plant roots, SA is incorporated in siderophore pseudomonine preventing the induction of SA-induced plant immunity (Djavaheri et al. 2012). Contrarily, a related strain of WCS374, *Pseudomonas* strain SS101, induces systematic resistance in *Arabidopsis* via SA-dependent pathway (Van de Mortel et al. 2012). The SA-dependent nature of strain SS101 is due to elevated SA in rhizosphere and is exhibited due to its lack of effective incorporation in pseudomonine. Strain SS101 also acts as cyclic lipopeptide massetolide A elicitor of ISR in tomato.

In general, PGPRs reach a high population in rhizosphere and protect the plants by outnumbering the pathogens. Protein secretion system, encoded by the genome of *Pseudomonas* strain WCS, is involved in rhizosphere competence. There are six protein secretion systems in bacteria that are involved in microbe–microbe and host–microbe interactions. In genome of WCS strains, type II, III, V, and VI secretion systems are responsible for the interaction. However, T1SS and T2SS are involved in secretion of AprA, phosphatase, lipase, and extracellular protease to facilitate nutrient acquisition (Putker et al. 2013; Bull et al. 1991). They are also involved in the secretion of bacteriocins (Parret and DeMot 2002) and cyclic lipopeptides (Raaijmakers et al. 2010). Strains WCS374 and WCS417 possess several genes and gene clusters to secrete effector proteins and encode T3SS in *P. fluorescens* strain (Zamioudis and Pieterse 2012; Rezzonico et al. 2005; Preston et al. 2001). WCS strains compete in rhizosphere by the T5SS-related genes that secrete toxins that cause a contact-dependent growth inhibition of surrounding bacteria (Ruhe et al. 2013). Similarly, T6SS competes in a specific cell contact-dependent manner with microbes exhibiting their bactericidal effect (Kapitein and Mogk 2013; Russell et al. 2013; Hood et al. 2010). Mutualistic microbes establish a strong relationship by evading the immune response that can, otherwise, be triggered in plant roots after the antigens are recognized (Zamioudis and Pieterse

2012). Millet and coworkers (2010) demonstrated that rhizobacterium WCS417 colonizes *Arabidopsis* roots and suppresses its immune response. This is mediated by the production of alkaline protease AprA. Interestingly, *P. syringae* and *P. aeruginosa* prevent flg22-triggered immunity by degrading flagellin monomers in *Arabidopsis* leaves (Pel et al. 2014; Bardoel et al. 2011). *Pseudomonas* strains suppress host immune responses via T3SS-mediated injection of effector proteins in a manner quite similar to that adopted by pathogenic microbes. However, non-pathogenic *Pseudomonas* have active T3SS gene clusters or T3SSs as they are associated with roots and do not cause any disease (Loper et al. 2012; Rezzonico et al. 2005; Preston et al. 2001).

3.2 Nodulation of Legumes by Rhizobia

Legumes establish a symbiotic relation with nitrogen-fixing bacteria called rhizobia. Rhizobia are involved in the formation of root nodules where these microsymbionts convert free, inert nitrogen into ammonia for plant consumption (Peters et al. 1986). This bioprocess is essential in sustainable agriculture as it provides protein-rich food and reduces requirement of exogenous nitrogen fertilizer (Wang et al. 2012). Rhizobium inoculates in soybean seed, enhances the fixation of atmospheric nitrogen, and helps in improving crop yield (Furseth et al. 2012). Although indigenous soil bacteria can form nodules in most legumes, the efficiency of nitrogen fixation varies among plant species (Schumpp and Deakin 2010). Knowledge of molecular machinery involved in symbiosis has helped to predict and control genetic factors responsible for symbiotic interactions, thereby improving the agronomic practices and crop yield.

Plants attain extra nitrogen to balance the loss of photosynthate during mutualistic association of rhizobia. The energy for nitrogen fixation comes from sucrose with expense of the photosynthate (Young and Johnston 1989). Consequently, the plants are benefited even in nitrogen-poor soils, whereas bacteria get the shelter and finally escape back to the soil during nodule senescence (Young and Johnston 1989). This process of nodulation is a coordinated effort of legumes and resident *Rhizobium* bacteria.

3.2.1 Legume Signals to Attract Bacteria

At the start of the signaling process, plants secrete flavonoids as signaling molecules that diffuse across the membrane of soil bacteria such as *M. truncatula* (Peters et al. 1986). On being detected by the bacterial receptor NodD, flavonoids generate chemotaxis that activates nodulation genes called *nod* genes. They encode for enzymes required in synthesis of Nod factors, the lipochitooligosaccharides essential in legume–rhizobia association (Oldroyd et al. 2011). This is followed by the transcription and translation process resulting in the expression

of NodD proteins that belong to LysR family of transcription regulators (Long 1996). This host–bacteria interaction is highly specific and regulated by very specific molecules as well. For instance, soybean produces daidzein isoflavonoids and genistein, both of which induce nod genes in *Bradyrhizobium japonica*. However, they inhibit the nod expression of *S. meliloti* (Peters et al. 1986). This mechanism enables the rhizobia to differentiate their specific host from other legumes. In promoter region of *nod* genes, nod boxes are present that comprise of conserved DNA motifs to which the NodD is generally bound (Perret et al. 2000). Flavonoid secretion also enhances the DNA binding of NodD1 with nod gene promoter in *Sinorhizobium meliloti* and induces *nod* gene to interact with the product of nod—a LysR-type regulator (Peck et al. 2006). Conformational changes activate bacterial NodD protein that acts as a sensor to recognize chemicals excreted by plant roots and induce gene expression thereafter (Russelle 2008). Expression of *nod* genes initiates synthesis of Nod factors called lipochitooligosaccharides that comprise four or five β-1,4 *N*-acetylglucosamines and terminal nonreducing sugar *N*-acylated 16–18-carbon fatty acid. Nod factors undergo chemical modification involving acetate, carbonyl, or sulfate functional groups or by sugars like fructose and arabinose (Perret et al. 2000).

Bender and coworkers (1988) demonstrated that *Rhizobium* sp. strain NGR234 recognizes a wide range of flavonoids from the host plant and transfers the nodD1 to *S. meliloti*. The bacterium has a narrow host range, and hence the protein mediates the formation of nodules in *Parasponia* nonlegume host (Bender et al. 1988). This transfer established the role of NodD in the regulation of legume and rhizobia recognition (Peck et al. 2006). In roots, a high concentration of flavonoids helps in promoting the colonization of rhizobia in vicinity of root hair (Russelle 2008). *Azorhizobium caulinodans*, *Methylobacterium nodulans*, and *Bradyrhizobium* are well-known nodule-forming symbionts (Jourand et al. 2005; Giraud and Fleischman 2004; Sy et al. 2001). On the basis of the genome, rhizobia are classified into six different genera. Their genome varies between 5.4 and 9.2 Mb (MacLean et al. 2007). The genomes contain specialized coding regions for regulatory and transportation genes that ensure their survival in soil. The genes, hence, adapt a number of strategies to develop symbiotic relations with legumes. For instance, they are involved in stress resistance, metabolic activities, transportation, and housekeeping. A few genes ensure the specificity of host selection like genes encoding for exopolysaccharides in *Sinorhizobium meliloti* and *celC2* genes for cellulose to allow bacterial entrance in *Rhizobium leguminosarum* (Robledo et al. 2008).

The Nod factors are detected and recognized by transmembrane surface receptors resulting in prompt response and abrupt disruption of cell wall which ensures the entrance of rhizobia in the plant cells. The Nod factors, hence, play an essential role in formation of nodules in legumes. The Nod factors in different rhizobia share a similar oligosaccharide backbone of chitin-like *N*-acetyl glucosamine with a fatty acyl chain at the nonreducing end. However, it varies in size, length, and chemical modification (sulfation, glycosylation, or saturation) of the backbone (Long 1996). Nod factors comprise of a core structure, synthesized by nodABC gene products. Any mutation in these genes, generally, results in the termination of symbiosis.

Some additional genes in Nod factor core are responsible for ensuring host specificity. For instance, in *R. leguminosarum*, host specificity is abolished by *Trifolium* species owing to the alteration in fatty acyl chain attached to Nod factor (Spaink et al. 1991). Contrarily, there is a boost in symbiosis specificity for *Vicia sativa* and *Pisum sativum* after the same exposure (Spaink et al. 1989; Djordjevic et al. 1985).

3.2.2 Nodule Formation

Nod factors are detected by a family of LysM family and serine or threonine receptor-like kinase known as Nod factor receptors (NFRs). In legumes, they are localized in the plasma membrane and contain LysM motifs in extracellular domain of epidermal cells in root hair. Examples of these are the NFP and LYK3 in *Medicago truncatula* and NFR1 and NFR5 receptors in *Lotus japonicus* (Arrighi et al. 2006; Madsen et al. 2010; Limpens et al. 2003; Radutoiu et al. 2003). LysM domains are directly bound to Nod factors owing to their abundance in chitin-binding proteins and peptidoglycan. The interaction further activates calcium-dependent signal transduction. This is followed by a fluctuation in the calcium levels within the nuclear-associated cytoplasm and nucleoplasm known as calcium spiking that results in expression of early nodulins (ENODs) (Oldroyd and Downie 2004).

Initial contact of rhizobia and host takes place at growing tip of root hair. Successful contact curls root hair to trap bacteria. Root hair undergoes growth changes as bacterial count increases, and there is an increased colonization of the nodule. Within the cytosol of root hair tip, Ca^{2+} level increases due to influx by Nod factors resulting in developmental changes. This causes a reorganization of microtubule and actin filaments in root hair (Felle et al. 1999). Rhizobia digest cell wall of root hair to form an infection thread. Stimulated root hair curls in response to attached bacteria and grows to form a narrow and long passage—the shepherd's crook. *nodL* and *nodF* genes are essential for biosynthesis of Nod factors. Any mutations in these genes result in abnormal infection threads (Bisseling et al. 2003). Similarly, in *M. truncatula*, LysM Nod factor receptors are involved in the formation of infection thread (Arrighi et al. 2006). Rhizobia in infection threads travel, divide, and spread through the cell into basal membrane ultimately releasing the bacteria into extracellular space and initiating the cell division in root cortex (Oldroyd et al. 2011). This spread of infection occurs after rhizobia breach the host cell wall by producing cellulose- and pectin-degrading enzymes to ensure its entry into the host cell (Brewin 2004; Robledo et al. 2011). Inside the nodule, rhizobia are released from infection thread in the droplets of polysaccharide. Although plant receptors are not completely defined and known, yet many resemble animal receptors to detect surface polysaccharides of bacteria.

Rhizobia are converted to bacteroides as infection travels from the tip of epidermal cells down to the root hair. This process is repeated until the bacteria reach its final destination that is the root cortex by forming additional branches of infection thread. On reaching the cortex, host cells start behaving as stem cells and give rise to new cells to form lateral organ nodule. Nodules can host single or multiple

strains of rhizobia. They can exist in determinate (spherical in nature and lack persistent meristem) or indeterminate (situated in the distal end of cylindrically shaped lobes) forms (Russelle 2008). On approaching nodule cells, bacteria are internalized via endocytosis. Plant develops peribacteroid membrane responsible for exchange of compounds with plants and bacteria; bacteroides immediately grow and develop around the droplet region by endocytosis. However, individual bacteria on ingestion differentiate into nitrogen-fixing organelles called symbiosome. The host plants restrict these microbes outside or in exocytoplasmic regions (Russelle 2008). The host barters fixed carbon for fixed nitrogen from bacteria through the symbiosome membrane. In rhizobial legume symbiosis, high specificity is a striking feature occurring at the initial stage of the interaction. It is associated to nodule development at early stage and nitrogen fixation later (Oldroyd et al. 2011). Bacteroides maintain a symbiotic relation with legumes by losing ammonium assimilatory capacity. In certain strains of rhizobia, NodS induces TtsI expression that encodes transcriptional regulator involved in binding tts boxes, upstream of operons. They code for T3S machinery and its effectors (Wassem et al. 2008). R genes in few ecotypes recognize the effector proteins and limit host range for bacteria (Fig. 3).

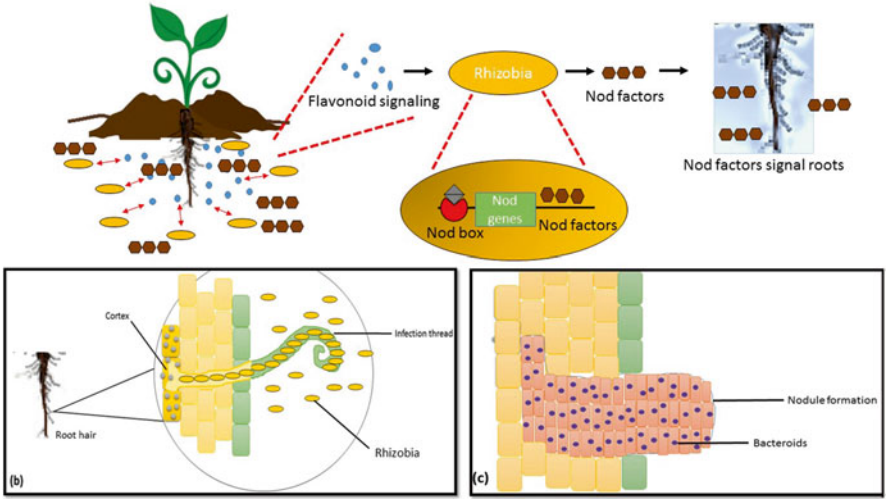

Fig. 3 Rhizobial nodule formation in legumes. (**a**) Plant releases flavonoids that are detected by receptors of rhizobial cell in soil environment and induce *nod* genes to synthesize Nod factors. These Nod factors are detected by NFRs in plants and entrap bacteria to colonize root hair. (**b**) Rhizobia digest cell wall of root hair to form infection thread that leads rhizobia to cortex of plant roots. (**c**) Rhizobia are converted to *Bacteroides* as infection travels. When bacteria reach the root cortex, host cells start behaving as stem cells and give rise to newly generated cells to form lateral organ nodule. In these nodules, rhizobia fix atmospheric N into usable form like ammonia and nitrates (Oldroyd et al. 2011)

3.3 Use of Bacterial Determinants in Plant Interaction

Plant immune system is highly sensitive and comprises a number of inducible barriers to prevent microbial attack (Jones and Dangl 2006). The polysaccharides present in the surface of bacteria that directly interact with the host cells include capsular polysaccharides (KPS), cyclic β-glucans, exopolysaccharides (EPS), and lipopolysaccharides (LPS). These microbial determinants of interaction are of prime importance in the induction of an effective symbiosis. Each determinant contributes in symbiosis, while any defect in these factors may lead to the abortion of symbiosis in early or later stages due to the alteration in conformation of EPS (Finan et al. 1985; Cheng and Walker 1998). Similarly during symbiosis, failure in production and transport of cyclic β-glucan results in inhibition of infection thread (Dylan et al. 1986). In the beginning, these microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) are perceived by host pattern recognition receptors (PRRs). This leads to the development of immunity called PAMP-triggered immunity (PTI). However, gram-negative microbes use T3SS and deliver effectors into plants' cell that dampens the PTI and allows the microbe to access the host. The secondary defense of plant is activated as resistant gene (R) product detects the presence of effector proteins leading to the activation of effector-triggered immunity or ETI. The rhizobial T3SS releases effector molecules that trigger ETI as perceived by host R genes and regulates specific nodulation (Deakin and Broughton 2009; Soto et al. 2009).

These defense-related responses occur during legume–rhizobia interaction. However, they are less prominent once symbiotic relationship has been established (Lohar et al. 2006). Rhizobia MAMPs, including surface polysaccharides and Nod factors, suppress the defense responses in specific compatible host (Shaw and Long 2003; Jones et al. 2008). Rhizobial MAMPs can also activate immunity in nonleguminous plants resulting in the induction of a strong response (Staehelin et al. 1994). This property establishes the host-specific role of rhizobial MAMPs to recognize compatible legume during symbiotic relation and evading the host defense responses by overcoming them.

Symbiotic rhizobia are similar to pathogenic bacteria as they use a similar approach to invade host immune surveillance (Dai et al. 2008; Kambara et al. 2009). They use T3SS to transfer effectors called nodulation outer proteins (Nops) into host cells (Deakin and Broughton 2009). Both Nod factors and effectors of rhizobial T3SS are regulated by bacterial transcription activator NodD and flavonoids. Bacterial pathogens utilize T3SS for eliciting defense response and pathogenicity (Buttner and He 2009). On the other hand, T3SS of rhizobia is responsible for nodulation and rhizobial infection. Rhizobial effectors are known to promote bacterial infection in case they are left undetected by host R genes.

3.4 *Mycorrhizal Associations*

Mycorrhizal fungi either exist as free-living organisms or get associated with roots of around 90 % of the plant species including many crops, wild grasses, and forest trees. In this symbiotic relationship, both partners exchange benefits with each other. Mycorrhizal fungi provide nutrients and minerals to plants and enhance the water absorption and disease resistance. In return, they are provided shelter for fungal growth and reproduction (Bonfante and Genre 2010; Smith and Read 2008). Mycorrhizal fungi spread its hyphal network in soil, which is often shared by the adjacent plants, allowing horizontal and efficient transfer of nutrients (Helgason et al. 1998). The areas where mycorrhiza develops and interacts with host plants are called symbiotic interfaces (Bonfante 2001; Harrison 2005; Parniske 2008). Mycorrhiza is classified broadly into two categories on the basis of anatomical aspects defining whether fungi colonize intercellular spaces of roots or develop inside root cells. They are, hence, named ectomycorrhiza and endomycorrhiza, respectively. The endomycorrhizas are further classified into arbuscular, ericoid, and orchid.

3.4.1 Arbuscular Mycorrhizal Fungi (AMF)

Arbuscular mycorrhizal fungi (AMF) are found in association with the roots of around 80 % of the terrestrial plants (Bago et al. 2003; Newman and Reddell 1987). In this type of association, root tip is affected. Germinating spores form hyphae and produce hyphopodium in root epidermis. This intraradical colonization continues intercellularly and intracellularly. As it reaches the inner circle of cortical cells, it networks into fungal tree branches called arbuscules (Bonfante and Genre 2010).

MF colonization is initiated by perception of particular root exudates from compatible hosts (Nagahashi and Douds 2003; Tamasloukht et al. 2003). Kosuta and his coworkers (2003) reported that the signaling molecules from fungi activate plant genes when plant and fungal cells were placed in close proximity yet separated physically by impenetrable membrane. In *Medicago* system, Early Nodulation11 (ENOD11) gets activated by Nod factors of rhizobia (Journet et al. 2002), while AMF activates the pathway even at some distance from hyphae (Kosuta et al. 2003). The AMF interaction with host plant is mediated by signaling pathway similar to rhizobium–legume symbiosis (Oldroyd and Downie 2004; Parniske 2008). In this type of symbiosis, seven types of genes (SYM genes) are required and considered necessary. Some of these genes are specific to nodulation (LjNFR1/LjNFR5). As Myc factor is perceived by host plant through its specific receptors called Myc factor receptors (MFR), they elevate the cytosolic calcium levels (Oldroyd and Downie 2004). In *Lotus japonicus*, certain receptor-like kinases are responsible for perception of direct or indirect signals from AMF and rhizobia. These receptors are referred to as LjSYMRK secondary membrane proteins, for example, the MtDMI2 in *Medicago truncatula* (Markmann et al. 2008).

The MtDMI2 is responsible for signal transduction mediated by a kinase domain that phosphorylates the substrate. The nuclear localization of SYM pathway elements showed a rapid transduction of signal. The appearance of a nuclear pore complex suggests the involvement of nucleoporins in this pathway including LjNUP85 and LjNUP133. Another protein, LjPOLLUX or LjCASTOR, located in the nuclear envelope, is responsible for cation channel opening and calcium spiking. Similarly, MtDMI1 and MtDMI2 proteins in *M. truncatula* are responsible for controlling the regulation of $Ca2^{+}$ concentration in perinuclear cytoplasm (Oldroyd and Downie 2004; Kosuta et al. 2008).

LjCCaMK is a calcium- and calmodulin-dependent protein kinase that forms a complex with product of SYM gene by phosphorylating it. This protein is located in the nucleoplasm where it performs the decoding of calcium oscillation. In AM-mutated fungi, LjCCaMK is known to restore root colonization for SYM genes located upstream of Ca^{2+} spiking (Hayashi et al. 2010; Madsen et al. 2010). Another nuclear protein, LjCYCLOPS, leads to gene regulation during colonization (Yano et al. 2008). There are certain alternate signaling pathways responsible for early response of AM fungi. In case of rice-98 and *M. truncatula*, SYM-independent regulation is responsible for AM-induced gene expression (Kuhn et al. 2010). The AM fungi, thereafter, generate biologically active molecules that further mediate various signaling pathways. In the life cycle of mycorrhizal fungi, hyphal branching is a critical step as branch-inducing factor triggers morphogenesis of hyphae to ensure host root contact in order to establish symbiosis (De Carvalho-Niebel et al. 2002). In *Lotus japonicus*, root exudates are involved in symbiotic cross talk. The sesquiterpenes in dormant mycorrhizal fungi trigger hyphal branching (Akiyama et al. 2005). Flavonoid biosynthetic pathway in *M. truncatula* encodes for enzymes including chalcone synthase (CHS) and phenylalanine ammonia lyase (PAL) in arbuscules containing cells. Otherwise, the same pathways are involved in regulating the isoflavone reductase (IFR)—the defense-related enzymes. Induction of these enzymes triggers high production of flavonoids that enhance mycorrhizal growth rather than antimicrobial phytoalexin production that inhibits fungal growth (Harrison 2005; 1999). Mycorrhizal fungi on perceiving chemical response from roots get stimulated and spread out to invade root tissues. However, this branching is limited to the cortex of root tissues which shows control of host plants in fungal proliferation (Garcia-Garrido and Ocampo 2002).

Resting spore germinates into short explorative mycelium that increases the direct contact with host. Similarly, plant roots also receive fungal exudates that activate SYM pathway to trigger calcium spiking. This signal transduction activates cellular and transcriptional responses. Plant–fungi interaction takes place by attachment of hyphopodium on epidermal and cortical cells of roots that trigger assembly of prepenetration apparatus (PPA). PPA is an aggregate of cytoplasm in these cells that helps in the development of penetration assembly. Once fungi colonize intracellularly in epidermis, it follows PPA's route to the inner cortex and allows the development along the root axis. This PPA mechanism is repeated on reaching the internal portion of cortical cells and allows branching on small scale. Ultimately, arbuscule forms an extensive network by branching

and occupying a huge volume of cell. This strategy allows an efficient nutrient exchange (Bonfante and Genre 2010). Under this scenario, mycorrhiza-associated roots modulate plant defense responses that were activated on microbial invasion (Garcia-Garrido and Ocampo 2002). Moreover, other defense responses such as antioxidants, phenylpropanoid biosynthesis, and PR genes are also activated. These responses are weak, short termed, and strictly localized yet differ for each pathogen (Gianinazzi-Pearson 1996).

3.4.2 Ectomycorrhizal Fungi

Ectomycorrhizal fungi surround the outer region of plants; for example, in root tip, they form a thick mantle of hyphae around the epidermal cells (Bonfante and Genre 2010). Ectomycorrhizal fungi also respond to flavonoids excreted via root exudates such as rutin that promotes hyphal growth in *Suillus bovinus*. Similarly, rutin in *Eucalyptus globulus* spp. *bicostata* stimulates growth of *Pisolithus* species. Other trees, however, failed to respond to *Pisolithus* species infection (Lagrange et al. 2001). Kikuchi and coworkers (2007) demonstrated that picomolar concentration of rutin is sufficient to trigger symbiosis and stimulate the growth of putative symbionts. The fungi are highly specific to the stimulation for particular flavonoids. Luteolin, biochanin A, and quercetin, for instance, had no effect on germination of *S. bovinus*. In contrast, flavonoids, rutin, hesperidin, naringenin, quercetin, and genistein, while luteolin, biochanin A, and quercetin enhance their germination (Kikuchi et al. 2007).

Ectomycorrhizal fungi comprise of cell wall enzymes such as pectin lyases and pectinases that target plant cell wall. As fungi develop symbiosis with the plant tissue, certain genes of cell wall-degrading enzymes are upregulated. The nature of these enzymes shows saprotrophic role of EM fungi. *L. bicolor* retains substrate-degrading enzymes including chitinases, glucanases, glycosyl hydrolases, and proteases in order to degrade organic compounds and polysaccharides of microfauna (Martin et al. 2010). Around 15 genes of *L. bicolor* encode for hexose transporters. However, the genes for enzyme invertase, which converts sucrose to glucose and fructose, are absent. This suggests that the host plant depends on fungi for its glucose requirement. *T. melanosporum* possesses one invertase gene that allows its access to sucrose pool. Other fungi like *Hebeloma cylindrosporum* and *Amanita muscaria* metabolize fructose. Similarly, in maize, a biotrophic pathogen, *Ustilago maydis*, causes smut disease. The fungal plasma membrane comprises of sucrose transporter to acquire plant sucrose without secreting invertases (Wahl et al. 2010). These mutualistic interactions help both partners to fulfill nutrition requirement by exchanging nutrients. Research studies have shown that around 491 genes encode for the transporter proteins in *L. bicolor*. In comparison, saprotrophic and pathogenic fungi have 381 less abundant genes in *T. melanosporum*. Upregulation of these genes during symbiosis exhibits an intense traffic of amino acids, polyamines, and oligopeptides via symbiotic interface (Martin and Nehls 2009).

The genome study of EM revealed a high capacity of mycelium to import inorganic and organic nitrogen sources from soil such as peptides, ammonium, and nitrate via urea permease or ammonium transporter. These functions are common to various EM fungi including *Paxillus involutus* (PiDur3) and *A. muscaria* (AmAMT2) (Martin and Nehls 2009). Mycelium transfers nitrogenous compounds to the mantle and the Hartig net for further exportation to the plant. In this case, ammonium and glutamine are ideal candidates to cross symbiotic interface (Martin and Nehls 2009). Ammonium ions are directly released from Hartig net hyphae which upregulate ammonium importer in fungal roots or integrate ammonium ions into amino acids for translocation into the plant cells (Couturier et al. 2007). However, in *L. bicolor*, the discovery of upregulated 300 small cysteine-rich secreted proteins during interaction with *Douglas fir* and *poplar* has presented new scenarios. These proteins act as effectors during early plant–fungi interaction or establish symbiotic interface by MISSP7 proteins in hyphal mantle (Martin et al. 2008). Earlier studies have shown the inhibition or stimulation of phytopathogenic fungi by various agents like pisatin, a flavonoid having antifungal activity (Perrin and Bottomley 1961). Other chemicals are reported to hinder the development of phytopathogenic fungi like pterocarpans (e.g., medicarpin, maackiain, trifolirhizin) and pterocarpans by isoflavone betavulgarin that restricts growth of sugar beet pathogens (Rao 1990). Similarly, the hyphal growth of phytopathogenic fungi is inhibited by galangin and flavones—hyperoside (Afolayan and Meyer 1997; Li et al. 2005). Moreover, allelopathic rice releases tricin from root exudates that reduces level of cultivable fungi in soil (Kong et al. 2008). Spore germination is either stimulated or inhibited by the release of various flavonoids, for example, naringenin and kaempferol, that inhibits spore germination in *Pyricularia oryzae*, a rice pathogen (Padmavati et al. 1997). Spore germination is inhibited in *Verticillium dahliae* by rutin (El Hadrami et al. 2011). Pterocans stimulate spore germination in *Fusarium solani* (Ruan et al. 1995).

4 Negative Plant–Microbe Interactions

4.1 Self-Defense

The primary steps of communication between plants and microbes occur irrespective of the nature of relationship, that is, whether it is symbiotic or pathogenic. During this interaction, recognition is the initial event that involves signal perception and transduction followed by up- or downregulation of genes and subsequent activation of biochemical pathways. The end products act as signaling molecule for host leading to chemotaxis effect on microbe to either establish as pathogenic or in symbiotic association.

Plants possess multilayered defense mechanisms that recognize microbial infection. One of the layers is involved in detecting conserved molecules of microbes known as PAMPs/MAMPs. These are detected by specific receptors similar to that

of symbiotic microbe receptors (PRRs) that can sense bacterial lipopolysaccharides (LPS) or fungal chitin, quorum sensing factors, peptidoglycans (PGN), and flagellin (Boller and Felix 2009). In contrast, plants have receptor-like kinases (RLKs) and receptor-like proteins (RLPs) with functional domains located in plasma membrane. RLPs comprise of a transmembrane (TM) and an extracellular domain (ECD). They lack any specific signaling domain but possess a small cytosolic domain. RLKs, on the other hand, comprise of an intracellular kinase domain, a single-pass TM domain, and an ECD (Maekawa et al. 2011). When a pathogen tries to invade the plant, local defensive immunity is activated rapidly activating either of the two types of recognition. Plant recognizes microbe surface antigen MAMP by surface PRR to trigger PTI (Jones and Dangl 2006). This mechanism helps the plants to prevent pathogenic microbes. In addition to recognizing microbial determinants, PRR also recognizes other components such as cell fragments and plant peptides that act as danger signal and are known as damage-associated molecular patterns (DAMPs). They are produced by pathogens during wound or infection and cause a physiological change. Binding of DAMPs and PAMPs with PRR activates PAMP-triggered immunity (PTI) to initiate the production of reactive oxygen species (ROS). This causes a calcium burst and activates calcium-dependent associated kinases (CDPKs) and mitogen-associated protein kinases (MAPKs) leading, ultimately, to the imitation of transcriptional reprogramming (Nicaise et al. 2009; Tena et al. 2011).

Another bacterial determinant, the flagella, has a major component flagellin to induce PTI in majority of plants. Any mutation in flg22 epitope prevents its recognition by FLS2 receptor and results in dominated pathogenic or symbiotic relation (Boller and Felix 2009). PAMPs are essential for microbial life in order to evade host immune defenses by continuously undergoing negative selection of immune epitopes (McCann et al. 2012). However, PTI in plants actively blocks the infection of microbes. A loss of PRR causes disease susceptibility for both non-adopted and adopted pathogens (Boller and Felix 2009). Additionally, PTI can be suppressed or evaded if adapted pathogen releases any of the specific effector proteins (Dodds and Rathjen 2010). Nevertheless, secondary defense mechanisms involve resistance (R) genes that code for receptors located in cytoplasm. They recognize the effectors released by the pathogens and activate effector-triggered immunity (ETI) (Jones and Dangl 2006).

5 Genomics of Plant Hormone Signaling in Plant–Microbe Interaction

The protection of vulnerable roots depends on the level of chemical attack mounted by the pathogens. It is often mediated by plant secretions such as defensive proteins and chemicals (Bais et al. 2002; 2004a, b; Flores et al. 1999). These chemicals either function against a broad range of microbes or against certain specific pathogens. Rosmarinic acid (RA) is caffeic acid ester having strong antimicrobial activity for

soilborne microbes and opportunistic pathogen *Pseudomonas aeruginosa*. Extracts of *Phytophthora cinnamomi* have been shown to activate Rosmarinic Acid in root exudates of cultured roots of sweet basil, *Ocimum basilicum* (Bais et al. 2002).

Other chemicals like phytoalexin and phytoanticipins are involved in restricting the onset of disease owing to their antimicrobial activity. In vivo phytoalexin concentration cannot be measured due to direct contact with pathogens. However, a number of studies have provided the concentration of various classes of phenylpropanoids at organ and cellular level in root exudates of *Arabidopsis*. It has been shown that phenylpropanoid concentration levels were high when encountered with nonhost *Pseudomonas syringae* strains as compared to *P. syringae* pv. tomato DC3000 (host pathogen). Host pathogens show a resistance to these chemicals resulting in the onset of disease which establishes the potent antimicrobial activity of phytochemicals against nonhost pathogens (Bais et al. 2005).

Another class of phytohormones is also involved in plant defense during interaction with pathogens. These chemicals include salicylic acid (SA) that provides resistance against hemibiotrophic and biotrophic pathogens. Similarly, ethylene (ET) and jasmonic acid (JA) combinations are involved in fighting necrotrophic pathogens (Glazebrook 2005). The two pathways work antagonistic to each other, i.e., a high necrotrophic resistance causes high susceptibility to biotroph, while biotroph resistance elevates necrotroph susceptibility. Some pathogens are also specialized in mimicking plant hormones. Coronatine, a jasmonate (JA)–isoleucine, is similar to plant JA. It is synthesized by strains of *P. syringae* via unrelated pathways of coronamic acid (CMA) ligation with polyketide coronafacic acid or CFA (Fonseca et al. 2009). In *Pseudomonas* strains, coronatine production is regulated by HrpL which regulates TTSS. This is responsible for delivering type three effectors (TTS) into host cells. Coronatine works antagonistically to SA and similar to JA. Coronafacic acid ligase, encoded by genome of *Pectobacterium atrosepticum*, functions to ligate cfa and cma (Toth et al. 2006). *Pectobacterium carotovorum* and *Dickeya* sp. have a small portion of *cfa* and *ligase* homologous genes. In contrast, tested strains of *P. atrosepticum* contain *cfa* instead of *cma* biosynthetic gene cluster (Slawiak and Lojkowska 2009).

In plant–pathogen interaction, hormonal cross talk results in effective systemic immunity. Gibberellic acid (GA) degrades DELLA protein growth repressors causing an accumulation of ROS and SA. It also regulates the JA signaling (Achard et al. 2003; Navarro et al. 2006). Brassinosteroid is involved in providing resistance against biotroph–hemibiotroph (Nakashita et al. 2003). Similar response is also noted against abiotic stresses via NPR1 (nonexpressor of pathogenesis related 1) that also regulates SA signaling (Divi et al. 2010; Dong 2004). Brassinosteroid also induces PR1 expression independent of NPR1 (Divi et al. 2010). Under biotroph attack, cytokinin enhances response of SA via NPR1 in contrast to active auxin signaling that suppresses synthesis of SA (Robert-Seilaniantz et al. 2007). Active synthesis and signaling of abscisic acid (ABA) increase susceptibility in plants for several pathogens (Ton and Mauch-Mani 2004). ABA also plays a central role in abiotic stresses as environmental factors may weaken plant immunity leading to increased tendency for disease outbreak (Asselbergh et al. 2007)

Certain pathogens synthesize plant-related hormones and are capable of inducing similar symptoms due to hormonal imbalance (Bari and Jones 2009; Grant and Jones 2009, Robert-Seilaniantz et al. 2007). One such example is formation of galls in *Agrobacterium tumefaciens* that transfers its T-DNA and initiates production of cytokinin and auxins (Akiyoshi et al. 1983). Gall is an outgrowth in plants due to imbalance of auxins and cytokinins (Robinette and Matthysse 1990). Similarly, *P. syringae* pv. tomato delivers TTE which is capable of inducing production of auxin and ABA (Schmelz et al. 2003). Expression of AvrRpt2, in vulnerable *Arabidopsis*, increases concentration of auxin. In *Brassica*, clubroot disease caused by *Plasmodiophora brassicae* enhances expression of auxin receptors and downregulates CK degradation (Siemens et al. 2006). *Xanthomonas campestris* TTE AvrBs3 causes cell hypertrophy due to the induction of auxin-responsive genes (Marois et al. 2002). *In planta* expression of Pst AvrPtoB downregulates PAMP genes and increases ABA genes. This results in an elevated bacterial growth with weak response of basal defense (de Torres-Zabala et al. 2007). The exact mechanism of disrupting ABA signaling networks by AvrPtoB is still unknown. ABA increases in response to effector proteins as this is a core virulence mechanism that enhances bacterial and fungal resistance in ABA biosynthetic mutants (Asselbergh et al. 2007; Ton and Mauch-Mani 2004). The susceptibility of NCED5 (9-cis-epoxycarotenoid dioxygenase 5) overexpressers is also increased concurrently (Fan et al. 2009).

Similarly, *Pseudomonas syringae* B728a invades the host and starts the production of auxins via the conversion of indole acetonitrile into indoleacetic acid by utilizing nitrilase (Howden et al. 2009). This production of phytohormones by pathogens is an indication of the onset of disease. *Erwinia chrysanthemi* (soft rot pathogen) also produces auxins inside plant cells (Yang et al. 2006). However, mutations in auxin biosynthetic pathway allow the growth of pathogens. They attenuate the virulence factors; for example, they reduce the impact of TTE on cell wall degradation. *Erwinia herbicola* pv. *gypsophilae*, a gall-inducing microbe, carries auxin synthesis components including indole pyruvate pathway (IPyA) and indole acetamide pathway (IAM). It also contains a cytokinin synthesis component. Disruption in these pathways (IAM or CK pathway) reduces the gall size. However, IPyA pathway reduces the population of pathogen instead of affecting gall size (Manulis et al. 1998).

6 Conclusions and Future Perspective

Plant production needs to be optimized to fulfill the growing demand under the dynamic environmental conditions. Intensive breeding of crops has led to the loss of beneficial microbes in soil environment. There is a wide range of plant microbes that can be used for extracting beneficial outcomes. However, many aspects of this relationship remain unexplored and unexploited. Genetic circuit regulation between the symbiont and the host plant can be optimized to enhance crop production. There

are numerous and diverse opportunities to produce bioenergy crops by exploiting plant–microbe interaction. Similarly, low-cost biomass can be used to harness fossil fuel that can help in avoiding the fluctuation in fuel prices. A major challenge is to meet the growing needs of food for an exploding human population. Energy requirement can be sorted out by growing perennial crops on unfit land. Moreover, endophytes can be employed in the phytoremediation process. These strategies may help in reducing cost spent on production of various agents while meeting the demands of the society. These benefits of endophytes can be exploited in promoting plant development and making agriculture more sustainable. Understanding mechanisms of plant–microbe interaction may help to obtain greater biotechnological benefits and employ them in a wide range of applications including phytoremediation of contaminated soils and production of bioenergy crops. Similarly, phytohormones or secondary metabolite-producing microbes can be exploited in promoting plant growth and as biopesticides or bio-fertilizers.

The study of genomics can be integrated with proteomics and transcriptomics to determine the symbiotic genes of the microbes interacting with the plants. Similarly, low-tech application can be used in layering microbial biofilm inoculation to aid the plants in their growth and development. Moreover, a number of techniques can be employed for manipulating the bacterial response in order to regulate the rhizosphere environment. The interaction in this zone is still unexplored and uncharacterized. Therefore, monitoring endophyte population and soil and plant interaction may help in unveiling the hidden secrets of plant–microbe interaction. Nevertheless, computational methods, genome sequencing, and high-tech applications are required to elucidate the complexity of plant–microbe interaction. They can be employed for gaining an understanding of the expression of genes and their role in multiple pathways of this communication. However, unleashing the molecular mechanisms in rhizosphere interaction may pose challenges due to the dynamic and complex nature of interaction within the system (Badri et al. 2009; Farrar et al. 2014). Efforts, hence, need to be made to investigate and materialize the outcomes of research studies on the genomics of plant–microbe interaction.

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Soil Microbe Diversity and Root Exudates as Important Aspects of Rhizosphere Ecosystem

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Abstract The rhizosphere is an area of soil surrounding plant roots in which soil's most reactions take place. The term "rhizosphere" was coined by Lorenz Hiltner, and it is 1–2 mm wide. The rhizosphere is divided into three zones: endorhizosphere, rhizoplane, and ectorhizosphere. The two dynamic properties of soil rhizosphere are root exudates and soil microbes. Root exudates are the chemical compounds that are secreted by roots and act as a source of food for soil microbes and play a pivotal role in soil microbe and plant interaction. These are low- and high-molecular-weight compounds. The root exudates are important for root-microbe and root-root communication. The other important aspect of rhizosphere is soil microbes. The soil microbes include bacteria, fungi, and actinomycetes. These organisms are important for both soil and fungi. The main aspect of this chapter is to give brief information about the underground world, and its future perspective is to understand soil microbe and plant interaction for enhancing sustainable agriculture. Studies on gene expression in the

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rhizosphere and the use of other molecular techniques like m-RNA, proteomics, labeled root compounds, stable isotope probes, and reporter technology will help in exploring underground undiscovered world.

Keywords Rhizosphere • Roots • Exudates • Soil microbes • Allelochemicals • Ecosystem

1 Introduction

The rhizosphere is the area of soil roots where most of the reactions are affected by plant roots. The rhizosphere is about 1–2 mm wide with no distinct boundaries (Brimecombe et al. 2007). Lorenz Hiltner is a German scientist who coined the term "rhizosphere" to explain plant root association. At Munich in 2004, a meeting was organized in his memory. The term rhizosphere is from the Greek words "*rhiza*" which means root and "*sphere*" which means field or area of influence (Hartmann et al. 2008). The rhizosphere is broadly classified into the following three zones, viz., endorhizosphere, rhizoplane, ectorhizosphere (Clark 1949; Lynch 1987; Pinton et al. 2001a). The endorhizosphere consists of root tissues including cortical cells and the endodermis. Rhizoplane is the area of root surface where soil microbes and soil particles interact. It comprises of the cortex, epidermis, and mucilage. The third zone is ectorhizosphere which is formed from soil particles adjacent to roots. In addition to these three fundamental zones, few other layers are also found which include the mycorrhizosphere, rhizosheath, and bulk soil (Linderman 1988; Curl and Truelove 1986; Gobat et al. 2004). Mycorrhizosphere is the mycorrhizal association of plants. Rhizosheath is the strongly adhering dense layer and consists of root hairs, mucoid layer, soil particles, and soil microbes. Bulk soil is the portion of soil which is not the component of rhizosphere (Brundrett 2009; Lambers et al. 2008) (Figs. 1 and 2).

The rhizosphere is called the hot spot of soil microbes (Brimecombe et al. 2007). In Kashmiri Language, we may call rhizosphere as Wazwan point for soil microbes. The rhizosphere is also called as human gut microbiome for plants (Mendes et al. 2011). Rhizosphere is considered as the spot where soil genesis actually starts (Pate et al. 2001). To soil microorganisms, rhizosphere is the lush oasis in the desert. Because it is underground, rhizosphere is considered as the last frontier in agriculture. Soil microbial community also has greater reservoir of biological diversity in the world (Curtis et al. 2002; Chaparro et al. 2013; Philippot et al. 2013; Buée et al. 2009). The rhizosphere soil contains up to 10^{11} microbial cells/g (Egamberdiyeva et al. 2008) and over 30,000 prokaryotic species. The combined genome of the rhizosphere is greater than that of plant and thus is called plants' second genome (Bron et al. 2012). The eelworms are being used to quantify the extent of rhizosphere because they are highly in peculiar in reacting to chemicals exudated by plant roots (Bolton et al. 1992) (Table 1).

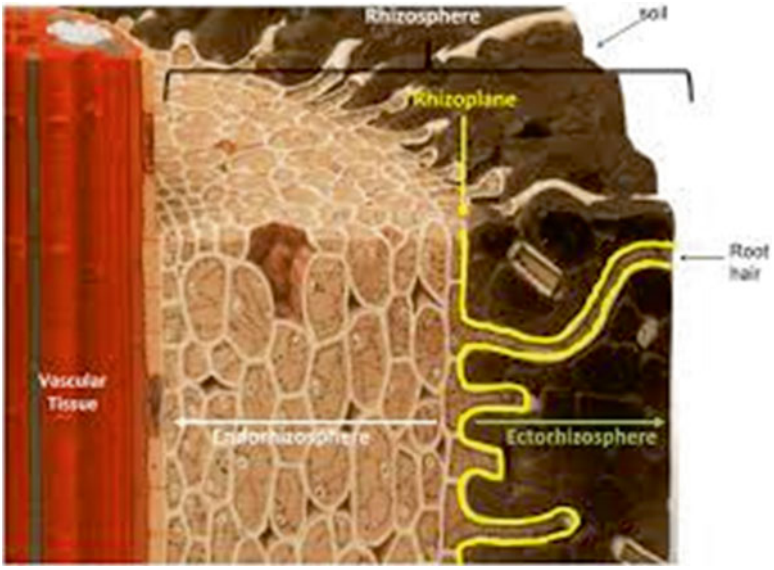

Fig. 1 Ectorhizosphere of the soil root ecosystem

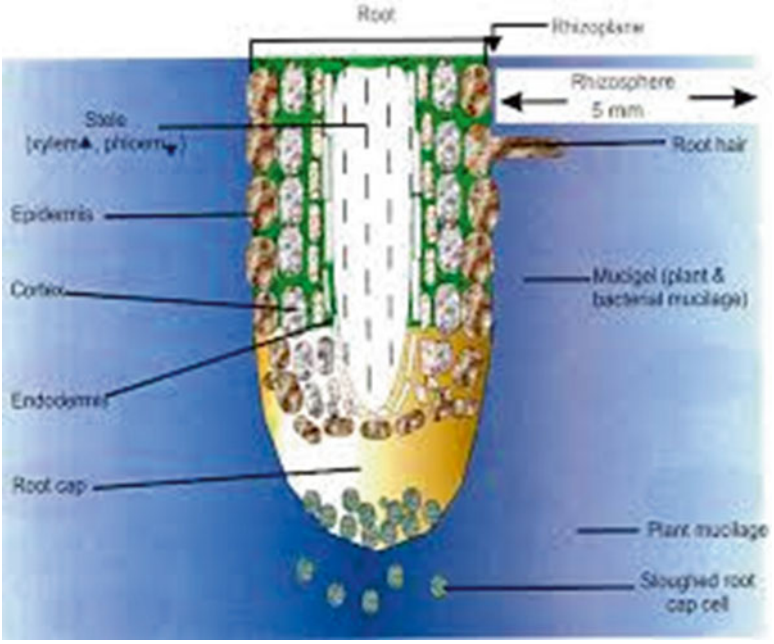

Fig. 2 The endorhizosphere and its different components

Table 1 Similarity between the human gut and rhizosphere

Characters	Human gut	Rhizosphere	References
Important for nutrient uptake	Microbes help in the breakdown of food and generate essential nutrients, such as vitamins B and D. In reward, microbes of the human gut get the carbon source from mucin	Soil bacteria, fungi, and actinomycetes assist plants to the uptake of nitrogen, phosphorus, potassium, and other important nutrients for their growth. The microbes also help in genesis and formation of minerals and degradation of organic matter. The root exudates and other rhizodeposits provide the energy source of soil microbes	Bais et al. (2006), VanDer et al. (2008), Derrien et al. (2010), Fagundes et al. (2012)
Restricts colonization of pathogens	Certain interactions of beneficial microorganisms include nutrient competition, inhibitory protein production, alteration of receptor sites, and modification of toxins	Certain soil beneficial microorganisms suppress plant pathogens by certain interactions like nutrient competition, antibiotic and lytic enzyme production, and consumption of pathogen stimulatory compounds	Lugtenberg and Kamilova (2009), Doornbos et al. (2012), Fagundes et al. (2012)
Modulate host immunity	The microbes stimulate host innate immunity system that not only affect intestinal mucosa but also produce immune responses in the respiratory tract. Also the development of microflora in the gut of humans during first year of life is very important for the development of the immune system	The rhizobacteria suppress diseases and systemically boost defense system of plants. The rhizobacteria trigger plant resistance to the pathogens. Most important chemicals are jasmonates	Bron et al. (2012), Ent et al. (2009), Fagundes et al. (2012), Ichinohe et al. (2011)

Distinguish friend from foe	Pathogens and symbionts have similar molecular patterns which are perceived by immune system, but the mechanism of immune system response is still unknown. Many mechanisms are present to prevent stimulation of immune system like physical barrier of mucous and reduced pathogen receptors in epithelial cells. Regulatory T cells suppress immune response of commensal gut microbes	Pathogens and symbionts have similar molecular patterns but are recognized by the immune system which is still unknown and thus differentiates friend from foe. Both pathogens and beneficial soil microorganisms suppress plant immune system and promote their own colonization through secretion of effector molecules	Chinen and Rudensky (2012), Lathrop et al. (2011), Zamioudis and Pieterse (2012)
Microbiome density and diversity	Microbial diversity is very high in the human gut ranging from 10^{11} to 10^{12} cells per ml of intestinal fluid, but its phylogenetic diversity is very low having only 7 of 55 described bacterial phyla which mostly include firmicutes and bacteroides. It is seen that more than 500–1000 sp. of bacteria exist in the human gut. There occurs stratified type of microbial variation and certain category of another community known as enterotypes	The rhizosphere microbial density is higher than bulk soil, and it ranges from 10^{8} to 10^{9} cells/g. These microbial communities are considered most diverse communities in the world with 10^{4} bacterial sps./g of soil. Rhizosphere microbes vary between plant species if grown in same soil	Weinert et al. (2011), Arumugam et al. (2011), Roesch et al. (2007), Weller et al. (2002)

2 The Dynamic Properties of Rhizosphere Are Root Exudates and Soil Microbes

2.1 Root Exudates

The knowledge of roots and its biology, biochemistry, and genetic evolution has considerably increased during the last few years, but the certain processes occurring in the rhizosphere by the roots such as root exudates and root border cells are still unknown (Benfey and Scheres 2000; Hawes et al. 2000). The plant roots provide mechanical anchorage to the plants and assist in water and mineral nutrient uptake. Some special functions including synthesizes, secretion, and accumulation of diverse group of chemical compounds are also performed by the plant roots (Flores et al. 1999). These compounds exudated by the plant roots play a pivotal function as chemical attractants in the soil root ecosystem (Estabrook and Yoder 1998; Bais et al. 2001). These chemical compounds are referred as root exudates. A diverse group of these chemical compounds have been found exudating from intact and healthy roots. These compounds include sugars, amino acids, peptides, vitamins, nucleotides, organic acids, enzymes, fungal stimulants, and inhabitants and also some other compounds which help in plant water uptake, plant defense, and stimulation (Pate et al. 2001; Pate and Verboom 2009; Taylor et al. 2009). Sugars, organic acids, coumarins, lipids, flavonoids, enzymes, amino acids, proteins, aliphatics, and aromatics are examples of primary substance found within the roots (Shukla et al. 2011). Among these, the organic acids are of considerable importance because of its role in providing substrate for microorganisms and also acting as intermediate in biological and chemical reactions in the soil (Philippe 2006; Wutzler and Reichstein 2013). The ability of plant roots to produce a wide range of chemical compounds is the most striking feature of plant roots with nearly 5–21 % of all photosynthetically fixed carbon being transferred to rhizosphere through root exudation (Marschner 1995). These root exudates are being classified as low-molecular-weight compounds and high-molecular-weight compounds. Sugars, amino acids, phenols, organic acids, and various other secondary metabolites are included in low-molecular-weight compounds, whereas mucilage and proteins are included in high-molecular-weight compounds (walker et al. 2003). These root exudates are relatively important in mediating the communication of plants with soil microbes (Bais et al. 2004; 2006; Weir et al. 2004; Broeckling et al. 2008). Root exudation is an important element of rhizodeposition and is a primary source of soil organic carbon released by the roots (Hutsch et al. 2000; Nguyen 2003). Whipps and Lynch (1985) first coined the term rhizodeposition as materials lost from roots, which include lysates, insoluble exudates, soluble material, and certain gases like carbon dioxide and ethylene.

2.1.1 Interaction Studies of Root Exudates

Another important function of root exudates is that it acts as a messenger that initiates and intimates physical and biological communication between the soil microbes and plant roots. Root-mediated rhizospheric communication is grouped into two categories: negative and positive interactions (Weller et al. 2002; Mendes et al. 2011; Elsas et al. 2012). Positive interactions involve communication of plant roots with certain plant growth-promoting rhizobacteria (PGPR). These plant roots produce certain chemicals that act as signals and attract certain microbes and stimulate chemotaxis (Thimmaraju et al. 2008). Positive interactions of root exudates also include growth enhancers that enhance growth of neighboring plants and help in cross-species signaling. The negative interaction of root exudates includes secretion of insecticidal and nematicidal compounds, phytotoxins, and secretion of antibiotics (Bais et al. 2006).

Root Rhizosphere Communication

Performance of plant species depends upon its ability to recognize and receive changes in environment and to respond to these changes for acclimatization. These changes are very important for growth and development of plants and microbes (Chaparro et al. 2014). Root exudate is a major food source of soil microbes that communicate with the plants and is considered most diverse ecosystem on earth (Vogel et al. 2009). These interactions of soil microorganisms and plant roots are categorized into root-root communication and root-microbe communication.

Root-Root Communication

When roots communicate with neighboring roots of other plant species, they prevent their invadence by release of certain chemical messengers (Ahmed et al. 2007). Allelopathy is the phenomena which involve beneficial, harmful, direct, and indirect effect on plants through secretion of secondary metabolites (Li et al. 2010). Allelopathy is known for more than 2000 years with respect to plant interference (Callaway and Aschehoug 2000; Ridenour and Callaway 2001; Weston and Duke 2003). Allelopathy also has importance in agriculture, because the allelochemicals produced by the plants control weed population (Haribal and Enwick 1998). The most important allelochemicals in the plant ecosystem include phenolic compounds. Phenols are the chemical compounds having a hydroxyl group (–OH) attached to an aromatic hydrocarbon group (Zeng et al. 2008). Phenolic compounds which play an important role in allelopathy are produced from pentose phosphate pathway. 4-Phosphate erythrose and phosphoenolpyruvic acid undergo certain condensation reaction with sedoheptulose 7-phosphate and generate phenolic compounds. There occurs a series of reactions in shikimic and acetic acid metabolic pathway. The phenolic allelochemicals have adverse impact on the photosynthesis and respiration

of other plant species by weakening their oxygen absorption capacity and by reducing their photosynthetic rate by reducing chlorophyll content. Patterson (1981) reported that 10–30 μmol/l caffeic acid, ferulic acid, vanillic acid, coumaric acid, and cinnamic acid could considerably reduce growth of soybean. Bais et al. (2002) reported (+) catechin and (−) catechin as root phytotoxin. + catechin was produced invasive behavior of knapwood and (−) catechin was inhibitory to the soilborne bacteria. It has also been seen that certain allelochemicals released by the host roots stimulate haustoria formation (Estabrook and Yoder 1998; Yoder 2001). Allelochemicals produced by the black walnut causes growth inhibition and is one of the earliest classical examples of allelopathy (Bais et al. 2006). The naphthoquinone, juglone (5-hydroxy-1, 4- naphthoquinone), is responsible for the walnut toxicity. Juglone is generally found in nontoxic form, but when exposed to air it becomes oxidized and thus becomes toxic. Juglone is extracted from fresh bark of stripped roots or from fresh fruit hulls (Kocacali et al. 2009). Many other close relative species of black walnut like butternut or white walnut (*Juglans cinerea*) also produce juglone, but in limited quantities. Wheat is also known to produce root exudates with allelopathic activity. Due to simple phenolic compounds like p-coumaric, p-hydroxybenzoic, ferulic acid, vanillic acid, and syringic acid, the presence of hydroxamic acids is responsible for wheat allelopathy (Yongqing 2006). Sorghum roots also secrete a mixture of hydrophobic substances which are biologically active and include sorgoleone, characterized as (2-hydroxy-5-methoxy-3-pentadecatriene)-p-benzoquinone. Sorgoleone is used as an important bioherbicide which is used for broadleaf and grass weeds at concentrations below 10 μM in hydroponic bioassays (Xiaohan et al. 2004). Some plants also secrete secondary metabolites that suppress growth of specific plants (autotoxicity). Autotoxicity is a phenomenon mostly applicable in agricultural crops and weeds, as well as in some plants that inhabit natural systems. Phytotoxic root exudates play an important role in mediating autoinhibition in some species like *Cucumis sativus* (garden cucumber), *Centaurea maculosa* (spotted knapweed) (Perry et al. 2005), and *Asparagus officinalis* (garden asparagus) (Yu et al. 2003).

Root Microbiome Communication

In the unseen underground ecosystem, some complex communication occurs which includes root- root and root-microbe interaction which has both beneficial and harmful outcomes (Bais et al. 2006). The sophisticated processes include root-microbe interaction which includes both mutualistic and pathogenic relationship, metabolic processes including parasitic plants and root secretion, energy transfer which comprises electric potential, and resource distribution and information transfer which include quorum sensing. These processes play a critical role in terrestrial ecosystem (Bouwmeester et al. 2007; Gewin 2010). Some microbial bioactive compounds which function within the belowground ecosystem play a dynamic role in plant life. Roots of the plants continuously secrete organic compounds which help in harnessing beneficial microbes and suppressing plant pathogens (Berg and Smalla

2009; Marschner and Timonen 2005). Thus, these roots have stimulatory or inhibitory influence on soil microbes which help in their community structure development as they increase their competition for nutrients and other resources (Cesco et al. 2010, 2012).

There occurs a dynamic interaction between soil microbes and plants in nature which is based on coevolutionary pressures (Klironomos 2002; Dobbelaere et al. 2003; Duffy et al. 2004; Morrissey et al. 2004; Morgan et al. 2005; Reinhart and Callaway 2006). Consequently, the microbial communities in the rhizosphere vary due to certain factors like different species of plant (Batten et al. 2006; Innes et al. 2004), their genotypes (Kowalchuk et al. 2006), and their different developmental stages (Mougel et al. 2006; Wei et al. 2007). Microbial community in soil is closely related to highly diverse plant communities, but their connecting link is still unclear and it is believed that their close relationship occurs due to widely occurring habitat heterogeneity or enhanced plant biomass. It may be also due to different carbon substrates which act as signaling compounds as they are secreted by the plant roots. These compounds belong to a class called flavonoids which are responsible for specific microbe-host interactions. These flavonoids act as signaling molecules and are highly present in symbiotic and pathogenic microbes; there occur a large number of flavonoids in the plants and a greater number of flavonoids are identified in legumes. More than 4000 different flavonoids occur which mediate host specificity (Perret et al. 2000). In several *Fusarium* plant interactions, flavonoids help in micro- and macroconidia germination but have no effect on hyphal growth during infection. Certain isoflavonoid compounds are also present in legume crops. Soya bean (*Glycine max*) produces genistein, daidzein, and, isoflavonoids which effectively stimulate *Bradyrhizobiumjaponicum* nod genes but have negative effect on the *Sinorhizobium meliloti* nod gene expression. *S. meliloti* nod gene expression gets stimulated by luteolin (Juan et al. 2007). This phenomenon helps rhizobia to differentiate between hosts and other legumes. The specific flavonoid produced by the legumes not only stimulates nod gene expression but also has its effect on rhizobial chemotaxis (Bais et al. 2006). Strigolactones recently have been identified as important signaling molecules in the AMF-plant interaction and thus are "hot issues" in the mycorrhizal study. Ectomycorrhizal fungi are also stimulated by Brassicaceae (Zeng et al. 2003). There occurs a specific interaction between rhizobia and legume allowing only few rhizobial strains to nodulate with specific host legumes. *Medicago*, *Melilotus*, and *Trigonella* genera are nodulated with *S. meliloti*, whereas *Rhizobium leguminosarum* bv. viciae stimulates nodulation in *Pisum*, *Vicia*, *Lens*, and *Lathyrus* genera (Bais et al. 2006). Scientists demonstrated that plant roots secrete L-MA (malic acid) which acts as effective signaling molecule to establish beneficial rhizobial communities (Thimmaraju et al. 2008). *Arabidopsis thaliana* and *Medicago truncatula* are the two model plant species which are unable to maintain nonresident soil fungal populations, but maintain resident soil fungal populations. These phenomena occur largely due to root exudates. In vitro-generated root exudates applied to the soil fungi show similar results to that of plants growing in same soil (Yanhong et al. 2009).

2.2 *Rhizosphere Soil Microbes*

Someone has rightly said that rhizosphere microorganisms have two faces like Janus, the Roman god of doors and gates who symbolizes changes and transitions from one condition to another (one part of the face looks at the plant roots and the other at the soil; the ears and nose sense other gods, and the mouth is wide open for swallowing). It is also well established that soil is a good medium for plants and microbes, but the plants and their associated microbes help in genesis and weathering of soil (Pate et al. 2001; Pate and Verboom 2009; Taylor et al. 2009; Pausch et al. 2013). Soil formation occurs due to weathering process which primarily occurs due to soil microbes (Raven and Edwards 2001; Beerling and Berner 2005; Taylor et al. 2009). The soil microflora includes bacteria, fungi, actinomycetes, protozoa, and algae (Raaijmakers and Weller 2001; Singh et al. 2007; Grayston et al. 1998; Broeckling et al. 2008). Recently the nucleic acid analysis revealed enormous diversity in the soil (Nannipieri et al. 2003a, b; Suzuki et al. 2006).

2.2.1 Microorganisms and Their Mode of Action

The soil microbes can generally be divided into beneficial, harmful, and neutral microbes. The beneficial soil microbes can further be divided into three categories. The first category helps in nutrient supply (Ma et al. 2003; Robin et al. 2008; Michaud et al. 2008). The second group includes those that stimulate plant growth by suppressing activity of phytopathogens. The third group of microbes directly promotes growth of plants by secreting phytohormones (Welbaum et al. 2004; Brimecombe et al. 2007) (Fig. 3).

2.2.2 Nutrient Availability and Plant Growth Promotion

The most population in the rhizosphere is occupied by the bacteria. Those rhizosphere bacteria which enhance plant growth are called plant growth-promoting rhizobacteria (PGPR) (Kloepper JW Schroth 1978; Lucy et al. 2004). The most dynamic function of PGPR is secretion of phytohormones. A diverse group of PGPR are inoculated on the crops which include *Azospirillum* (Cassan and Garcia 2008), *Bacillus* (Jacobsen et al. 2004), *Pseudomonas* (Loper and Gross 2007), *Rhizobium* (Long 2001), *Serretia* (De Vleeschauwer and Hofte 2007), *Stenotrophomonas* (Ryan et al. 2009), and *Streptomyces* (Schrey and Tarkka 2008). Some fungi belonging to genera *Ampelomyces*, *Coniothyrium*, and *Trichoderma* have also beneficial effects (Harman et al. 2004). The mode of action of PGPR involves complex mechanism which promotes plant growth, development, and protection. The most versatile functions of PGPR are biofertilization, phytostimulation, and biocontrol (Morgan et al. 2005; Muller et al. 2009; Chet and Chernin 2002). The success of plant-microbe interaction depends on colonization (Lugtenberg et al. 2002;

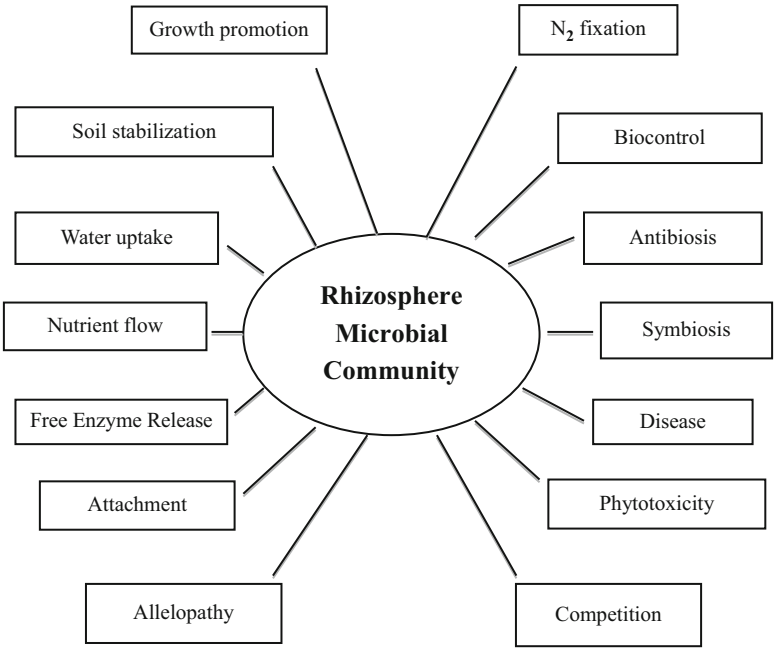

Fig. 3 Shows role of rhizosphere microbial community and its harmful and beneficial effects

Kamilova et al. 2005). The steps of colonization include attraction, recognition, adherence, invasion, colonization, and growth (Pinton et al. 2007; Berg 2009).

Plant growth in the agriculture is enhanced by certain abiotic and biotic factors. The abiotic factors comprise light, temperature, water, and air. The biotic factors include PGPR which help in plant growth by secreting enzymes (Lynch 1990; Marilley and Aragno 1999; Garcia et al. 2001). Interestingly the inoculation of PGPR increases the crop yield and plant growth (Farzana et al. 2009). Some plant growth-promoting rhizobacteria have more than one trait (Joseph et al. 2007; Yasmin et al. 2007; Egamberdiyeva 2007). These PGPR release volatile compounds like 2,3-butanediol and acetoin that help in growth and development of Arabidopsis thaliana (Ryu et al. 2003). There have also been reports that diazotrophical bacterial application in the soil increases crop yield, plant height, and microbial population in the soil (Anjum et al. 2007). Due to certain combination of PGPR, carbohydrates, and IBA (double and triple combinations), there occurs increased rooting capacity of apple (Karakurt et al. 2009). PGPR are the most effective model organism that can replace pesticides and other harmful supplements which cause soil and environmental pollutions. These PGPR also act as biofertilizers and bioenhancers and reduce use of

chemical fertilizers (Ashrafuzzaman et al. 2009). Utilization of PGPR with alternative use of chemical fertilizers reduces pollution, preserves environment, and increases agricultural productivity (Ştefa et al. 2008). Combination of PGPR and arbuscular mycorrhizal fungi enhances nutrient use efficiency of plants and allows low rate of application of fertilizers (Adesemoye et al. 2009; Tanvir et al. 2015). The bacteria and archaea are responsible for biological nitrogen fixation. These include symbiotic nitrogen fixers like rhizobium, which are obligate symbionts in legume plants and *Frankia* in nonleguminous plants and certain free-living forms like cyanobacteria, azospirillum, azotobacter, and diazotroph.

2.2.3 Pathogen Inhibition

Soil microbes live around plant roots and feed on root secretions and dead root cells. Root colonization not only results in high plant growth-promoting rhizobacterial population densities but also functions as antagonistic metabolites (Shoda 2000; Raaijmakers et al. 2002). The different mechanisms involved are antibiosis, parasitism, and induced systemic resistance. Antibiosis is the phenomenon where microbial growth gets inhibited by different compounds like antibiotics, toxins, biosurfactants, and volatile organic compounds. Parasitism is the phenomenon where cell wall-degrading enzymes such as chitinase and β-1,3-glucanase are secreted which degrade cell wall (Compant et al. 2005; Haas and Defago 2005). A wide range of antifungal metabolites such as zwittermicin-A, kanosamine, and lipopeptides are secreted by *Bacillus subtilis*. These antifungal metabolites include surfactin, iturin, and fengycin families (Emmert and Handelsman 1999; Ongena and Thonart 2006). Competition for the carbon source of energy is responsible for fungi inhibition by reducing fungal spore germination (Chin et al. 2003; Alabouvette et al. 2006). Another mechanism of pathogen inhibition is induced resistance. The induced resistance involves the use of beneficial bacteria that not only reduces the activity of pathogenic microorganisms through antagonism but also stimulates plant defense mechanism (Shoda 2000; VanLoon 2007). In some instances, the mechanism of induced systemic resistance coincides with systemic acquired resistance. Both induced systemic resistance and systemic acquired resistance enhance the resistance of plant which depends on signaling compounds like ethylene, jasmonic acid, and salicylic acid (VanLoon 2007).

2.2.4 Rhizosphere Effect

The rhizosphere effect is determined by dividing the number of microorganisms per gram of rhizosphere soil by the number of microorganisms in a gram of control soil (Wasaki et al. 2005; Herman et al. 2006). The rhizosphere effect greatly reduces as we move away

from roots. For bacteria and fungi, R:S value ranges from 5 to 20. Actinomycetes is a less effected group of soil microorganisms having R:S effect of 2–12 (Curl and Truelove 1986; Foster 1986; Lynch 1990; Rovira 1991; Pinton et al. 2001a, b; Whipps 2001).

3 Quorum Sensing: The Bacterial Communication

"Quorum sensing" (QS) is the communication of bacteria which includes cell density. It is cell-to-cell communication. The bacterial quorum sensing occurs by the binding of signals with their receptor proteins. When binding occurs, it regulates gene expression in response to cell density (Gonzalez and Marketon 2003; Hong et al. 2012). The signaling molecules involved in quorum sensing are called autoinducers. These autoinducers are synthesized at particular stage of life cycle or may be synthesized for stimulating response, once the signaling molecule has reached at a particular concentration (Gonzalez and Marketon 2003). The quorum sensing is a cell density level: once a particular cell density is achieved, the concentration of quorum-sensing signals becomes enough to induce gene expression, either directly through transcriptional regulator or indirectly by signaling cascade activation (Fuqua et al. 2001). *N*-acyl homoserine lactone (AHL) is the most studied quorum-sensing signal molecule (Williams et al. 2007). AHL signals are highly preserved in nature having same homoserine lactone moiety, but differ in length and structure of acyl side chain. The *N*-acylated side chains have fatty acids. These chains have varying degrees of saturation, different chain lengths (4–18 carbons), and presence of different groups (hydroxy, oxo-, or no substituent at the C3 position) (Swift et al. 1997; Schuster et al. 2013). LuxI synthase gene using intermediate of fatty acid biosynthesis and *S*-adenosyl methionine synthesizes AHL molecules. The AHL molecules will incorporate LuxR protein and regulate downstream gene expression. Each LuxR protein is specific for its AHL signal molecules (Parsek and Greenberg 2000). AHL regulates many target genes, but basic mechanism of gene regulation and AHL biosynthesis seem to be specific in quorum-sensing bacterial species (Dong et al. 2002). QS mechanism with LuxI/LuxR signal molecules in *Agrobacterium tumefaciens* causes crown gall disease of plants. *Agrobacterium tumefaciens* with tumor-derived opines and transcriptional factor OccR or AccR regulate gene expression of LuxR homologue TraR (Oger et al. 1998; Zhu and Winans 1988). *Pseudomonas aeruginosa* uses LasI/R and RhlI/R to promote regulation and expression of virulence factors and biofilm formation (Glessner et al. 1999). Another class of homoserine lactone known as *p*-coumaroyl-homoserine lactone (pC-HSL) has been discovered to be produced by the bacteria *Rhodopseudomonas palustris*. The intracellular fatty acid is not used as precursor in the synthesis of pC-HSL molecules, and synthesis occurs due to RpaI and LuxI by using environmental *p*-coumaric acid (Schaefer et al. 2008). Many bacteria use QS to gain maximal competition advantages, and to measure the advantages of QS some organisms use quorum quenching (QQ) (Lin et al. 2003; Rodolfo et al. 2015). This QS widely occurs in prokaryotes and eukaryotes and plays an important role in pathogen-host and microbial interactions (Dong et al. 2002).

4 Conclusion

In this chapter, we discussed an overview of ecology of various organisms and the root exudates. Various microorganisms are present in the rhizosphere, and they form a complex community which is connected with each other and with the external environment. The genetic and functional diversity of soil microbes is very important for both plant and soil health. The major challenge ahead of rhizosphere is the discovery of new signaling molecules that occur between different organisms; these discoveries are very important to enhance our knowledge to deal with the new pest and disease problems in the sustainable manner. There also occurs challenges to adopt new crops and cropping systems which absorb most of the nutrients from soil particularly nitrogen and phosphorus because our phosphorus sources are getting diminished. Today rhizosphere is considered a new research field with many exciting challenges. These challenges can be both fundamental and applied. There are some major developments in biogeochemistry and ecology of rhizosphere which need a global consideration. In symbiotic association, a great achievement has been made, but there still occurs a great lacuna of knowledge in other biological interactions. Rhizodeposition is considered the central concept in rhizosphere ecosystem and beyond rhizosphere ecology. Rhizodeposition is very important for terrestrial ecosystem biodiversity and functioning. In rhizosphere studying of gene expression is used for understanding certain processes like inducing microbial activity, biological control, nutrient competition, and certain molecular interactions between roots and roots and roots and microorganisms. Some techniques have been developed to characterize m-RNA (Nannipieri et al. 2003a, b), but soil proteomics is still not so developed (Nannipieri 2006; Ogunseitan 2006). Stable isotope probe (SIP) has also been used in understanding functional activity and community structure in soil (Radajewski et al. 2000; Manefield et al. 2006). Labeled root exudate compounds and monitoring microorganisms of rhizosphere also involve the use of stable isotope compounds (Manefield et al. 2006). At single cell level, reporter technology is to be used to assess functions of rhizosphere soil including gene expression (Sorensen and Nybroe 2006). The increasing knowledge of the promoter, regulator, and reporter gene insertion techniques shall allow use of reporter gene technology for regulation, expression, and induction of any gene in rhizosphere. The methodological improvement of new technology will allow designing of new reporter bacteria to respond to specific root exudates.

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An Insight into the Legume–*Rhizobium* Interaction

G. Yamal, Ankita Bidalia, Krati Vikram, and K.S. Rao

Abstract Active forms of nitrogen are limiting in soil, but the legume–*Rhizobium* interaction overcomes this barrier by biological nitrogen fixation and lessens the usage of fertilizers. An understanding exists between the two partners for symbiotic association to share their resources without either one becoming dominant. Certain compounds released by the host legume plants into the rhizosphere attract the rhizobia and activate the expression of rhizobial nod genes that in turn leads to the production and secretion of strain-specific NFs. NF signalling cascade and events of cell divisions in cortex and pericycle and bacterial infection occur in an orchestrated manner and give rise to a nodule. The nodule organogenesis can be studied under nodule formation and bacterial invasion. Depending on the persistence of meristem, nodules formed can be determinate or indeterminate, but ultimately it is the host plant species that determine the type of nodule formed. More than 90 % of arable land experience one or other kind of stress. Stress conditions affect the host plant, rhizobium and also the interaction between the two.

Keywords Legume • Rhizobium • Stress • Nodulation

1 Introduction

The earth's atmosphere consists of 78.1 % nitrogen gas, but the biologically active forms of nitrogen are limiting in soil and can restrict plant growth. Thus, it's imperative for plants to capture nitrogen, in the form of nitrates and ammonia, from the soil. Modern agriculture relies on application of industrially synthesized nitrogen fertilizers to maximize crop productivity. Production of nitrogen fertilizers is expensive and consumes a lot of fossil fuel. In addition to this, 30–50 % of applied nitrogen fertilizer gets leached out and leads to environmental problems. The reliance on chemical fertilizer can be reduced by biological nitrogen fixation (BNF), wherein the atmospheric nitrogen is converted to ammonia by an enzyme called nitrogenase. The

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process of BNF was discovered by Beijerinck in 1901 (Wagner 2011). This process has ecological and agronomical importance and accounts for 65 % of the nitrogen used in agriculture worldwide. It is estimated that roughly 200 million tons of nitrogen is fixed annually by the symbiotic association between rhizobia and legume (Graham and Vance 2003; Peoples et al. 2009).

Legumes are a large group of angiospermic plants skilled with the ability to establish symbiotic association with the nitrogen-fixing bacteria called rhizobia (including the genera *Azorhizobium*, *Allorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*) that are widespread under different edaphic and climatic conditions. The Fabaceae or Leguminosae family includes three subfamilies: Faboideae (or Papilionoideae), Mimosoideae and Caesalpinioideae. Members of these families can grow in nitrogen-poor soils; therefore, they play a crucial role in the sustainable development of the agriculture (Pislariu et al. 2015). One of the identified roles of BNF is in poverty alleviation. However, applying the knowledge of the mechanism of BNF and providing training to the farmers about the inoculation, providing the efficient strains at the cheaper cost to the farmers, could be a step towards sustainability (O'Hara et al. 2008).

The rhizobium and host legume develop a metabolic cooperation which forms an exchange-control system that enables the two partners to share their resources without either one becoming dominant (Lodwig et al. 2003). An intricate dialogue occurs between the two partners, and the compatibility is tested, after which the host plants and rhizobia exchange signals. The rhizobia enter and infect the roots of compatible legume plants either by the root hair or through cracks present in root epidermis. Rhizobial signalling to host root hairs then leads to root hair deformation, branching and curling, mitosis in the root cortex and ultimately the formation of specialized structures called nodules.

Thus, this two-way molecular conversation between the host legume and the bacteria leads to morphological, anatomical, physiological and cytological changes in plants. Depending on the shape and maintenance of a meristematic region, two kinds of nodules, viz. determinate and indeterminate, are formed by host plants upon infection by rhizobia, but ultimately it is the plant that determines the type of nodule formed. In general, the indeterminate nodules are formed by temperate legumes, and determinate nodules are formed by tropical legumes (Lotocka et al. 2012). So far various functional genomics resources have been developed for the two model legumes *Medicago truncatula* and *Lotus japonicus* (Pislariu et al. 2015).

Plants being sessile often have to face a wide variety of environmental conditions. Like other plants legumes also face one or other kind of stress as more than 90 % of arable land experience stress. The presence of stress effects the growth of the host plant, the rhizobia and their interaction. Thus, stress changes the physiological state of host plant, rhizobial survival in the soil, infection and nodule establishment and ultimately the nodule functioning. In the present chapter, effect of stresses such as soil pH, soil moisture, salinity, temperature, nutrients, predation, etc. on the legumes, rhizobium and legume–*Rhizobium* interaction has also been discussed. The present chapter is an attempt to give an overview of process of nodule formation and the changes that occur in the host plant, along with the different types of nodules formed and the effect of natural stresses on plant, rhizobium and their interaction.

2 Nodule Organogenesis

The history of the study of root nodule formation dates back to the sixteenth century in the drawings of Fuchs and Dalechamps; however, Malpighi proposed that the swellings (the word used by Malpighi for nodules) are due to insect larvae. Interestingly, the necessity of bacteria for nodule formation was demonstrated during the nineteenth century by Frank and Beijerinck, Frank showed that soil sterilization prevented nodule formation, and Beijerinck prepared pure cultures of nodule occupants and used them to infect legumes (Bond 1948; Pueppke and Broughton 1999). Root nodule is a structure, unique to leguminous plants and is of great interest for several reasons. The nodules can be formed only after bacterial infection and possess differentiated tissues in a definite arrangement. However, today it is well established that nodule formation involves a two-way dialogue between the host legume and the bacteria rhizobium. During the dialogue, various signalling molecules are exchanged that regulate the specificity of legume and rhizobium interaction for initiation, differentiation and functioning of nodules. This specificity has ecological and evolutionary importance as it allows infection by symbiotic friend and not a pathogenic foe (Long 2015). The nodule organogenesis can be studied into two subheadings: nodule formation and bacterial invasion.

2.1 Process of Nodule Formation

Plants release certain signal compounds (such as lectins, flavonoids) in the rhizosphere that favour the initiation of nodule development. In the 1970s, the lectin recognition hypothesis was proposed according to which the plant lectins and rhizobium exopolysaccharides (EPS) mediate specificity in symbiosis (Hirsch 1999; Long 2015). In the rhizosphere, the exuding phenolic flavonoid compounds from the roots of the host plant attract the rhizobia and activate the expression of rhizobial nod genes that in turn leads to the production and secretion of strain-specific NFs (Gage 2004; Ferguson et al. 2010). In the free-living rhizobium, nodD gene shows its expression, but the rest of the nod genes (nodABC, nodE, nodH, nodPQ) are expressed only in the presence of plant. There are 'common' nod genes, viz. nodABC, that are essential for nodulation and certain 'host specific' such as nodE (in *R. leguminosarum* for nodulation in peas or clover), nodH and nodPQ (in *R. meliloti* for nodulation in alfalfa). However, the ability of rhizobia to nodulate several legumes is linked not only to the presence of different nod genes in its genome but also with the legume promiscuity (Roche et al. 1991). Therefore, certain bacteria can nodulate only specific legume host, while others such as NGR234 and USDA257 (strains of genus *Ensifer*) have a broad host range (Pueppke and Broughton 1999). The infection of varied legumes by several strains of rhizobia occurs due the presence of several copies of the nodD gene, which in turn permit them to respond to different types of flavonoids produced and secreted by plants (Cooper 2007; Gibson et al. 2008).

The NFs are lipochitooligosaccharides (LCO), the derivative of chitin but differ in the number of GlcNAc units (2–4), in the length and degree of unsaturation of the fatty acid chain, as well as the presence of various substitutions on the oligosaccharide backbone with various substitutions at the (non)reducing-terminal and/or nonterminal residues (Haeze and Holsters 2002; Fliegmann and Bono 2015). These variations are characteristic for each rhizobium and are involved in the specific recognition between the legume plant and its symbiont (Ferguson et al. 2010). Nod factors are responsible for nodule organogenesis and control infection, leading to the formation of root nodules. Thus, it is the nod genes, NFs and EPS, that determine the host range.

Rhizobia enter into the plant root either by the root hair or through cracks present in root epidermis. However, root hair infection at the tips is the most common, because they have thinner and less cross-linked cell walls which further allow the rearrangement of underlying microtubules, change vesicle trafficking to the growing tip and allow easy penetration by microsymbiont. Root hair deformation gets started within 6–8 h after the attachment of rhizobia (Ferguson et al. 2010). Rhizobial signalling to host root hairs leads to root hair deformation, branching and curling and mitosis in the root cortex, which culminates in the formation of nodule primordium. The nodABC genes are essential for root hair curling as well as infection, for eliciting mitosis in the root cortex and for nodule formation (Haeze and Holsters 2002).

2.2 Process of Bacterial Invasion

Bacteria invade the plant cells through tubular structures termed ‘infection thread’. Infection thread is a composite structure, as it has parts contributed by the two symbiotic partners (Gage 2004). The infection thread is comprised of plant cell wall components (esterified and unesterified pectins, xyloglucans and cellulose) and encapsulates the dividing bacteria in small quantity, thus facilitating the passage of bacterial cell into the cortex. This microcolony of rhizobia contains high concentration of NFs and the cell wall-degrading enzymes. The rhizobial microcolony penetrates through the cell wall and the plasma membrane remains intact, which is followed by re-synthesis and redigestion (Gage 2004; Ferguson et al. 2010).

The increased NF levels produced by the invading rhizobia lead to the mitotic division of root cortical cells. These divisions of cortical cells in the roots result in the formation of nodule primordium. In the tip region of the extending thread, active cytoplasmic streaming along with plant cytoskeleton has been proposed to play a role in the growth of infection threads. The infection threads reach into the cortical zone of the root and then to newly induced dividing cells via root hair. Bacteria get released into an infection droplet in the host cell cytoplasm through the growing tip of the infection thread. The bacteria remain enclosed within peribacteroid membrane derived from plasma membrane of host plant; this structure is

known as symbiosome (Udvardi and Day 1997). In symbiosome, these bacteria divide continuously even before they get differentiated into bacteria and start to fix nitrogen (Ferguson et al. 2010).

Reduction of nitrogen to ammonia occurs within bacteroids; this reaction is catalyzed by oxygen-sensitive enzyme nitrogenase. The level of oxygen in root nodules is regulated by leghaemoglobin, providing legume nodules a pink tinge. O'Brian and co-workers (1987) revealed that the host plant encodes the haemoglobin apoprotein, whereas the heme group is primarily synthesized by the bacterium. These researchers used a heme biosynthesis mutant strain LO505 of *Bradyrhizobium japonicum*. The mutant strain was deficient only in protoporphyrinogen oxidase activity and thus could not catalyze the penultimate step in heme biosynthesis. As a result of this, the mutant strain formed small root nodules. The bacteroids isolated from these nodules lacked protoporphyrinogen oxidase activity, and nodules contained no detectable leghaemoglobin in the nodule cytosol. These results suggest that bacterial heme synthesis is required for leghaemoglobin formation in soybean root nodules.

The leghaemoglobin remains localized within the host plant cytosol and not in the peribacteroid membrane. Thus, in nodules atmospheric nitrogen gets reduced to ammonia that subsequently assimilates into plants after its conversion into glutamine via glutamine synthase. Further, glutamate synthase enzyme converts glutamine into glutamate. The process of nodulation brings several changes in the root of the plants at morphological, anatomical, physiological and cytological levels (Ferguson et al. 2010; Gordon et al. 1992).

3 Changes in Root During Infection Thread Growth

Infection threads are considered to be tip-growing structures and develop from growing root hairs (Gage 2004). These probably elongate by using at least some of the machinery that was supporting root hair growth before infection took place. Since understanding the processes that contribute to tip growth in root hairs should enhance the understanding of the processes involved in infection thread growth, the same has been discussed in the following sections.

3.1 Cytological Changes During Root Hair Development

The root hair elongates through polarized secretion of vesicles to the tip region, with concomitant yield of the tip wall under the influence of internal turgor pressure (Hepler et al. 2001; Ketelaar and Emons 2001; Smith 2003; Rounds and Bezanilla 2013). Turgor pressure that provides the uniform stress or force in all the directions is a scalar property that irreversibly deforms the cell wall and ultimately leads to root growth but plays no role in determining the direction of growth (Kropf et al. 1998).

The directional growth of the cell depends on unequal mechanical properties of the cell wall which in turn depends on the unequal deposition of the wall matrix at certain sites of root while confining others and orientation of the cellulose fibrils (Kropf et al. 1998; Rounds and Bezanilla 2013). Under stress conditions the less viscous cell wall deforms at the faster rate in comparison to the more viscous cell wall. However, the viscosity and thickness of the wall at the tip of the cell result from several factors such as the net deposition, expansion and the variations in cross-linking within several components (Rounds and Bezanilla 2013). In root hairs, the vesicles that fuse at the tip are derived from Golgi bodies, located at a short distance behind the growing tip. These vesicles supply membrane and cell wall components that get incorporated into the plasma membrane, cell wall and extracellular matrix. During tip growth, vesicles and other organelles reach to the apical region of the cell by actin-dependent cytoplasm streaming. The cytoplasm typically moves towards the growing tip along the outside of the cell and then moves back towards basal regions via the centre region of the cell. This pattern of movement (most commonly observed in pollen tubes) is referred as 'reverse fountain streaming' (Iwanami 1956; Hepler et al. 2001; Rounds and Bezanilla 2013). The region which lies immediately adjacent to the tip does not show any cytoplasmic streaming and is devoid of organelles. This region termed as clear zone contains the vesicles that fuse with tip and provides material needed for growth. The transportation of vesicles from the base of clear zone to their site of fusion near the tip of the root hair is supposed to be mediated by diffusion, as the vesicles are delivered at the base of the clear zone and consumed at the apex (Miller et al. 1997; Lhuissier et al. 2001).

3.2 Roles of Actin and Microtubule Cytoskeleton in Tip-Growing Cells

Tominaga et al. (1997) experimentally revealed the mechanism involved in organization of actin filaments (AFs) in root hair cells (site of reverse fountain streaming) of *Hydrocharis*. Both microtubules (MTs) and AFs lie longitudinally within the cortical region of the root hair cell. However, in the transvacuolar strand, only AFs were present and MTs were entirely absent. The double inhibitor experiment using AFs inhibitor cytochalasin B, and MTs inhibitor propyzamide, showed that cytochalasin B reversibly inhibited cytoplasmic streaming while propyzamide alone had no effect. Removal of cytochalasin B after treating root hair cells with these inhibitors together failed to recover cytoplasmic streaming. However, after removal of propyzamide, both cytoplasmic streaming and original organization of AFs were recovered, suggesting that MTs play a vital role in the organization of AFs.

Similarly, in *Medicago truncatula*, the effects of microtubule stabilizing and destabilizing drugs on the morphology of the growing root hair have revealed the role of MTs in maintaining the normal structure of the subapical cytoplasmic dense region. These experiments imply that MTs play important role in maintaining the normal distance between the nucleus and the growing tip of the root hair during root hair growth and in actin maintenance (Lloyd et al. 1987; Tominaga et al. 1997; Sieberer et al. 2002).

3.3 Roles of Ca^{+2} in Tip-Growing Cells

Calcium is a versatile signalling component involved in root hair elongation (Robbins et al. 2014). Experiments conducted with ion-specific dyes have exhibited that root hairs show tip focus gradients of calcium like that of pollen tubes. The concentration of this ion at the tip of growing root hairs, just below the plasma membrane, is about 1 μM and varies up to a basal concentration of 100 nM within 20 μm, but the non-growing root hairs do not show such a gradient (Wymer et al. 1997). Necessity of calcium channel activity for root hair tip growth and maintenance was demonstrated by the use of calcium channel blocker, verapamil. 50 μM verapamil caused the dissipation of elevated calcium ion concentration and cessation of root hair growth. Re-establishment of calcium gradients shows that the highest calcium ion concentration promotes root hair growth. Change in the direction of root hair growth reorients the calcium ion gradient, and the gradient again changes/reverts when root hair growth is returned to the original direction.

4 Types of Nodules: Determinate and Indeterminate

According to the mode of development, nodules formed by *Rhizobium* are of two kinds, viz. determinate and indeterminate, but ultimately it is the host plant that determines the type of nodule formed (Franssen et al. 1992; Maunoury et al. 2008; Ferguson et al. 2010). In general, the indeterminate nodules are formed by temperate legumes, and determinate nodules are formed by tropical legumes (Nap and Bisseling 1990). The shape, site of first internal cell division, maintenance of a meristematic region and form of the mature nodule are the features that can be used to distinguish the two kinds of nodules (Newcomb and Peterson 1979; Ferguson et al. 2010). In general a nodule consists of an inner central region and an outer cortex that acts as an oxygen diffusion barrier (Witty et al. 1987; Parsons and Day 1990). Thus, the legume nodule anatomy is characterized by a central infected region, surrounded by a cortex of uninfected cells and a dichotomously branching vascular system. The vasculature system for sucrose/photosynthate delivery to nodule is present in cortex only. On the other hand, the central region of mature nodule consists of two types of cells: one those infected with rhizobia and other uninfected cells (which are generally less in numbers) (Gordon et al. 1992; Brown and Walsh 1994). Immunogold labelling studies using polyclonal antibodies to sucrose synthase were conducted by Gordon and co-workers (1992). They found a greater intensity of labelling in the cytosol of uninfected interstitial cells of the central nodule region compared with the cytosol of the infected cells. Thus, in indeterminate nodules starch is stored in both infected and uninfected cells, whereas it is rarely or never found in infected cells in determinate nodules (Gordon et al. 1992). In both determinate and indeterminate nodules, the epidermal responses are similar, but cortical responses are different, viz. in determinate nodules initials arise from outer/midcortical cells of the root, whereas in indeterminate nodules, the initials arise from inner cortical cells (Subramanian 2013).

The tissues that surround the infected tissue consist of three different cell layers. First layer is known as outer cortex, followed by nodule endodermis, which is one cell layered and inner cortex or 'nodule parenchyma' forms the third layer (Brown and Walsh 1994). Thus, the inner and outer cortex are separated by a 'common endodermis' termed as nodule endodermis (Frazer 1942; Bederska et al. 2012). The inner cortex or nodule parenchyma houses vascular bundles, each with its own endodermis (Brown and Walsh 1994). The xylem of the vascular bundles provide water and the photo assimilates are supplied to the nodule by phloem (Bederska et al. 2012). Thus vascular system guarantees import of nutrients from the host and export of nitrogenous products from nodules, as effective nitrogen fixation depends on the balance between the import of photo assimilates and the export of nitrogenous solutes (Walsh et al. 1989; Streeter 1993; Schulze 2004; Bederska et al. 2012). Inner cortex is also important to protect the nitrogenase (a key enzyme in nitrogen fixation, which is highly sensitive to oxygen) by forming a diffusion barrier. However, this barrier is strongly dependent on the nodule cortical anatomy (Brown and Walsh 1994; Sujkowska et al. 2011; Bederska et al. 2012). A physiological paradox occurs in the nodule where the aerobic requirements of bacteroid and the oxygen sensitivity of nitrogenase both have to be dealt with. Protection against oxygen is provided by the nodule environment through a cortical diffusion barrier. The main route of oxygen diffusion is through nodule apex, which generates a longitudinal oxygen gradient. As a result, the free oxygen concentration drops to less than 50 nM in the central nitrogen-fixing zone containing *Rhizobium* bacteroids. Interestingly, bacteroid respiration in the central zone is made possible by a high concentration of leghaemoglobin and induction of a high-affinity cbb3 oxidase. Therefore, these microorganisms fix nitrogen in a microaerobic, nitrogen-rich environment, and thus nif gene induction during symbiosis is regulated by N (Dixon and Kahn 2004).

The indeterminate nodules are generally elongated and have a persistent meristem, while the determinate nodules are globose or obovate and lack a persistent meristem (Walsh et al. 1992). Lotocka et al. (2012) suggested that the appropriate term for indeterminate nodules should be 'nodules with indeterminate growth meristem'. The first cell division occurs in the cortex in indeterminate nodules and is anticlinal, whereas in determinate nodules' first cell division takes place in outer cortex (Ferguson et al. 2010). In indeterminate and determinate nodules, the vascular elements are surrounded by one to several layers of pericycle and an endodermis, but in indeterminate nodules, the vascular elements continue to differentiate throughout the nodule life, towards the meristem (Walsh et al. 1992).

There are certain plants that have been used historically for studies on indeterminate and determinate nodules. These include *Medicago sativa* (alfalfa), *M. truncatula*, *Pisum sativum* (pea), *Vicia* sp. (vetches) and *Trifolium* sp. (cloves) for indeterminate nodule and G*lycine max* (soybean), *Vicia faba* (bean) and *Lotus japonicus* for determinate nodules (Cook 2000; Handberg and Stougaard 1992). However, *Lotus japonicus* and *Medicago truncatula* that develop determinate and indeterminate nodules, respectively, are considered as model organisms (Lopez et al. 2008). Both these plants had a common ancestor ~40 MY ago; still they

Table 1 Summary of differences between determinate and indeterminate nodules

Determinate nodules	Indeterminate nodules
Formed in tropical legumes	Formed in temperate legumes
Generally globose or obovate in shape	Elongated/cylindrical
No persistent meristem	Meristem is persistent, and the vascular elements continue to differentiate throughout the nodule existence
Starch is rarely or never stored in infected cells	Starch is present in both infected and uninfected cells
First cell division is periclinal and the initials arise from outer/midcortical cells	First cell division is anticlinal and the initials arise from inner cortical cells
No clear zonation	Five histological zones can be seen
Infected cells have minimal vacuolation	Infected cells are highly vacuolated
The bacteroids have high viability and are normal rod shaped and many per symbiosome	The bacteroids have low viability and are enlarged and branched and one per symbiosome
Bacteroids represent same genomic DNA content	Differentiation of bacteroids causes genome amplification
Model organism for studies *Lotus japonicus*	Model organism: *Medicago truncatula*

form different types of nodules (Lavin et al. 2005). Both these plants are diploid, have small genome and can be inbred to form genetically homogenous lines. Other characters which make these plants a favourite material are their short life cycle and prolific seed production. The genome sequencing has been done for both these plants, and thus mutants specific to symbiotic nitrogen fixation have been characterized, and the responsible genes have been isolated (Cannon et al. 2006). Table 1 summarizes the differences between determinate and indeterminate nodules.

4.1 Indeterminate Nodules

In the indeterminate nodules, the activity of meristem is persistent and new cells are constantly added to the distal end, forming cylindrical nodules. The nodules, growth and functioning occur simultaneously, and the differentiation can be observed in a single longitudinal section. During the development of indeterminate nodules, the first cell division is anticlinal and occurs in the inner cortex, and then in the endodermis and pericycle, the periclinal divisions take place and a primordium is formed. Being indeterminate nodules, the nodule meristem maintains its activity throughout the growth cycle and apical meristem continuously produces new cells that are being infected by bacteria, thus a gradient of developmental stages/zones can be identified while the nodule continues to grow. Five histological zones can be distinguished in the fully developed indeterminate nodules (Timmers et al. 2000; Ferguson et al. 2010; Bederska et al. 2012).

The apical part (Zone I) consists of meristem and produces cells of the nodule tissue, but no rhizobia. The distal part has an invasion or symbiotic zone. Adjacent to this part is the infection zone (Zone II) that has young symbiotic tissue. In this zone the cell division comes to an end and bacteria are released from the infection threads into the cytosol. These bacteria then undergo endocytotic internalization and differentiate into bacteroids to form symbiosomes. Bacteroid is different from the free-living, rod-shaped rhizobia (Paau et al. 1978, 1980; Vasse et al. 1990). This differentiation of bacteroids is linked to morphological and cytological changes, such as cell elongation, genome amplification, membrane permeabilization and loss of reproductive capacity (Mergaert et al. 2006).

Next to this is a transitional zone or interzone II/III with starch stored in cells, and here bacteroid-containing tissues undergo final differentiation. Bacteroid differentiation is orchestrated with dramatic changes in the invaded plant cells, which enlarge and are highly polyploid (Timmers et al. 2000). Zone III is the differentiated zone, where nitrogen fixation takes place. There is a senescent zone (Zone IV), where symbionts degenerate (Vasse et al. 1990; Hirsch 1992). The presence of a saprophytic zone (Zone V) was first time shown in alfalfa by Timmers and coworkers (2000). In this zone, rhizobia neither undergo differentiation into bacteroid nor they are surrounded by membrane envelope and possess features of free-living bacteria. This zone is of special interest as it forms an ecological niche where intracellular rhizobia take advantage of the interaction for their exclusive benefit and live as parallel saprophytic partners (Timmers et al. 2000). These rhizobia are returned to the soil after nodule decomposition (Lotocka et al. 2012).

4.2 Determinate Nodules

In determinate nodules, the nodule zonation is not clearly distinguished. The determinate nodules originate from mitotic activity of the root outer cortex. However, the mitotic activity stops during development, and increase in nodule size occurs mainly due to cell expansion. The inner region of determinate nodule has rhizobia-colonized cells where symbiotic nitrogen fixation occurs and relatively small rhizobia-infected (as yet uncolonized) cells in the surrounding region. During the development process, the infection thread branches and penetrates the cells of the central region, once the infection thread comes in contact with nodule primordia. The bacteria bud off from the tips of the infection threads into the plant cytoplasm and the bacteria become enclosed by the peribacteroid membrane. The bacteroids of determinate nodules represent the same cell size, genomic DNA content and reproductive capacity as the free-living bacteria. Thus at the end of symbiosis, these bacteria can return to a free-living lifestyle and recolonize the rhizosphere (Mergaert et al. 2006).

5 Molecular Conversation

Symbiotic nitrogen fixation genes can be broadly divided into nod, nif and fix genes (Fischer 1994). The nod gene products are required for the early steps in nodule formation (which is discussed subsequently). In free-living diazotrophs such as *Klebsiella pneumoniae*, nif genes exist and are structurally homologous to rhizobial nif genes. The conserved nif gene plays a similar role in rhizobia as in *K. pneumoniae* (Long 1989; Fischer 1994, Schmitz et al. 2002). Alternatively, the term 'fix gene' is used for genes that are essential for nitrogen fixation but do not have a homologous counterpart in *K. pneumoniae* (Long 1969; Fischer 1994). However, the homologues of some of the fix genes exist in bacteria that do not fix N (Dixon and Kahn 2004). Interestingly, both nif and fix gene mutants are able to cause nodule development, but the nodules do not fix nitrogen (Long 1969). Regulation of nif expression is a complex phenomenon, and the regulatory events that control the transcription of the nif genes in free-living and symbiotic diazotrophs have been well reviewed by Dixon and Kahn (2004).

5.1 NF Perception and Signalling Cascade

Roots of the host plant attract the rhizobia and activate the expression of rhizobial nod genes that in turn leads to the production and secretion of strain-specific NFs. There are two receptor-like kinases (RLK) that are located on the epidermal cells: LjNFR1 and LjNFR5 in *L. japonicus* and MtLYK3/MtLYK4 and MtNFP in *M. truncatula* (Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003; Arrighi et al. 2006). These NF receptors comprise of an intracellular kinase domain, a transmembrane domain and an extracellular region having lysin motif (LysM) domains. These LysM domains mediate recognition of different NAG-containing ligands and facilitate microbial infection and symbiosis (Gust et al. 2012). Most of the NF receptors are characterized by the presence of activation loop (the site of phosphorylation). The NF receptors, LjNFR1 and MtLYK3/MtLYK4, have a typical serine/threonine kinase domain, but LjNFR5 and MtNFP do not have any activation loop (Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003). The absence of an activation loop in the receptor of one of the kinase domains gives an indication that the two LysM RLKs form a heterodimeric receptor, with the active kinase domain functioning in downstream signal transduction. Thus, the NF that is perceived by two LysM RLKs leads to NF signalling cascade, cortical and pericycle cell division and bacterial infection events (Cardenas et al. 1998; de Ruijter et al. 1998).

The signalling cascade involves potassium ion (K^+) channel proteins located in the nuclear membrane (encoded by MtDML1, LjCASTOR and LjPOLLUX) (Ané et al. 2004; Imaizumi-Anraku et al. 2005; Riely et al. 2007), two nucleoporins (encoded by LjNup133 and LjNUP85) (Kanamori et al. 2006; Saito et al. 2007)

and a calcium- and calmodulin-dependent protein kinase (CCaMK) (encoded by MtDM13/PsSYM9) (Lévy et al. 2004; Mitra et al. 2004). Rapid influx of Ca^{+2}, followed by membrane depolarization involving efflux of Cl^- and K^+ (Felle et al. 1999), induces oscillation in cytosolic Ca^{+2} concentration, which is known as Ca^{+2} ion spiking (Wais et al. 2000). Ca^{+2} spiking signals are perceived by CCaMK (Oldroyd and Downie 2004). Various mutation studies suggest that NF LRR RLK, the ion channels and the nucleoporins act downstream of NF perception, but upstream of Ca^{+2} spiking, and CCaMK acts downstream of Ca^{+2} spiking (Ferguson et al. 2010). Downstream of CCaMK, many transcription factors necessary for nodulation and nodule inception get activated. These include nodulation signalling pathway 1 (NSP1) (Smit et al. 2005), NSP2 (Kaló et al. 2005), Ets2 repressor factor required for nodulation (ERN) (Middleton et al. 2007) and nodule inception (NIN) (Schauser et al. 1999; Borisov et al. 2003). Mutational studies of NSP1 and NSP2 have shown that these mutants exhibit normal Ca^{+2} responses when treated with NFs but fail to initiate transcription of the early nodulation (ENOD) genes localized in the epidermis. NSP1 and NSP2 get activated after Ca^{+2} spiking (Catoira et al. 2000; Oldroyd and Long 2003), but probably downstream of CCaMK (Ferguson et al. 2010). In addition to these genes, the ENOD gene shows its expression in the epidermal cells. Experimental evidences suggest that expression of ENODs in the epidermis is an orchestrated effort of NSP1, NSP2, ERN1 and NIN (Andriankaja et al. 2007; Hirsch et al. 2009; Ferguson et al. 2010). NF signalling cascade, cell divisions in cortex and pericycle and bacterial infection, all these events occur in a coordinated way and give rise to a nodule.

5.2 Epidermal and Cortical Responses During Early Stages of Nodulation

During root hair invasion, bacterial infection is regulated by epidermis, whereas formation of a nodule is controlled by cortex. However, in case of crack invasion, the epidermis is breached and the bacteria gain direct access to cortical cells. The developmental processes in the cortex and epidermis are different, but coordinated such that a nodule primordium occurs close to the site of bacterial infection.

Essentiality of a cytokinin receptor for nodule development indicates cytokinin as a key player in nodule organogenesis. Indeed, cytokinin may be the mobile signal communicating epidermal perception of NF to the inner root (Subramanian 2013; Ferguson et al. 2010). Abscisic acid (ABA) has already been proposed as a mobile signal and is known to have a role in both the epidermis and cortex (Ding et al. 2008; Biswas et al. 2009; Ding and Oldroyd 2009). ABA is a negative regulator of nodule development and other plant hormones such as auxin, brassinosteroids and gibberellins acting as positive regulators. Like the hormones, there are certain factors and signals that are required both in the cortex and in the epidermis. For example, CCaMK is required in the epidermis and the cortex but involves entirely different pathways. Similarly, NSP1 and NSP2, which act downstream of CCaMK in the epidermis, and

downstream of CCaMK and the cytokinin receptor in the cortex is thus required in both epidermis and cortex (Heckmann et al. 2006). Another transcription factor, NIN, also appears to have a role in both epidermal and cortical cells. NF perception in the epidermis causes rapid responses in the inner root. In *M. truncatula* within 16 h, cytoskeletal rearrangement takes place in pericycle cells (Timmers et al. 1999). Such rapid response in the inner root to rhizobia/NF suggests some form of signalling communication.

The activation of the mitotic cell cycle and regulators of the cell cycle in cortical cells play an important role during the formation of a nodule primordium. A cytokinin receptor, which has a histidine kinase domain (encoded by MtCRE1/LjLHK1), functions in the root cortex and is essential for cell division events (Gonzalez-Rizzo et al. 2006; Tirichine et al. 2007). Downregulation, or loss of function, of this receptor results in decreased nodule numbers, as nodule primordium is not formed by plant (Gonzalez-Rizzo et al. 2006; Murray et al. 2007). During such an event rhizobial infections take place, but the infection threads lose their direction and spread laterally rather than growing towards the root cortex (Murray et al. 2007). Thus, nodule primordia formation or the cytokinin receptor is not mandatory for bacterial infection events, but for guiding the growth of infection thread.

The loss-of-function Mtdmi3 mutants produce a non-nodulation phenotype as CCaMK activity is required in the epidermis. But gain-of-function mutants of CCaMK result in spontaneous nodulation due to controlled cell divisions in the cortex (Gleason et al. 2006; Tirichine et al. 2006). However, presence of functional copies of NSP1 or NSP2 is essential for nodulation in gain-of-function mutants of CCaMK (Gleason et al. 2006; Tirichine et al. 2007). Thus, NSP1 and NSP2 act downstream of CCaMK in the epidermis.

The transcription factor, NIN, has a role in both epidermal and cortical cells (Schauser et al. 1999; Borisov et al. 2003; Marsh et al. 2007). In the epidermis, the mutant NIN plants show show excessive ENOD11 expression (suggesting that NIN is not essential for NF-induced ENOD11 expression), excessive root hair curling, blocked rhizobial infection (Schauser et al. 1999; Marsh et al. 2007) and in the cortex, such mutants are unable to initiate cell divisions and nodule primordium formation (Schauser et al. 1999; Borisov et al. 2003). NIN acts as a negative regulator of NF signalling to regulate the spatial expression of ENOD11 in the root epidermis (Marsh et al. 2007). The expression of NIN is brought out by cytokinin or NF application (Gonzalez-Rizzo et al. 2006; Murray et al. 2007), further supporting the idea that NIN positively regulates cortical cell divisions.

6 Legume–*Rhizobium* Under Stress

Stress refers to any environmental condition that affects normal growth, metabolism and development of organisms. More than 90 % of arable land experience one or other kind of stress and causes more than 50 % of crop loss worldwide. In the symbiotic association of rhizobium and legume, it is important to discuss the factors that affect the microbe, the host plant and the functioning of the symbiotic association, as the N_2

fixation is strongly related to the physiological state of the host plant (Bordeleau and Prevost 1994; Zahran 1999). Stress factors may be from natural or anthropogenic sources (i.e. due to human activities). Natural stresses include soil pH, soil moisture, salinity, temperature, nutrients, predation, etc. Stress leads to the generation of reactive oxygen species (ROS). These ROS react with biomolecules like proteins, nucleic acids, membrane lipids, etc. and hamper their normal functioning in the cell. Plants respond to these stresses through synthesis of metabolites and antioxidant enzymes that enhance tolerance mechanisms in plants under stress (Latef and Ahmad 2015). The presence of stress changes the physiological state of host plant, rhizobial survival in the soil, infection and nodule establishment and ultimately the nodule functioning. In the following section, major environmental constraints and their effects on the legumes, rhizobium and legume–*Rhizobium* interaction are discussed.

6.1 Soil pH

Soil acidity alters the availability of phosphorous, calcium and molybdenum and determines the toxicity of iron, aluminium and manganese (Muthukumar et al. 2014). Highly alkaline soil has sodium chloride, bicarbonate and borate which are toxic to both legume and rhizobium. Neutral or slightly acidic pH in soil is required for most leguminous plants (Bordeleau and Prevost 1994). In both tropical and temperate areas, the acidic pH limits the growth and survival of rhizobium strains in soil and nodulation and ultimately constrains nitrogen fixation of legumes (Graham et al. 1994). In a study by Tang and Thomson (1996), they studied the effects of pH (4, 5, 6, 7 and 8) and bicarbonate (5 mM $KHCO_3$) on the growth and nodulation of 14 grain legume species supplied with N or reliant on N_2 fixation. Species shows a broader optimal pH range for growth when supplied with N, but showed sensitivity at low pH when reliant on N_2 fixation.

Thus, soil pH affects the number of nodules, the nitrogenase activity, the nodule ultrastructure and the fresh and dry weights of nodules to a greater extent (Vassileva et al. 1997; Zahran 1999). Generally, nodulation problems occur once the pH falls below 5.5, as the rhizobium attachment to root hair is hampered (Bordeleau and Prevost 1994; Zahran 1999).

Taylor et al. (1991) suggested that at low pH, the rhizobial population decreases. However, not all the strains exhibit pH sensitivity, and rhizobia appear to be more tolerant to alkalinity and acidity and then do their legume hosts, thus host legume is considered to be a limiting factor for creating rhizobium–legume symbiosis (Tang and Thomson 1996; Graham et al. 1994; Zahran 1999). *Bradyrhizobium* (slow-growing strain) is more tolerant to low pH than the fast-growing strains of rhizobium, with the exception of *R. loti* and *R. tropici*. In pH-tolerant strains, the cytoplasmic pH is not altered much by external acidity. Experimental evidences suggest that this tolerance can be attributed due to the differences in the lipopolysaccharide composition of strains, proton exclusion and extrusion, synthesis of acid shock proteins and high

cytoplasmic potassium and glutamate levels (Aarons and Graham 1991; Bhat and Carlson 1992; Fujihara and Yoneyama 1993; Zahran 1999).

Like rhizobial species, the legume species also differ in their response to low pH (Tang and Thomson 1996). Muthukumar et al. (2014) suggest three possible mechanisms that enable plants to tolerate acidic conditions: (1) exclusion of toxic ions (such as Al and Mn) from the root apex, (2) tolerance to toxic levels of Al and Mn through detoxification in the plant symplasm and (3) enhanced efficiency in the uptake of limiting nutrients from acid soils (Kochian et al. 2005; Bhalerao and Prabhu 2013).

Soil acidity also limits the phosphorous availability, reduces legume growth and indirectly limits nodulation (Bordeleau and Prevost 1994). Phosphorous deficiency affects the growth of the legume host as well as of the symbiont. Rhizobial strains differ in their capacity to tolerate phosphorous deficiency, and generally slow-growing strains are more tolerant than the fast-growing ones (Zahran 1999). The presence of heavy metals such as aluminium further aggravates the problem of low pH, as under these conditions phosphorous is precipitated and becomes unavailable to plant and rhizobium in the rhizosphere. Thus, such conditions result in the stunted root growth, low calcium uptake by plant and reduced nodulation and nitrogen fixation (Zahran 1999; Azooz and Ahmad 2015). Wood et al. (1984) observed the symbiosis of *Trifolium repens* var Huia–*Rhizobium trifolii* strain HP3 in axenic solution culture system that under 10 mM phosphate and 50 mM Al, aluminium inhibits the root elongation at pH < 6.0 and root hair formation at pH < 5, while nodulation and rhizobium multiplication at pH < 6.0. Ferreira et al. (2012) demonstrated that the rhizobial strains of *R. tropici*, viz. UFLA04-195, UFLA04-173 and UFLA04-20, have greater efficiency for plant growth, shoot nitrogen content and nodulation when compared to strain CIAT 899.

6.2 Soil Moisture

Moisture regime determines the distribution, survival and activity of the rhizobium in their microhabitats (Orchard and Cook 1983; Zahran 1999). Waldon et al. (1989) isolated 74 rhizobial strains from the nodules of the desert woody legumes. Rhizobia isolated from surface and deep phreatic soil were compared. Jenkins et al. (1989) worked on three warm desert ecosystems such as sand dune, Chihuahuan Desert of New Mexico and Sonoran Desert of Southern California and observed that fast-growing rhizobia exists as free-living populations from 0 to 8 m depth, while slow-growing rhizobia dominated the surface 1 m of soil. However, this distribution of rhizobia was related to the concentration of total soil salts in the soils. Such studies show that rhizobia can exist in soil with low moisture contents but with low population density, while legumes are sensitive to extreme water regimes (Jenkins et al. 1989; Waldon et al. 1989; Tate 1995; Bordeleau and Prevost 1994). Faba bean and pea are known to be drought sensitive, whereas lentil and chickpea are known as drought-resistant genera (Azooz and Ahmad 2015). Tolerant

legumes show osmotic adjustment (Ford 1984) by accumulating osmolytes such as glutamic acids (Botsford and Lewis 1990), trehalose, N-acetylglutaminylglutamine amide (D'Souza-Ault et al. 1993; Smith et al. 1994), proline (Kapuya et al. 1985) and pinitol (*o*-methylinositol) (Ford 1984).

Low moisture induces oxidative damage in legumes thereby effecting nodule performance (Azooz and Ahmad 2015). When 1-month-old alfalfa plants were inoculated with *Sinorhizobium meliloti* strains 102 F78 and subjected to drought, a decrease in plant growth due to decrease in leaf area and decrease in nodule dry mass was observed (Aranjuelo et al. 2007). Interestingly, Talbi et al. (2012) in their study observed that inoculation of *Phaseolus vulgaris* with a *R. etli* strain (which has enhanced expression of cbb 3 oxidase) increased the tolerance of *Phaseolus vulgaris–R. etli* symbiosis to drought and modulate carbon metabolism in nodules. The degree of water stress on the rhizobium and its activity also depends on the age and growth stage of the host plant. For example, in *Phaseolus vulgaris*, Pena-Cabriales and Castellanos (1993) demonstrated that water stress is more detrimental to nodulation at reproductive stage, rather than at the vegetative stage. Nodules initiated under sufficient water conditions show retarded growth, if exposed to dry conditions. In dry soil infection is restricted because of the short, stubby root hairs, which are inadequate for rhizobial infection (Bordeleau and Prevost 1994). Rate of nitrogen fixation in legume plants decreased under drought stress due to the accumulation of ureides in nodules and shoots and reduced shoot nitrogen demand, xylem translocation rate and metabolic enzyme activity (Azooz and Ahmad 2015).

6.3 Soil Salinity

Salinity is a major abiotic stress limiting agricultural production especially in arid and semiarid regions (Munns and Tester 2008). Rhizobial strains are found to be salinity tolerant than their partner legumes; the tolerance in this symbiotic nitrogen fixation depends on the plant as well as the rhizobium genotype (Dogra et al. 2013). Soil salinity adversely affects the microbial population mainly because of ion toxicity and osmotic stress (Tate 1995; Zahran 1999). The bacteria adapt to saline conditions by the intracellular accumulation of low molecular weight organic solute osmolytes (Csonka and Hanson 1991); increase in intracellular free glutamate and/or K^+ (Zahran 1999); release of osmoprotectants such as sucrose, ectoine, mannitol, lactose, etc. (Talibort et al. 1994; Ghittoni and Bueno 1995; Gouffi et al. 1999); accumulation of glycine betaine (Fougère et al. 1991); and increase in the content of polyamines (Fujihara and Yoneyama 1993). In a study by Sharma et al. (2013), the isolated rhizobial strains from the root nodules of three leguminous plants, namely, sesbania (*Sesbania sesban*), lablab (*Lablab purpureus*) and pigeon pea (*Cajanus cajan*), growing at a research farm in Dubai were able to nodulate in saline water 12 dS m^{-1} on 21-day-old seedlings.

Saline soils limit the productivity of legumes by adversely affecting the growth of the host plant, the symbiotic development of rhizobial root nodules and their

nitrogen fixating capacity (Dogra et al. 2013). Some legumes, e.g. *Vicia faba*, *Phaseolus vulgaris* and *Glycine max*, are more salt tolerant than others, e.g. *Pisum sativum* (Zahran 1999). Increase in soil salinity causes water stress, ion toxicity, nutritional disorders, oxidative stress, alteration of metabolic processes, membrane disorganization by displacing membrane Ca^{+2} by Na^{+}, reduction of cell division and expansion and genotoxicity in legumes (Azooz and Ahmad 2015). In another study by Latrach et al. (2014), salinity decreased the plant height, their dry mass and nodulation in the symbiotic combinations of two Moroccan alfalfa (*Medicago sativa*) populations (Demnate and Tata) and two rhizobial strains (rhLAr 1 and rhLAr 4). Oufdou et al. (2014) inoculated faba beans by rhizobial strains RhOF6 and exposed plants to 150 mM of NaCl. In the plant inoculated by RhOF6, glutathione S-transferase (GST) activity was generally increased while a decrease in glutathione peroxidase (GPOX), superoxide dismutase (SOD), ascorbate peroxidase (APOX) and monodehydroascorbate reductase (MDHAR) in roots of faba bean.

The initial steps of legume–rhizobium interaction are inhibited under salt stress (Zahran 1991). The legume–*Rhizobium* symbioses and nodule formation on legumes are more sensitive to salt or osmotic stress than are the rhizobia. Tu (1981) demonstrated that failure of nodulation in soybean occurs due to decrease in rhizobial colonization and shrinkage of root hairs. Salt stress reduces nodule respiration and cytosolic protein production, especially leghaemoglobin, thus depressing nitrogen fixation (Zahran 1999).

6.4 Temperature

Ideally, 28–31 °C is found to be the optimal temperature for rhizobial growth, and they are generally unable to grow at and above 37 °C. Heat-tolerant rhizobia are likely to be found in environments affected by temperature stress. For example, rhizobial isolates from southern Nile Valley of Egypt were tolerant to 35–40 °C, although they formed less effective symbiosis with their legume hosts (Zahran 1999). At high temperature, the rhizobial strains become ineffective and outnumber the infective rhizobia in the rhizosphere. Zahran et al. (1994) reported that the heat stress changes the lipopolysaccharide pattern of some strains of rhizobia.

In legume host, elevated temperature negatively affects photosynthesis, respiration, water relations and membrane stability and also modulates levels of hormones and primary and secondary metabolites. The enhanced expression of a variety of heat shock proteins and production of ROS constitutes major plant responses to heat stress (Azooz and Ahmad 2015). Increased temperatures adversely affect root hair formation, adherence of bacteria to hairs, root hair infection, sites of nodulation, bacteroid differentiation and nodule structure and functioning, but accelerate nodule senescence (Roughley 1970; Roughley and Dart 1970; Pankhurst and Gibson 1973; Sutton 1983). Hungria and Franco (1993), in their study, screened strains of *Rhizobium leguminosarum* bv. *phaseoli* and observed that some of the strains were

able to nodulate beans even when given a heat shock of 35 and 38 °C for 8 h in a day. However, the nodules formed were ineffective and nitrogen did not accumulate in plants. Thermal shocks of 40 °C at the time of flowering decreased the nitrogenase activity and nodule relative efficiency of plants but recovered only when new nodules were formed.

Michiels et al. (1994) compared heat-tolerant (CIAT899) and heat-sensitive (CNPAF512) strains of bean-nodulating rhizobia: 14 heat shock proteins were detected in CNPAF512 at 40 °C and 6 heat shock proteins in CIAT899 at 45 °C. In cowpea, Simões-Araújo and co-workers (2002) showed similarities in the transcripts – fragments derived after heat shock to those that encode for wound-induced proteins, disease resistance protein, heat shock proteins and xylan endohydrolase isoenzyme, as well as different housekeeping genes.

In temperate legumes, elevated temperatures delay nodule initiation and development, interfere with nodule structure and functioning, whereas nitrogen fixation efficiency is mainly affected in tropical legumes (Bordeleau and Prevost 1994). However, in a study on alfalfa (temperate legume) by Aranjuelo et al. (2007), elevated temperature decreased not only plant growth but also CO_2 and N_2 fixation rates and inhibited nodule activity. The results were obtained by inoculating alfalfa with *Sinorhizobium meliloti* strain 102 F78 grown under different temperature (25/15 or 28/18 °C, day/night) and water treatments.

6.5 Predation

Rhizobium bacteria have to adapt to soil conditions in the soil ecosystem, and spatial distribution of rhizobia in the soil influences their survival (Postma et al. 1990). The rhizobium is attacked by number of organisms such as insect larvae (for source of food), nematodes, bacteriophages, viruses, etc. (Andrés et al. 2012). In addition to the competition for resources with other organisms, rhizobia also have to cope with the predation by the protists (Jousset et al. 2006). This decreases bacterial number and also plays an important role in controlling bacterial populations (Jjemba 2001; Rønn et al. 2002). As a response to predation, bacteria have developed several adaptations such as morphological changes, increased motility, toxin production and membrane properties that make them unattractive (Jousset et al. 2006). In a study by Pérez et al. (2014), it was observed that in a cocultured medium of *Myxococcus xanthus* (a soil bacterium) and strains of *Sinorhizobium meliloti*, the predatory pattern is determined by the galactoglucan released by the rhizobial strain.

7 Epilogue

Understanding the role of biological N_2 fixation can help us to achieve agricultural sustainability worldwide. However, the relationship between plants, soil microorganism and soil is multifaceted. Plants and microorganisms, directly or indirectly, play a

crucial role in the major ecological processes such as nutrient cycling, soil formation, improving soil fertility and BNF, etc. The process of nodulation involves two-way molecular conversation between the host legume and the bacteria. A number of natural and anthropogenic factors affect the legume–*Rhizobium* interaction. However, there are varieties of strains that can tolerate harsh environmental conditions. Optimum utilization of BNF can help us to achieve agricultural sustainability worldwide. The use of new inventions and its access to the farmers could be a step towards sustainability and will help in improving the economic status of the farmers. Therefore, more research program across the world to identify the superior legume varieties and rhizobial strains for the human welfare are required.

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Role of Phytohormones in Stress Tolerance of Plants

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Abstract Environmental stresses, both biotic and abiotic, cause negative impact on plant growth and development, and plants need to adopt certain strategies for maintaining proper growth under stress conditions. These strategies include certain physiological, biochemical, and molecular mechanisms to cope with these stresses. These mechanisms include the production of hormones (phytohormones) and osmolytes. Phytohormones are organic molecules that affect various plant physiological processes like growth, development, and cell differentiation. Phytohormones regulate key physiological events under normal and stress conditions. They play a vital role for enhancing the ability of plants to adapt to the harsh environmental conditions by mediating a wide range of adaptive responses. These responses enable the plants to acclimatize to adverse soil conditions. Various types of phytohormones play an important function in plants individually or in coordination with each other. The nature and level of these hormones in plants are major factors that influence plant processes and functions. The present chapter describes the potential role of phytohormones for promoting plant growth and development under stress conditions. The major classes of plant hormones and their source of production have been described. Metabolism of phytohormones and their physiological responses with special reference to their concentration-dependent or negative impact on plant growth have been discussed in detail. The impact of these hormones on plant growth under stress conditions has been reviewed and discussed with selected examples. Also, the role of microbes in phytohormone production has been elaborated with examples. Future perspectives of the area have also been discussed.

Keywords Brassinosteroids • Gibberellins • Phytohormones • Stress tolerance

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1 Introduction

Stress conditions cause significant negative effect on crop productivity by disturbing plant processes owing to their impact on hormonal and nutritional imbalances. Some of the common stresses that cause negative impact on plant growth and development include salinity, drought, heavy metals, nutrient deficiency, and pathogens. These stresses affect the plant growth in one way or another. One stress may affect more than one plant processes by causing negative impact in a number of ways. For example, salinity affects plant growth by causing ion toxicity, oxidative stress, nutritional disorders, water stress, and hormonal imbalances (Munns 2002; Zhu 2007; Ashraf 2009; Nadeem et al. 2010a). In natural soil environment, plant develops certain mechanisms to cope with biotic and abiotic stresses in harsh environment. Multiple pathways of cellular signaling are activated to any given stimuli. These signals enhance the accumulation of phytohormones. Phytohormones are signaling molecules directing physiological and developmental processes in plants. The amount of hormones varies greatly depending upon certain biotic and abiotic factors. Even very low concentration of these hormones may cause significant impact on plant growth and development.

Hormonal signaling is critical for plant defenses against environmental stresses (Taiz and Zeiger 2010). Production of phytohormones plays central role in plant stress tolerance. The five major classes of phytohormones are auxin, cytokinins, ethylene, gibberellins, and abscisic acid. In addition to these well-known plant hormones, brassinosteroids, jasmonic acid, salicylic acid, and nitric acid have also been identified as chemical messengers present in trace quantities in plants (Rao et al. 2002). These hormones move throughout the plant body via the xylem or phloem transport stream.

Among these hormones, abscisic acid (ABA) is the most studied stress-responsive hormone that is involved in number of stresses including osmotic, drought, and cold stress (Peleg and Blumwald 2011; Wasilewska et al. 2008). Auxin is involved in the regulation of plant processes like organogenesis, embryogenesis, and vascular tissue formation (Petrasek and Friml 2009). Brassinosteroids, that is a new group of plant hormones, influence plant development processes like seed germination, flowering, and senescence (Rao et al. 2002).

These biochemical substances (phytohormones) are produced by plants (Santner et al. 2009), and it is a well-documented concept that phytohormones perform many functions in plants by influencing a number of physiological and biochemical processes of plant. These hormones also play an important role in mitigating the negative impact of various environmental stresses, both biotic and abiotic, on plant growth and development. These hormones integrate biotic and abiotic stress signals. Stress environment activates phytohormone signaling pathway that plays an important role in stress adaptation. It has been reported that adverse effect of salt stress on seed germination and plant growth was due to the decline in endogenous level of phytohormones (Wang et al. 2001; Debez et al. 2001). This argument was further supported when plant growth was enhanced under stress conditions by the exogenous application of phytohormones (Khalid et al. 2006; Egamberdieva 2009).

These and a number of other studies show that although plant itself has its mechanism to produce hormones to combat stress responses (Wasternack 2007; Xoconostle-Cazares et al. 2010; Kolaksazov et al. 2013), however its application from some other sources like inoculation with hormones producing bacteria and/or application of synthetic phytohormones may be useful for alleviating stress-induced impact on plant growth and development (Khan et al. 2004; Afzal et al. 2005; Egamberdieva 2009). This review highlights the importance of phytohormones in plant stress tolerance. The sources of phytohormones, its metabolism, and physiological impact of these hormones on plant growth particularly under stress environment have been reviewed and discussed in the following sections.

2 Sources of Phytohormones

Plant hormones or phytohormones are naturally occurring organic compounds that affect various physiological processes of plant. These hormones could be either synthesized by the plant or the microorganisms.

2.1 Plant Hormones

Plants have high plasticity for adaptation to certain environmental stresses by virtue of their specific mechanisms like their ability to synthesize endogenous hormones. Hormones are involved in response to certain environmental stimuli as well as for regulating internal development processes. Among phytohormones, auxin was the first hormone about which Charles Darwin in 1880 provided clue in his book entitled *The power of movement of plants*. Later on, in 1926, Dutch botanist Frits W. Went discovered auxin. L-Tryptophan is the precursor of the auxins, and root exudates are the main source of auxins in soil (Etesami et al. 2009).

These hormones serve as endogenous messengers against biotic and abiotic stresses. Initially, five plant hormones are identified including ethylene, abscisic acid, cytokinins, gibberellins, and auxins (IAA). These hormones are considered as classical phytohormones, and higher plants can synthesize all these five major classes of phytohormones. In addition to these well-documented hormones, brassinosteroids, jasmonic acid, polyamines, strigolactones, nitric oxide, and salicylic acid are also included in the list of phytohormones (Santner et al. 2009; Chen et al. 2009a). These hormones have been identified from a variety of plants (Table 1).

The synthesis of a plant hormone is tightly regulated and subject to positive or negative feedback mechanism and often affected by other hormones and environmental factors. A number of stress phytohormones can mediate stress tolerance in plants (Wasternack 2007). For example, drought tolerance limits the loss of water through abscisic acid-mediated closure of stomata (Xoconostle-Cazares et al. 2010). Also, an increase in content of jasmonates and salicylic acid in wheat has been observed under cold stress (Kosova et al. 2012; Kolaksazov et al. 2013).

Table 1 Phytohormones production by plants

Plant	Hormone	Reference
Barley (*Hordeum vulgare* L.)	Indole-3-acetic acid	Ayvaz et al. (2012)
	Abscisic acid	Ayvaz et al. (2012)
Alpine (*Arabis alpina*)	Jasmonate	Kolaksazov et al. (2013)
Wheat (*Triticum aestivum*)	Jasmonic acid	Kosova et al. (2012)
	Ethylene	Datta et al. (1998)
	Abscisic acid	Zhao et al. (2001)
	Salicylic acid	Kosova et al. (2012)
Arabidopsis (*Arabidopsis thaliana*)	Indole-3-acetic acid	Bartling et al. (1994)
	Gibberellins	Kobayashi et al. (1994)
	Abscisic acid	Xiong et al. (2001)
	Cytokinins	Takei et al. (2001)
	Auxin	Wang et al. (2015)
Rice (*Oryza sativa*)	Gibberellins	Helliwell et al. (2001)
Maize (*Zea mays*)	Gibberellins	Spray et al. (1996)
	Abscisic acid	Tan et al. (1997)
Bean (*Phaseolus vulgaris* L.)	Brassinosteroids	Yokota et al. (1987)
Conifer (*Cryptomeria japonica*)	Brassinosteroids	Watanabe et al. (2000)
Tomato (*Lycopersicon esculentum*)	Brassinosteroids	Yokota et al. (2001)
	Ethylene	Mayak et al. (2004)
Chick pea (*Cicer arietinum*)	Ethylene	Kukreja et al. (2005)
Potato (*Solanum tuberosum* L.)	Salicylic acid	Coquoz et al. (1998)

Brassinosteroids are considered as the sixth group of phytohormones that was isolated from pollen of rape plant (*Brassica napus* L.) (Rao et al. 2002) and confers resistance to biotic and abiotic stresses (Sasse 2003; Mussig 2005). Brassinosteroids are present in plants at extremely low concentration, and their level varies in plant tissues with higher concentration of brassinosteroids in young tissues compared to mature ones (Yokota and Takahashi 1986). In addition to this, occurrence of jasmonic acid and salicylic acid is also reported in plants which are involved in various developmental processes like seed germination, root growth, and senescence (Creelman and Rao 2002; Chen et al. 2009b; Wasternack and Hause 2002, 2013).

Phytohormones move throughout the plant body and are distributed within plant tissues from cell to cell via the xylem or phloem transport stream. These hormones can cause significant impact on plant physiological processes. The excess amount of hormones may be stored in plant tissues as conjugates for further use. Plant gene and phytohormones interact with each other. Some genes activate the plant hormones, whereas certain hormones activate the genes as well. Many genes are involved in hormone perception and signaling pathways that control the production and activity of hormones by expression level of relevant gene. In plants, there are hormone receptors with high affinity responding to the phytohormones. The activities of phytohormones are affected by different parameters that include the properties and affinity of the receptors as well as the cytosolic Ca^{2+} (Weyers and

Paterson 2001). Most plant cells have receptors for different hormones. These cells recognize the hormones, and when a hormone meets the right receptor, it triggers a response.

The release of hormones is a normal physiological process of a plant during its life cycle and is also mediated by environmental conditions. After the release of hormones, these may act either close to or remote from their sites of synthesis to regulate responses to environmental stimuli (Davies 2004). Plant hormones operating at low concentration are able to translocate within the body and bind to a specific receptor protein.

2.2 *Microbial Hormones*

It is well established that two types of hormones are available to plants, one is endogenous production by plants and second is exogenous production by microorganisms. Like plants, a number of microorganisms residing in the soil also produce phytohormones; however, their pathways for hormone production may be different from plants. So far, a number of bacterial and fungal strains have been evaluated for their ability to produce phytohormones, and some selected examples have been mentioned in Table 2.

Table 2 Microbial production of phytohormones

Microbe	Hormone	Reference
Bacterial phytohormones		
Stenotrophomonas maltophilia SSA	Indole-3-acetic acid, gibberellic acid, trans-zeatin riboside, and abscisic acid	Naz and Bano (2012)
Pseudomonas mendocina Khsr2, *Pseudomonas stutzeri* Khsr3, and *Pseudomonas putida* Khsr4	Indole-3-acetic acid, gibberellic acid, trans-zeatin riboside, and abscisic acid	Naz and Bano (2012)
Bradyrhizobium japonicum	Indole-3-acetic acid	Minamisawa and Fukai (1991)
Bacillus subtilis IB-22	Cytokinins	Kudoyarova et al. (2014)
Pseudomonas putida	Indole acetic acid	Gravel et al. (2007)
Azospirillum spp.	Abscisic acid, gibberellins	Cohen et al. (2009)
Rhizobium phaseoli	Gibberellins, indole-3-acetic acid	Atzorn et al. (1988)
Bradyrhizobium japonicum	Indole-3-acetic acid, gibberellic acid, abscisic acid	Boiero et al. (2007)
Pseudomonas putida	Indoleacetic acid	Patten and Glick (2002)
Rhizobium leguminosarum	Indole-3-acetic acid, ethylene	Dazzo et al. (2000)
Pseudomonas putida	Indole-3-acetic acid, ethylene	Mayak et al. (1999)
Azotobacter chroococcum	Gibberellin	Pati et al. (1995)
Azospirillum brasilense	Auxin, abscisic acid	Kolb and Martin (1985)
Bacillus pumilus, *Bacillus licheniformis*	Gibberellins	Gutierrez-Manero et al. (2001)

(continued)

Table 2 (continued)

Microbe	Hormone	Reference
Azospirillum brasilense	Indole acetic acid (IAA) and gibberellic acid	Kumaran and Elango (2013)
Pseudomonas sp.	Indole acetic acid	Malik and Sindhu (2011)
Fungal phytohormones		
Cladosporium sp.	Gibberellins	Hamayun et al. (2010a)
Penicillium citrinum	Indole acetic acid	Khan et al. (2008a, b)
Paecilomyces formosus LHL10	Gibberellins, indole acetic acid	Khan et al. (2012a, b)
Fusarium oxysporum	Gibberellin and auxin	Hasan (2002)
Phoma glomerata LWL2 and *Penicillium* sp. LWL3	Gibberellins, indole acetic acid	Waqas et al. (2012)
Aspergillus fumigatus sp. LH02	Gibberellins	Khan et al. (2011b)
Aspergillus fumigatus	Gibberellins	Hamayun et al. (2009)
Trichoderma atroviride	Indole acetic acid	Gravel et al. (2007)
Pisolithus tinctorius	Indole-3-acetic acid	Frankenberger and Poth (1987)
Cyanobacteria/algal phytohormones		
Nostoc OS-1	Indole-3-acetic acid	Hussain et al. (2015)
Nostoc PCC 73102	Indole-3-acetic acid	Sergeeva Prasanna et al. (2002)
Anabaena	Indole-3-acetic acid	Prasanna et al. (2010)
Oscillatoria annae	Indole-3-acetic acid	Varalakshmi and Malliga (2012)
Scenedesmus obliquus	Indole-3-acetic acid	Correa et al. (2011)
Haematococcus pluvialis	Abscisic acid	Kobayashi et al. (1997)
Chlorophyta (Cyanobacteria)	Cytokinins	Ordog et al. (2004)
Cyanophyta (Cyanobacteria)	Cytokinins	Stirk et al. (2002)
Dunaliella sp.	Abscisic acid	Tominaga et al. (1993)
Dunaliella salina	Abscisic acid	Cowan and Rose (1991)
Hydrodictyon reticulatum	Brassinosteroids	Yokota et al. (1987)

Among this microbial population, beneficial bacteria commonly known as plant growth-promoting rhizobacteria (PGPR) are the major contributor of phytohormones. These PGPR promote the plant growth and development by the production of phytohormones and act as biostimulators (Glick et al. 1998; Jimenez-Delgadillo 2004). A number of workers have reported the production of phytohormones by a significant population of bacteria (Arshad and Frankenberger 1998; Rao et al. 2002; Baca and Elmerich 2003; Khalid et al. 2006; Egamberdieva 2009). The type and amount of hormones produced by microorganisms are variable depending upon microbial community as well as suitable substrate or precursor available to the microorganisms. For example, Khalid et al. (2004) reported that among 30 bacterial isolates, 22 were able to use precursor of indole-3-acetic acid (IAA). They further reported that in the presence of IAA precursor, i.e., L-tryptophan, the bacterial efficiency

to synthesize IAA enhanced manyfold. Likewise, mevalonic acid is the substrate for the microbial synthesis of gibberellins and abscisic acid; however, formation of different intermediate compounds during the growth phase of a microorganism determines what will be the final compound, i.e., a gibberellin or an abscisic acid. It is worth noting that the presence of a precursor in the environment can stimulate/enhance the synthesis of phytohormones and it may or may not be the basic requirement for microbial synthesis of hormones. A PGPR strain can also produce a phytohormone in the absence of a substrate in the soil environment as it has been observed in the case of IAA production by certain bacteria without precursor (Khalid et al. 2006).

Free-living bacteria and bacteria living in association with plants produce phytohormones. For example, free-living *Azospirillum brasilense* and symbiotic *Bradyrhizobium japonicum* have been reported to produce indole-3-acetic acid, gibberellic acid, and zeatin (Cassan et al. 2009). Similarly, an endophytic bacterium *Sphingomonas paucimobilis* ZJSH1 has also been found to produce indole acetic acid, salicylic acid, abscisic acid, and zeatin (Yang et al. 2014).

Apart from bacterial population, a number of fungi present in soil environment are also able to produce growth hormones. Akhtar et al. (2005) reported that 78, 83, 89, and 72 % fungal strains isolated from wheat, maize, potato, and tomato rhizosphere, respectively, were able to produce ethylene in the presence of L-methionine. Reports about phytohormone production by endophytic fungi are also available (Khan et al. 2008a, b; Hamayun et al. 2010a). Khan et al. (2011a) reported gibberellin production by endophytic *Aspergillus fumigatus* sp. LH02. Brassinosteroids have also been identified in unicellular green algae *Chlorella vulgaris* (Bajguz 2009; Stirk et al. 2013). Kim et al. (2005) reported more than 50 naturally occurring brassinosteroids from the entire plant kingdom.

3 Metabolism of Phytohormones

In an earlier study, Bont et al. (1979) observed that ethylene metabolism in *Mycobacterium* involves the epoxidation of the double bond by a mono-oxygenase. Wiegant and DE Bont (1980) found a new route for the degradation of ethylene glycol via acetaldehyde and acetate. They found that ethylene glycol was not an intermediate in ethylene metabolism.

It has been observed that in addition to synthesize IAA, some strains of *B. japonicum* are also able to catabolize IAA. Jensen et al. (1995) studied the catabolism of indole-3-acetic acid and 4- and 5-chloro-indole acetic acid by two strains of *Bradyrhizobium japonicum* (strains 61A24 and 110). They observed that both strains metabolized IAA with different efficacies and IAA was metabolized via dioxindole-3-acetic acid, dioxindole, isatin, and 2-aminophenyl glyoxylic acid (isatinic acid) to anthranilic acid. They reported that degradation of 4-Cl-IAA apparently stopped at the 4-Cl-dioxindole. The metabolism of IAA by peroxidases has been reported by oxidizing IAA via two different mechanisms: one is conventional mechanism that requires H_2O_2 (Schulz et al. 1984) and the other one is not dependent on H_2O_2 and requires O_2 instead of H_2O_2 (Savitsky et al. 1999).

Cytokinins are present in plants both as free base and the corresponding nucleosides and nucleotides. In earlier studies, first of all, Paces et al. (1971) demonstrated the oxidative cleavage of cytokinins in a crude tobacco culture. Later on Whitty and Hall (1974) named this cleavage as enzyme cytokinin oxidase. Mok et al. (2000) reviewed the synthesis and metabolism of cytokinin and reported that a number of enzymes were involved in cytokinin metabolism and these enzymes were not cytokinin specific. Cytokinin dehydrogenase is the enzyme that catalyzes irreversible inactivation of cytokinins. For years, it was assumed that molecular oxygen was essential for the activity of cytokinin dehydrogenase; however, the work of Galuszka et al. (2001) and Frebortova et al. (2004) showed that other electron acceptors, especially quinone types such as 2,3-dimethoxy-5-methyl-1,4-benzoquinone, also functioned efficiently other than oxygen. In addition to many plant species, the activity of this enzyme has also been reported in few lower organisms like moss, slime mold, and yeast (Gerhauser and Bopp 1990; Armstrong and Firtel 1989; Van Kast and Laten 1987).

4 Physiological Effects of Phytohormones

Phytohormones are naturally occurring substances that are produced by the plant and play a very important role in certain physiological processes of plant. These are released by the plant during its life cycle in normal conditions and also in response to some environmental stimuli. These signal molecules are present in trace quantities and are actively involved in many biochemical processes (Ogweno et al. 2010). Among these hormones, ethylene, jasmonic acid, and salicylic acid play a role in biotic stress tolerance, and abscisic acid plays a role in regulating the abiotic stress tolerance (Ton et al. 2009; Bailey et al. 2009; Kavroulakis et al. 2007; Hadi and Balali 2010). However, some of these phytohormones are also equally effective for promoting biotic and abiotic stress tolerance in plants like jasmonates and salicylic acid (Hadi and Balali 2010; Hara et al. 2012; Khan and Khan 2013; Kazan 2015).

These hormones affect almost all the processes of plant life cycle and also play a critical role in plant defense system against environmental stresses both biotic and abiotic (Taiz and Zeiger 2010; Williams 2010). The effectiveness of the hormone depends upon its suitable concentration, its production at the right place and time, as well as its interaction with specific receptor. Depending upon their nature and concentration, they may cause positive and negative impacts on plant growth and development. They exert their influence on target cells where they bind transmembrane receptors, and depending upon the context, they are subject to positive or negative feedback control. Some of the major positive and negative impacts of these hormones on plant growth are discussed in the following sections.

4.1 Positive Effects

The role of phytohormones for accelerating plant growth and development is well documented. Hormones affect almost all physiological processes of plant. These hormones also enhance the plant resistance against unfavorable conditions and protect the plant from negative impact of a number of biotic and abiotic stresses. Among these hormones, ABA serves as an endogenous messenger in biotic and abiotic stress responses of plants (Adie et al. 2007; Ton et al. 2009). Gibberellic acids have been shown to have an effect on reactive oxygen and antioxidant activities (Tian et al. 2011; Wang et al. 2012).

Schumacher and Chory (2000) reviewed the role of brassinosteroids and reported that these hormones were required for a wide range of plant developmental processes including shoot and root elongation, seed development and germination, and development of vascular tissue. Gibberellic acids are also involved in stem and leaf elongation, flower induction, trichome, anther, seed germination, and fruit and seed development (Pharis and King 1985; Singh et al. 2002). Jasmonic acid (JA) induces resistance to a broad range of herbivores and is known to reduce the growth and survivorship of many insects. Fan et al. (2014) reported the role of JA to enclose the invading nematodes at the initial site of infection and then inhibit nematode multiplication and spread. The application of cytokinins also proved useful for regulating the plant response to environmental stress (Ha et al. 2012).

Auxin is a well-known group of phytohormones that plays a significant role in the initiation of primary root growth and promotion of root hair and lateral root formation (Takahashi 2013). The involvement of auxin in plant-microbe signaling is also known (Berg 2009). Recently, Kovaleva et al. (2015) observed that the addition of IAA to the nutrient medium increased the content of actin cytoskeleton (F-actin) in the apical and subapical zones of pollen tubes that might be responsible for the stimulation of pollen growth. This argument is supported by further observations such as the decrease in the content of endogenous IAA, inhibited germination, and/or blocked male gametophyte polar growth. It is also evident from the work of Tian et al. (2008) who observed that root inhibition due to high nitrate concentration was closely related to the reduction of IAA level in roots.

This positive impact of auxin is not only observed due to naturally occurring auxin compounds but also with the application of synthetic ones. It was observed from the work of Bajguz and Piotrowska-Niczyporuk (2014) that the application of natural as well as synthetic auxins caused significant impact on the growth, metabolite content, and antioxidant response of green alga (*Chlorella vulgaris*).

These phytohormones work individually and in coordination with each other and cause impact on plant physiological processes. An increase in ABA level has been observed in green algae when exposed to heat stress in the presence of brassinosteroids (Bajguz 2009). The synergistic role of ABA in regulating plant growth and development with brassinosteroids, gibberellic acid, and auxin has also been reported by workers (Zhang et al. 2009; Achard et al. 2006). Similarly, physiological activity of brassinosteroids is largely consistent with physiological influences

exerted by auxins. Stimulation of cell proliferation and endogenous accumulation of proteins, chlorophylls, and monosaccharides has been in algal cell by this synergistic interaction (Bajguz and Piotrowska-Niczyporuk 2013). Although the basic mechanisms of these interactions are not much clear however, it has been reported that these interactions are possibly mediated through various metabolic pathways (Kudryakova et al. 2013; Bajguz and Piotrowska-Niczyporuk 2014). Along with auxins and gibberellins, brassinosteroids promote cotton fiber initiation and elongation in the cultured ovule system (Sun et al. 2005; Shi et al. 2006).

These hormones in addition to controlling intrinsic growth also mediate adaptation of plant development to changing environmental conditions (Tuteja and Sopory 2008; Wolters and Jurgens 2009). Javid et al. (2011) reviewed the role of phytohormones in alleviating salt stress in crop plants. They concluded that the concentration of auxin, cytokinins, gibberellins, and salicylic acid decreased in the plant tissues under salinity stress, while an increase was observed in abscisic acid and jasmonate level. They demonstrated that changes in hormonal level is the cause of growth reduction under salinity stress and this negative impact can be diluted by the application of plant growth regulators. Cabello-Conejo et al. (2014) evaluated the phytoextraction capacity and growth of four Ni hyperaccumulating species (*Alyssum malacitanum*, *Alyssum corsicum*, *Alyssum murale*, and *Noccaea goesingense*) in the presence of four phytohormones (B, C, K, and P) based on gibberellins, cytokinins, and auxins. They observed that plant species were varied regarding biomass production and depend on the type of PGR and its rate of application. A significant increase in plant biomass and Ni accumulation was observed with the application of phytohormones, and most effective results were obtained in case of Ni accumulation with auxin-based product.

4.2 Negative Effects

Phytohormones cause positive effects on a number of plant processes; however, certain negative impacts on plant growth have also been observed due to these hormones. As discussed in previous sections, the effects on plant growth by phytohormones may be variable which depends upon their concentration, environmental factors, and physiological status/process of the plant. A phytohormone enhances plant growth up to a particular concentration, and growth inhibition may occur if concentration increases from that particular level. For example, ethylene that plays significant role in a number of plant processes also causes negative impact on plant growth and development due to its elevated level particularly under stress environment (Nadeem et al. 2010a). At low concentration, promotion of root growth while at high concentration inhibition of root elongation has been observed (Mattoo and Suttle 1991; Belimov et al. 2002). Ethylene plays an important role in legume-rhizobia association and causes significant impact on rhizobial infection in legumes (Penmetsa and Cook 1997). Inhibition of nodulation with ethylene has been observed whether it was applied directly as a gas or in the form of its precursor like

ACC (Yuhashi et al. 2000). This argument is further supported when nodulation was restored after treating with ethylene inhibitor (Guinel and Sloetjes 2000). Ethylene also affects the plant growth negatively by causing certain disorder or many other disorders such as leaf abscission, senescence, epinasty, and chlorophyll destruction (Shibli et al. 2007; Nadeem et al. 2010b). In addition to ethylene, indole acetic acid and abscisic acid are also known to modulate abscission (Suttle and Hultstrand 1993; Sexton and Roberts 1982).

Kukavica et al. (2007) observed that IAA inhibited the root elongation of hydroponically grown pea plants. IAA induced the disappearance of peroxidase isoforms and hydroxyl radical formation in the root and the root cell wall. Malik and Sindhu (2011) while studying the impact of co-inoculation of indole acetic acid producing *Pseudomonas* sp. with *Mesorhizobium* on chickpea (*Cicer arietinum*) growth and nodulation observed that exogenous seed treatment with higher concentration of IAA (10.0 μM) inhibited the growth of seedlings.

Similar response was also observed in the case of other hormones. For example, the phytotoxicity caused by some bacteria and fungi was due to the suppression of root growth by secretion of IAA at high concentration (Barazani and Friedman 1999a; Ditengou and Lapeyrie 2000). ABA positively affected the leaf size and bud dormancy and negatively influenced the size of guard cells and internode length (LeNoble et al. 2004). Severe inhibition of pollen germination and pollen tube growth was observed due to the application of gibberellins to grape flowers before or during anthesis (Kimura et al. 1996).

The phytohormones interact with each other, and this interaction may be a negative one, as observed in the case of jasmonic acid and gibberellins (Heinrich et al. 2013) where a high level of jasmonic acid antagonizes the biosynthesis of gibberellins. This decrease in gibberellins results in the inhibition of stem elongation of *Nicotiana attenuata*. They reported that this inhibition of gibberellins was due to high level of jasmonic acid.

5 Environmental Stresses and Plant Growth

The growth and productivity of plants are affected by various biotic and abiotic stresses. The plant growth is affected by osmotic stress, ionic toxicity, nutrient, and hormonal imbalances (Ashraf 2004; Munns et al. 2006; Ashraf and Foolad 2007). The important environmental stresses that effect plant growth and development include drought, salinity, high temperatures, freezing, flooding, and mechanical impedance.

Among environmental stresses, soil salinity is one of common problems of various arid and semiarid regions. Salinity causes an adverse effect on soil by degrading its quality, reducing the area of crop cultivation, and minimizing the crop yield (Sadiq et al. 2002). In arid and semiarid regions, 50 % reduction in the yield of major crops has been observed owing to salinity (Munns 2005; Keshtehgar et al. 2013). Growth inhibition is one of the primary impacts of salinity on plants that

might be due to its negative impact on photosynthesis as well as cellular disruption and oxidative disintegration under saline environment (Zhu 2007). Almost all morphological and physiological processes of plants are affected by salinity. In salty conditions, among various ions that cause a negative impact on plant growth, the effect of sodium is more pronounced that interferes the potassium uptake (Zhu 2007) that results in potassium deficiency in plant (Nawaz and Ashraf 2010). This increased Na/K ratio causes certain cellular and nutritional imbalances in plants like reduction in soluble sugars and essential nutrients (Ibrahim et al. 2006). Lowering of membrane stability of vital cell organelles was also observed due to high Na/K ratio (Gadallah 1999; Heuer 2003). In addition to Na^+, higher chloride (Cl^-) concentrations also disturb the plant metabolic activities by affecting the activities of certain enzymes. For example, plant losses its ability to maintain their osmotic pressure due to high chloride ion in the cytoplasm (Misra and Saxena 2009). In saline environment, inhibition of RNA and DNA synthesis has been observed due to reduced production of certain amino acids and respective nitrogenous bases required for this purpose (Chen et al. 2003; Song et al. 2006).

For plants, water availability is considered as a major dictating factor for their production. Under salinity stress, due to the increase in soil osmotic potential, soil water becomes unavailable to plants and extraction of water from soil becomes difficult (Nawaz et al. 2010) that result in the disturbance of certain cellular and metabolic activities of plants that leads to improper plant growth and development (Munns 2005). Under water-deficit conditions, plant growth is adversely affected due to alteration in many key physiological processes related to growth and reproduction (Manivannan et al. 2008). Plants respond promptly to water stress, and the consequences of even a short-term drought at any growth stage cause negative impact on plant's whole life cycle. Drought affects almost every morphological, physiological, and biochemical aspect of plant and poses severe limitations for crop production (Aroca 2012). The literature revealed that drought mainly affects key processes regarding cell division, water relations, nutrient uptake, nutrient assimilation, energy transfer, carbon fixation, and photosynthesis (Yamance et al. 2003; Gomes et al. 2010; Taiz and Zeiger 2010; Asrar and Elhindi 2011). The reduction in water contents of cytoplasm restricts the cell division, elongation, and differentiation primarily due to decrease in turgor pressure, metabolic activity, and inhibition of energy transfer (Taiz and Zeiger 2010). The inhibition of cell multiplication adversely affects the vegetative and reproductive growth due to lower biomass accumulation leading to stunted root and shoot growth, poor flowering, and fruit development (Asrar and Elhindi 2011). Under water-deficit conditions, plants show limited nutrient uptake and become nutrient deficient. Several reports indicate that under water shortage, plants show significant reduction in all macro- and micronutrients in roots and shoots, especially nitrogen, potassium, and phosphorus (McWilliams 2003; Subramanian et al. 2006; Asrar and Elhindi 2011).

Temperature affects every physical, chemical, and biological process in living cells. A slight increase or decrease in temperature can cause irreversible damage to crop plants. High temperature is the most extreme form of temperature stress which

is challenging the plant's survival in extreme climatic conditions. Plants under high temperature stress show a variety of responses at cellular and molecular level. High temperature adversely affects the growth, phenology, biochemistry, physiology, and anatomy of plant (Wahid et al. 2012).

Like other stresses, the effect of high temperature on plant growth can be observed at any stage from germination to seed production. Ren et al. (2009) reported that high temperature inhibits the germination due to alteration in expression of protein profiles. At germination, plants are more sensitive to temperature change that severely inhibits the seedling emergence and its development (Egli et al. 2005). Specific enzymes involved in germination are denatured by high temperature that inhibits the growth of germinating embryos (Wahid et al. 2012). Photosynthesis is also affected by heat stress as high temperature destroys the mesophyll cell followed by deshaping and swallowing of chloroplasts, stroma, and lamella, severely affecting the activity of photosystem II (Carpentier 1999).

The chilling injury refers to the extreme low temperature but slightly above freezing point, while freezing injuries occur when freezing temperature prevails and solutions in plants start freezing followed by crystallization resulting in complete ceasing of biochemical machinery and rupture of membrane structures (Sokolnik 2012). The disturbance in biochemical mechanisms also results in the production of reactive oxygen species (ROS) that induces oxidative stress, as low temperature hinders the functioning of oxido-reductive enzymes, e.g., catalase inhibition leads to higher accumulation of H_2O_2 and free radicals (Los and Murata 2004; Sun et al. 2010).

At present, due to the rapidly increasing industrialization and urbanization, environmental pollution is becoming a serious issue. The most toxic pollutants that prevail in the environment are heavy metals that are toxic to every living, and reports showed that almost every ecosystem has been contaminated with these pollutants (Wei and Yang 2010; Azizullah et al. 2011). The release of heavy metals in soil and water is becoming a serious limitation for crop production not only in area surrounding the industrial locations, but heavy metal stress is also becoming a serious issue even in remote areas as the injudicious and blind use of chemicals for crop production severely contaminated the soils with heavy metals (Hjortenkrans et al. 2006; Nada et al. 2007). These heavy metals pose cytotoxic, genotoxic, and mutagenic effects on plants. Most of heavy metals are actively uptaken by plants and transferred into food chain resulting in serious health issues in animals and humans as well (Flora et al. 2008).

The above discussion showed that plants faces a number of stresses in soil environment. All these stresses affect the plant growth and development by causing negative impact on various plant physiological processes. These stresses also interact with each other, and the intensity of their impact may be increased. The intensity of these stresses may vary with plant species as well as growth stage. One stress may be more detrimental at particular growth stage, and control of this negative effect at that stage could be beneficial for proper plant growth and development.

6 Mitigation of Stress-Induced Impacts on Plant Growth Through Phytohormones

The use of plant growth regulators is an effective approach to promote plant growth and development. Owing to their growth promotion abilities, phytohormones are being used effectively for enhancing crop production under normal as well as stress conditions. There are certain reports which show their effectiveness in agricultural production (Saeedipour 2013; Bano and Yasmeen 2010; Kovaleva et al. 2015; Afzal et al. 2005).

6.1 Mechanism of Action

A variety of mechanisms are adopted by plants to cope with stress conditions. Among these, one of the effective and comprehensive mechanisms includes the biosynthesis of plant growth regulators or phytohormones. Production of these organic metabolites is a primary tool for plants to mediate a wide range of adaptive response systems (Santner et al. 2009) and be involved in regulating various plant processes under normal as well as adverse soil conditions necessary for normal plant growth and development (Kaya et al. 2009).

The phytohormones cause impact on all phases of the plant throughout its life cycle. Movement of phytohormones throughout the body takes place via the xylem or phloem transport stream. In order to exert their response, phytohormones bind transmembrane receptors or endoplasmic reticulum. Hormonal concentrations and tissue sensitivity regulate the physiological process that causes profound effects on plant growth (Taiz and Zeiger 2010). In response to stress conditions, plants tend to accumulate high concentrations of phytohormones like auxins and IAA (Javid et al. 2011; Wang et al. 2001). This high concentration of hormones might be helpful for mitigating the negative impact of stress. For example, under low soil water potential, auxins accumulation in plant roots takes place which are transported to leaves. At the surface of cell membrane of stomatal cells, these auxins bind to receptors that result in enhanced stomatal conductance (Babu et al. 2012). It is reported that IAA has influence on oxidative phosphorylation in respiration and enhances oxygen uptake. It has been assumed that growth enhancement by IAA might be due to increased energy supply.

Gibberellin biosynthesis is also greatly influenced by developmental and environmental stimuli that disturb the level of hormone in the plant (Yamaguchi and Kamiya 2000; Olszewski et al. 2002). A decrease in endogenous level of gibberellic acid (GA) and reduced crop yield have been observed under salinity stress (Xie et al. 2003; Hamayun et al. 2010b). It might be due to decrease GA biosynthesis in plant and due to the activity of oxidases that cause distraction of hormones in plant. Similarly, there are many reports which show that in order to get immunity against stresses, plants accumulate significant amount of cytokinins in their body. Regulation of carbon and nitrogen assimilation with the accumulation of cytokinin that

enhanced drought tolerance in rice has been reported by Reguera et al. (2013). Rivero et al. (2007) also reported tobacco tolerance against drought through accumulation of cytokinins. It has been hypothesized that cytokinins enhance wheat salt tolerance through interacting with other plant hormones by regulation and detoxification of toxic ions and reactive oxygen species. Abscisic acid and cytokinins interact antagonistically causing opposite effects on various plant developmental processes including stomatal conductance, seed germination, and cotyledon expansion (Blackman and Davies 1984; Thomas 1992). It is also reported that high CK accumulating in plants show improved nutrient efficiency during nutrient deficiency (Rubio-Wilhelmia et al. 2011). It has been observed that cytokinins influence the nitrogen metabolism (Sakakibara et al. 2006) by enhancing nitrate reductase activity in plants (Sykorova et al. 2008).

6.2 Phytohormones and Plant Stress Tolerance

Endogenous and exogenous applications of phytohormones play a significant role in enhancing plant ability to maintain their growth under stress conditions. In addition to endogenous plant hormones, exogenous application of phytohormones also plays an important role in improving plant tolerance against adverse conditions (Table 3). A number of reports are available that are demonstrating the positive effects of endogenous and exogenous application of phytohormones on plant growth and development (Fan et al. 2014; Afzal et al. 2005; Fassler et al. 2010; Kumar et al. 2014; Shaddad et al. 2013).

Auxins are well-known phytohormone that play an important role in plant tolerance against various environmental stresses like salinity, waterlogging, and soil acidity (Salama and Awadalla 1987; Ribaut and Pilet 1991; Gadallah 1994, 1995). In many plants and under certain environmental conditions, endogenous phytohormone production may be lower which is not sufficient to mitigate the negative impact of stress environment (Wyn Jones and Storey 1981; Yancey 1994; Subbarao et al. 2001). Exogenous application of phytohormones under such conditions may be helpful in reducing adverse effects of stress (Makela et al. 1998a, b; Yang and Lu 2005). For example, exogenous application of auxins caused significant increase in crop yield by reducing the adverse effect of water stress (Abdoli et al. 2013a, b). Similar response was also observed in the case of heavy metal stress where the application of IAA minimizes the negative impact of heavy metal toxicity on plant growth by regulating metal accumulation and decreasing oxidative damage (Gangwar and Singh 2011; Gangwar et al. 2014). It is achieved by enhancing the activity of antioxidant enzymes as reported by Kumar et al. (2012a, b). They demonstrated that regulation of heavy metal uptake by auxin application was due to enhancement in enzymatic and nonenzymatic antioxidant activity. In water-deficit environment, the closing of stomata reduces the CO_2 fixation that disturbs the photosynthetic activity of the plants (Chatrath et al. 2000); however, reverse has been observed by application of IAA under such condition (Khalid et al. 2013).

Table 3 Effect of phytohormones on plant growth

Crop	Hormone	Response	Reference
Tomato (*Lycopersicum esculentum*)	Jasmonic acid	Proved as a useful disease control agent by inhibiting the multiplication of nematode and enclosing the invading nematodes at the initial site of infection	Fan et al. (2014)
	Salicylic acid	Decreased disease symptoms and 73 % reduction in the infection symptoms on the potato tubers were observed. With increase in concentration from 0.2 to 2 mM, the number of potato tubers was enhanced	Hadi and Balali (2010)
Petunia (*Petunia hybrida* L.).	Auxin and cytokinin	Stimulation of polar growth due to the intensification of the cytoplasm flow. Auxin and cytokinin regulate the pollen tube polar growth via their effects on actin polymerization and spatial organization	Kovaleva et al. (2015)
Black gram (*Phaseolus mungo* L.)	Indole acetic acid	Reduction in protein content and nitrate reductase activity were observed that was alleviated by IAA application and crop yield improved	Guru Devi et al. (2012)
Maize (*Zea mays*)	Cytokinins	Enhanced germination and pollen tube growth of maize (Zea mays L.) under sodium chloride salinity	Dhingra and Varghese (1985)
	GA3 and IAA	Seed priming with phytohormones enhanced germination and radicle and plumule length. 8 h was more effective than 16 h in all aspects	Saeedipour (2013)
	GA3 and IAA	EDTA significantly reduced the plant growth and dry biomass, while application of phytohormones improved it. Combined application of EDTA, GA3, and IAA was more effective, and significant increase in Pb uptake and its translocation into shoot were observed	Hadi et al. (2010)
	Indole-3-acetic acid, gibberellic acid, and *trans*-zeatin	Significant improvement in growth and their P status. GA3 and *t*-Z promoted shoot/root growth and morphological changes. IAA affected the chemical composition of the rhizosphere	Wittenmayer et al. (2008)
	Auxins	Diluted the impact of cold stress by reducing the morphological and physiological changes in cold-stressed plants	Battal et al. (2008)

Pepper (*Capsicum annuum*)	Indole-3-acetic acid	An increase in spermine and a decrease in putrescine in leaves of pepper were observed with IAA application	San-Francisco et al. (2005)
Sunflower (*Helianthus annuus*)	Indole-3-acetic acid	Significant increase in Zn uptake and alleviation of toxic effects of Pb and Zn on plant root and shoot growth. Enhanced phytoextraction potential of treated plants	Fassler et al. (2010)
	Cytokinins	Enhanced phytoextraction by improving biomass production that might be due to stimulation of cell division and shoot initiation	Tassi et al. (2008)
Hyperaccumulating species (*Alyssum corsicum*, *Alyssum malacitanum*, *Alyssum murale*, and *Noccaea goesingense*)	Gibberellins, cytokinins, and auxins	Enhanced Ni extraction efficiency and improved growth and biomass production of tested species	Cabello-Conejo et al. (2014)
Coriander (*Coriandrum sativum* L.)	GA3 and 2,4-D	Improved growth parameters and decreased proline contents were observed with increasing concentration of applied hormones	Kumar et al. (2014)
Wheat (*Triticum aestivum*)	Abscisic acid and benzyladenine	Decrease in IAA and GA and increase in proline and ABA under water stress. Application of said hormones played a role in osmoregulation by the production of proline. ABA was more effective at the later stages while benzyladenine at early stages	Bano and Yasmeen (2010)
	Gibberellins	Seed soaking with gibberellins enhanced the seedling vigor under salinity stress	Afzal et al. (2005)
	Gibberellic acid	Improved Ca^{2+} and K^{+} uptake and enhanced growth and yield parameters of wheat	Iqbal and Ashraf (2013)
	Jasmonic acid	Enhanced activities of antioxidant enzymes and the concentration of antioxidative compounds to reduce the excessive reactive oxygen species	Qiu et al. (2014)
	Auxins	Grain yield improved under drought stress by the application of auxins	Abdoli et al. (2013a, b)
	Auxins	Improvement in plant antioxidant defense system occurred that reduced the negative impact of heavy metal stress	Kumar et al. (2012a)
	Gibberellins	Enhanced root and shoot dry matter. Chlorophyll and carotenoid contents improved	Turkyilmaz (2012)

(continued)

Table 3 (continued)

Crop	Hormone	Response	Reference
Rice (*Oryza sativa*)	Auxin	Application of IAA precursor L-TRP improved the growth and yield. Enhanced the uptake and translocation of cadmium	Farooq et al. (2015)
	Auxins	Enhanced carbohydrate accumulation in grains	Javid et al. (2011)
Roselle (*Hibiscus sabdariffa*)	Gibberellic acid	Improved photosynthetic pigments, growth, and osmotic relations under salinity stress	Ali et al. (2012)
Strawberry (*Fragaria ananassa*)	Gibberellins	Application of phytohormone improved the fruit quality	Qureshi et al. (2013)
Groundnut (*Arachis hypogaea*)	Gibberellins	Positively increased growth and yield parameters	Khan et al. (2011a, b)

Gibberellins play an important role in plant growth and development owing to their impact on seed germination, root/shoot elongation, as well as flowering and fruit patterning (Fleet and Sun 2005; Shani et al. 2013). Gibberellins are significantly focused phytohormones by researchers to be used as stress protectant (Basalah and Mohammad 1999; Hisamatsu et al. 2000). An enhancement of wheat growth has been observed under saline condition by the application of gibberellins (Parasher and Varma 1988). According to the findings of Maggio et al. (2010), exogenous application of gibberellic acid under stress conditions reduces the stomatal resistance and increases water use efficiency. Afroz et al. (2005) reported the improvement in photosynthetic efficiency and nitrogen metabolism of salt stress mustard plant due to the application of gibberellins. Yield enhancement due to seed priming with gibberellin is attributed to the regulation of ion uptake and their partitioning (Kumar and Singh 1996; Iqbal and Ashraf 2010). Kaya et al. (2006) demonstrated that maize drought tolerance can also be improved by the application of gibberellic acid. According to their findings, it was due to enhancing chlorophyll and leaf water content as well as maintaining membrane permeability and nutrient concentrations in plant body. This can also be obtained by enhancing the activity of antioxidant enzymes against reactive oxygen species (Falkowaska et al. 2010). Plants under high temperature stress show increased acidulation of extracellular solution and decreased proteolysis level that can be regulated by the application of gibberellins (Aleksandrova et al. 2007).

The fundamental role of cytokinins is considered to maintain the indeterminate property of shoot apical meristems (Davies 2004; Hopkins and Huner 2008). Cytokinins also regulate the assimilate partitioning, sink strength, and source/sink relationships (Kuiper 1993; Ronzhina and Mokronosov 1994; Roitsch 1999). Like other phytohormones, cytokinins also affect the plant responses to environmental stresses, and this effect may be a direct and indirect one (Wilkinson et al. 2012). For example, under drought stress, the decrease in cellular contents of cytokinin results in an increase of abscisic acid (Davies and Zhang 1991) which caused the closing of stomata resulting to low photosynthetic activity of the plant (Rivero et al. 2010). In certain cases, stress-induced cytokinin synthesis like in tobacco (Rivero et al. 2009), cotton (Kuppu et al. 2013), and peanut (Qin et al. 2011) protects the plants from adverse effects of different stresses that cause negative impact on plant physiology (Reguera et al. 2013). Barciszewski et al. (2000) also reported the plant tolerance against salinity and drought due to accumulation of cytokinins. Other workers also reported the role of exogenous application of cytokinins in plant stress tolerance (Wang et al. 2001; Gupta et al. 2003; Yang et al. 2003) which is considered as an economical and easy strategy for inducing stress tolerance in many crops (Torres-Garcia et al. 2009). For example, seed priming with cytokinin is reported to increase germination and seedling survival under salt stress (Iqbal et al. 2006). Improvement of wheat seedlings and potato plants with cytokinin application under salt stress has been observed (Naqvi et al. 1982; Abdullah and Ahmad 1990). Cytokinins also act as protectant agent against plant pathogens as reported by Ketabchi and Shahrtash (2011). where the application of cytokinin significantly reduces the negative impact of *Fusarium moniliforme* on maize seedlings.

Plants synthesize different phytohormones under stress conditions depending upon the strength of their defense mechanism. The exogenous application of different phytohormones has been proven as an effective method to cope with stressful environments. It is concluded that the exogenous application of phytohormones to stress-sensitive plants can induce stress tolerance in plants.

7 Enhancing Plant Stress Tolerance Through Phytohormone-Producing Microbes

As described earlier, plant growth regulators (PGRs) or phytohormones are organic compounds and produced by plants in very minute quantities and translocated to different tissues. These PGRs play a primary role in the coordination of physiological processes related to growth, reproduction, and stress management. In rhizosphere, a number of microbes also synthesize these hormones as signaling agents for phytostimulation (Egamberdiyeva 2005). For example, auxins are important group of phytohormones, which are synthesized by soil microbes in abundance. Under stress conditions, inoculation of PGR-synthesizing microbes enhances the stress tolerance of plants. Use of PGR-synthesizing microbes as biofertilizers has been proven as a highly effective technique for enhancing crop production under normal as well as stress conditions (Ahemad and Kibret 2014). A number of microbial strains produce a variety of phytohormones including IAA, gibberellic acid, proline, and zeatin (cytokinins). All these hormones play a pivotal role in the enhancement of plant growth and productivity not only under normal conditions but also under stress conditions especially salinity, drought, temperature, and oxidative and photogenic stresses (Cassan et al. 2009). The role of PGPR for promoting plant growth owing to their ability to produce phytohormones has been reviewed in Table 4.

Literature indicates that PGR-synthesizing rhizobacteria greatly help in protecting plants under stressful conditions (Khalid et al. 2006; Egamberdiyeva 2005; Nadeem et al. 2010b). A number of bacterial strains are capable of synthesizing different phytohormones (Lugtenberg and Kamilova 2009). Commercially available PGRs are also being used exogenously to induce different plant responses and enhance plant growth under stress conditions. But, these artificially synthesized PGRs are not only very expensive but are also less efficient than PGRs from microbes (Khalid et al. 2006). Under stress, the use of PGR-producing microbes as biofertilizer is reported to improve plant growth and production by the ameliorating action of phytohormones, secreted by rhizospheric microbes, that helps plant to regulate their osmotic potential, hormonal balance, and level of toxic ions in cytoplasm.

Leinhos and Bergmann (1995) reported that phytohormone-producing microbes enhance plant growth and induce drought tolerance in plants by producing IAA that is taken up by plants and performs a significant role in alleviation of adverse effects of drought on plants. San-Francisco et al. (2005) also reported in pepper plants that inoculation of auxin-producing microbes enhanced the growth and physiology of pepper plants growing under nutrient stress conditions. The induction of stress tolerance was attributed to the production of free polyamines and enhanced levels of

Table 4 Effect of phytohormone-producing microbes on plant growth

Crop	Microbe	Response	Reference
Maize (*Zea mays*)	*Stenotrophomonas maltophilia*	Significant improvement in growth occurred. Root and shoot proline content enhanced under normal and salinity stress	Naz and Bano (2012)
	Azospirillum lipoferum	ABA and GA inhibitors negatively affected the growth of plants; however, inoculation with phytohormone-producing bacteria completely reversed this effect. The relative water contents of inhibitor-treated plants and drought-stressed plants were significantly lower, and this effect was completely neutralized by inoculation	Cohen et al. (2009)
Rice (*Oryza sativa*) and wheat (*Triticum aestivum*)	*Nostoc*OS-1	Efficiently colonized the roots of both crops and improved growth	Hussain and Iqbal (2015)
Wheat (*Triticum aestivum*)	*Pseudomonas aurantiaca*, *Pseudomonas extremorientalis* TSAU6, and *Pseudomonas extremorientalis* TSAU20	Significantly increased root growth. Response was more significant under salinity stress	Egamberdieva (2009)
	Bacillus subtilis IB-22, *B. subtilis* IB-21	*B. subtilis* IB-22 increased amino acid rhizodeposition, while *B. subtilis* IB-21 did not significantly affect amino acid concentrations and failed to accumulate cytokinins in culture media	Kudoyarova et al. (2014)
	Rhizobium spp.	Through production of IAA, *Rhizobium* inoculation enhanced water and micronutrients by improving root growth. Overall plant growth was improved	Etesami et al. (2009)
Soybean (*Glycine max* L.)	*Penicillium funiculosum*	Significant enhancement in soybean growth parameters. Less endogenous abscisic acid and elevated jasmonic acid contents in treated plants under salt stress	Khan et al. (2011a)
	Aspergillus fumigatus sp. LH02	Significant improvement in growth parameters including shoot length and shoot fresh and dry biomass under salt stress. Chlorophyll contents and photosynthetic rate also enhanced	Khan et al. (2011b)
Corn (*Zea mays* L.) and soybean (*Glycine max* L.)	*Azospirillum brasilense* Az39, *Bradyrhizobium japonicum* E109	Inoculation with PGPR singly or in combination promoted seed germination, nodule formation, and early development of corn and soybean seedlings	Cassan et al. (2009)

(continued)

Table 4 (continued)

Crop	Microbe	Response	Reference
Fodder (*Galega orientalis* Lam.)	*Rhizobium galegae*, *Pseudomonas* sp.	Application of IAA producing *Pseudomonas* with IAA absent *R. galegae* enhances rhizobia-legume interactions acting as "rhizobium helper bacteria"	Egamberdieva et al. (2010)
Indian mustard (*Brassica juncea*)	*Pseudomonas* sp. and *Bacillus megaterium*	Enhanced plant growth and protected the plant from Ni toxicity. Nonsignificant effect on Ni extraction, however, produced significant biomass	Rajkumar and Freitas (2008)
Cucumber (*Cucumis sativus*)	*Paecilomyces formosus* LHL10	Significantly enhanced cucumber growth parameters. Dilute the negative impact of salinity by accumulating proline and antioxidants and maintaining plant water potential	Khan et al. (2012a, b)
	Burkholderia sp.	Significantly enhanced cucumber growth parameters. Soluble sugar and crude protein contents were significantly higher in inoculated plants	Kang et al. (2010)
	Phoma glomerata LWL2 and *Penicillium* sp. LWL3	Under stress, growth was affected that was regulated by endophytes inoculation through altering abscisic acid, jasmonic acid, and salicylic contents	Waqas et al. (2012)
Tomato (*Lycopersicum esculentum*)	*P. putida* and *T. atroviride*	Inoculation caused significant improvement in the fresh weight of both root and shoot in the presence of L-tryptophan	Gravel et al. (2007)
Chickpea (*Cicer arietinum*)	*Pseudomonas* sp., *Mesorhizobium* sp.	Co-inoculation significantly increased nodule number and nodule biomass. The response was more significant in case of co-inoculation compared to *Mesorhizobium*-inoculated plants alone	Malik and Sindhu (2011)

spermine and decreased levels of putrescine that efficiently regulated the mineral uptake in plants and reduced the stress intensity. Similarly, Wilmowicz et al. (2008) reported that several microbes were capable of producing abscisic acid, which is a vital phytohormone. The inoculation of such microbes to plants resulted in significant improvement in reproductive growth including flowering and fruiting (Zhang et al. 2006). Under stress conditions, microbial-derived phytohormones also help plants to withstand stress-induced oxidative destruction through maintaining the equilibrium between antioxidants and ROS (Arbona et al. 2005).

Under high nitrate supply plant shows reduced root growth due to nitrate stress. However, the introduction of IAA producing microbial inoculants increases the concentration of endogenous IAA which improves root growth under nitrate stress (Forde 2002; Tian et al. 2008). Liu et al. (2007) reported that the uptake of bacterial synthesized phytohormones increased the heavy metals chelation which reduced the severity of heavy metal stress on plants. Ahmad et al. (2014) reported the inoculation with auxin-producing halo-tolerant PGPR. Ahmad et al. (2013) improved the productivity of mung bean grown under salinity stress.

Some soil microbes synthesize phytohormones from their precursors and release the soil solutions that are taken up by plants. The abundant availability of these precursors enhances the ability of plants to produce larger concentrations of phytohormones. Zahir et al. (2010) reported that the inoculation of auxin-producing rhizobacteria and application of L-tryptophan (a precursor of auxin) alone and in combination enhanced the endogenous auxins and significantly improved yield of maize. The combined application was more effective for promoting plant growth and yield. Similar results were obtained by Arkhipchenko et al. (2005) as growth and biomass production were significantly improved in legume fodder when applied with L-tryptophan-synthesizing microbes under drought conditions. L-tryptophan results in higher production of auxins in plant's body resulting in improved water relations and vascular conductance (Barazani and Friedman 1999b; Taiz and Zeiger 2000).

Under stress conditions, the production of ethylene is a fundamental phenomenon that obstructs major plant physiological processes. Ethylene is a growth regulator, and higher production of this restricts plant growth and induces early senescence (Nadeem et al. 2010a). On the other hand, soil microbes having 1-aminocycloprop ane-1-carboxylate (ACC) deaminase restrict the activity of ethylene, produced under stress conditions. Several microbes have the ability to biosynthesize ACC-deaminase and help the plants in maintaining adequate levels of ethylene (Glick et al. 2007; Nadeem et al. 2007; Ahmad et al. 2011). Cheng et al. (2007) reported that the application of soil microbes having the ability to produce ACC deaminase was found to enhance plant growth under low temperature and salinity stress.

It may be concluded that phytohormones are unique substances involved in plant growth and stress regulation. However, the biosynthesis of these substances varies from species to species as not all the plants can produce ample concentrations of these hormones under stress conditions. In this case, soil microorganisms take part in plant stress tolerance mechanisms and provide them with already synthesized phytohormones which are highly effective for plant growth promotion under stress conditions so that plants can grow and produce better even under hostile conditions without any laborious input.

8 Conclusions and Future Prospects

The above discussed review shows the importance of plant growth regulators in plant growth promotion. In soil environment, plant faces various biotic and abiotic stresses that affect a number of plant physiological processes. To cope with these stresses, plant develops certain strategies, and the production of hormones is one of them. These phytohormones play an important role in plant growth and development by accelerating plant processes. Not only the endogenous plant hormone but its exogenous application and microbial synthesized phytohormones are equally effective for promoting plant growth. It is also evident from the above discussed review that these phytohormones also interact with each other and this interaction may be positive or negative. Most of the negative responses by phytohormones are due to high concentration that directly affects the particular plant process or antagonizes the production of other hormones that results in impaired plant growth. This elevated level of growth hormone might be due to the result of some environmental stimuli or overproduction of a particular hormone by inoculated strains.

The work of a number of researchers discussed here shows that concentration of a hormone, its use for particular purpose, as well as application of phytohormone-producing strain are some of the major factors that should be kept in mind for improving plant growth and development under normal and stress conditions. In order to clear our understanding about these aspects, there is a huge gap that should be filled by conducting research on gross root level. Research should be focused on rate and timing of phytohormone application, their stability, as well as their bioavailability in soil environment. The selection and evaluation of potential strains that have the ability to produce phytohormones need further research so that a suitable strain for a particular purpose can be used effectively.

By using biotechnological and molecular approaches, efforts could also be focused in developing genetically engineered plants that have the ability to synthesize particular hormones which enable them to withstand and maintain their growth in adverse soil conditions. These transgenic plants would be able to grow under various conditions with minimal yield losses.

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Soil Pollution and Remediation

Sameen Ruqia Imadi, Zeshan Ali, Hamna Hasan, and Alvina Gul

Abstract The planet Earth is suffering from an ever-escalating rate of pollution. It was not until the twentieth century that mankind was seriously concerned about pollution. But now pollution has reached to such a significant level that is influencing all ecological compartments. There are many types of pollution. Among these most important are i.e. soil pollution, air pollution, noise pollution, and water pollution. Concerns about soil pollution have increased in the recent decades. Soil pollution has deteriorated large areas of agricultural land around the globe. It is due to soil pollution that soil biodiversity is declining. Human health is also at risk due to high concentration of pollutants found in soil. Vegetation grown on polluted soil is also contaminated to varying degrees. Simple and cost effective solution to soil pollution is bioremediation. It is an efficient technique in which hyper-accumulator plants and native plants along with bacteria and other microorganisms are grown in polluted soils. These organisms absorb and or degrade pollutants and enhance soil quality. As the bioavailability of nutrients increase, soil functioning improves. Bioremediation can be performed using a large number of techniques including biostimulation, bioaugmentation, phytoremediation, mycoremediation etc. This chapter deals with soil pollution, its possible causes and adverse environmental effects. The chapter is concluded with bioremediation as a potential alternative for soil cleanup with possible future recommendations.

Keywords Phytoremediation • Bioremediation • Environmental pollution • Human Health • Agricultural soils

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1 Introduction

On the planet Earth, one of the most vital, life-supporting system is soil. Estimates are made in which it is claimed that almost 25 % of the soil worldwide is highly degraded, whereas 44 % soil is moderately degraded. This degradation of soil has been attributed to pollution caused by metals, radionuclides, metalloids, pesticides, and persistent organic pollutants. New soil pollutants are also emerging with industrialization and globalization. These pollutants are antibiotics, nanoparticles, disinfectants, and flame retardants (Tripathi et al. 2015). Soil quality can be defined as the capability of soil to perform its specific functions. Microbial properties of soil are being used for a long time to indicate the quality of soil. This is because soil microbial properties have a quick response and are highly sensitive. Microbial properties of a certain soil are undoubtedly the most ecologically relevant indicators of soil quality (Gómez-Sagasti et al. 2012).

Soil pollution is basically caused by poor management of municipal effluents as well as industrial wastes (Fernández-Caliani 2012). Many economic activities are involved in enhancement of soil pollution. Among these anthropogenic activities, mining and smelting are at top. Mining takes place where high concentration of metals or minerals is present. The presence of large concentration of heavy metals causes impacts on water resources, crops, soils, and vegetables. Eating crop and vegetables grown on such soil may be hazardous to human health (Zhang et al. 2012). It is due to ever-increasing urbanization and industrialization which have posed serious social and environmental threats to the world. Some of these threats include soil pollution and ecological degradation (Wang et al. 2014).

Sewage sludge, mine tailing, industrial wastewater, waste rock piles, and acid mine drainage have resulted in contamination and pollution of large cultivable as well as fallow lands. This not only causes pollution but also leads to soil deterioration. It is difficult to reestablish vegetation in polluted soil due to toxic elements, harsh climatic conditions, organic contaminants, and acidic constituents of soil (Anawar et al. 2015). Soil pollution can be determined using biological as well as chemical approaches. Different plant species can be used as bioindicators of soil pollution especially heavy metal pollution. Some of these plants include *Oenanthe* sp., *Callitriche* sp., *Juncus* sp., and *Typha* sp. (Ladislas et al. 2012). Some of the common wastes which lead to the development of soil pollution include domestic wastes, hazardous wastes, commercial wastes, nonbiodegradable wastes, ashes, biodegradable wastes, animal wastes, sewer, biomedical wastes, industrial solid wastes, and construction wastes (Demirbas 2011).

2 Causes of Soil Pollution

2.1 Coal Ash

Large-scale production of coal ash from thermal power plants around the world is now causing a serious threat to the environment by polluting the soil. This increases the soil toxicity by increasing heavy metal content of the soil; moreover,

fly ash has a slow rate of degradation due to which it pollutes the soil (Bhattacharya et al. 2012). Coal burning leads to emission of chromium, cadmium, and lead. An experiment was conducted in China in which it was observed that the total yearly emissions of chromium, cadmium, and lead have significantly increased in recent years. This leads to soil pollution as well as air pollution (Tian et al. 2012). Coal combustion also yields mercury in the coal ash (Sun et al. 2014). Extraction of coal from mines and combustion of coal result in damaging the soil and environment. Coal ash acts as a deteriorating agent of soil (Srivastava et al. 2014).

It can be rightly said that every step of life cycle of coal from extraction, transport, processing to combustion enhance the pollution. It carries a vast variety of hazards to health as well as the environment (Epstein et al. 2011). It has been observed that soils which are located in the vicinity of coal-fired thermal power stations are polluted not only by ashes but also by heavy metals including arsenic, nickel, strontium, chromium, copper, cobalt, mercury, cadmium, barium, beryllium, and vanadium (George et al. 2015). Residues of coal combustion are considered as hazardous solid waste around the globe (Verma et al. 2014).

2.2 Sewage and Industrial Waste

Random dumping of hazardous waste generated by industrial area can be a major cause of soil contamination and eventually pollution. It has been observed that random dumping of industrial wastes is the main source of heavy metal pollution in surrounding areas (Bhagure and Mirgane 2011). Severe soil pollution can be caused due to poor management of effluents and industrial wastes. Anthropogenic sources lead to deterioration of soil in the worst way possible (Fernández-Caliani 2012; Ali et al. 2015a, b). Industrial wastes basically comprise heavy metals including zinc, lead, arsenic, chromium, mercury, copper, cobalt, nickel, and cadmium. Soil around the world is under serious threat by heavy metal toxicity (Kodom et al. 2012). Rapid industrial development has posed serious threats to soil. These threats are in the form of heavy metal accumulation. Soil, polluted due to industrial wastes and sewage, largely contains lead, copper, zinc and cadmium. Different kinds of industries add different types of waste materials in soil. For example, it has been observed that metal producing industrial wastes and smelting industrial wastes have large amount of lead, arsenic, zinc, iron, nickel, and copper. Similarly the waste of textile industry includes manganese and cadmium, whereas that of leather industry includes chromium (Kabir et al. 2012).

Solid waste management is becoming a complicated problem day by day. This complication is due to the increase in population, changes in lifestyle and industrialization. In developing countries, industrial wastes and sewage are dumped in open dumps, whereas in developed countries, it is dumped in landfills or recycled. Severe environmental problems like soil pollution may be caused by open dumping and landfilling (Singh et al. 2011).

2.3 Pesticides and Herbicides

Pesticides are widely being used in agriculture. Pesticides help in prevention from pests, diseases, weeds, and plant pathogens. They also contribute in maintaining high quality and ensure increase in yield (Damalas and Eleftherohorinos 2011). Enhanced use of pesticides might lead to the development of soil pollution through accumulation of nitrogen and different chemicals in cultivable soils. This results in deteriorating soil and soil biota (Sun et al. 2012). Concerns about pesticide pollution of the environment have increased in previous decades (Van Toan et al. 2013). DDT and HCH are potential contaminants which are largely used on agricultural lands. These compounds are used because they have low cost and high effectiveness. They have high stability due to which they stay in soil and result in soil pollution (Mishra et al. 2012).

Soil pollution due to pesticides and herbicides can be easily measured by manipulating soil enzyme activities. It has been observed that activities of enzymes like phenol oxidase act as early indicator of soil pollution. Further enzymes which can be used to detect pesticide soil pollution are arylamidase and beta-glucosidase (Floch et al. 2011). During studies it has been observed that there are several groups of pesticides which largely pollute the soil. Among these groups are organochlorine pesticides which include dichlorodiphenyltrichloroethanes (DDT's), hexachlorocyclohexanes (HCH's), and chlordane (Syed et al. 2013).

Herbicides like oxyfluorfen pollute the soil. They not only pollute the soil but also have adverse effects on soil biodiversity, i.e., act as fatal agents for a variety of earthworm species which are living in soil. Earthworms including *A. molleri*, *E. fetida*, and *L. terrestris* accumulate oxyfluorfen present in soil. This compound also alters soil biochemical properties by polluting it (Tejada et al. 2016). Other herbicides which pollute the soil include mesotrione and atrazine. They are degraded by soil and produce a large number of degradation products. These products include deethylatrazine, hydroxyatrazine, desethyldesisopropylatrazine, and deisopropylatrazine. These herbicidal products add to the pollution of soil. High levels of these products pose toxicity to soil biodiversity (Barchanska et al. 2012).

2.4 Heavy Metals

Heavy metals are released in the environment due to their mobilization through extraction from ores and processing. These elements are nonbiodegradable; hence, they accumulate in the soil (Ali et al. 2013; Ali et al. 2015a). Soil pollution caused by heavy metals is a worldwide problem. Among heavy metals, the most prominent is arsenic pollution. It usually occurs in soils which are at the river basin. Human disturbance is associated with increase in arsenic and lead contents in the soil. Besides lead and arsenic, the main pollutant heavy metals include copper, nickel, iron, cobalt, cadmium, manganese, mercury and zinc (Zhao et al. 2014b). Industries including chemical, paint, machinery, plastic, packing, electric, cosmetics, metal, food, textile, automotive supply, and wood increase significant amounts of heavy

Table 1 Standard permissible concentration of metals in soil

Sr. No	Heavy metal	Standard concentration in soil (mg/kg)
1	Cadmium	5
2	Arsenic	5
3	Barium	1
4	Chromium	8
5	Lead	5
6	Mercury	0.2
7	Selenium	5
8	Silver	5
9	Zinc	12
10	Copper	5
11	Molybdenum	15
12	Nickel	1.75

metals in soil (Yaylali-Abanuz 2011). Heavy metal pollution has affected many parts of the world especially developing areas (Li et al. 2014). High levels of chromium might leach in soil due to mining, corrosion inhibitors in cooling water, metal plating, tanning and textile industries, wood preservation, glass and ceramics, ink manufactures, dye, and pigments (Dhal et al. 2013). Standard permissible concentrations of heavy metals in soil are given in Table 1.

Soil contamination due to heavy metals is of serious concern because heavy metals possess intense toxic effects (Ali et al. 2015c). Soil is polluted by metals which are largely produced from wastewater treatment plants, agricultural/industrial, activities and treated/untreated wastewater (Rahman et al. 2012). It has been observed that the cadmium pollution of soil is the result of industrial wastewater and agricultural practices (Hani and Pazira 2011). Application of large levels of phosphorus fertilizers on soil causes arsenic pollution (Hartley et al. 2013). Mining is one of the greatest contributors of soil heavy metal pollution (Jin and You 2015).

2.5 Traffic Activities

Heavy traffic significantly contributes in soil pollution. In an experiment conducted in Mexico, it was observed that cesium, zinc, and arsenic contents are significantly high in urban soils as compared to rural ones. These concentrations are much more than the standard guidelines of the US Environmental Protection Agency and are not without harmful effects on biodiversity and human health (Mireles et al. 2012). Heavy traffic contributes to soil pollution with heavy metals like copper, lead, and zinc. This anthropogenic activity also adds cadmium in the soil (Zhao et al. 2014a). It has been observed that heavy traffic also emits zinc and lead. Increase in concentration of zinc and lead can overcome the threshold and result in soil pollution. Soil pollution with polycyclic aromatic hydrocarbons is also due to intense traffic near agricultural lands (Gunawardena et al. 2012). Agricultural soils which are present near heavy traffic roads are observed to be greatly affected by lead pollution (Hu et al. 2013).

3 Effects of Soil Pollution

Effects of soil pollution were observed in a biomarker response of earthworms. Earthworm samples were collected from different areas, and it was observed that high levels of soil pollution cause significant health hazards in earthworms. Histopathological and biochemical changes are observed. In all the samples examined, epithelial cell lining was enlarged, hyperplasia was caused in muscle cells, and mucus secretion was also enhanced. It was also seen that structural integrity of circular and longitudinal muscles was lost. Dilation and vacuolization was seen in chloragogenous tissue. Affected earthworms also showed tissue necrosis. These examinations show that soil pollution has biological effects. It also predicts and warns about ecological changes which will soon affect human health (Kiliç 2011). Soil is a very important medium of the environment which is closely linked to humans and biodiversity. The presence of harmful materials in soil leads to the development of problems in the environment (Li et al. 2011).

Polluted soils possess ecotoxicological effects on all terrestrial, groundwater, and aquatic ecosystems. These effects can be fatal to a large number of organisms. Large groups of organisms are on verge of extinction due to soil pollution (Machender et al. 2013).

3.1 Harmful Effects on Health

Soil pollution is observed to increase risks of diseases. Cancer risks are seen to be enhanced by the excess of arsenic in soil. If polluted soil gets a dermal contact, then the probability of cancer further increases (Fernández-Caliani 2012). Significant harmful effects on human health are caused by inorganic chemicals present in soil as pollutants. Major contaminants to cause harmful health effects are arsenic and fluorine (Farooqi 2015). The existence of a high amount of heavy metals in soil possesses serious negative influences on human health (Zhao et al. 2011). Soil polluted with heavy metals is the cause of high carcinogenic as well as noncarcinogenic risks to humans especially children and women (Li et al. 2014). Cadmium present as soil pollutant shows potent human health risks (Zhao et al. 2012). It has been observed that the people living in areas in which soil is polluted by zinc and cadmium have a high risk of high blood levels of lead, arthralgia, and osteomalacia and high amount of cadmium in urine (Zhang et al. 2012).

Human health is prone to serious effects by chromium toxicity (Dhal et al. 2013). Some heavy metals are carcinogenic, endocrine disruptors, mutagenic, and teratogenic. They are also involved in causing neurological and behavioral changes in children (Ali et al. 2013). Heavy metals cause serious health effects in diet dominated pathways. Non-cancer risks are increasingly caused by chromium and lead, whereas cadmium is involved in posing serious cancer effects (Liu et al. 2013). Soil contaminated through coal-fired thermal power stations may lead to the levels of pollution

which have devastating effects on human health (George et al. 2015). Studies predict that non-carcinogenic risks to children are mainly posed by soil which is polluted with chromium, manganese, lead, and arsenic. These heavy metals had carcinogenic risks which were almost 30–200 times higher than the safe level. These risks are largely attributed to soil pollution with these heavy metals (Cao et al. 2014).

Studies were conducted in paddy fields grown in heavy metal-polluted soils. It was observed that edible parts of paddy also contain large concentration of cadmium, lead, and zinc. Eating paddy which is grown under polluted soil might cause significant damage (Luo et al. 2011). Pesticides including HCH- and DDT polluted soils possess carcinogenic as well as non-carcinogenic toxicities on children and women (Hu et al. 2011). It has been observed that soil polluted by antimony is a reason behind a large number of toxicities and carcinogenicities. High levels of antimony exposure may lead to the development of cardiovascular diseases, liver diseases, respiratory system diseases, and skin diseases (Jin and You 2015). Toxicities caused by heavy metals are mentioned in Fig. 1.

It is known that exposure to heavy metal-polluted soil results in significant carcinogenic and non-carcinogenic effects. The cancer risks in adults have been seen, and it was observed that chromium contributes to 93.8 % cancer risks, whereas lead may lead to the development of cancer in 6.19 % cases (Luo et al. 2012). Soil polluted with thallium is toxic to human health. Excessive thallium in soil causes chronic thallium poisoning. It is also involved in urinary tract infections (Xiao et al. 2012). It has been concluded through studies that dermal contact with soil polluted by arsenic derived from sewage irrigation can result in carcinogenic risks. Lead-polluted soil however causes non-carcinogenic effects on children (Qiao et al. 2011). Polycyclic aromatic hydrocarbons may have significant carcinogenic effects to human health (Wang et al. 2013).

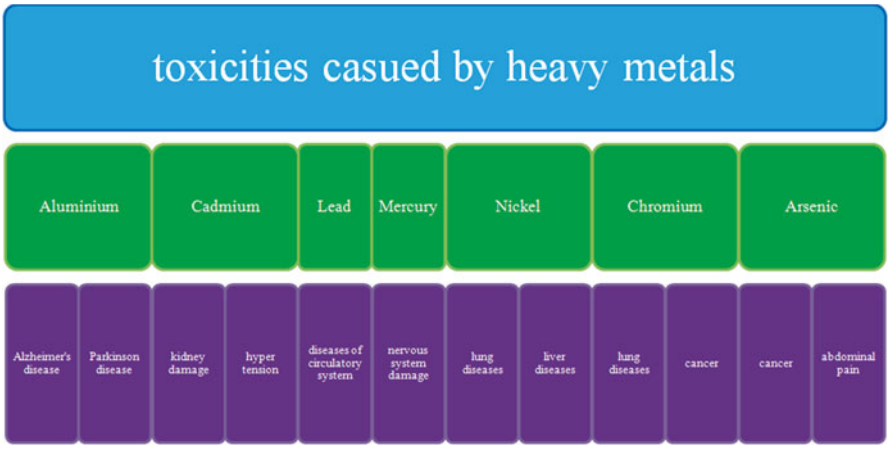

Fig. 1 Toxicities caused by heavy metals in humans

3.2 Devastating Effects on Environment and Biodiversity

Soil biodiversity is a prerequisite for environmental stability and sustainability. It is the plant biodiversity on which richness of soil biota depends. Soil biota functions to keep the soil living. It also helps in nutrient cycling and storage, formation of soil organic matter, and its turnover. Soil pollution affects soil biodiversity in worst way possible. It kills most of the living organisms as well as it alters their metabolism and biochemical pathways which lead to the ultimate degradation of soil (Thiele-Bruhn et al. 2012). The toxic effects which are produced by soil pollution reach soil biota, thus affecting microbial community biomass and metabolic activities. Although almost all of the communities of a certain society respond to soil pollution, it is the microbiota community which are most affected (Wahsha et al. 2013).

Soils polluted with pesticides are a risk to biodiversity. They may leach into water and hence are a cause of death of marine organisms. Pesticides are also observed to have detrimental effects on wildlife and plants. Risk assessment of pesticide-based toxicities can be different depending upon the type of pesticide used, amount of pesticide used, and time of exposure (Damalas and Eleftherohorinos 2011). Heavy metal pollution gives rise to severe harms to ecological environment and soil biodiversity (Jin and You 2015). Petroleum hydrocarbon pollution in soil may cause severe toxic effects on earthworms and plant growth (Tang et al. 2011). The presence of petroleum components in soil is toxic to humans, microorganisms, plants, as well as soil (Besalatpour et al. 2011).

4 Soil Remediation

It was not until the last quarter of the twentieth century that environmental pollution was considered as a threat. The present scenario of soil pollution needs an immediate attention and research toward remediation of polluted soil and detoxification of deteriorated soil. Hazardous agents need to be cleaned from the soil to ensure sustainable environmental growth (Wasi et al. 2013). Bioremediation is known to be a natural process of gaining soil functioning back by using soil microorganisms and higher plants. These plants and microbe change bioavailability of metals and metalloids present in contaminated soil (Park et al. 2011). Soil remediation can be performed using aquatic as well as dominant terrestrial plants (Kumari et al. 2016).

Soil remediation can be obtained through biochar. Biochar is known to be a stable carbon rich by product which is synthesized as a result of pyrolysis or carbonization of plant and animal-based biomass (Ahmad et al. 2014; Barrow 2012). It has been observed that using biochar as a soil remediating agent helps in enhancing the emergence ability of seed and productivity of crop. It also increases nutrients in soil, soil water holding capacity, and microbial diversity. Biochar is also observed to possess properties which ameliorate the deteriorated soils (Anawar et al. 2015). The soil which has concentration of highly stable as well as mobile agrochemicals

including pesticides and herbicides can be remediated using biochar (Uchimiya et al. 2012). Biochar has been used to remediate the soils which are polluted both by heavy metal and organic pollutants. Further studies on biochar need consideration before its commercialization as remediating agent because it might have some negative effects on activity of pesticides and herbicides (Tang et al. 2013).

Bioremediation of lead- and cadmium-polluted soil can be performed by waste oyster shells. In this way, oyster shells can be used instead of wasting and soil can also be remediated. Shells contain large amounts of calcium carbonates which are healthy remediating agents. It is known that the polluted soil with lead and cadmium most of the times gets acidic. This acidic nature of soil can be cured by using basic calcium carbonate from oyster shells (Ok et al. 2011). Earthworm species including *A. molleri* can be used to bioremediate the soils which are polluted by herbicides like oxyfluorfen. These earthworms accumulate excessive herbicide and decrease its concentration in soil (Tejada et al. 2016). Petroleum hydrocarbon contaminants can easily be removed using cost-effective bioremediation technique. As it is known that major components of most of crude oil are biodegradable, hence they can easily be treated by bioremediation (Thapa et al. 2012).

Another method to enhance bioremediation is aerobic remediation of petroleum sludge. In this method microbial community composition of soil is changed to degrade petroleum compounds into organic compounds and release nutrients. It has been observed that populations of certain bacterial communities increase during bioremediation. Among these communities are Proteobacteria which are observed to be almost 50 % of the total bacterial population. Around 16.6 % *Firmicutes* were also present in this population. Hence, it can be said with ease that these organisms have role in bioremediation of petroleum sludge-polluted soil (Reddy et al. 2011). Soil polluted with heavy metals and radionuclides, organic compounds which include chlorinated solvents, for example, TCE, petroleum hydrocarbons, and atrazine pesticides, can be remediated with microorganisms (Das and Adholeya 2011).

5 Techniques Used in Bioremediation

Many techniques can be used for remediation of soil. Heavy metal remediation is the most important aspect of soil amelioration after pollution. Among the heavy metals, mercury is of serious concern because it may cause high toxicity even in small amounts. Soil mercury remediation can be performed using a large number of techniques which are named as stabilization/solidification (SS), phytovolatilization, immobilization, phytoextraction, vitrification, phytostabilization, thermal desorption, electro-remediation, nanotechnology, and soil washing (Wang et al. 2012). Research has explored that richness of plant species in soil and availability of wide variety of nutrients enhance the activity of plant soil microbes. This has an apparent effect on counteracting soil toxicity by heavy metals (Stefanowicz et al. 2012). Natural bioremediation potentials to get rid of soil pollution can be activated by a technique known as bioaugmentation (Sprocati et al. 2012).

5.1 Biostimulation

Diesel-polluted soil was remediated with Fenton combined with NPK (nitrogen, phosphorus, and potassium) fertilizer. It has been observed that coupled treatment of Fenton and NPK fertilizers results in increased efficiency of soil to get rid of petroleum hydrocarbons. Percentage removal of petroleum hydrocarbons as a result of this treatment was measured to be 58 % as compared to natural attenuation which was 49 % in surface layer. Similarly removal of petroleum hydrocarbons from saturated layer and non-saturated layer was also enhanced. It has been observed that application of Fenton alone might reduce natural biodiversity of soil, but as it was accompanied with NPK fertilizer, hence no negative effects on biodiversity were observed. This biostimulation technique also enhances degrading microbiota of soil. This technique can be considered as an efficient technique to remediate hydrocarbon-polluted soils (Silva-Castro et al. 2013).

Biostimulation of crude oil-contaminated soil was performed using Fenton coupled with nitrilotriacetic acid in a ratio of 1:1. After 20 weeks of remediation, it was observed that soil in which Fenton was coupled with nitrilotriacetic acid had reduced the amount of the total petroleum hydrocarbons by up to 88.9 % as compared to 55.1 % which was the result of treatment only with Fenton (Gong 2012). In situ metal stabilization technique can be used to remediate the contaminated soil. Long-term stability of copper and arsenic in coal ash-polluted soil helps in regaining the soil functioning (Tsang et al. 2014).

5.2 Phytoremediation

Phytoremediation which is performed through phytoextraction as well as phytostabilization is considered to be a potent technology through which we can get rid of soil pollution. Soil quality can be restored by the use of remediation technologies (Olaniran et al. 2013). Metal hyper-accumulators are being explored to be used as phytoremediating agents. Phytoremediation is performed using plants and microflora (Ali et al. 2013; Farid et al. 2014). Dominant terrestrial plants which basically include ferns can be used for remediation of soil in a synergistic way. Contaminated wastelands can be restored using ferns (Kumari et al. 2016). Among different tree species, *Acacia auriculiformis*, *Vetiveria zizanoides*, *Albizia lebbeck*, *Cymbopogon flexuosus*, and *Dalbergia sissoo* are considered to be the best to improve physiochemical properties of soil. They not only remediate soil pollution caused by coal ash but also help in improving the functioning of soil (Srivastava et al. 2014). A study suggests that *Alhagi maurorum Desv* and *Tamarix* sp. are important plants which can help in remediation of polluted soil in coal ash landfill area (Pen-Mouratov et al. 2014).

Soils which are contaminated by heavy metals are very hard to restore. In this case phytoremediation can be performed using hyper-accumulators. Plants accumulate heavy metals, thus remediate the soil. It is one of the most successful and

environment-friendly approaches of modern era to get rid of soil heavy metal pollution by plants (Shakoor et al. 2013). Phytoremediation is the best-known alternative for conventional remediation technologies. It is not only cheap but also an efficient technique (Witters et al. 2012). Microbe-assisted phytoremediation helps in detoxifying harmful soil and restoring biodiversity of degraded land to a large extent. This technology termed as MAP can be applied to various lands deteriorated by metals, pesticides, and hydrocarbons (Juwarkar 2012).

Experiments have been conducted in cold regions. It has been seen that treatment of petroleum-polluted soils with native tree species and grasses is an effective way of phytoremediation. It not only remediates the soil but also promotes ecological recovery. Phytoremediation possesses long-term positive effects on soil (Leewis et al. 2013).

5.3 Mycoremediation

Mycoremediation is a technique in which mycorrhizal fungi are used to ameliorate the soil from pollution (Ali et al. 2015c). Mycoremediation is considered to be one of the most suited techniques for remediation of heavy metal-polluted soil. The use of arbuscular mycorrhizal fungi has emerged as an interesting choice for remediation. This fungus helps in regaining soil potential by contributing to plant growth and nutrient acquisition. Arbuscular mycorrhizal fungi help the plants to grow and develop in soils which are highly toxic with heavy metals because they accumulate these heavy metals themselves and hence promote remediation (Meier et al. 2012).

6 Conclusion and Future Prospects

Analysis of soil pollution is considered to be a prerequisite for ecological restoration and management of soil (Qian et al. 2012). In the developing regions, comprehensive assessment of soil metal pollution originating from municipalities, acid mine drainage, agricultural activities, and industries is required (Guillén et al. 2012). This will help in identification of recommended policies to mitigate pollution and save the environment (Li et al. 2014). Biochar can be produced from industrial wastes and domestic sewage to improve soil condition and help in enhancement of soil biodiversity. Research has to be done to use industrial wastes and sewage properly and convert them in useful substances (Paz-Ferreiro et al. 2012). There are many anthropogenic sources which cause important environmental risks. Remediation of polluted soil has been performed using physical and chemical methods, which are largely not suited due to operational costs and technical inputs involved (Tripathi et al. 2015). In future, appropriate measures need to be taken to control levels of heavy metals in cultivatable soil. This will in turn result in enhancement of life quality and human health (Zhao et al. 2011). In future, vermicomposting as a huge bioremediation technique can be performed.

Vermicomposting of municipal solid waste before it affects the soil can be a sustainable waste management option (Singh et al. 2011). Phytoremediation is a promising alternative for commercial soil cleanup technologies because it is cost-effective as well as efficient, but its use is limited because it takes long period for remediation of soil. Profitable phytoremediation strategy should be planned in which biofuel crops can be used to remediate the soil. In such a case, not only the soil will be remediated, but these crops will also produce increased levels of biofuels. Further research has to be done to grow biofuel crops on contaminated soil (Oh et al. 2013).

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The spelling of the second editor, Mohd Sayeed Akthar's name was incorrect in the Table of contents and Chapter opening pages of chapters 4 and 7. The name should read as Mohd Sayeed Akhtar.

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