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Preface

One major challenge for the twenty-first century will be the production of sufficient food – the United Nations Population Fund estimates that the global human population may well reach 10 billion by 2050 (www.unfpa.org). This means increasing agricultural productivity of food crops, as plants form the basis of every food chain. If global food production is to keep pace with an increasingly urbanized and growing population while formulating new food production strategies for developing countries, the great challenge for modern societies is to boost plant productivity in an environmentally sustainable manner. Habitat-imposed abiotic and biotic stress is a serious condition causing major problem for crop productivity. About 20 % of cultivable and at least half of irrigated lands around the world are severely affected by environmental stresses. However, in these conditions, there are plant populations successfully adapted and evolutionarily different in their strategy of stress tolerance. Vascular plants do not function as autonomous individuals, but house diverse communities of associated microbes. The role of these microbes can no longer be ignored. To date, improvements in plant quality and production under abiotic and biotic stresses have relied largely on manipulating plant genomes by breeding and genetic modification. Increasing evidence indicates that the function of microbes seems to parallel more than one of these characteristics. Besides developing mechanisms for stress tolerance, microorganisms can also impart some degree of tolerance to plants toward abiotic stresses like drought, chilling injury, salinity, metal toxicity, and high temperature.

Plant growth-promoting microorganisms (PGPM) are capable of alleviating environmental stress and elicit tolerance in plants to promote their growth. Several PGPM elicit physical and/or chemical changes related to plant defense in the form of induced systemic resistance (ISR) under biotic stress. Researchers emphasized that PGPM-elicited ISR has suppressed plant diseases caused by a range of pathogens in both the greenhouse and field. PGPM-elicited physical and chemical changes in plants result in enhanced tolerance to drought, salt, and other factors that have been described in the form of induced systemic tolerance (IST) under abiotic stress. This project will focus on recent research concerning interactions between PGPM and plants under biotic and abiotic stresses. Consequently, continued research is needed to develop new approaches to ameliorate the efficiency of PGPM and to understand the ecological, genetic, and biochemical relationships in their habitat.

Exploitation of plant-microbe interactions can result in the promotion of plant health and can play a significant role in low-input sustainable agriculture applications for both food and nonfood crops. An understanding of the mechanisms enabling these microbes to interact with plants will be worthwhile to fully achieve the biotechnological potential of efficient partnerships for a range of applications. The most important and promising area of research for future studies is developing microbes to promote the sustainable production of cultivable crops in stresses (abiotic and biotic). In addition, the ability of microbes to confer stress resistance to plants may provide a novel strategy for mitigating the impacts of global climate change on agricultural and native plant communities. Microbes could play a significant role in stress management, once their unique properties of tolerance to extremities, their ubiquity, and genetic diversity are understood and methods for their successful deployment in agriculture production have been developed. These microorganisms also provide excellent models for understanding stress tolerance mechanisms that can be subsequently engineered into crop plants.

In the present world of rapidly growing population, it's a pressing need to produce a large quantity of crop yield to fulfill the basic requirements of life, and that's why it became more and more dependent on the use of agrochemicals in the form of fertilizers and pesticides. Although it shows instant effect on the growth and disease control, it is not long-lasting. In addition to this it also lowers the fertility of the soil. Benign microbe is proven as the best alternative of these agrochemicals with a lot of positive benefits. Besides promoting plant growth, microbes defend it from different disease-causing agents. Hence, in this book emphasis has been given to the role of bacterial strains in the abiotic/biotic stress, and their implication has been shown in the form of ISR.

In this book, editors compiled researches carried out by researchers in the form of compendium with elaborate description that relate with the "role of microbes in plant health upon biotic stress."

Chapter 1, "Changes in Phytochemicals in Response to Rhizospheric Microorganism Infection," describes the reports of recent investigations and infers that inoculation with rhizospheric microorganisms including plant growth-promoting rhizobacteria and arbuscular mycorrhizal fungi could change the production and accumulation of plant pharmacologically active compounds.

Chapter 2, "*Bacillus*-Mediated Induced Systemic Resistance (ISR) Against *Fusarium* Corm Rot," describes that *Bacillus* species have been very effective BCA due to their ability to produce heat and desiccation-resistant spores, to withstand high temperature, unfavorable pH, and lack of nutrients or water, and the ease of stable formulation preparation. This specie can display almost all the mechanisms of a biocontrol and bio-stimulation/fertilization agent.

Chapter 3, "Plant Growth-Promoting Rhizobacteria: Key Mechanisms of Action," describes an understanding of the direct and indirect mechanisms of action of PGPR, and their various benefits to plants are summarized and discussed.

Chapter 4, "Priming of Plant Defense and Plant Growth in Disease-Challenged Crops Using Microbial Consortia," describes that priming is known to aid in acclimation to various types of stress – abiotic and biotic in microorganisms – and gaining insights into the mechanisms and metabolites involved represents another challenging area. This compilation provides an overview of the recent developments in this field, highlighting the significance of the findings toward developing a "greener" agricultural scenario.

Chapter 5, "Seed Priming-Mediated Induced Disease Resistance in Arid Zone Plants," describes that seed priming allows plants to activate defense responses more quickly and effectively against plant pathogens without affecting growth of the plant and has the potential to emerge as a strategic tool for modern plant protection. It summarizes the current knowledge of the seed priming and its relevance for plant protection with special reference to bio-priming.

Chapter 6, "*Trichoderma* Secondary Metabolites: Their Biochemistry and Possible Role in Disease Management," focuses on the use of *Trichoderma* as a biocontrol agent in the present agriculture system and its advantages over traditional pesticides and fertilizers.

Chapter 7, "Induced Systemic Resistance in Rice," describes that plants possess a plethora of defense mechanisms that respond to both biotic and abiotic stresses. The response of a plant to various pathogens and pests can vary depending on factors such as host variety, strain, as well as environmental factors. In rice biotic stresses have been known to activate the JA/ETH and auxin pathways. Due to the higher levels of endogenous SA in rice, the SA-independent pathways are a preferred way of inducing resistance within the rice host.

Chapter 8, "Plant Growth-Promoting Rhizobacteria-Mediated Acquired Systemic Resistance in Plants Against Pests and Diseases," describes PGPR-mediated acquired systemic resistance against plant pathogens and insect pests, mechanisms of action, and results of field applications confirming their substantial role in inducing systemic resistance in crop plants.

Chapter 9, "Acyl Homoserine Lactone-Producing Rhizobacteria: Elicit Systemic Resistance in Plants," focuses on the role of AHLs produced by bacteria that play a unique role in altering the expression of plant defense genes.

Chapter 10, "Biological Control of Chickpea *Fusarium* Wilt Using Rhizobacteria 'PGPR,'" focuses that ISR has recently gained considerable importance in the control of *Fusarium* wilt of chickpea diseases both in greenhouses and in the fields.

Chapter 11, "AM Fungal Effect on the Growth of Selective Dicot and Monocot Plants," focuses on the arbuscular mycorrhizal status of selective dicot plants such as chickpea (*Cicer arietinum* L.), cowpea (*Vigna unguiculata* L.), and green pea (*Pisum sativum* L.) and selective monocot plants such as *Triticum aestivum* (L.) and *Pennisetum glaucum* and its beneficial effect on the efficiency of morphological and physiological changes in such plants grown under greenhouse condition.

Chapter 12, "*Trichoderma* spp.: Efficient Inducers of Systemic Resistance in Plants," describes the role of *Trichoderma* spp. as BCA and established plant root colonizers and their biocontrol nature primarily due to mycoparasitism and antibiosis mechanisms against various pathogens. Progress in research in plant immunity induced by beneficial microorganisms suggests that other than mycoparasitism and antibiosis, *Trichoderma* spp. are potent inducers of ISR in plants.

Chapter 13, "Induced Systemic Resistance by Rhizospheric Microbes," focuses that ISR has been developed as a significant and imperative means and way by which the selected and potential plant growth-promoting microbes in the rhizosphere influence the whole plant structure for higher and better defense against the broad range of pathogens and insect herbivores.

Chapter 14, "Combinations of PGPR for Initiation of Systemic Resistance Against Tree Diseases," discusses the PGPR and emphasizes the need to have unvarying significances for tree (i.e., *Eucalyptus*) that comprises the ISR against any pathogenic attack.

Chapter 15, "Plant Growth-Promoting Microbial-Mediated Induced Systemic Resistance in Plants: Induction, Mechanism, and Expression," focuses on recent research study concerning interaction between PGPMs and plants under biotic stress condition.

Last but not least we'd like to express our gratitude to contributors upon their consent to be a part of this book.

Noida, India

Devendra K. Choudhary
Ajit Varma

Contents

Retracted Chapter: Changes in Phytochemicals in Response to Rhizospheric Microorganism Infection

1

Mehrnaz Hatami and Mansour Ghorbanpour

Abstract

Plants are considered as source of commercially important phytochemicals that include metabolites of primary and secondary metabolism wherein primary metabolites are present throughout the plant kingdom and secondary metabolites have a more limited distribution and specialized function. The secondary metabolites are of major interest because of their different functions and their impressive biological activities ranging from antimicrobial, antibiotic, insecticidal, and hormonal properties to highly important pharmacological and pharmaceutical activities. The plant secondary metabolites, therefore, are widely used in aromatic, therapeutic, or chemical industries. This chapter surveys the reports of recent investigations involving rhizospheric microorganisms especially plant growth-promoting rhizobacteria and arbuscular mycorrhizal fungi that could change the production and accumulation of plant pharmacologically active compounds.

The original version of this chapter was revised: The chapter was retracted as it contains significant parts plagiarizing another publication. The erratum to this chapter is available at https://doi.org/10.1007/978-981-10-0388-2_16

The editors have retracted this chapter by Hatami and Ghorbanpour [1] because of overlap with a previously published article by Pedone-Bonfirm et al. [2].

Author Ghorbanpour does not agree with this retraction. Author Hatami did not respond to correspondence about this retraction.

[1] Hatami M, Ghorbanpour M (2016) Changes in phytochemicals in response to rhizospheric microorganism infection. In: Choudhary D, Varma A (eds) Microbial-mediated induced systemic resistance in plants. Springer, Singapore

[2] Pedone-Bonfim MVL, da Silva FSB, Maia LC (2015) Acta Physiol Plant 37:27. https://doi.org/10.1007/s11738-015-1781-3

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1.1 Introduction

It is consensus that higher plants use primary metabolites, namely, carbohydrates, amino acids, and lipids, to produce different secondary metabolites that are directly involved in the growth and development. Secondary metabolites are compounds produced in other metabolic pathways that, although important, are not essential to the functioning of the plant. However, secondary plant metabolites are useful in the long term, often for defense purposes, and give plants characteristics such as color. Secondary plant metabolites are also used in signaling and regulation of primary metabolic pathways and synthesize upon different biotic and abiotic elicitors stress. Numerous physiological traits and genetic diversity, viz., environmental conditions, geographic variation, and

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evolution are among the main factors that affect the accumulation and composition of secondary metabolites (Figueiredo et al. 2008). Pathway for secondary metabolites induced upon infection by microorganisms and abiotic factors such as osmotic stresses in plants (Sanchez et al. 2004). The function of secondary metabolites can be classified as mediators in the interaction of the plant with its environment, such as plant-insect, plant-microorganism and plant-plant interactions (Harborne 2001; Dixon 2001).

The production of secondary metabolites helps in the plant's defense system that includes the constitutive production of antifeedants and phytoanticipins and the inducible phytoalexins (Dixon 2001). In addition, there are several phenomena wherein secondary metabolism plays a role in plant development, e.g., reproduction (in attracting pollinators including male fertility). Furthermore, secondary metabolites determine perception for human food quality (taste, color, and smell) along with plant pigments required for the diversity of ornamental plants and flowers. Besides, plant secondary metabolites are used for the production of medicines, dyes, insecticides, flavors, and fragrances and further divided into three chemically distinct groups, namely, terpenes, phenolics, and nitrogenated compounds. The terpenes/terpenoids constitute the largest class of secondary metabolites which follow acetyl-CoA/glycolytic intermediates. Terpenes exhibited delineate functions in plant growth/development and can be considered primary rather than secondary metabolites. The basic functions of terpenes which include the plant hormones and the carotenoids (play a role in the growth/development and photosynthesis), the essential oils (as insect repellents) are important during pollination, and possess important antibacterial, antifungal, analgesic, anti-inflammatory and antioxidant properties, and the triterpenic saponins (as detergent, foaming, emulsifying, antimicrobial and antioxidant properties) (Heldt 2005).

Plants produce a large variety of secondary compounds that are considered phenolics and are synthesized mainly via the shikimic acid pathway. Plant phenolics are a chemically heterogeneous group of numerous individual compounds wherein some are absolute soluble in organic solvents, some are water-soluble carboxylic acids and glycosides, and others are large, insoluble polymers. They are considered as defensive compounds against herbivores and pathogens, pollinator attractors/seed dispersers, and protectors against ultraviolet radiation and for signaling in various interactions between plants and microorganisms (Taiz and Zeiger 2004; Steinkellner et al. 2007). Among them, several compounds are important for human health as they showed antioxidant, anti-inflammatory, antibacterial, antiproliferative, and antiviral properties (Martens and Mithofer 2005; Santos and Mello 2007). A large variety of plant secondary metabolites originated from amino acids and/or have nitrogen as part of their structure acting as anti-herbivore defenses that include alkaloids and cyanogenic glycosides, which are of considerable interest because of their toxicity to humans as well as their medicinal properties (Taiz and Zeiger 2004). The production of secondary metabolites in plants is influenced by various factors that include seasonality, day/night cycle, longevity/development of the plant, availability of nutrients, pluviometric index, temperature, ultraviolet radiation, altitude, atmospheric composition, CO_2 concentration, mechanical stimuli, and pathogenic attacks (Gobbo-Neto and Lopes 2007; Ghasemzadeh et al. 2010).

1.2 Elicitation of Secondary Metabolites in Soil Microorganism-Inoculated Plants

Plant growth-promoting rhizobacteria (PGPRs) and arbuscular mycorrhizal fungi (AMF) are two main groups of soil microorganisms that colonize the rhizosphere and substantially improve plant growth and efficiency through different mechanisms. However, PGPRs and AMF have been shown to interact during their processes of root colonization and exhibited cross support as AMF influenced PGPRs and PGPRs influenced AMF. Accumulation of secondary metabolites in plant is induced by microbe-plant interaction including physiological/genetic factors and environmental conditions. Some of the PGPRs, AMF, and their combination verified to be biotic elicitors for production of bioactive compounds in medicinal and aromatic plants are presented in Table 1.1.

Table 1.1 Effect of biotic elicitors on production of secondary metabolites in various plant species

Type of bio-elicitor	Plant species	Elicitation of phytochemicals	Reference
PGPRs			
Hormonema ssp.	*Brugmansia candida*	Hyoscyamine and scopolamine	Pitta-Alvarez et al. (2000)
Trichoderma viride	*Catharanthus roseus*	Ajmalicine	Namedo et al. (2002)
Pseudomonas fluorescens	*Catharanthus roseus*	Ajmalicine	Jaleel et al. (2007)
Pseudomonas aeruginosa and *Pseudomonas fluorescens*	*Pisum sativum*	Phenolic compounds (gallic, cinnamic, and ferulic acid)	Bahadur et al. (2007)
Pseudomonas fluorescens and *Bradyrhizobium* sp.	*Origanum majorana* L.	Terpinene-4-ol, *cis*-sabinene hydrate, *trans*-sabinene hydrate, and α-terpineol	Banchio et al. (2008)
Bacillus subtilis	*Crocus sativus* L.	Picrocrocin, crocetin, and safranal	Sharaf-Eldin et al. (2008)
Bacillus subtilis	*Ocimum basilicum*	α-Terpineol and eugenol	Banchio et al. (2009)
Pseudomonas fluorescens	*Catharanthus roseus*	Serpentine	Jaleel et al. (2009)
Bacillus cereus	*Salvia miltiorrhiza* Bunge	Tanshinone	Zhao et al. (2010)
Pseudomonas fluorescens, *Bacillus subtilis*, and *Azospirillum brasilense*	*Origanum x majoricum*	*Cis*- and *trans*-sabinene hydrate, gamma-terpinene, carvacrol, and thymol	Banchio et al. (2010)
Bacillus subtilis and *Pseudomonas fluorescens*	*Pelargonium graveolens*	Essential oil yield	Mishra et al. (2010)
Pseudomonas fluorescens, *Bacillus subtilis*, and *Azospirillum brasilense*	*Mentha piperita*	(+) Pulegone and (−) menthone	Santoro et al. (2011)
Pseudomonas putida and *fluorescens*	*Hyoscyamus niger* L.	Hyoscyamine and scopolamine	Ghorbanpour et al. (2013a, b)
Pseudomonas fluorescens and *Azospirillum brasilense*	*Tagetes minuta*	Monoterpenes and phenolic compounds	Cappellari et al. (2013)
Bacillus polymyxa, *Pseudomonas putida*, *Azotobacter chroococcum*, and *Glomus intraradices*	*Stevia rebaudiana*	Stevioside	Vafadar et al. (2014)
Pseudomonas putida and *fluorescens*	*Salvia officinalis* L.	*Cis*-thujone, camphor, 1,8-cineol, total phenolics, flavonoids, and antioxidant and antimicrobial compounds	Ghorbanpour et al. (2015)
AMF			
Rhizophagus intraradices	*Castanospermum australe*	Castanospermine	Abu-Zeyad et al. (1999)
Glomus margarita			
Glomus macrocarpum and *Rhizophagus fasciculatus*	*Anethum graveolens* L. and *Trachyspermum ammi* L.	Essential oil	Kapoor et al. (2002a)
Glomus macrocarpum	*Coriandrum sativum* L.	Essential oil	Kapoor et al. (2002b)
Rhizophagus fasciculatus			
Funneliformis caledonium, *Funneliformis mosseae*	*Cucumis sativus* L.	Triterpenoids	Akiyama and Hayashi (2002)
Glomus rosea	*Prosopis laevigata*	Trigonelline	Rojas-Andrade et al. (2003)

(continued)

Table 1.1 (continued)

Type of bio-elicitor	Plant species	Elicitation of phytochemicals	Reference
Glomus versiforme	*Lycopersicon esculentum* Mill.	Soluble phenols	Zhu and Yao (2004)
Rhizophagus clarus, *Claroideoglomus etunicatum*	*Mentha arvensis* L.	Essential oil	Freitas et al. (2004)
Gigaspora margarita			
Glomus macrocarpum, *Rhizophagus fasciculatus*	*Artemisia annua* L.	Essential oil and artemisinin	Chaudhary et al. (2008)
Funneliformis mosseae	*Ocimum basilicum* L.	Rosmarinic and caffeic acid and phenols	Toussaint et al. (2008)
Funneliformis mosseae	*Trifolium pratense* L.	Total isoflavones	Khaosaad et al. (2006)
Rhizophagus fasciculatus	*Azadirachta indica* L.	Azadirachtin	Venkateswarlu et al. (2008)
Mix-(*Claroideoglomus claroideum* (=*Glomus claroideum*), *Funneliformis mosseae* (=*Glomus mosseae*), *Glomus microaggregatum*, *Rhizophagus intraradices* (=*Glomus intraradices*)	*Allium cepa* L.	Total phenols	Perner et al. (2008)
Dentiscutata heterogama (=*Scutellospora heterogama*)	*Zingiber officinale* L.	Oil-resin	Silva et al. (2008)
Gigaspora decipiens			
Acaulospora koskei			
Entrophospora colombiana			
Mix of the four isolates			
Rhizophagus intraradices	*Echinacea purpurea* L.	Phenols	Araim et al. (2009)
Rhizophagus intraradices, *Funneliformis mosseae*, *Glomus* mix	*Cynara cardunculus* L.	Phenols and antioxidant activity	Ceccarelli et al. (2010)
Rhizophagus intraradices	*Fragaria* x *ananassa* Duch	Flavonoids	Castellanos-Morales et al. (2010)
Funneliformis mosseae, *Rhizophagus intraradices*, mix (six *Glomus* species)	*Valeriana officinalis* L.	Sesquiterpenic acids	Nell et al. (2010)
Glomus aggregatum	*Catharanthus roseus* L.	Indole alkaloids	Ratti et al. (2010)
Rhizophagus fasciculatus			
Rhizophagus intraradices			
Funneliformis mosseae			
PGPRs + AMF			
Glomus aggregatum, *Bacillus coagulans*, and *Trichoderma harzianum*	*Solanum viarum*	Total phenols, ortho-dihydroxy phenols, tannins, flavonoids, and alkaloids	Hemashenpagam and Selvaraj (2011)
Glomus mosseae, *Bacillus coagulans*, and *Trichoderma viride*	*Begonia malabarica* Lam.	Total phenols, ortho-dihydroxy phenols, tannins, flavonoids, and alkaloids	Selvaraj et al. (2008)

(continued)

Table 1.1 (continued)

Type of bio-elicitor	Plant species	Elicitation of phytochemicals	Reference
Arbuscular mycorrhizal and phosphate-solubilizing bacteria	Rose-scented geranium (*Pelargonium* sp.)	Citronellol, geraniol, geranial, and 10-epi-γ-eudesmol	Prasad et al. (2012)
Rhizophagus fasciculatus	*Lactuca sativa* L. var. longifolia	Soluble phenols	Baslam et al. (2011)
Commercial inoculants (*Funneliformis mosseae* and *Rhizophagus intraradices*)			
Rhizophagus fasciculatus, *Rhizophagus intraradices*	*Chlorophytum borivilianum*	Saponins	Dave and Tarafdar (2011)
Funneliformis mosseae			
Claroideoglomus lamellosum (=*Glomus lamellosum*), *Claroideoglomus etunicatum*	*Origanum onites* L.	Essential oil	Karagiannidis et al. (2011)
Rhizophagus intraradices, *Funneliformis mosseae*	*Hypericum perforatum* L.	Anthraquinone derivatives	Zubek et al. (2012)
Mix (*Funneliformis constrictum*, *Funneliformis geosporum*, *Funneliformis mosseae*, and *Rhizophagus intraradices*)			
Rhizophagus intraradices	*Solanum lycopersicum* L. var. money maker	Lycopene	Giovannetti et al. (2012)
Funneliformis mosseae, *Rhizophagus fasciculatus*	*Vitis vinifera* L.	Quercetin, total phenols	Eftekhari et al. (2012)
Rhizophagus intraradices			
Mix of the three species			
Acaulospora longula, *Gigaspora albida*	*Myracrodruon urundeuva*	Total phenols and total flavonoids	Oliveira et al. (2013)
Rhizophagus fasciculatus	*Stevia rebaudiana* (Bertoni)	Stevioside and rebaudioside A	Mandal et al. (2013)
Glomus sp.	*Fragaria* x *ananassa* var. Selva	Anthocyanin	Lingua et al. (2013)
Rhizophagus fasciculatus	*Lactuca sativa* L. var. longifolia and var. capitata	Anthocyanins	Baslam et al. (2013a, b)
Commercial inoculant (*Funneliformis mosseae* and *Rhizophagus intraradices*)			
Acaulospora longula + *Gigaspora albida*	*Anadenanthera colubrina*	Total phenols, total flavonoids, and total tannins	Pedone-Bonfim et al. (2013)
Funneliformis mosseae	*Cucumis sativus* L.	Phenols, flavonoids, and lignin	Chen et al. (2013)
Gigaspora albida, *Acaulospora longula* *Claroideoglomus etunicatum* (=*Glomus etunicatum*)	*Libidibia ferrea*	Total flavonoids	Silva et al. (2014)
Gigaspora albida	*Passiflora alata* Curtis	Total phenols and total flavonoids	Oliveira et al. (2014)
Rhizophagus irregularis, *Funneliformis mosseae*	*Viola tricolor* L.	Flavonoids, phenolic acids	Zubek et al. (2015)

1.3 Plant Growth-Promoting Rhizobacteria (PGPRs)

PGPRs are a specific group of soil microorganisms that efficiently colonizes the rhizosphere and rhizoplane and substantially improve plant health. There are several important bacterial genera that have been reported as PGPRs including *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*, and *Serratia* (Gray and Smith 2005). PGPRs may be considered as biotic elicitors, which have the potential to stimulate the production of secondary metabolites in plants. Ghorbanpour et al. (2013a) reported the effects of inoculation with two rhizobacteria strains, *Pseudomonas putida* (PP) and *Pseudomonas fluorescens* (PF), on tropane alkaloid (hyoscyamine and scopolamine) production in *Hyoscyamus niger* under three drought stress levels, i.e., 30 % (W1), 60 % (W2), and 90 % (W3) water depletion field capacity. The results showed that maximum hyoscyamine and scopolamine contents were recorded in PF-treated plants under W3 conditions. In contrast, the highest root and shoot alkaloid yield were obtained in plants bacterized with PP against W1 conditions. Although PP was the most effective strain under low WDS, PF had the highest efficiency in improving alkaloid production in the presence of severe (W3) water deficit stress (Ghorbanpour et al. 2013a).

Upon attack by microbial pathogens, terrestrial plants activate various defense mechanisms that include hypersensitive responses (a mechanism used by plants to prevent the spread of infection by microbial pathogens), production and accumulation of antimicrobial products called phytoalexin-/pathogenesis-related proteins with potential antimicrobial properties, and production and oxidative cross-linking of cell wall polymers (Penninckx et al. 1998). Secondary metabolism in plants is highly regulated by the presence of biotic/abiotic elicitors in the environment. Ghorbanpour et al. (2015) characterized PGPRs such as *Pseudomonas fluorescens* (*Pf* Ap1, *Pf* Ap18) and *Pseudomonas putida* (*Pp* Ap9, *Pp* Ap14) strains as well as their role (individually or in consortium) on biosynthesis of secondary metabolites, essential oils, total phenolics, and flavonoids in *Salvia officinalis* L. The antioxidant and antibacterial properties of the extracts and essential oils obtained from the inoculated plants were also investigated. Results indicated that different PGPR strains varied in their efficiency for production of auxin, siderophore, 1-aminocyclopropane-1-carboxylate deaminase, and phosphate solubilization. All inoculated plants showed significantly higher phosphorus content, essential oil yield, and total phenolics as well as flavonoid values compared to uninoculated control plants. The major constituents of essential oils include *cis*-thujene, camphor, and 1,8-cineol and increased upon inoculation with reference PGPRs. The extract from all inoculated plants exhibited improved antioxidant activity, which is remarkable for the *Pf* Ap18 strain that showed lowest IC_{50} value across treatments. Antibacterial assay of various essential oils and their major constituents against pathogenic bacteria showed that the highest activity was observed against *Staphylococcus aureus* using essential oils of *Pp* Ap14 source. Based on the results, it has been suggested that individual inoculation with effective PGPR strains can substantially improve plant growth and secondary metabolism in *S. officinalis* plants (Ghorbanpour et al. 2015). In other experiments, it has been found that *Hyoscyamus niger* plants inoculated with PGPRs exhibited higher value of hyoscyamine than scopolamine (Ghorbanpour et al. 2013a, b, c), which could be due to the high antimicrobial activity of hyoscyamine. Furthermore, antifungal activities of hyoscyamine and scopolamine against 40 fungal strains associated with *Hyoscyamus muticus* and found that all fungal strains were tolerant to scopolamine but sensitive to hyoscyamine (Abdel-Motaal et al. 2009).

Banchio et al. (2008) studied the effects of PGPR strains *Pseudomonas fluorescens*, *Bacillus subtilis*, *Sinorhizobium meliloti*, and *Bradyrhizobium* spp. on essential oil yield and constituents in *Origanum majorana*. The obtained results demonstrated that inoculation with PGPRs enhanced production of certain terpenes. Plants inoculated with *Bradyrhizobium* spp. and/or *P. fluorescens* showed significant increase in total essential oil yield by 10 and 24-fold, respectively. Based on the results, it was suggested that increases in total essential oil yield in response to inoculation were not merely due to increased biomass and might have resulted from increased biosynthesis of terpenes. The main compounds affected by inoculation with *P. fluorescens* were terpinen-4-ol, *cis*-sabinene hydrate, *trans*-sabinene hydrate, and α-terpineol as the concentrations of these compounds in inoculated plants were >1000-fold higher than uninoculated controls. Furthermore, the lack of effect of the *B. subtilis* and *S. meliloti* strains tested was attributed to their poor adaptation to root exudates and/or insufficient root colonization (Banchio et al. 2008).

The effects of single inoculation and co-inoculation with two PGPR strains (*P. fluorescens* and *Azospirillum brasilense*) on essential oil composition and phenolic content in Mexican marigold (*Tagetes minuta*) were studied (Rosario Cappellari et al. 2013). They observed that essential oil yield increased by 70 % in *P. fluorescens*-inoculated and co-inoculated plants in comparison with uninoculated controls, without altering the essential oil composition. The biosynthesis of the major essential oil components was increased in the inoculated plants. The total phenolic content was twofold higher in singly inoculated or co-inoculated treatments than in uninoculated control plants. Accordingly, they suggested that considering the economic importance of monoterpenes and phenolic compounds for a variety of applications in food and cosmetic industries, *P. fluorescens* and other suitable PGPRs have clear potential for improving the essential oil yield and productivity of cultivated medicinal plants (Rosario Cappellari et al. 2013).

1.4 Arbuscular Mycorrhizal Fungi (AMF)

AMF are eukaryotic fungi belonging to the *Glomeromycota* phylum and make an association with the roots of about 80 % of higher plants, forming a mutualistic symbiosis involving the exchange of products of photosynthesis, nutrients, and water (Berbara et al. 2006; Nagahashi et al. 2010). Upon inoculation with AMF, plants produced bioactive compounds that reflect the activity of plants with medicinal potential. Furthermore, studies have shown that accumulation of metabolites such as essential oils and some cyclohexanone derivatives is directly related to mycorrhization (Vierheilig et al. 2000; Ceccarelli et al. 2010; Zeng et al. 2013). It has been reported that treatments with AMF significantly improved the values of sesquiterpenes ($C_{15}H_{24}$) in valerian, and *Gigaspora margarita* as well as *Gigaspora rosea* increased the content of eugenol ($C_{10}H_{12}O_2$) in basil (Nell et al. 2010; Copetta et al. 2006). Moreover, phenolic compounds, such as flavonoids and several alkaloids, were found significantly higher following inoculation with AMF (Meng and He 2011; Yu et al. 2010; Rosa-Mera et al. 2011). The inoculation with *Glomus macrocarpum* and *Glomus fasciculatum* on *Artemisia annua* significantly enhanced the amount of artemisinin content (Chaudhary et al. 2008). However, some studies have shown that AMF do not have positive effects on the content of constituents. For example, inoculation of basil plant with AMF resulted in an increase in anthocyanin contents, but failed to alter the polyphenolic values (Lee and Scagel 2009). Moreover, Nell et al. (2009) reported that inoculation of *Salvia officinalis* by AMF had no effect on total phenolics and rosmarinic acid content in the roots (Nell et al. 2009). Also, no significant effect was found on essential oil composition of oregano (Khaosaad et al. 2006), and no effect was observed on the content of total terpene and single monoterpene in *Artemisia annua*, while production of specific sesquiterpenes in leaves was altered in mycorrhizal plants compared to control untreated plants (Rapparini et al. 2008). The effects of AMF symbiosis on plant proteome and

metabolism were previously studied and reported that AMF induced changes in phytohormone balance in inoculated plants (Torelli et al. 2000; Fitze et al. 2005) and the production of pathogenesis-related proteins as well as bioprotective effects for plant defense mechanisms (Toussaint et al. 2007).

1.5 Combination of PGPRs and AMF

The synergistic effects of combined inoculation of PGPRs with AMF have been reported on production of essential oil in plants. Prasad et al. (2012) found that the chemical composition of geranium oil was significantly affected by inoculation with phosphate-solubilizing bacteria and/or AMF. The content of linalool, geranial, 10-epi-γ-eudesmol, and citronellol in the geranium oil increased and that of *cis*- and *trans*-rose oxide decreased by inoculation with phosphate-solubilizing bacteria alone or in combination with AMF as compared to uninoculated controls. The changes in various constituents in the essential oil of all inoculated and fertilized geranium plants could be related to the enhanced uptake of phosphorous and divalent metallic cations in plant tissues (Prasad et al. 2012). Furthermore, tissue culture-regenerated plantlets of *Stevia rebaudiana* Bertoni were transferred to pots and subsequently inoculated with PGPRs (*B. polymyxa*, *P. putida*, and *A. chroococcum*) and an arbuscular mycorrhizal fungus (*G. intraradices*); the highest stevioside value was obtained in plants dually inoculated with *G. intraradices* + *A. chroococcum*, followed by *G. intraradices*+ *B. polymyxa* and *A. chroococcum*+ *P. putida*, respectively. Triple inoculations had less positive effects compared to dual inoculations, probably due to higher competition between microorganisms (Vafadar et al. 2014).

The root system of Italian oregano (*Origanum* x *majoricum*) was subjected to inoculation with three PGPRs (*P. fluorescens*, *B. subtilis*, *A. brasilense*), and the essential oil content was measured (Banchio et al. 2010). Obtained results showed that the total essential oil yield for plants inoculated with *P. fluorescens* or *A. brasilense* was approximately 2.5-fold higher than controls wherein the major essential oil compounds *cis*- and *trans*-sabinene hydrate, γ-terpinene, carvacrol, and thymol showed increased biosynthesis. Nonpathogenic PGPRs have been shown to stimulate the biosynthesis of secondary metabolites in plants through a mechanism termed induced systemic resistance (VanLoon and Glick 2004). It is well established that biological agents can act as effective elicitors of key enzymes involved in biosynthetic pathways of secondary metabolites which are clearly related to plants defense responses against pathogenic agents despite being induced by nonpathogenic bacteria (Chen et al. 2000; Kloepper 1993). The effect of combined inoculation for plant *Begonia malabarica* Lam. (Begoniaceae) by an arbuscular mycorrhizal fungus (*G. mosseae*), a PGPR strain (*B. coagulans*), and *Trichoderma viride* was studied on production of secondary metabolites. Plants inoculated with microbial consortium consisting of *G. mosseae* + *B. coagulans* + *T. viride* showed the highest increase in leaf secondary metabolites (total phenols, ortho-dihydroxy phenols, flavonoids, alkaloids, and tannins) followed by the plants dually inoculated with *G. mosseae* + *B. coagulans* (Selvaraj et al. 2008). Plant co-treated with *G. mosseae* and *B. subtilis* strain produced 25–103 % more artemisinin than in other treatments, indicating a synergistic effect between both inocula (Awasthi et al. 2011). Synergy has also been found between *G. fasciculatum* and a beneficial *Pseudomonas* in improving yield and forskolin content of *Coleus forskohlii* tubers under field conditions (Singh et al. 2013). In a similar study, the effects of the arbuscular mycorrhizal fungus *G. aggregatum*, the PGPR strain *B. coagulans*, and *T. harzianum* were evaluated on secondary metabolite content of *Solanum viarum* seedlings. Triple inoculation of *G. aggregatum* + *B. coagulans* + *T. harzianum* resulted in maximum secondary metabolites including total phenols, ortho-dihydroxy phenols, flavonoids, alkaloids, saponins, and tannins. Here, the higher secondary metabolite values in inoculated plants were attributed to the enhanced mycorrhizal colonization and

improved nutrient status of the host plants (Hemashenpagam and Selvaraj 2011).

1.6 Mechanisms Underlying Elicitor-Mediated Biosynthesis of Secondary Metabolites in Plants

Production of secondary metabolites depends largely on primary metabolism including photosynthesis and oxidative pathways for carbon and energy supply (Singh et al. 1990). As a result of enhanced nutritional status, it has been suggested that the net photosynthesis of PGPR-inoculated plants increased (Giri et al. 2003). Factors affecting plant dry matter accumulation and biomass may influence the interrelationship between primary and secondary metabolism, leading to increased production of secondary metabolites and greater substrate availability for monoterpene biosynthesis, respectively (Harrewijn et al. 2001). The increased value of monoterpenes in PGPRs hosting plants may also be due to growth-promoting substances released by the microorganisms, which affect metabolic pathways in plants. The mechanism of AMF-induced changes in the phytochemicals of plant tissues may be multidirectional, while the exact mechanisms by which AMF change the biosynthesis of secondary metabolites still remain unclear (Toussaint et al. 2007). It has been suggested that increase in the production of phenolics, terpenes, and nitrogen-containing compounds in mycorrhizal infected plants may be due to the vegetative response to the fungal colonization, which may involve different metabolic processes that can be mediated by a better absorption of nutrients (phosphorous and nitrogen) enhanced by the symbiosis (Zubek et al. 2010).

The modification of metabolite concentration including flavonoids and alkaloids in roots may be the consequence of signaling mechanisms between the host plant and fungi. Elicitation of terpenes, steviosides and rebaudiosides, in mycorrhizal *S. rebaudiana* is associated with the increase of jasmonic acid (Larose et al. 2002; Rojas-Andrade et al. 2003; Mandal et al. 2013). Many studies have shown that the high production of phenolics may be related to improved activities of the enzymes, chalcone synthase and chalcone isomerase, involved in the synthesis of flavonoids and phenylalanine ammonia-lyase, responsible for catalyzing the deamination of phenylalanine, which is a key regulating stage in the formation of phenolics (Ibrahim and Jaafar 2011). In addition to increased activities of related enzymes for secondary metabolism, namely, phenylalanine ammonia-lyase, glucose-6-phosphate dehydrogenase, and shikimate dehydrogenase, the AMF also increased the expression of genes involved in the regulation of the phenylpropanoid pathway (Chen et al. 2013). Altered gene expressions in hosts as a result of AMF colonization influence their metabolism and lead to the induction of chemical defense. Moreover, a positive correlation was observed between the density of glandular trichomes, the main structure for essential oil secretion, and the amount of artemisinin in mycorrhizal leaves of *Artemisia annua* L. (Kapoor et al. 2007). Also, cytological variations including increase in the number of plastids and mitochondria were observed in the AMF-inoculated plant, leading to activation of the tricarboxylic acid cycle and the plastid biosynthetic pathways thereby increasing the biosynthesis of phytochemicals (Lohse et al. 2005; Strack and Fester 2006). Molecules produced upon biotic elicitation play an important role in pathways linked to the biosynthesis of secondary metabolites. For example, growth regulators, volatile organic compounds, jasmonic acid, ethylene, nitric oxide, and reactive oxygen species (ROS), which increase plant immunity by activating defense pathways, have long been observed to be transducers of elicitor signals in production of plant secondary metabolites. Multiple signaling pathways and important mechanisms of action of elicitor in production of plant secondary metabolites are presented in Fig. 1.1.

Signal perception is regarded as the first committed step of the elicitor signal transduction pathways in plants. Following perception, plant receptors are activated initially and then in turn

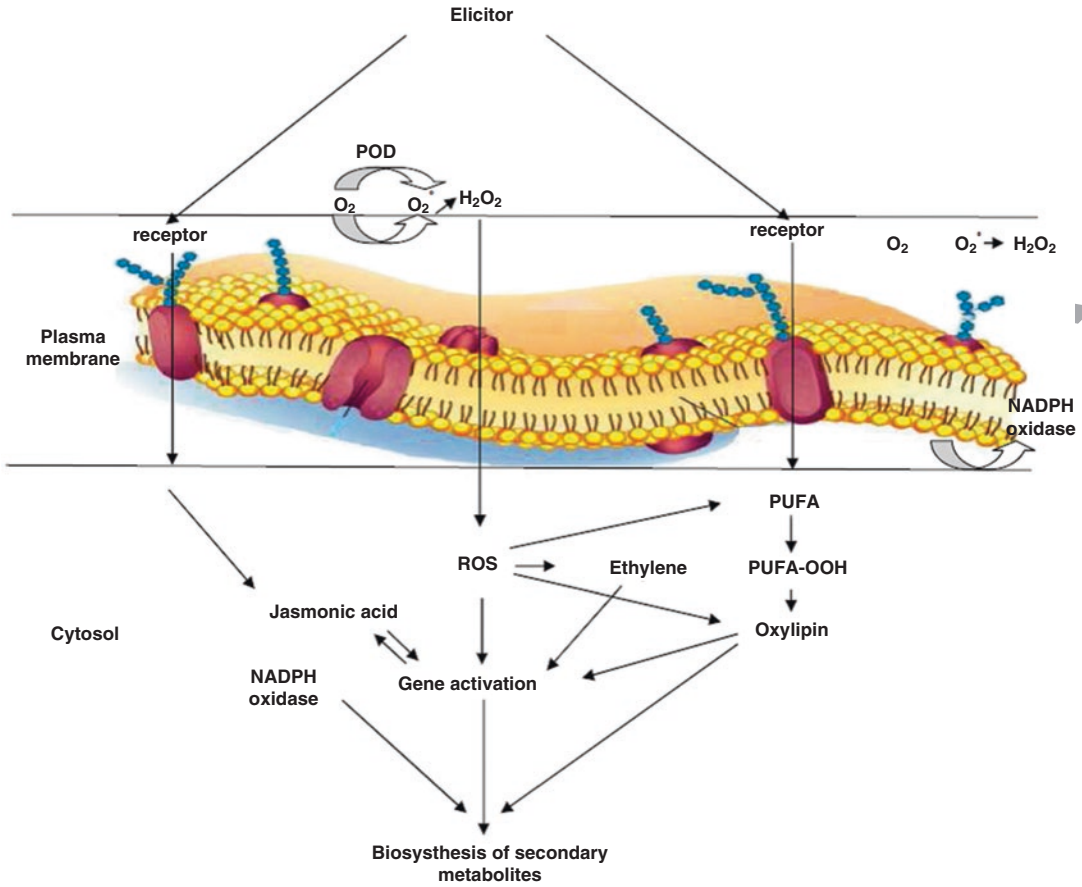

Fig. 1.1 A model for signal transduction events by elicitor, leading to the expression of genes encoding enzymes involved in the biosynthesis of secondary metabolites in plants. Under elicitor treatment, the interaction of elicitor-cell activates specific enzymes including peroxides (*POD*) and NADPH oxidases, resulting in the generation of reactive oxygen species (*ROS*). ROS generated from nanomaterial-induced oxidative stress oxidize polyunsaturated fatty acids (*PUFAs*) to polyunsaturated fatty acid hydroperoxides (*PUFA-OOH*), which are converted to oxylipins, leading to the expression of genes involved in the biosynthesis and accumulation of secondary metabolites in plant cells

activate their effectors such as ion channels and protein kinases. The activated effectors transfer the elicitor signals to second messengers, which further amplify the elicitor signal to other downstream reactions and expression (Zhao et al. 2005). Secretion of volatile organic compounds by PGPRs can be another possible mechanism for enhancing production of plant secondary metabolites. All organisms produce volatile organic compounds, which are characterized by low molecular weight and high vapor pressure, and play important roles in communication within and between organisms. Bacterial volatile organic compounds have been reported as a rich source for new natural compounds that may increase crop productivity and essential oil yield in medicinal and aromatic plants. For example, the effects of volatile organic compounds produced by three PGPRs (*P. fluorescens*, *B. subtilis*, and *A. brasilense*) on essential oil constituents of *Mentha piperita* L. were studied. Santoro et al. (2011) reported that production of monoterpenes increased twofold in plants inoculated with *P. fluorescens*, showing increased biosynthesis of the two major essential oil, (+) pulegone and (−) menthone. Menthol in *A. brasilense*-inoculated plants was the only major essential oil that showed a significant decrease. It has also been

reported that the PGPR strain *B. subtilis* GB03 releases volatile organic compounds that elevate essential oil production in *Ocimum basilicum* (Banchio et al. 2009). The two major essential oil components, R-terpineol and eugenol, increased by two- and tenfold, respectively, in plants exposed to GB03 volatile organic compounds or with root inoculation, as compared to uninoculated control (Banchio et al. 2009).

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Bacillus-Mediated-Induced Systemic Resistance (ISR) Against *Fusarium* Corm Rot

2

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Abstract

Fungi constitute the largest group of plant pathogens responsible for a range of serious plant diseases wherein fungal rot is a major disease associated with the pre- and post-harvest produce of plants. One of the most widely spread rot-causing fungi is *Fusarium* spp. that include *F. oxysporum*, *F. graminearum*, and *F. solani* that infect bulbs, tubers, rhizomes, and corms and lead to the decomposition of the tissue and finally death of the plant. Under low temperature, fungal infection usually remains dormant which under favorable climatic conditions converts in to disease. As there is a large decline in the annual yield of the crop plant due to *Fusarium* rots, so this has been an issue of concern since long. Earlier only chemical pesticides were used to control these infections but due to their ill effects on soil fertility, the focus has shifted to the use of biological control agents (BCA). Among BCA, a group comprised of bacilli, pseudomonads, and actinomycetes, together with nonpathogenic organisms *Fusarium*, *Trichoderma*, and *Streptomyces*, played an important role against phytopathogens. BCA helps in plant disease control and growth mainly by two methods: (i) secretion of antimicrobial compounds and (ii) induction of systemic resistance in plants. *Bacillus* species have been very effective BCA due to their ability to produce heat and desiccation-resistant spores and to withstand high temperature, unfavorable pH, lack of nutrients or water, and the ease of stable formulation preparation. This species can display almost all the mechanisms of a bio-control and bio-stimulation/fertilization agent.

2.1 Introduction

Plants by virtue of being capable of fixing solar energy are the main players in a complex food web together with their ability to innate defensive capacity against pests and pathogens.

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Microorganisms living in associations with the plants, however, alternatively transit between pathogenic and symbiotic states depending on plant health and environmental conditions. Among interactions between plant and microbes, the symbiotic association plays an important role in nitrogen fixation, growth promotion, protection from pathogens, nutrient mobilization, and subsequent uptake of essential nutrients by plant to develop its full genetic potential. The root system/rhizosphere is a hot spot of plant-microbe interaction, is a narrow zone surrounding the roots, and is influenced by root secretions and associated microbes, making this small zone one of the most energy-rich habitats on earth wherein up to 40 % of the plant's photosynthetically fixed carbon is deposited into the rhizosphere (Compant et al. 2005; Lynch and Whipps 1991). Plants select and enrich certain bacterial communities by secreting certain compounds that selectively stimulate/repress growth of specific microbe. Pathogenic microorganisms associated with plants cause many diseases, leading to major loss in the yield. Many bacterial, fungal, and viral species are known to be pathogenic to plants, and the fungal rot is one of the major diseases associated with the pre- and post-harvest produce of plants. *Fusarium oxysporum*, *Fusarium graminearum*, and *Fusarium solani* are some of the common *Fusarium* pathogens causing plant rots in leaves, roots, tubers, corms, stems, etc. (IAC 1992). Pathogenic *Fusarium* sp. infection results in deformations of bulbs, corms, tubers, and rhizomes, including blue-gray to purple-brown discoloration, spongy decay under the outer scales (Ma et al. 2013).

It is our consensus that chemical pesticides have been used through times to control these infections, but due to their ill effects on soil fertility, there is a need for alternative biological agents. In plant pathology, the term "biological control" applies to the use of microbial antagonists to suppress diseases as well as the use of host-specific pathogens to control weed populations (Compant et al. 2005). A biological control agent (BCA) helps in plant disease control and growth by employing two methods which include, first, secretion of antimicrobial compounds and, second, induction of systemic resistance in plants (Ramamoorthy et al. 2001). Induced resistance can be described as resistance put by plants against any biotic stress triggered by biological (insects, pathogens, or microbes) or chemical inducers which is regulated by a network of interconnected signaling pathways, wherein plant hormones play a major regulatory role. Induced resistance is not only expressed locally at the site of infection but also systemically in plant parts that are spatially separated from the infection, hence the term induced systemic resistance (ISR). Many bacterial species belonging to pseudomonads, bacilli, actinomycetes and fungi belonging to various species of nonpathogenic *Fusarium*, *Trichoderma*, and *Streptomyces* are used as biocontrol agents that induce systemic resistance in plants (Choudhary and Johri 2009).

2.2 *Fusarium* as a Causative Agent of Plant Rots

Plant diseases are generally caused by biotic and abiotic factors wherein biotic factors include diseases caused by living organisms like bacteria, viruses, and fungi and abiotic factors cover nonliving components like nutrient deficiency and weather. Pathogens such as bacteria, fungi, viruses, nematodes, insects, parasitic plants, and phytoplasmas (phloem-dwelling prokaryotes) attack the various organs of the plant during different stages in its growth cycle. Biotic elicitation hampers the photosynthesis by killing the leaves of the plant (diseases of the foliage), while others interfere with the water and mineral absorption from the soil (diseases of the roots and stem base), some impair the translocation of sugars produced during photosynthesis to the grain (systemic virus diseases), and others completely destroy the developing grains (disease of the head and kernel) (http://www.croppro.com.au/crop_disease_manual/ch01s01.php).

Fungi constitute the largest number of phytopathogens and are responsible for a range of serious plant diseases. Most fungal bodies consist of thin delicate filaments called hyphae that

grow inside/on the host's tissues and reproduce through spores which are often visible as outgrowth on the affected parts of the plants (Singh 1983). Diseases caused by fungi include rots (*Botrytis* rots, *Fusarium* rots, *Sclerotinia* rots, *Rhizoctonia* rots, etc.), rust, and mildews (downy mildews, powdery mildews, etc.) (http://ausveg.com.au/intranet/technical-insights/cropprotection/fungal-diseases.html). The most common and destructive among the fungal diseases are the plant rots. Rots are characterized by plant decomposition and putrefaction. The decay due to rot may be hard, dry, spongy, or slimy and may affect any plant part. Rot disease caused by various species of *Fusarium* affects many perennial and annual plants. *Fusarium* is an omnipresent fungus in soil, water, and air and can be recovered from plant inflorescence to the deepest roots in the soil. Most of *Fusarium* species are harmless and are saprotrophic, while some produce mycotoxins (e.g., moniliformin (MON), fumonisins(FBs)) in cereal crops that can affect human and animal health by entering in the food chain (Gräfenhan et al. 2013; Nayaka et al. 2010). *Fusarium*-caused diseases include wilts, blights, rots, and cankers of many horticultural, field, ornamental, and forest crops in both agricultural and natural ecosystems. *Fusarium* infection may initiate in roots or corms from soilborne inoculums/in aboveground plant parts via air/water, e.g., *F. oxysporum* first penetrate the roots asymptomatically and then it colonizes vascular tissue and triggers massive wilting, necrosis, and chlorosis of aerial plant parts. On the other hand, *F. graminearum*, the major cause of *Fusarium* head blight of cereals worldwide, infects floral tissues during anthesis and spreads into uninfected flowers through the central axis of the inflorescence, eventually damaging kernels and contaminating them with toxins (Ma et al. 2013). Pathogenic *Fusarium* has a wide range of host specificity but in this chapter we focus on plants that seed through bulbs and corms (modified stems) or tuberous roots (modified roots) such as saffron, banana, potato, gladiolus, lily, narcissus, and tulip. The rot in gladiolus is caused by *F. oxysporum* f. *gladioli* that survives in soil indefinitely. *Fusarium oxysporum* f. *gladioli*, *F. solani*, *Penicillium stromatinia gladioli*, *Bacillus croci*, and *Burkholderia* are some of the pathogens associated with *Crocus* corm rot (Fiori et al. 2012) wherein *F. oxysporum* is the most destructive pathogen, causing severe losses in most saffron fields. These plants when infected show a dark discoloration within underground parts that commonly extends into the leaf bases. Seedlings of all flowering plants may suddenly wilt, collapse, and die. On the surface of infected stems, bulbs, or corms, masses of *Fusarium* spores (white or pinkish) are formed in fungal fruiting bodies called sporodochia (Fig. 2.1) (https://ipm.illinois.edu/diseases/rpds/650)

2.2.1 Mechanism of Infection

Fusarium infect the crops through various organs including the root (wheat, bare root, etc.), corm (saffron, gladiolus), stem (soyabean and potatoes), and crown (head of wheat plant, banana); however, in present case the focus is on corm rot. Healthy plants can become infected by *Fusarium* if the soil in which they are growing is contaminated with the fungus. Plants can become infected in the field at the time of germination of spores/mycelia. The fungus can invade a plant either with its sporangial germ tube/mycelium by invading the plant's roots. The corms get infected directly through the root tips/wounds in the corms caused by wind, hail, mechanical damage, or insect feeding (Palmero et al. 2014). *Fusarium* genome encodes cell wall degrading and other hydrolytic enzymes presumed to be deployed during infection to gain access to nutrition. Infection can spread from mother corm to cormlets (daughter corm). Once inside the plant, the mycelium grows through the root or corm cortex intercellularly. The fungus along with other opportunistic pathogens decomposes the tubers by inhibiting protein translation and promotes cell death. On reaching the xylem, the mycelium invades the vessels through the xylem's pits. At this point, the mycelium remains in the vessels, where it usually advances upward toward the stem and crown of the plant. *Fusarium* species does not need to produce any aboveground fruiting

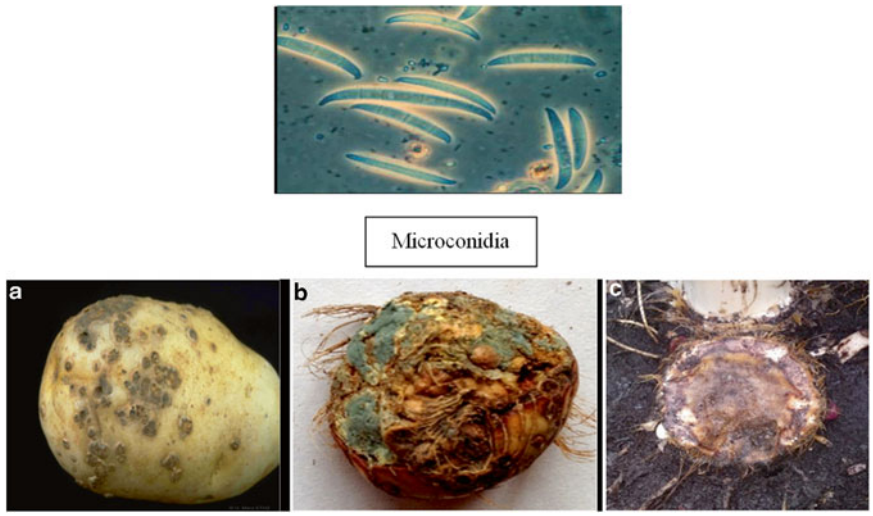

Fig. 2.1 Symptoms of rot in various belowground plant organs. (**a**) Potato tuber rot, (**b**) saffron corm rot, (**c**) banana corm rot

bodies to spread the disease. Only formations of microconidia (Fig. 2.1) are necessary later in the season that are carried upward within the vessel by way of the plant's sap stream. When the microconidia germinate, the mycelium can penetrate the upper wall of the xylem vessel, enabling more microconidia to be produced in the next vessel (http://www.potatodiseases.org/dryrot.html).

2.2.2 Disease and Symptoms

There are mainly three types of *Fusarium*-induced rot symptoms associated with corms: brown rot (most common), vascular rot, and basal dry rot. The symptoms common to all three forms are a firm, brown to black rot; yellowing, browning, and premature dying of the leaves; and a browning and destruction of the roots. The corms may rot before digging, in storage, or after planting (Partridge 2003). In brown rot no vascular distortion occurs. Brown to black lesions appear on the surface of corm tissue, typically near the base. The rot can extend deeply into the corm flesh. The corm tissue remains firm and becomes scaly. Vascular rot develops occasionally. In this form the disease involves the vascular tissue. When the bulbs are cut open, the dark core is visible extending from the base toward the top. Brown spots can also appear on the surface when the infected vascular bundles extend completely through the flesh and reach the outer corm layer. The basal dry rot differs from the brown-rot form mainly in the position and thickness of the lesions. It occurs only on the base of the corms and usually is restricted to the first and second internodes and rarely extends more than 2–4 mm into the flesh.

The *Fusarium* rot spot (round or oval) becomes depressed as rotten tissue dries. The surface shrinks and often forms concentric rings. The rotted tissue becomes compressed and tough on drying. Planting a severely infected corm can produce feeble shoots that die early. Less severely infected corm gives rise to normal plant until in the late season when the tip of the leaves turns yellow and starts dying gradually. However, these symptoms may vary greatly in different varieties and different stocks. For example, in banana corm rot, the leaves do not turn yellow but remain green and die in severe infection. The affected plants appeared weak, small, and stunted. The *Fusarium* infection in corm may be latent, and disease manifestation depends on air and soil temperatures. Symptoms are usually absent or mild at temperatures below 21–24 °C (70–75 °F) and are most severe in temperature range of 29–32 °C (80–90 °F). Plants at low temperature

may be infected yet show no symptoms until the temperature rises (https://ipm.illinois.edu/diseases/rpds/650).

2.3 Management of *Fusarium* Rot Disease

The management of rot diseases caused by *Fusarium* species is very important so as to decrease the chances of yield loss in many of the economically important crops. Currently the management of this disease depends entirely on chemical fungicides in most of the areas because of their ease of utilization, effectiveness, and industrial production. The most commonly used fungicides include dichlorodiphenyltrichloroethane, captan, benlate, and thiabendazole (Cohn et al. 2007). These fungicides have been reported to inhibit the germination of rot-inducing organisms including *F. moniliforme*, *Botryodiplodia theobromae*, and *Penicillium sclerotigenum*. These chemicals are heavy-duty chemicals whose demerits have already been reported in literature including pollution and toxicity. Multiple fungicide applications have increased the economic cost and public concerns of using fungicides. These chemicals also affect the beneficial microflora associated with the plant and put selection pressure for evolution of resistant pathotypes. Consequently, there is an immediate demand for more safe, rational, sustainable, and eco-friendly strategies. Biological control offers an environmentally friendly alternative to pesticides for controlling plant diseases, thereby reducing the pesticide risks, pollution and toxicity, and resistance development (Thakore 2006). Bacteria from the rhizosphere and bulk soil communities have been reported to be effective biocontrol agents and also play a role in suppressive soil phenomenon. The biocontrol agents/biopesticides and other nonconventional pesticides can be grouped into the following categories: The first is semiochemicals which are message-bearing substances produced by a plant or animal or a functionally similar synthetic analogue which evokes a behavioral response in individuals of either same or different species, e.g., allomones, kairomones, pheromones, and synomones. Other nonconventional pest control products are substances other than the above category and meeting the following criteria: (i) low inherent toxicity to nontarget organisms, (ii) not persistent in the environment, (iii) pesticidal action should not be the result of toxicity to the organism, (iv) less likelihood of selecting for pest resistance, and (v) widely available to the public with a history of safe use under conditions for human exposure and the environment. Common food items, extracts, preservatives or additives, and plant extracts and oils are commonly included in this category. The second category is microbials which are microorganisms, bacteria, alga, fungus, protozoan, virus, mycoplasma, or rickettsia and related organisms and any associated metabolites, which have the pest control effects attributed to them. Plant growth-promoting bacteria (PGPB) and plant growth-promoting fungi (PGPF) are being exploited commercially for plant protection. Treatment of seed with PGPRs causes cell wall structural modifications and biochemical/physiological changes ultimately resulting in the synthesis of proteins and chemicals involved in plant defense mechanism.

In recent years different research groups tried to prove the intimate relation between plant health and microbial association. Evidence provided for the fact that selected bacterial strains can promote plant health by stimulating plant immune system. Many studies allowed authors conclude that disease resistance was caused by a plant-mediated immune response known as microbial-induced systemic resistance (ISR). Since the first review on microbial-mediated-induced systemic resistance, significant progress has been achieved in understanding the molecular basis of triggering, signaling, and expression of microbial-induced systemic resistance. Consortium of different PGPRs has increased efficacy by induced systemic resistance against several pathogens. Lipopolysaccharides, siderophores, and salicylic acid are the major components of PGPR-mediated-induced systemic resistance (Pieterse et al. 1996). *Bacillus* species, *Pseudomonas* species, *Trichoderma* species, and nonpathogenic *Fusarium species* are the most

commonly employed species as biocontrol agents which protect plant from pathogens by inducing systemic resistance.

2.3.1 *Bacillus*-Induced Systemic Resistance

Bacillus has been one of the key genera used for the biocontrol of plant diseases due to the advantage of producing heat- and desiccation-resistant spores, ability to withstand high temperature, unfavorable pH, lack of nutrients or water, and the ease of stable formulation. *Bacillus* spp. is among the most commonly commercialized PGPB with direct and indirect growth promotion, easy dispersal, and long shelf life of their spores. The popularity of *Bacillus* as a biocontrol agent is also because of it being, firstly, a well-studied genus, and, secondly, the US food and drug administration (USFDA) has granted "generally regarded as safe" (GRAS) status to *Bacillus* species (Harwood and Wipat 1996). Besides the spore-forming capacity of *Bacillus*, the ability to move and survive as a facultative anaerobe in the rhizosphere of plant makes it more effective as a biopesticide (Piggot and Hilbert 2004). Another important reason for interest in *Bacillus* species is due to the diversity in their mode of action. This species can display almost all the mechanisms of a biocontrol and bio-stimulation/fertilization. *Bacillus* species such as *B. amyloliquefaciens*, *B. subtilis*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. mycoides*, and *B. pumilus* are most widely used in bioformulation preparation wherein *B. amyloliquefaciens* FZB42 is a producer of three families of lipopeptides, surfactins, bacillomycin D, and fengycins, which are well-known secondary metabolites with antifungal activity (Arguelles-Arias et al. 2009; Chen et al. 2009; Rückert et al. 2011) (Table 2.1).

2.3.2 Mechanisms Involved in Biocontrol by *Bacillus*

Bacillus species usually colonize leaf surfaces, root systems, and their surrounding soil layers thereby taking advantage of the exudates and nutrients released by the plants. In return they provide protection from the pathogenic organisms. Broadly this protection is of three types: (i) by competing for an ecological niche or substrate, (ii) production of inhibitory allelochemicals, and (iii) induction of systemic resistance in host plants. Inability of some PGPR strains to exert antagonist effect against pathogens directly shed new light on the diversity of their modes of action. The indirect suppression of disease without direct contact with the pathogen suggested that such strains may act as efficient biocontrol by activating defense system in the host plant. This stimulation of plant immune system allowing an accelerated activation of defense response upon pathogen attack was termed as induced systemic resistance (ISR). Induced systemic resistance is a three-step process involving sequentially:

(i) The perception of elicitors by plant cells produced by the inducing agents that initiate the phenomenon
(ii) Signal transduction required to propagate the induced state systemically through the plant
(iii) Expression of defense mechanisms that limit or inhibit pathogen penetration into the host tissues

ISR relies on pathways regulated by jasmonate and ethylene. Interconnected signaling pathways regulate induced defenses of plants against pathogens (Pieterse et al. 1996). The primary components of the network are plant signal molecules, namely, salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and nitric oxide (NO) (Delledonne et al. 1996). In response to a pathogen attack, there is an increase in SA levels, and exogenous application of SA can induce a set of pathogenesis-related genes and establish systemic acquired resistance (SAR) (Uknes et al. 1992). JA and ET are low-molecular-weight volatile compounds produced by microbes in the rhizosphere of the plant that can also trigger induced systemic resistance (Thomma et al. 2001; Yan et al. 2002). ET signaling is required in the translocation of ISR signals. JA and ET act together in the activa-

Table 2.1 Commercially available bioformulations based on *Bacillus* species (Cawoy et al. 2011)

Bacillus species	Product	Target organism	Crop
B. amyloliquefaciens	Rhizo-vital 42	Soilborne pathogens, *Fusarium* species	Potato, corn, strawberry, tomato, cucumber, and ornamental plants
B. subtilis	Cease	*Fusarium* species	Several crops
B. subtilis	Companion	*Fusarium oxysporum*	Pea, soyabean, corn, and others
B. pumilus	Yield shield	–	Soyabean
B. subtilis	Highstick, Promix	*Fusarium* species	Soyabean, ornamental, and other crops
B. pumilus	Ballad	–	Cereals, oil plants, and sugar beet
B. subtilis	FZB42WB	*Fusarium oxysporum* and other *Fusarium* species	Several crops
Bacillus spp.	Sublic	–	Several crops
B. subtilis	Biosubtilin	*Fusarium* species	Cotton, cereals, vegetables, and ornamental plants

tion of defense responses. In addition to SA, JA, and ET, NO has also been recently characterized as a signaling molecule in plants. NO promotes seed germination and leaf extension, and root growth along with it also delays leaf senescence and fruit maturity. In addition NO activates many genes and triggers resistance-associated hypersensitive cell death (Romero-Puertas and Delledonne 2003). In one of the experiments, *B. amyloliquefaciens* NJN-6 has been used as a biocontrol agent against pathogenic *F. oxysporum*. In this example, the antifungal effect of volatile compounds triggering induced resistance in plants against *F. oxysporum* was studied. A total of 36 volatile compounds from *B. amyloliquefaciens* NJN-6 were isolated, and on the basis of GC-MS analysis, 11 compounds completely inhibited fungal growth. The antifungal results of these compounds suggested the important role played by volatile compounds in the suppression of *F. oxysporum* (Yuan et al. 2012).

2.4 Conclusion

Discovery of selected beneficial soilborne *Bacillus* spp. accumulated a wealth of knowledge on mechanisms underlying induced systemic resistance against corm rot diseases. The immune system of the host plant plays an important role, whereby on one hand, it can be activated to ward off the enemies, and on the other hand, it can be suppressed to accommodate the mutualists. Host plant is so much affected by the associated microbe that sometimes it is also designated as plant's second genome. A continuous increase in our knowledge on the molecular and genetic basis of plant-microbe communication in terms of evolutionary and ecological relevance will be highly instrumental for the development of sustainable future crops with maximum profits.

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3 Plant Growth-Promoting Rhizobacteria: Key Mechanisms of Action

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Aurenívia Bonifacio,
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and Fabio Fernando de Araujo

Abstract

Plant growth-promoting rhizobacteria (PGPR) have gained worldwide importance and acceptance for their agricultural benefits through the application of combinations of different mechanisms of action, which allows increases in crop yield. This is due to the emerging demand for reduced dependence on synthetic chemical products and to the growing necessity of sustainable agriculture within a holistic vision of development and environmental protection. The use of selected plant-beneficial rhizobacteria may represent an important biotechnological approach to alleviate the negative effects of stress and to optimize nutrient cycling in different crops. Recent progress in our understanding of their action mechanisms, diversity, colonization ability, formulation, and application should facilitate their development as reliable components in the management of sustainable agricultural systems. In addition, numerous studies indicate increased crop performance with the use of these microorganisms. In this chapter, an understanding of the direct and indirect mechanisms of action of PGPR and their various benefits to plants are summarized and discussed.

Keywords

PGPR • Phytohormones • Effectiveness • Crop • ISR • Catabolic enzymes

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3.1 Introduction

During the past couple of decades, the use of plant growth-promoting rhizobacteria (PGPR) for sustainable agriculture has increased tremendously in various parts of the world. Significant increases in the growth and yield of agronomically important crops in response to inoculation with PGPR have been repeatedly reported (Kloepper et al. 1980; Chanway 1997; Vessey 2003; Gray and Smith 2005; Araujo 2008; Figueiredo et al. 2010; Kang et al. 2010; Rodrigues et al. 2013; Chauhan et al. 2015). Studies have also shown that the growth-promoting ability of some bacteria may be highly specific to certain plant species, cultivars, and genotypes (Bashan 1998).

PGPR can affect plant growth by various direct and indirect mechanisms (Kloepper and Schroth 1978; Glick et al. 1995; Cattelan et al. 1999; Gupta et al. 2000; Li et al. 2000; Hayat et al. 2010; Saraf et al. 2011; Minaxi et al. 2012; Kavamura et al. 2013; Ahemad and Kibret 2014). These mechanisms can probably be active simultaneously or sequentially at different stages of plant growth (Chaparro et al. 2013). Some examples of these mechanisms are (a) increased mineral nutrient solubilization and nitrogen fixation, making nutrients available to the plant; (b) phytohormone production, such as indole–3–acetic acid, abscisic acid, gibberellin, cytokinins, and ethylene; (c) antagonism against phytopathogenic bacteria by producing siderophores, β-1,3-glucanase, chitinases, antibiotics, fluorescent pigments, and cyanide; (d) the ability to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, a vital enzyme that reduces ethylene levels in the roots of developing plants, thereby increasing root length and growth; and (e) improving plant stress tolerance to salinity, metal toxicity, and drought through the production of exopolysaccharides (EPSs), biofilm formation, and osmolyte reduction to avoid cell water loss.

The modes of action of the PGPR and their various benefits to plants range from the simple occupation of empty biological spaces to ecological relationships such as antibiosis, competition, predation, and symbiosis, among others (Kloepper et al. 2004; Avis et al. 2008). The use of selected microorganisms may represent an important biotechnological approach to decrease the deleterious effects of stress in crops (Egamberdieva et al. 2013; Nadeem et al. 2014). An effective strategy to alleviate the negative effects of stress in plants is the co-inoculation of seeds with different PGPR species, such as *Rhizobium* and *Azospirillum* (Figueiredo et al. 2008; Bashan and de-Bashan 2015). The presence of *Azospirillum* sp. in the rhizosphere was reported to elicit or activate the hydrolysis of conjugated phytohormones and flavonoids in the root tissue, thus bringing about the release of compounds in their active forms (Saikia et al. 2010).

Overall, the ability of microbes to confer stress tolerance to plants may provide an eco-friendly strategy for mitigating the impacts of global climate change on agricultural and native plant communities, as well as provide excellent models for understanding stress tolerance mechanisms that can be subsequently engineered into crop plants (Choudhary 2012).

3.2 PGPR: Current Perspective

In search of more sustainable agriculture, PGPR have been used extensively worldwide (Choudhary et al. 2011). PGPR benefit plant growth and development when present in the rhizo- and endosphere (Lugtenberg and Kamilova 2009; Compant et al. 2010; Choudhary et al. 2011; Duca et al. 2014). In an effort to elucidate the concept of PGPR, Bashan and Holguin have proposed to divide them into two groups: PGPB and biocontrol-PGPB. According to this classification, PGPB would encompass bacteria capable of synthesizing growth-promoting substances; fixing atmospheric nitrogen; providing phosphate, potassium, iron, and other nutrients; and mitigating the deleterious effects of abiotic stresses, whereas biocontrol-PGPB are able to decrease or prevent the deleterious effects of soil plant pathogens (Bashan and Holguin 1998). Gray and Smith (2005) have shown that PGPR associations depend on the degree of bacterial

proximity to the root and the intimacy of the association. In general, these associations can be separated into two categories: (1) extracellular (ePGPR) associations, which exist in the rhizosphere, on the rhizoplane, or in the spaces between the cells of the root cortex, and (2) intracellular (iPGPR) associations, which exist inside root cells in specialized nodular structures. Most of rhizobacteria belonging to this group are Gram-negative rods with a lower proportion being Gram-positive rods, cocci or pleomorphic (Bhattacharyya and Jha 2012).

PGPR are widely distributed in the Bacteria domain, mainly in the phyla *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, and *Proteobacteria* (Figueiredo et al. 2010). When present in soil (cultivated or noncultivated), these bacteria are responsive to chemical attractants—a diverse group of compounds that are synthesized, accumulated, and secreted by plant roots; these compounds are generically referred to as root exudates (Huang et al. 2014). The root exudates modify the chemical and physical properties of the soil and regulate the bacterial community that is present in the area surrounding the root surface (Dakora and Phillips 2002). In fact, the chemicals present in root exudates act as substrates and chemotactic or signaling molecules and mediate the selection of the microbial community that will interact with the plant (Chaparro et al. 2014).

In addition to root exudates, quorum-sensing molecules are increased in response to bacteria present in the rhizo- and endosphere (Compant et al. 2010; Chaparro et al. 2013). Quorum-sensing molecules, which are defined as a group of molecules responsible for cell-to-cell communication between plants and bacteria, allow bacteria to share information about their cell density (Badri et al. 2009). This sharing of information regulates the expression of various genes (mainly in the roots) that are linked to plant development (Badri et al. 2008). *N*-Acyl-homoserine lactone (AHL) is the most important quorum-sensing molecule and is generally found in Gram-negative bacteria that live in association with plants (Babalola 2010). Interestingly, AHL has a differential influence on the interaction between *Methylobacterium mesophilicum* and rice and *Eucalyptus*, using different metabolic routes for each plant host (Dourado et al. 2013). In *Azospirillum lipoferum*, quorum-sensing molecules are associated with rhizosphere competence and adaptation during the plant-host interaction (Boyer et al. 2008).

Once near the roots, PGPR can stimulate plant performance and development through direct or indirect mechanism (Ahemad and Kibret 2014). The direct mechanisms involve nutrient acquisition and the synthesis of phytohormones (Compant et al. 2010). Bacterial populations present at high density in the rhizosphere stimulate nutrient uptake by plant roots; this has been observed for *Azospirillum*, *Bacillus*, and *Rhizobium* (van Loon 2007). The most studied and longest exploited PGPR are the rhizobia (including the *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*) for their ability to fix N in their legume hosts (Vessey 2003). Furthermore, the free-living diazotrophic *Azospirillum* supplies its host plant, mainly maize, wheat, and sugarcane, with nitrogen through nitrogen atmospheric fixation and positively affects plant growth (Bashan and de-Bashan 2010, 2015; Duca et al. 2014).

Nitrogen, phosphorus, and potassium are most important macronutrients for plant development and their deficiencies reduce plant yield. Similar to nitrogen, the uptake of potassium and phosphorus may be mediated by PGPR when interacting with their host plant (Lugtenberg and Kamilova 2009; Richardson and Simpson 2011). Co-inoculation of pepper and cucumber plants with *Bacillus megaterium* var. *phosphaticum*, a phosphate-solubilizing bacterium, substantially increased the availability of phosphorus for these plants, whereas co-inoculation with *B. mucilaginosus*, a bacterium that solubilizes potassium, significantly improved the availability of potassium to both pepper and cucumber (Han et al. 2006). As a result, the increased phosphorus and potassium availability improved the growth of pepper and cucumber plants. Consequently, the use of phosphate and potassium PGPR solubilizers as a biofertilizer source represents an ecologi-

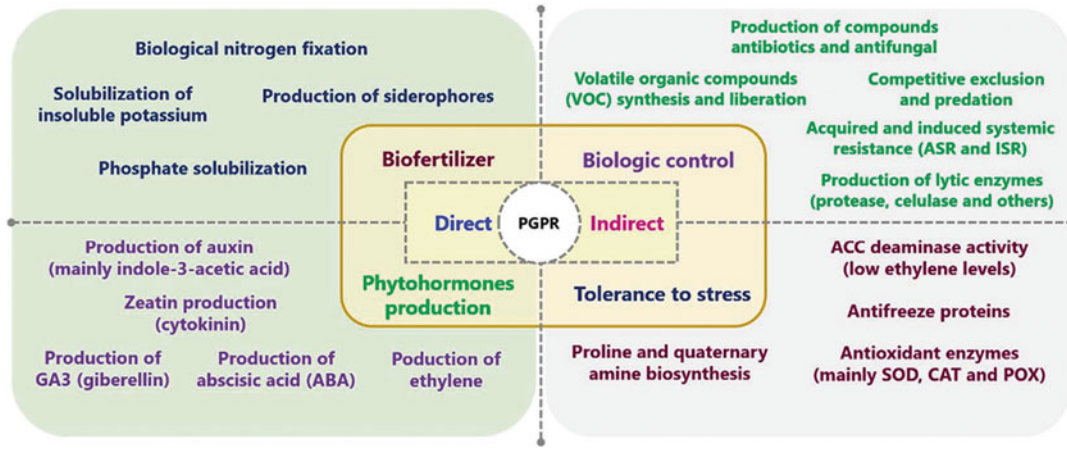

Fig. 3.1 Direct and indirect mechanisms mediated by plant growth-promoting rhizobacteria (PGPR) with beneficial effects on host plants (Chauhan et al. 2015; Pii et al. 2015)

cal solution for soil fertilization and the improvement of plant nutrition and production (Vessey 2003).

PGPR can produce or modulate phytohormone levels and thereby affect the hormonal balance of the host plant (Duca et al. 2014; Glick 2014). Auxin, gibberellin, cytokinins, ethylene, abscisic acid, and brassinosteroids are classical phytohormones with key roles in plant development (Huang et al. 2014). Phytohormone synthesis and release by *Azospirillum* strains present in the rhizosphere are considered the major mechanisms for the modification of root architecture and the increase in nutrient uptake by plants (Cohen et al. 2008; Cassan et al. 2014). Auxins are the most important phytohormone produced by *Azospirillum*, *Bacillus*, *Paenibacillus*, and *Pseudomonas*, while gibberellins are strongly synthetized by *Acetobacter*, *Azospirillum*, *Bacillus*, *Herbaspirillum*, and *Rhizobium* (Babalola 2010; Cassan et al. 2014). *Burkholderia* and *Paenibacillus* exhibited ethylene production and secretion linked with plant growth and biocontrol activity (Vacheron et al. 2013).

The improvement of plant development is often related to the presence of rhizobacteria responsible for fixing atmospheric nitrogen, solubilizing potassium and phosphate, or producing phytohormones (Fig. 3.1). Moreover, the production and secretion of lytic enzymes and antibiotics as well as iron sequestration are indirect mechanisms mediated by rhizobacteria that result in plant growth promotion (Badri et al. 2009; Huang et al. 2014). Under iron-deficient conditions, bacteria synthesize siderophores and can supply the host plant with chelated iron (Saha et al. 2013). Iron-chelating siderophores produced by PGPR in the rhizo- and endosphere may suppress soilborne plant pathogens (Compant et al. 2010). Species of *Bacillus*, *Paenibacillus*, *Serratia*, *Enterobacter*, and *Pantoea* use lytic enzymes, such as amylase, chitinase, β-1,3-glucanase, and protease, to destroy the cell walls of soilborne pathogens (Backman and Sikora 2008; Nimnoi et al. 2010; Jha et al. 2013; Chauhan et al. 2015).

The stimulation of plant development by PGPR can probably be activated at different stages of plant growth (Figueiredo et al. 2010). The plant-microbe interaction specific to each plant age can be useful to combat pathogenic microorganisms or to improve nutrient uptake by plants (Chaparro et al. 2014). Species of *Azospirillum*, *Bacillus*, *Burkholderia*, *Herbaspirillum*, *Nitrobacter*, and other nitrogen-fixing bacteria, which directly or indirectly provide nitrogen to plants, are mainly attracted by root exudates released by plants in later stages of development, e.g., when greater quantities of nitrogen are required for flowering and grain filling (Franche et al. 2009). The roots of *Arabidopsis* plants released more defense-related compounds

at later stages of life; these compounds attracted rhizobacteria that were antagonistic to many plant pathogens (Chaparro et al. 2013; Ahemad and Kibret 2014).

In addition to preventing deleterious effects caused by phytopathogens, the use of PGPR can positively affect plant growth and development under stressful situations (Yang et al. 2009; Hayat et al. 2010; Carmen and Roberto 2011). Under drought or salt stress, strains of *Azospirillum* change plant development and behavior to cope with these stressful environments (Arzanesh et al. 2011). Under limiting conditions, plants often adjust their endogenous phytohormone levels to decrease the negative effects of environmental stressors (Hayat et al. 2010; Glick 2015). The co-inoculation of common bean plants with *Paenibacillus polymyxa* and rhizobia alleviated the adverse effects of drought stress and maintained plant growth and development (Figueiredo et al. 2008). These findings indicate that the use of PGPR in association with plants represents an effective and promising tool to increase plant yield worldwide.

3.3 PGPR: Direct Mechanisms of Action

PGPR directly affect plant metabolism by providing nutrients that are usually scarce in the rhizosphere, such as nitrogen (Ahmad et al. 2008; Babalola 2010). The capture and subsequent release of nitrogen to plants is carried out by bacteria present in the rhizo- and endosphere through a diverse set of processes. PGPR may convert nitrogen trapped in the molecular or atmospheric form (N_2) into biologically useful forms in a process known as biological nitrogen fixation (BNF). Only diazotrophic bacteria execute BNF, as the nitrogenase enzyme is present only in these organisms (Bhattacharjee et al. 2008). Members of the genera *Anabaena*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Clostridium*, *Klebsiella*, *Nostoc*, *Paenibacillus*, and *Rhodobacter* are examples of free-living diazotrophic bacteria that provide available nitrogen to several plants (Grobelak et al. 2015).

Nitrogen is the nutrient that is required in the highest amount, and its availability is a major factor that limits plant development (Courty et al. 2015). Globally, considerable attention has been given to the shortage of nitrogen in agricultural soils, which reduces plant yield capacity, and new technologies have been developed and tested to prevent the use of chemical fertilizers in cultivated areas (Bhattacharjee et al. 2008; Figueiredo et al. 2013). The combination of species of *Anabaena*, a free-living diazotrophic bacterium that fixes nitrogen, and *Azolla* is a natural means of providing nitrogen to waterlogged rice plants (Bhuvaneshwari and Kumar 2013; Fosu-Mensah et al. 2015). In this case, the free-living diazotrophic *Anabaena* may be referred to as a "biofertilizer," i.e., a beneficial microorganism that helps to maintain soil quality and plant health through its biological activity. Biofertilization of rice with *Anabaena* contributes high nitrogen amounts (up to 50 kg ha^{-1}), reduces nitrogen loss via ammonia volatilization, and stimulates plant growth (Bhuvaneshwari and Kumar 2013).

Various *Azospirillum* species enhance plant growth, mainly those with the C4 photosynthetic pathway, through atmospheric nitrogen fixation (Bhattacharyya and Jha 2012). Additionally, the biosynthesis and liberation of ammonium ions, nitric oxide (NO), and phytohormones in soil solution are other mechanisms that are activated by *Azospirillum* and which have positive impacts on plant growth (Molina-Favero et al. 2008; Pii et al. 2015). *A. brasilense* and *A. lipoferum* are the major *Azospirillum* species studied worldwide and are frequently used for the inoculation of rice, maize, and sugarcane (Bashan and de-Bashan 2010). *A. brasilense* is able to alter plant root architecture by increasing the formation of lateral and adventitious roots and root hairs (Bashan et al. 2014; Bashan and de-Bashan 2015) and displays the ability to synthesize NO by different pathways (Molina-Favero et al. 2008). NO is required for root organogenesis, root hair formation, and the growth of adventitious and lateral roots (Molina-Favero et al. 2008; Pii et al. 2015), which enhance nitrogen uptake by plants.

In a study conducted on *Arabidopsis* inoculated with *A. brasilense* Sp7 under nitrogen-

limited conditions, a significant increase in plant gene expression of high-affinity transport systems (HATSs) was observed (Ahmed 2010). Inorganic nitrogen may be taken up by HATSs localized in root cells (Courty et al. 2015). These systems, which are predominant in the micromolar range, are able to capture ammonium or nitrate ions, resulting in more effective nitrogen capture by the host plants (Pii et al. 2015). The modulation of HATS function is linked to alterations in plant growth and development (Richardson et al. 2009; Ahmed 2010). Furthermore, through an active process that occurs in the root cell wall, plants may absorb nitrate ions generated by nitrifying bacteria after the release of ammonium produced by *Azospirillum* (Marulanda et al. 2010; Courty et al. 2015; Pii et al. 2015).

In addition to nitrogen, phosphorus and potassium are important nutrients provided to plants by PGPR under nutrient-limited conditions (Babalola 2010; Sharma et al. 2013; Courty et al. 2015). The mechanisms involved in phosphorus uptake by PGPR remain poorly understood (Pii et al. 2015). Phosphorus is found in soil mainly in an organic form, principally phytate or insoluble inorganic phosphate, and is commonly found as calcium phosphate, hydroxyapatite, and/or rock phosphate (Richardson et al. 2009). PGPR act as phosphate solubilizers and convert inaccessible phosphorus into forms that can be absorbed by plants through phytase action or the production of organic acids (Sharma et al. 2013). Phytase (myoinositol hexakisphosphate phosphohydrolase) is an enzyme that is active in *Bacillus*, *Enterobacter*, *Klebsiella*, and *Pseudomonas* (Jorquera et al. 2011; Sharma et al. 2013; Vacheron et al. 2013). For this reason, these PGPR are collectively referred to as phosphate-solubilizing or phytase-producing bacteria (PPB).

The capacity to mineralize phytate in combination with other PGPR qualities, e.g., siderophore and phytohormone production, increases the potential use of PGPR in soils with high organic phosphate contents (Pii et al. 2015). Moreover, phosphate-solubilizing PGPR that provide phosphates through the release of organic acids are important in modern agriculture (Sharma et al. 2013). *Bacillus*, *Burkholderia*, *Erwinia*, *Paenibacillus*, *Pseudomonas*, *Rhizobium*, and *Serratia* are described in literature as possessing phosphate-solubilizing ability through the release of organic acids (Öğüt et al. 2011). The release of organic acids, mainly acetate, oxalate, and citrate, by PGPR enhances proton efflux and acidifies the rhizosphere; consequently, inorganic phosphate is solubilized from mineral sources (Bhattacharyya and Jha 2012). The use of phosphate-solubilizing PGPR is considered an environmentally friendly alternative to phosphorus supplementation and improves plant growth (Fig. 3.2).

PGPR can effectively promote the absorption of other nutrients, in addition to nitrogen and phosphorus, as well as promote plant growth (Ahmad et al. 2008). The inoculation of wheat with *Pseudomonas* sp. or *Bacillus* sp. resulted in significant increases in potassium, calcium, and magnesium uptake in a calcareous soil without fertilization (Öğüt et al. 2011). These PGPR species deliver potassium through the solubilization of insoluble potassium sources through the production and liberation of organic acids (oxalate, succinate, and citrate) in the rhizosphere, similar to PGPR phosphate solubilizers (Sharma et al. 2013). The solubilization of potassium by PGPR improves soil fertility and the bioavailability of soluble potassium to plants and is thus considered an important plant growth-promotion mechanism under field conditions (Sharma et al. 2013).

Bacillus megaterium increased calcium, phosphorus, boron, copper, iron, manganese, and zinc uptake and increased biomass in trefoil plants under water-limited conditions (Marulanda et al. 2010). Iron is a micronutrient that is involved in various metabolic pathways, and its deficiency disrupts essential processes in plant metabolism, such as respiration or photosynthesis (Radzki et al. 2013). Under iron-limiting conditions, *Bacillus*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Rhodococcus*, and other rhizobacteria produced siderophores, small iron chelator molecules that enable the transport of iron to the root cells (Raza and Shen 2010). This process helps to maintain plant growth and creates an unfavorable environment for phytopathogens that cannot grow under

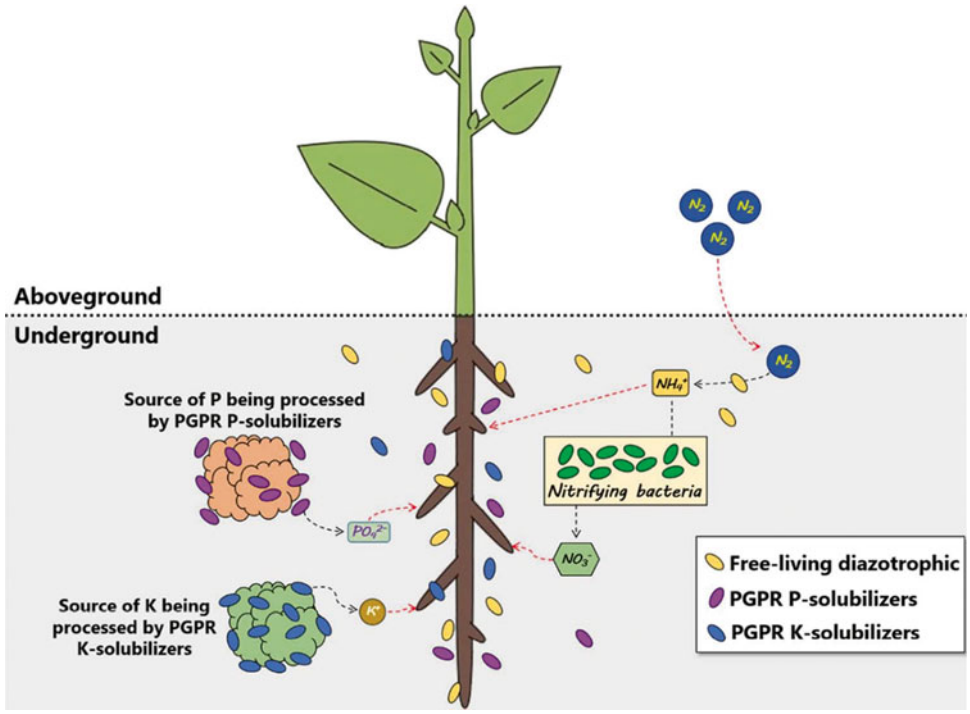

Fig. 3.2 Modes of nitrogen (N), phosphorus (P), and potassium (K) improvement for soil and plants mediated by plant growth-promoting rhizobacteria (PGPR). Free-living diazotrophic bacteria are able to capture nitrogen from the atmosphere (aboveground) and release it to plants as ammonium (NH_4^+) or nitrate (NO_3^-). Other PGPR act as K or P solubilizers and release K or P in forms that can be absorbed by plants

iron-deficient conditions (Pii et al. 2015). The multi-facets of PGPR provide effective uptake of macro- and micronutrients associated with phytohormone production, which enables plant growth under various environmental conditions.

In addition to improved plant nutrition, the biosynthesis of phytohormones is also considered to directly stimulate plant growth (Hayat et al. 2010; Spence and Bais 2015). Auxin, gibberellin, cytokinin, ethylene, and abscisic acid are examples of phytohormones produced and released by numerous members of the genera *Alcaligenes*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Bradyrhizobium*, *Brevibacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Mycobacterium*, *Pseudomonas*, *Rhizobium*, and *Serratia* (Egorshina et al. 2012; Spence and Bais 2015). Among the phytohormones produced by PGPR, the effects of auxin are the most commonly studied and described in the literature. Auxins are produced in meristematic areas and regulate numerous plant processes linked to cell elongation. Alteration in root morphology and development is the most modified trait in plants inoculated with PGPR auxin producers (Glick 2014; Spence and Bais 2015).

Wheat seedlings treated with spores of *Bacillus subtilis* 11BM exhibited growth stimulation as well as a transient increase in indole–3–acetic acid (IAA), the auxin that is most commonly studied worldwide (Egorshina et al. 2012). *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109 synthetize IAA in concentrations that are adequate to induce morphological changes and promote growth in maize and soybean (Cassan et al. 2009). IAA production by *Mesorhizobium* sp. and/or *Pseudomonas aeruginosa* positively stimulated potassium and phosphorus uptake by chickpea inoculated with these microorganisms (Verma et al. 2013). These increases in nutrient uptake are related to better access to soil nutrients as a

consequence of the increase in the root surface or intensifications in root exudation that provide additional nutrients for plants and maintain the growth of PGPR in the rhizosphere (Hayat et al. 2010).

A considerable number of PGPR secrete phytohormones in the rhizosphere; however, among these, PGPR gibberellin producers remain poorly understood (Pii et al. 2015). Gibberellins (GAs) are a group of phytohormones associated with alterations in plant morphology, mainly in stem and root tissues (Spence and Bais 2015). *Acinetobacter calcoaceticus*, *Bacillus pumilus*, *Bacillus licheniformis*, *Burkholderia cepacia*, *Herbaspirillum seropedicae*, and *Promicromonospora* sp. are examples of bacteria that produce gibberellins and result in positive effects in the endogenous GA of their host plants (Richardson et al. 2009; Figueiredo et al. 2010; Vacheron et al. 2013).

B. siamensis is a bacilli species that is able to produce GA and promote increases in the growth of banana plants (Ambawade and Pathade 2015). Different types of GA are produced by PGPR (Ahemad and Kibret 2014). In *Azospirillum*, GA3 is the major GA type identified and appears to be involved in promoting plant growth (Cassan et al. 2009).

Phytohormones are involved in practically all steps of plant growth. *Pseudomonas fluorescens* is a PGPR proficient in synthesizing the phytohormone cytokinin and solubilizing organic phosphorus, and their association with *Azospirillum brasilense* is able to improve the biomass and grain yield of wheat (Naiman et al. 2009). *Bacillus megaterium* induces the genes linked to cytokinin receptors in *Arabidopsis* before specifically initiating growth stimulation (Ortíz-Castro et al. 2008). These authors showed that growth promotion by *B. megaterium* is strongly related to three cytokinin receptors that are necessary for normal *Arabidopsis* growth. The production and elongation of root hairs are cytokinin-regulated growth responses (Werner and Schmülling 2009), and root system architecture of *Arabidopsis* elicited by *B. megaterium* is probably also linked to other phytohormones, such as auxin and ethylene (López-Bucio et al. 2007).

1-aminocyclopropane-1-carboxylate (ACC) is a direct ethylene precursor exuded by roots. ACC may be metabolized by PGPR that possess ACC deaminase, an enzyme that converts ACC in α-ketobutyrate and ammonium and therefore reduces ethylene amounts (Hayat et al. 2010; Glick 2014). At low concentrations, ethylene facilitates root elongation in plants under normal and stressful conditions. Considering that bacterial ACC deaminase reduces ethylene levels, the modulation of ACC levels in hosts may mitigate detrimental effects of biotic and abiotic stresses (Glick 2014). In addition to ethylene, abscisic acid (ABA) also modulates plant development under stressful conditions (Glick 2015; Spence and Bais 2015). *Achromobacter xylosoxidans*, *Bacillus licheniformis*, *B. pumilus*, *B. subtilis*, *Brevibacterium halotolerans*, *Lysinibacillus fusiformis*, and *Pseudomonas putida* are ABA-producing bacteria that positively influence plant homeostasis (Sgroy et al. 2009; Glick 2014).

Currently, there is a growing need to increase food production and minimize applications of chemical fertilizers; in this context, the employment of sustainable agriculture is extremely important (Bhattacharyya and Jha 2012). For sustainable agriculture, different cropping systems can be employed, among which the use of PGPR is promising. Nutrient uptake and phytohormone production are considered as direct mechanisms of PGPR (Ahemad and Kibret 2014).

3.4 PGPR: Indirect Mechanisms of Action

Several lines of evidence indicate that rhizospheric microorganisms are protective agents against soil pathogens (Melo 1991; Kloepper 1999). Rhizobacteria can suppress diseases that develop through various mechanisms of action, e.g., antagonism related to the production of antifungal antibiotics such as iturin by *B. subtilis* (Araujo et al. 2005); competition for space and nutrients with phytopathogens and other harmful microorganisms in the rhizosphere (Robin et al. 2008); production of volatile organic molecules, such as hydrogen cyanide (HCN) and ammonia

(Kai et al. 2009); production of molecules that can degrade cell walls such as chitinases and biosurfactants (Zhao et al. 2014); and induced resistance (Wall and Sanchez 1993).

Recent studies have indicated that biofilm formation in the rhizosphere is of considerable importance in the mechanisms of action of rhizobacteria on root pathogens. The presence of high concentrations of bacterial cells in biofilms results in the release of various metabolites such as toxins and antibiotics in their periphery, which has an inhibitory effect on phytopathogens in the soil. The biofilm of *Bacillus subtilis* is composed of compounds of a family of surfactins, i.e., cyclic molecules with amino acids and lipids, which act as powerful biosurfactants with antifungal and antibacterial activity. Moreover, biofilm of *B. subtilis* can participate in the induction of resistance in plants (Kwon and Kim 2014).

Catabolic enzymes (proteases, β-1,3-glucanase, and chitinases) and small molecules can be secreted by various microbial species and can contribute to the suppression of soilborne plant pathogens. Studies using electron microscopy show details of the antagonist effect on *Fusarium* hyphae (Fig. 3.3), highlighting the obvious abnormality of the mycelial growth, which can be attributed to the effect of cell wall-degrading enzymes such as chitinases, produced by rhizobacteria (Zhao et al. 2014). In addition, antibiotics and various compounds toxic to phytopathogens have been recovered from the metabolites of *Bacillus* strains (Esikova et al. 2002). *B. subtilis* produces lipopeptide antibiotics of the iturin and surfactin group that can suppress several plant diseases. Antagonism involving competition for space and nutrients within an ecological niche also plays an important role in the rhizosphere. This was proven in studies on *B. megaterium*, which can competently colonize roots and suppress *Rhizoctonia solani* (Zheng and Sinclair 2000).

The research conducted on the *Bacillus* genus has contributed significantly to the biological control of diseases especially that conducted on certain species of this genus, such as *B. subtilis* (Araujo 2008). It has been reported that *B. subtilis* can produce 66 different types of antibiotics (Katz and Demain 1977), mostly polypeptides with inhibitory effect against pathogenic bacteria and fungi. Side effects related to the biological control of fungi have been highlighted in recent years, such as the reduction of toxic metabolites in food and the production of enzymes such as chitinases, which can degrade the cell wall of fungi (Zhao et al. 2014).

The production of siderophores is a secondary effect of rhizobacteria. These molecules have the ability to sequester Fe^{3+} ions, which are considered essential for metabolism and cell growth. In this sense, the bacteria that colonize plant roots can compete for available iron in the soil and may inhibit the growth of other microorganisms in the rhizosphere. Siderophore-producing rhizobacteria can prevent the proliferation of pathogenic microorganisms around the root (Kumar et al. 2015).

Plants have a natural basal defense system against phytopathogens, but other systems that increase the resistance of plants can be activated or induced (Bonas and Lahaye 2002). The two commonly studied forms of resistance induction are acquired systemic resistance (ASR) and induced systemic resistance (ISR). ASR occurs when plants are exposed to an inducer agent (such as a pathogenic organism), resulting in the activation of defense mechanisms at the induction site, which displays alterations (necrosis), as well as other distant sites, resulting in the plant being systemically protected against subsequent infections caused by a broad spectrum of pathogens (Romeiro 2000). ASR is accompanied by an increase in the concentration of salicylic acid and the accumulation of proteins related to pathogenesis (PRPs), which are mechanisms involved in plant defense (Moraes 1998). ISR can be triggered by nonpathogenic microorganisms in the rhizosphere and does not involve the signaling pathway of salicylic acid or the induction of PRPs; rather, this type of resistance is activated by resistance-signaling pathway of jasmonic acid and ethylene (Pieterse et al. 1998).

When rhizobacteria colonize the root system, the constituent molecules of the bacterial cell or those synthesized by the bacteria act as elicitors of a biochemical signal. This signal is translocated to sites that are distant from the original

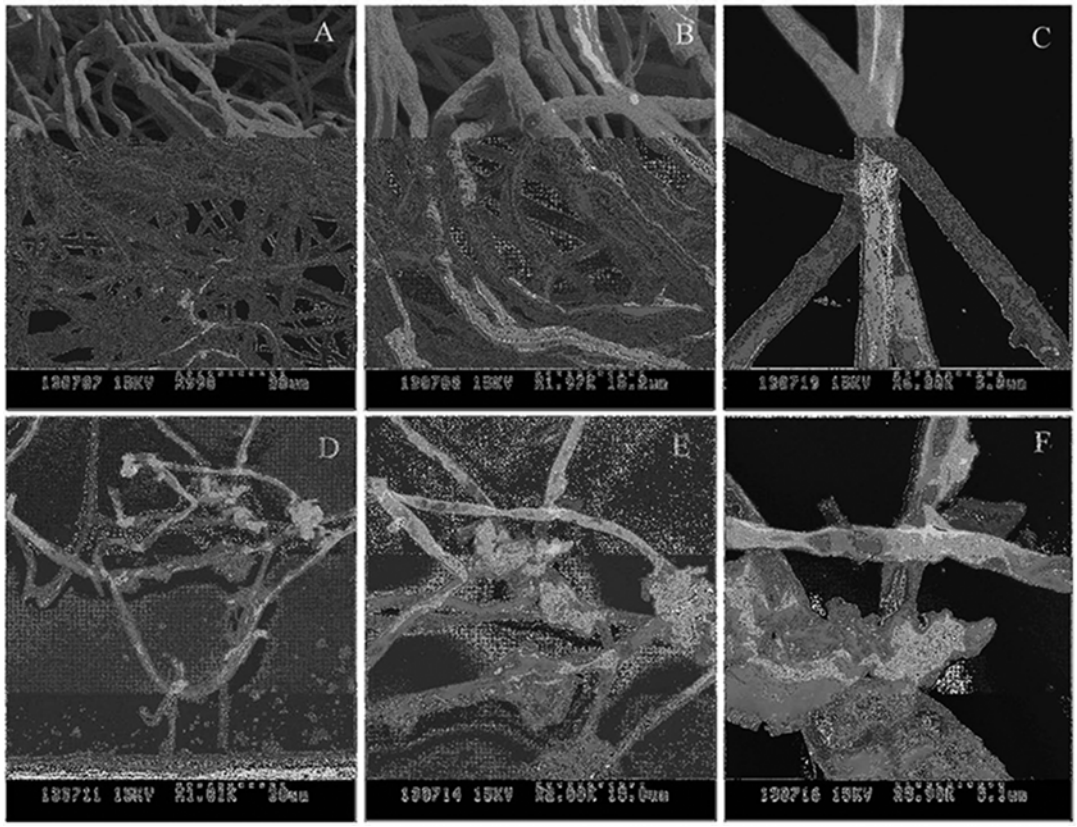

Fig. 3.3 Scanning electron microscope analysis of antagonistic bacteria interacting with hyphae of pathogens on PDA medium on the fifth day after incubation at 28 °C. Normal hyphae of *Fusarium graminearum* are depicted in (**a**–**c**), while abnormal hyphae of *F. graminearum* are shown in (**d**–**f**) (Zhao et al. 2014)

location, resulting in the activation of genes that dynamically code for the synthesis of resistant components and, consequently, the expression of induced systemic resistance (Romeiro 2000). Recent studies aimed to identify these elicitor molecules, which are components of the cell wall, such as lipopolysaccharides, or are released during the energy metabolism of cells. Of these, the most studied are the volatile organic compounds, such as butanediol and acetoin, which are released during anaerobic fermentation and actively participate as elicitors in induced systemic resistance (Choudhary et al. 2008). To confirm the role of butanediol in *Arabidopsis* growth promotion under biological conditions, mutant strains of *B. subtilis* genetically blocked in the production of butanediol were compared with their parental to examine the effect on plant-growth promotion. In this case, the butanediol-synthesis mutants reduced plant growth, whereas the controls did not (Ryu et al. 2003).

The identification of compounds produced by *B. subtilis* during its secondary metabolism is important to clarify the beneficial effects that the bacteria provide to plants (Phae and Shoda 1991). The large number of mechanisms involved in producing these compounds may be the reason why *B. subtilis* has been assessed in a wide range of agricultural crops under different conditions (Kilian et al. 2000). Within the major metabolic pathways studied and those involving the participation of rhizobacteria, stands out the metabolism involved in the production of phytohormones in the rhizosphere environment (Araujo et al. 2005), and the induction of resistance to biotic and abiotic stresses (Kang et al. 2010) has been

reported. The accumulation of proline in plants acts as an osmoprotectant, maintaining the water potential under deficit conditions and facilitating water uptake from the soil (Hanson et al. 1979). Rampazzo (2013) observed that proline accumulation in sugarcane was affected by inoculation with rhizobacteria: plants inoculated and subjected to water stress had a 2.2-fold increase in the concentration of proline in leaves compared with plants inoculated in the absence of stress.

Plants produce a range of antioxidant enzymes such as catalases, peroxidases, and superoxide dismutases involved in scavenging free radicals (Simova-Stoilova et al. 2008). The introduction of growth-promoting bacteria to the rhizosphere can greatly contribute to the production of antioxidant enzymes in plants. Inoculation with *Bacillus subtilis* increased the concentration of detoxification enzymes in plants, which is characterized as another beneficial effect resulting from the inoculation of these bacteria (Li et al. 2008). In tomato, the inoculation of *B. subtilis* was reflected as an increase in the peroxidase activity in plants (Araujo and Menezes 2009). Similarly, in corn plants inoculated with *Piriformospora indica*, the activity of catalase and superoxide dismutase was increased, and the effect of biotic stress was reduced (Kumar et al. 2009).

The presence of antioxidant enzymes in food is beneficial to the health of consumers because antioxidants are nutraceutical molecular components of functional foods, according to Andlauer and Fürst (2002). The term nutraceutical defines a wide variety of foods and food components with medical or health benefits. Nutraceutical action ranges from the supply of essential minerals and vitamins to protection against various infectious diseases (Hungenholtz and Smid 2002). Antioxidants can act directly in neutralizing the action of free radicals or can indirectly participate in enzymatic systems involved in this function (Moraes and Colla 2006). The main antioxidant compounds with nutraceutical characteristics in plants are flavonoids, which act as potent antioxidants and metal chelators, and are also well known for their anti-inflammatory, antiallergic, antiviral, and anticarcinogenic properties (Tapas et al. 2008).

3.5 Concluding Remarks

There is an urgent need for research to clearly define what bacterial traits are useful and necessary for different environmental conditions and plants so that optimal bacterial strains can be selected. Different compounds related to the presence of these microorganisms confer benefits to protect plants against pathogens and stressful conditions that may occur during cultivation. Foods derived from these plants can have a healthier chemistry for consumers. Due to direct and indirect mechanisms used by PGPR, the use of microbes in the cultivation of plants of agronomic interest is considered a useful tool in modern agriculture and therefore represents the core of eco-friendly agricultural practices. In this context, the increased use of PGPR is one of the major pathways to maintain or increase yield as well as reduce the environmental footprint via elucidation of different mechanisms involved that will help to make these plant-beneficial rhizobacteria a valuable partner in agriculture to develop future insights.

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Priming of Plant Defense and Plant Growth in Disease-Challenged Crops Using Microbial Consortia

4

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Abstract

Increasing concern regarding the significant environmental footprint due to excessive use of chemicals in agriculture has led to emphasis on sustainable and environmentally friendly agricultural practices. Microorganisms facilitate and catalyze the transformations of essential major and minor elements in biogeochemical cycles and, hence, represent a dynamic constituent of our environment. Their role as producers of allelochemicals and signaling molecules has been well investigated, but the complexity of interactions involved has made them lag behind in their race as biocontrol agents, as compared with chemical pesticides. Plant-microbe interactions represent one of the most investigated and intriguing areas of research, and their role in priming or as producers of signaling molecules such as jasmonic acid and defense enzymes or their role in eliciting various modes of resistance is gaining new dimensions in the last few years. Priming is known to aid in acclimation to various types of abiotic and biotic stress in microorganisms, and gaining insights into the mechanisms and metabolites involved represents another challenging area. This compilation provides an overview of the recent developments in this field, highlighting the significance of the findings toward developing a "greener" agricultural scenario.

4.1 Introduction

Crop plants experience numerous types of biotic and abiotic stress in the field and deploy various means to overcome them. As an example, biotic stress such as plant diseases can be managed by modifying agronomic practices, use of chemical agents against the particular pathogen, or use of disease-resistant varieties or through biocidal/biocontrol agents. Insect pests can also be man-

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aged by similar practices. The use of chemicals to control plant diseases and insect pests is widely considered as an effective management strategy. But these crop protection chemicals/pesticides contain substances that do not occur naturally and may cause harm to human health due to their persistence in the soil. Moreover, pesticides have a negative impact on soil-dwelling microbes which are beneficial in various ways, such as nutrient cycling, production of important plant hormones, and protection against soilborne pathogens. Hence, there is a need for alternate management strategies to protect crop plants against various biotic and abiotic stresses. One such strategy is the use of biological control agents which are not immediate in their effect and may be slow in action but have long-lasting beneficial effects and have the major advantage as an eco-friendly option.

Application of chemicals or microorganisms initiates a dialogue with plants, as they possess innate immunity. Methods to induce plants to express this immunity quicker and in a more efficient way are called "priming." The present compilation deals with the basic concepts of priming and methods of priming of plants against biotic and abiotic stress. Mechanisms underlying priming and the benefits of priming to crop plants are also dealt elaborately.

4.2 Types of Abiotic and Biotic Stress Experienced by Crop Plants

Abiotic stress is a collective term which refers to several environmental factors affecting severely the growth and hence the yield of crop plants. Among these environmental factors, the major constraints to crop production include drought, salinity, and extremes of temperature (Ashraf and Foolad 2007). Drought is a meteorological term which refers to a significant period of time without precipitation. Drought stress occurs in crop plants when available moisture content in the soil reduces to such an extent that transpiration by crop plants exceeds water absorption due to excessive light and heat (Farooq et al. 2009). Drought stress leads to impaired germination, reduction in growth rate, stem elongation, leaf expansion and stomatal movements, and hence a poor overall crop establishment. It also causes changes in various physiological and biochemical processes in a way that plant growth and productivity is affected. Drought-induced yield loss is reported in crops including maize, wheat, cotton, rice, soybean, and peas (Alexieva et al. 2001; Farooq et al. 2009).

Soil salinity, characterized by toxic levels of different salts, is also a major constraint to crop production alongside drought and other abiotic stress. Salinity affects plant growth by decreasing the uptake of various other nutrients like phosphorus, potassium, nitrate, and calcium and also leads to ion toxicity and osmotic stress (Tuteja et al. 2012). Salt stress in plants affects all major processes like photosynthesis, protein synthesis, and energy and lipid metabolism. Photosynthesis rates are normally lower in plants exposed to salinity especially sodium chloride. At the whole-plant level, detrimental effect due to high salinity can be observed as death of plants and/or decrease in yield (Parida and Das 2005).

Extremes of temperatures like excessive heat and cold also affect plant growth and hence the productivity. Like other abiotic stress, heat stress causes multiple adverse effects in plant growth as it is detrimental to various physiological processes of plants like germination, growth, development, reproduction, and yield. High temperature leads to many adverse effects in crop plants, most significant being the generation of excess reactive oxygen species. Physiological response includes denaturation of proteins and enzymes, membrane instability leading to metabolic imbalance. At the extreme level, heat stress causes cellular damage leading to collapse of cellular organization (Hasanuzzaman et al. 2013). Low temperature, like other abiotic stress, affects plant growth and productivity causing severe yield losses. Most plants of tropical origin exhibit various symptoms like chlorosis and necrosis leading to death (Sanghera et al. 2011). Freezing temperature induces ice formation in the intercellular spaces and cell wall leading to lowered water potential eventually leading to dehydration

of crop plants. Dehydration is a common effect due to freezing, drought, and salt stress. Hence, these three stress share many genes in common (Xin and Browse 2000). Apart from these three common abiotic stresses, other stresses like mineral nutrient deficiencies, heavy metal toxicities, and high soil acidity may also exhibit effects on the metabolism.

Biotic stress in crop plants includes diseases caused by plant pathogens, injury caused by insect pests, and competition exhibited by weed infestation. All crop plants are subjected to diseases both in the field and postharvest, the major groups of pathogens being viruses, fungi, bacteria, nematodes and parasitic plants. Such pathogens can at times become a serious threat to food security, such as the cases of Irish famine caused by *Phytophthora infestans* and Bengal famine caused by *Helminthosporium oryzae* (Strange and Scott 2005). Fungi cause the majority of plant diseases in agricultural fields, and fungal genera like *Rhizoctonia*, *Fusarium*, *Verticillium*, *Phytophthora*, and *Sclerotium* contain the major soil borne plant pathogens known, with members of this genera affecting a number of important crops which includes rice, wheat, maize, cotton, vegetables, and temperate fruits (Mehta et al. 2014). Some bacteria belonging to the *Proteobacteria*, *Mollicutes*, and *Actinomycetes* cause different plant diseases; a few of them have devastating effects on yield and quality. Major plant pathogenic bacteria belong to the genera *Pseudomonas*, *Xanthomonas*, *Erwinia*, *Ralstonia*, and *Streptomyces* (Van Der Wolf and De Boer 2015). Plant viral diseases cause serious devastations economically, and many viral diseases determine or change the season of planting or sowing major crops in various parts of the world. Viruses, when compared to other organisms are relatively simple with very little genetic makeup, yet the mechanisms by which they cause diseases in many crop plants are largely unknown (Kang et al. 2005). For example, barley yellow dwarf virus (BYDV) alone causes disease symptoms in most of the staple food crops like wheat, maize, rice, barley, and oats (Strange and Scott 2005). The other important viruses causing devastating plant diseases are cassava mosaic virus, tobacco mosaic virus, and citrus tristeza virus.

4.3 Effects of Stress on Crop Plants

Plants regulate gene expression by sensing the stress and responding to it by various mechanisms to increase the survivability in hostile conditions. Stress response elicited by crop plants varies depending on the degree and type of stress, such as drought, salinity, temperature extremes and pH extremes, and results in complex cellular responses. Abiotic stresses such as drought, low temperature, and excessive salts in soil result in poor uptake and transport of water leading to changes in stomatal functioning. This leads to a cascade of events starting from changes in carbon cycle, photosynthetic rate, and transpiration. Related biochemical pathways are also affected eventually leading to poor growth and development of plants (Bohnert and Sheveleva 1998). Plants first sense the stress once any of the biotic or abiotic factors deviate from optimum. This is followed by activation of signaling pathways that confers resistance to stress. Simultaneously, a drop in photosynthetic rate, transport of metabolites, and ion translocation to various parts of the plant is also observed (Duque et al. 2013).

Most plants possess physical barriers to protect against pathogens and insect pests (Dangl and Jones 2001). Yet plants are infected by variety of plant pathogens like fungi, bacteria, virus, and nematodes. Plants do not possess somatic adaptive immunity, and hence they depend on innate immunity at cellular level (Ausubel 2005). Current understanding of the plant immune system can be explained in a four-phased zigzag model. In phase I, pathogen recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs) leading to PAMP-triggered immunity (PTI) that can halt further progression of infection. In phase II, effectors are deployed by successful pathogens which can interfere with PTI. This results in effector-triggered susceptibility (ETS). In phase III, plant proteins recognize these effectors leading to effector-triggered

immunity (ETI) which is characterized by hypersensitive cell death response at the site of infection. In phase IV, successful pathogens avoid ETI by employing fresh and additional effectors that can suppress ETI (Jones and Dangl 2006).

Apart from jasmonic acid (JA) and salicylic acid (SA), other plant hormones like auxins, abscisic acid, cytokinins, ethylene, and brassinosteroids also play vital role in plant systemic signaling. Primary resistance against plant pathogens both biotrophic as well as hemibiotrophic is triggered by SA, while JA and ethylene trigger resistance against necrotrophic plant pathogens (Robert-Seilaniantz et al. 2011). It is possible to trigger plant immune response through various means, so that plant response to stress is quicker and more efficient than innate response. Such triggering mechanisms are dealt in future sections of this chapter.

4.4 Priming and Induced Resistance (IR)

Plants possess innate defense traits and also have developed inducible defenses against pathogens, insect pests, and even higher plants (weeds) which lead to regulation of gene expression and synthesis of defensive secondary metabolites and defense-related proteins (Dorantes-Acosta et al. 2012). However, plants can also be sensitized/primed for faster and more intense defense responses leading to enhanced resistance to biotic and abiotic stress. The physiological state in which plants are able to show more rapid and enhanced/better defense responses is called the primed state of the plant, and the mechanisms which induce such a physiological state in plant are called priming (Conrath et al. 2006). Priming-induced defense is depicted in a simple manner in Fig. 4.1.

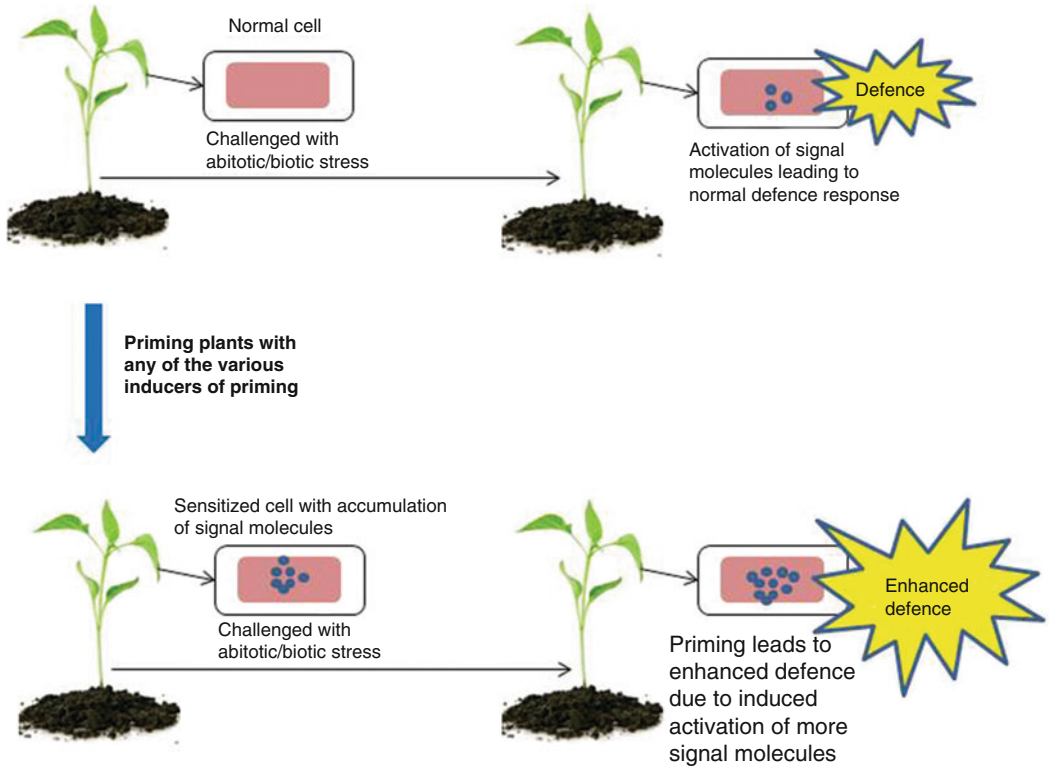

Fig. 4.1 Amplification of defense responses as a result of priming in plants

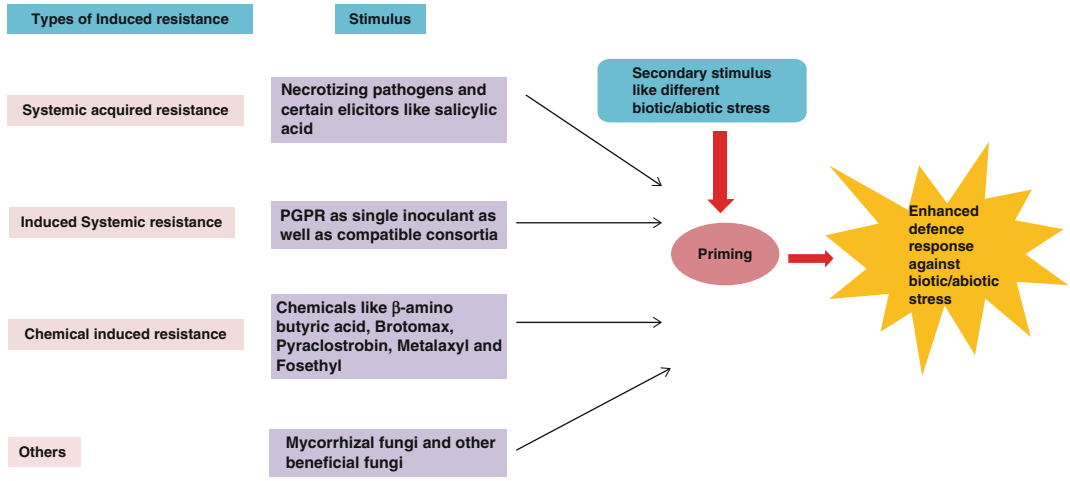

Fig. 4.2 Overview of various types of induced resistance and stimuli involved in priming

The term induced resistance (IR) is a generic one, and it refers to the state of resistance in plants triggered by pathogens, plant growth-promoting rhizobacteria, physical injury, or any chemical inducers which protects plant parts against future pathogenic attack, insect infestations, or any kind of abiotic stress (Pieterse et al. 2014). IR is of different types: systemic acquired resistance (SAR), induced systemic resistance (ISR), chemical-induced resistance, and wound-induced resistance (Conrath 2009). Various types of induced resistance and priming stimuli are depicted in Fig. 4.2. Resistance responses induced by pathogens, beneficial bacteria, chemical analogs, and wound are associated with priming for more rapid and more intense defense responses upon future pathogen challenge or abiotic stress (Beckers and Conrath 2007). Although the phenomenon of priming as a part of induced resistance is known for years, it did not attract much until the 1990s (Conrath et al. 2002). Initial investigation of priming employed a parsley cell culture and a pathogen-associated molecular pattern (PAMP) from the cell wall of *Phytophthora sojae* to understand molecular aspects of priming and PAMP-induced defense responses. It was established that besides pathogens, salicylic acid, its analogs, and several other chemical and biological agents can induce priming, which is often associated with enhanced resistance to variety of biotic and abiotic stress (Conrath et al. 2006).

4.5 Priming in Systemic Acquired Resistance (SAR)

Plants when infected with necrotizing pathogens develop resistance to numerous pathogens. Induced resistance may be established locally (at the site of infection) and are called localized acquired resistance. Often resistance is induced at a distal point from the infected tissue and is called systemic acquired resistance (Conrath 2009). SAR is typically characterized by high levels of plant hormone salicylic acid, accompanied by activation of pathogenesis-related proteins (PR) with antimicrobial activity. PR-1 is the best characterized PR proteins and is generally used as a marker for SAR (Pieterse et al. 2014). Accumulation of SA in plant tissues increased to an enormous level after pathogen infection, and several studies have shown that exogenous application of SA resulted in resistance to broad range of pathogen through SAR. Different studies indicated that priming is a major mechanism in SAR response in most plant species. The first in-depth study on priming was carried out in tobacco plants expressing chimeric PR-1:: GUS or PAL-3::GUS defense genes. It was reported that mere treatment with salicylic acid (SA) did not cause significant activation of defense genes. But once infected with *Pseudomonas syringae* or after wounding, activation of defense genes (as shown

by the activation of reporter gene) was much stronger in plants treated with SA, than in the plants that did not receive SA pretreatment (Mur et al. 1996). An experiment with soybean cell culture treated with SA and challenged with *Pseudomonas syringae* pv. *glycinea* showed an enhanced induction of defense gene transcripts (Shirasu et al. 1997). Nonpathogenic strains of *Fusarium oxysporum* (np*Fo*) have been shown to induce SAR against *Fusarium oxysporum* f.sp. *asparagi* (*Foa*) in asparagus. An experiment conducted with SA pretreatment in asparagus have been shown to potentiate peroxidase and phenylalanine ammonia-lyase when challenged with *Foa* (He and Wolyn 2005) It has also been shown that pretreatment with nonpathogenic strain, np*Foa*, primed asparagus for potentiated defense response to *Foa* (He et al. 2002). Acibenzolar is known to induce SAR in many plants. Acibenzolar-S-methyl (ASM) has been shown to induce resistance in sunflower against rust caused by *Puccinia helianthi*. It has been shown that ASM treatment has induced production of coumarins in sunflower which is known to affect the germination of uredospores (Prats et al. 2003). ASM pretreatment of cucumber have been shown to potentiate PAL (phenylalanine ammonia-lyase) when challenged with *Colletotrichum orbiculare* (Cools and Ishii 2002). ASM pretreatment followed by challenging with *Colletotrichum destructivum* in cowpea primes two key enzymes of phenylpropanoid/flavonoid pathway, PAL and chalcone isomerase (Latunde-Dada and Lucas 2001). In *Arabidopsis*, priming with benzo(1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) has shown stronger expression of PAL protein when challenged with *Pseudomonas syringae* pv. tomato DC3000, mechanical wounding, and osmotic stress through infiltration with water (Kohler et al. 2002). BTH, an analogue of SA, has been shown to prime and induce SAR in pepper against bacterial leaf spot caused by *Xanthomonas axonopodis* and *cucumber mosaic virus* in field conditions (Yi et al. 2012). The study indicated that priming defense genes plays a critical role in protection of plants against pathogens under natural conditions. Priming through various SAR inducers like SA, BTH, and *Colletotrichum falcatum* elicitor has been shown to differentially regulate five genes in the resistant gene analogues (RGA) and seven genes in phenylpropanoid pathways (Selvaraj et al. 2014). Based on these studies, it can be concluded that priming plays an important role in systemic acquired resistance, and SAR can be induced through not only the necrotizing pathogens but also through salicylic acid and various other chemical analogues.

4.6 Priming in Induced Systemic Resistance (ISR)

Several species of the bacterial genera *Pseudomonas*, *Bacillus*, and other rhizobacteria can induce resistance in crop plants. Such induced resistance effected by beneficial rhizobacteria is termed as rhizobacteria-induced systemic resistance, PGPR-induced systemic resistance, or simply induced systemic resistance. They resemble SAR, but in stark contrast to SAR, ISR is not associated with PR gene expression and SA independent rather dependent on jasmonic acid (JA) and ethylene (ET) signaling (Choudhary et al. 2007). A common feature of ISR response induced by beneficial microorganisms is priming. Defense responses are not activated directly, but once encountered with pathogen, pest, or any other stress, defense responses are accelerated, resulting in enhanced resistance (Van Wees et al. 2008). Van Peer et al. (1991) gave the first evidence of the role of priming in defense responses in PGPR-mediated ISR with the experiments in carnation. Inoculation with *F. oxysporum* f. sp. *dianthi* of carnation plants displaying ISR led to a faster rise in phytoalexin levels than in noninduced control plants (Conrath 2009). *Pseudomonas putida* LSW17S elicited ISR against fusarium wilt and pith necrosis in tomato. LSW17S confers disease resistance in *Arabidopsis* ecotype col-0 against *Pseudomonas syringae* pv. tomato DC3000. Cellular and molecular studies have revealed that LSW17S primes *Arabidopsis* for NPR1, ethylene, and jasmonic acid-dependent disease resistance. LSW17S treatment exhibited typical phenome-

non of priming which lasted more than 10 days (Ahn et al. 2007). *Arabidopsis* colonized by *Bradyrhizobium* sp. strain ORS278 was shown to exhibit ISR against *Pseudomonas syringae* pv. tomato DC3000 when challenged. Results suggested that ISR is exhibited through priming of defense-related genes influenced by JA and ET (Cartieaux et al. 2008). Some beneficial bacteria like *Paenibacillus alvei* K165 activate for enhanced SA-dependent defenses, while others, like endophytic *Actinobacteria*, are able to activate both the SA and the JA/ET pathway. Studies on ISR mutants have also suggested priming is important in ISR (Van Wees et al. 2008). *Pseudomonas azotoformans* GC-B19 and *Paenibacillus elgii* MM-B22 that induced ISR against *Colletotrichum orbiculare* in cucumber is mediated by priming of defense-related enzymes like β-1,3-glucanase, chitinase, and peroxidase (Sang et al. 2014). These studies and other accumulating evidences suggest that priming is the major phenomenon in ISR. Besides PGPRs, priming-based systemic resistance is induced by beneficial fungi and mycorrhiza. Endophytic fungus *Piriformospora indica* confers systemic resistance in barley against various root and leaf pathogens (Waller et al. 2005). *Trichoderma asperellum* T34 is shown to confer systemic resistance in *Arabidopsis* against a wide range of pathogens (Segarra et al. 2009). Mycorrhiza-mediated priming of systemic resistance has been observed in wide variety of crops against a broad spectrum of pathogens (Benhamou et al. 1994; Pozo et al. 2002, 2010; Jaiti et al. 2007; Hao et al. 2012).

4.7 Priming Mediated by Microbial Consortia

Plant defense can be induced by microbial consortia in more effective and efficient way as compared to single microbial inoculant. A consortium of biocontrol agents consisting of *Trichoderma harzianum*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* induced host-mediated defense responses against *Sclerotinia sclerotiorum* and *Sclerotium rolfsii* in pea plants. Consortium of these three compatible microorganisms enhanced defense parameters of the treated pea plants up to 1.4–2.3-fold as compared to 1.1–1.7-fold increment when treated with individual microbes (Jain et al. 2012). Similarly, a microbial consortium consisting of *T. harzianum*, *P. aeruginosa*, and *Mesorhizobium* sp. was shown to induce defense response against *S. rolfsii* in chickpea. Co-inoculation of *P. aeruginosa* and *Azospirillum* sp. was found to have synergestic effect on yield and suppression of root rot disease caused by *Rhizoctonia bataticola* (Marimuthu et al. 2013). Blast disease of rice was effectively managed using a consortium of two PGPR strains *P. fluorescens* Aur 6 and *Chryseobacterium balustinum* Aur 9 (Lucas et al. 2009). Enhanced activation of phenylpropanoid pathway leading to sudden increase in concentration of phenolics was achieved when plants were inoculated with consortium of beneficial microbes (Sarma et al. 2002; Singh et al. 2011). Co-inoculation of *P. aeruginosa*, *T. harzianum*, and *Mesorhizobium* sp. was found to increase shikimic acid accumulation four to ten times as compared to individual microbes. Other phenolics such as myricetin, ferulic acid, syringic acid, and quercetin were also accumulated in higher amounts (nearly 1.5–2 folds) in the leaves of consortium-treated chickpea plants after pathogen challenge compared to untreated control plants and single microbial treatments (Singh et al. 2014). Considering the abilities of various microbes in a compatible consortium to activate phenylpropanoid pathway, there is ample opportunity to harness this mechanism in controlling yield-threatening diseases in crop plants. Microbial consortium is also known to activate antioxidant mechanisms and systemic acquired resistance.

Cyanobacteria are commonly used as inoculants for rice and more recently for a number of other crops, including wheat, cotton, vegetables, and leguminous crops (Prasanna et al. 2012, 2013a, 2015b). Cyanobacterial consortia (*Anabaena-Azotobacter* biofilm) and *Anabaena* sp.-*Providencia* sp.) elicited defense responses in a set of maize hybrids, leading to enhanced activity of defense enzymes such as peroxidase, PAL, and PPO in roots, which also showed a pos-

itive correlation with Zn concentration in the flag leaf and increased crop vigor and yields (Prasanna et al. 2015a). *Cyanobacteria* exhibit activity of hydrolytic enzymes and produce a number of fungicidal molecules and show promise as biocontrol agents, particularly against soil borne phytopathogenic fungi in several crops, including cotton and vegetables (Manjunath et al. 2010; Dukare et al. 2011). They reduced the mortality by elevating the levels of hydrolytic and defense enzymes in the roots and shoots of plants, as evidenced by biochemical assays and DNA-based profiles (Prasanna et al. 2013b; Babu et al. 2015). A consortium of *P. aeruginosa*, *T. harzianum*, and *B. subtilis* is shown to increase H_2O_2 accumulation which regulates host defense response against *S. sclerotiorum* in pea (Jain et al. 2013). Various biocontrol agents are reported to induce pathogenesis-related (PR) proteins which play an important role in SAR. Microbial consortium of *T. harzianum*, *B. subtilis*, and *P. aeruginosa* induced synthesis of chitinases and β-1,3-glucanases in pea under the challenge of *S. sclerotiorum*. The activities of chitinases increased by 1.4e1.8-fold whereas β-1,3 glucanases by 1.4e4.6-fold in triple microbe consortium treatment compared to individual microbial treatments in pea (Jain et al. 2012). One of the main precautions while deploying consortium of PGPR against diseases is the compatibility among the strains. There are reports on negative impacts of microbial consortia which are attributed to the antagonistic activities of component biocontrol agents against one another (Bora et al. 2004; Elliott et al. 2009; Xu et al. 2010). Hence, there is a need for complete evaluation of compatibility among different biocontrol agents and PGPR and the development of effective consortium and management of different biotic and abiotic stress faced by crop plants.

4.8 Priming Induced by Chemicals

Besides pathogens and beneficial microorganisms, systemic resistance can be induced by various chemicals. These include salicylic acid (SA), its analogs, and various amino acids. SA analogs used widely as inducers of priming include 2,6-dichloroisonicotinic acid and its methyl ester (both referred as INA) and benzo(1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH; synonym, acibenzolar S-methyl, ASM) (Conrath 2009). Priming-mediated SAR induced by SA and its analogs has largely been dealt in the previous section, and it has been shown through various studies that priming is the major mechanism of SAR induced by SA and its analogs.

Among the amino acids, β-aminobutyric acid (BABA) received a lot of attention as inducers of priming. Research on the mechanism of BABA-induced resistance in *Arabidopsis* has shown that this type of IR, just like SAR and ISR, is frequently associated with priming for various pathogen-induced defense responses (Conrath 2009). BABA-induced resistance against necrotic pathogens was shown to be based on primed callose accumulation in *Arabidopsis* (Ton and Mauch-Mani 2004). BABA was shown to induce resistance in grapevine against downy mildew. A comparison among BABA, JA, BTH, ABA, and SA was done, and BABA was shown as the best protectant against downy mildew (Hamiduzzaman et al. 2005). BABA is known to reduce disease symptoms in various crops like sunflower, pepper, grapevine, cauliflower, and tobacco (Justyna and Ewa 2013). BABA at 10 mM concentration resulted in major accumulation of PR proteins and upregulation of several enzymes involved in the sesquiterpene phytoalexin biosynthesis (Bengtsson et al. 2014). The other chemicals used for priming plant defenses are Brotomax, Pyraclostrobin, Metalaxyl, and Fosethyl (Conrath et al. 2006).

4.9 Abiotic Stress Alleviation by Priming

For sustainable agriculture, improvement of plant tolerance in stress conditions is an important task, and microorganisms have the ability to impart tolerance to various abiotic stresses like drought, salinity, heat, and nutrient-limiting con-

ditions. Injury to plants due to the abiotic stresses mainly happen as a result of the oxidative damage by the free radicals produced in the plant cell. Such reactive molecules attack the vital cellular components like DNA and cellular membranes resulting in damage to the normal cellular mechanisms. Antioxidant enzymes like catalase and peroxidases have the capability to neutralize these reactive molecules, thereby, protect the cells from potential damage (Scandalios 1994). Many rhizobacterial species were demonstrated to have the ability to improve the activity of these enzymes. A study conducted by Kohler et al. (2008) showed that inoculation of PGPR *Pseudomonas mendocina* and arbusclar mycorrhizal fungi *Glomus intraradices*/*G. mosseae* elicited higher antioxidant enzyme catalase in lettuce crop under severe drought conditions. Inoculation of *P. fluorescens* pf1 in green gram plants under water stress conditions exhibited higher catalase and peroxidase activity compared to uninoculated control (Saravanakumar et al. 2011). In another study, inoculation of rhizobacterial strain *Pseudomonas putida* GAP-P45 resulted in improvement of plant biomass, leaf water potential, relative water content, free amino acids, and proline and sugars of maize plants under drought stress (Sandhya et al. 2010). Researchers in the recent past have concluded that bacteria belonging to genera such as *Bacillus*, *Rhizobium*, *Paenibacillus*, *Pseudomonas*, *Azospirillum*,*Methylobacterium*,*Achromobacter*, *Enterobacter*, *Burkholderia*, *Pantoea*, etc. can have promising results in the alleviation of abiotic stress in different crops (Grover et al. 2010). In a similar way, many endophytic and mycorrhizal fungi can alleviate the environmental stresses in plants by mutualism with various crop plants (Egamberdieva and Kucharova 2009; Rodriguez et al. 2008). Waller et al. (2005) reported that *Piriformospora indica* confers resistance against abiotic stress such as drought and salinity, besides promoting uptake of nitrate and phosphate.

Bano and Fatima (2009) investigated the effect of co-inoculation of *Rhizobium* and *Pseudomonas* on salt tolerance in *Zea mays* and concluded that co-inoculation resulted in decreased electrolyte leakage and maintained leaf water content leading to enhanced salt tolerance. Studies carried out by Egamberdieva et al. (2015) on the influence of *Pseudomonas putida* R4 in cotton for salinity tolerance showed that under salinity stress conditions, the root growth can be improved by the bacterium through production of IAA, which also confers resistance to *Fusarium* rot. Tolerance to the stress conditions is conferred by the production of plant hormones like IAA, GA, leading to an increased root growth and enhanced uptake of nutrients under these conditions (Egamberdieva and Kucharova 2009).

Phytohormone ethylene is involved in numerous physiological functions in plants. Its production is influenced by the various environmental stresses like high temperature, drought, salinity, flooding, etc. and results in senescence of plants and eventually lead to death of plants. Plant growth-promoting bacteria alleviate plants from these abiotic stresses by the production of ACC deaminase enzyme that metabolizes the aminocylopropane 1-carboxylate, thereby, reducing the stress levels in the affected plant. Mayak et al. (2004) concluded that *Achromobacter piechaudii* ARV8 produced ACC decarboxylase and resulted in induced systemic tolerance against drought and salinity in pepper and tomato crops. Studies carried out by Yim et al. (2012) demonstrated the synthesis of ACC deaminase by methylotrophic bacterium *Methylobacterium* spp. and their positive effect on tomato and red pepper. Several organisms were studied in detail for the past few decades including species belonging to *Pseudomonas*, *Bacillus*, *Achromobacter*, *Mesorhizobium*, etc. for their ability to reduce ethylene synthesis and aid in plant growth promotion under stress conditions (Glick 2014). Singh and coworkers (2015) reported that inoculation of ACC deaminase producing *Klebsiella* sp. SBP-8 conferred induced systemic tolerance against salt stress in wheat crop.

Arabidopsis thaliana plants inoculated with the *Paenibacillus polymyxa* resulted in enhanced drought tolerance by augmenting transcriptions of a drought-response gene, *EARLY RESPONSE TO DEHYDRATION 15* (*ERD15*) (Timmusk and Wagner 1999). Three plant growth-promoting

rhizobacterium (PGPR) strains (*Bacillus cereus* AR156, *Bacillus subtilis* SM21, and *Serratia* sp. XY21) were evaluated for their abiotic stress alleviation in cucumber plants. It was reported that the treated plants showed darker green leaved and lighter wilt symptoms than control plants after 13 days of withholding watering to plants. These PGPR bacteria conferred induced systemic tolerance to cucumber plants under drought stress by protecting plant cells, maintaining photosynthetic efficiency and root vigor, and enhancing level of antioxidant enzymes without involvement of ACC deaminase activity (Wang et al. 2012). Timmusk et al. (2014) evaluated PGPB strains *Bacillus thuringiensis* AZP2 and *Paenibacillus polymyxa* B from stress environments for enhancement of wheat (*Triticum aestivum*) drought tolerance in sand soil and reported increased photosynthetic activity, plant biomass, and five fold enhanced plant survival under severe drought. Further, emission of drought-associated volatile organic compounds (VOC) such as benzaldehyde, geranyl acetone, and b-pinene was also decreased in the plants treated with PGPR bacteria.

Under abiotic conditions, production of auxins, gibberellins, cytokinins, and antioxidants by some PGPR resulted in accumulation of abscisic acid and degradation of reactive oxygen species. The antioxidants reduce the oxidative stress on plants posed by various abiotic stresses (Stajner et al. 1997; Spaepan et al. 2008; Egamberdieva and Kucharova 2009). Bacteria capable of producing exopolysaccharides help plants in alleviating drought stress condition by improving the water holding capacity of the soil through improvement in soil structure.

PGPB enhances the level of osmoprotectant proline in the plants exposed to abiotic stresses. Proline scavenges the reactive oxygen molecules by antioxidant activity and protein stabilization through help of molecular chaperones in the stressed cells and protects them from the ill effects of reactive oxygen species (Bano and Fatima 2009; Kohler et al. 2009; Jha et al. 2011; Verbruggen and Hermans 2008). Volatile organic compounds (VOCs) emitted by rhizobacteria could both upregulate as well as downregulate the expression of *hkt1* expression in plants and maintain lower sodium levels under salt stress conditions (Zhang et al. 2008; Yang et al. 2009). Lucas et al. (2014) evaluated beneficial rhizobacteria for resistance against salt stress and bacterial blight in rice and reported that the inoculation of PGPR bacteria such as *Pseudomonas* sp. resulted in increased antioxidant enzymes and enhanced chlorophyll retention, apart from improvement in plant growth parameters in rice crop under salt stress conditions.

4.10 Insect Resistance by Priming

Among biotic stresses, damage by insects is a crucial component that needs to be addressed for achieving sustainable crop production. Plants can also benefit in terms of tolerance to insects when interacting with such beneficial microbes (Bennett et al. 2006; Vannette and Hunter 2009). By improving the plant's ability to regrow after insect damage by improved plant nutrient and water uptake, the beneficial microbes play a vital role in compensation of crop losses in the presence of insects (Herman et al. 2008; Kempel et al. 2009). Several soil microorganisms such as plant growth-promoting rhizobacteria and arbuscular mycorrhizal fungi help the plant combat biotic and abiotic stresses. These microorganisms interact in a bidirectional manner via plant with the insects (Pineda et al. 2010). Upon infection, the plant immune system is activated after recognizing the invasion of pathogen through the microbe-associated molecular patterns (MAMPs) or microbe-based biomolecules (Burketova et al. 2015). Arbuscular mycorrhizal association with plant roots may influence the performance of the above ground insect indirectly by means of changes in the quality and quantity of plants (Koricheva et al. 2009). They can even affect the natural enemies of the insects and pollinators by change in their behavior (Wooley and Paine 2011; Cahill et al. 2008) thereby influencing the food chain and web in ecosystems. In general, plant resistance to root feeders and common insects is increased by mycorrhizal colonization. On the other hand, they may increase the suscep-

Table 4.1 Some of PGPR microbes eliciting resistance to insects in various crops

Plant	Priming by	Against	References
Solanum lycopersicum	Arbuscular mycorrhizal fungi	*Meloidogyne incognita*	Vos et al. (2013)
Vitis spp.	AM fungi	*Xiphinema index*	Hao et al. (2012)
Oryza sativa	*Pseudomonas fluorescens*	*Cnaphalocrocis medinalis*	Saravanakumar et al. (2008)
Solanum lycopersicum	Arbuscular mycorrhizal fungi	*Helicoverpa armigera*	Song et al. (2013)
		Bemisia tabaci	
Brassica oleracea	*Acremonium alternatum*	*Plutella xylostella*	Raps and Vidal (1998)
Solanum lycopersicum, *Vicia faba*	*Acremonium strictium*	*Helicoverpa armigera*, *Trialeurodes vaporarium*, *Aphis fabae*	Vidal (1996), Jallow et al. (2004), and Jaber and Vidal (2009, 2010)
S. lycopersicum	*Bacillus subtilis*	*Bemisia tabaci*	Venezuela-Soto et al. (2010) and Herman et al. (2008)
		B. argentifolii	
Cucumus sativus	*Pseudomonas putida*	*Acalymma vittatum*	Zehnder et al. (1997)
Glycine max	*Lecanicillium* sp.	Aphids, white flies, cyst nematode	Goettel et al. (2008)

tibility of sucking insects and specialist insects (Hartley and Gange 2009). The arbuscular mycorrhizal fungi (AMF) have showed biocontrol potential against nematodes in tomato (Vos et al. 2013). Differential effectiveness of insect resistance has also been reported by Van oosten et al. (2008) in *Arabidopsis thaliana*. The specialist feeder insect *Pieris rapae* was found not affected, whereas a generalist feeder insect *Spodoptera exigua* was affected by the priming by *Pseudomonas fluorescens*. Recent reports on various plant defenses against pests are given in Table 4.1.

4.11 Signaling Pathways of Priming

Induced systemic resistance (ISR) is primed by various PGPR by the presence of elicitor molecules called microbe-associated molecular patterns (MAMPs), which include flagellin, peptidoglycans, lipopolysaccharides, and chitin, and also called as pathogen-associated molecular patterns (PAMPs). Upon interaction with these MAMPs, the plant activates a primary response to these molecules by recognizing the corresponding pattern receptors to MAMPs. The resultant primary immune response is called as MAMP-triggered immunity (Schwessinger and Zipfel 2008; Zamioudis and Pieterse 2012). Several signaling cascades are activated by these molecules in plants which are orchestrated by phytohormones like ethylene, jasmonic acid, and salicylic acid. These are the major regulators in inducible plant defense reactions (Pieterse et al. 2012). These MAMPs are implicated in the induction of ISR in plants by various PGPR bacteria including *Pseudomonas* and *Bacillus* spp. Besides these MAMPs, there are other molecules such as secreted bacterial components like iron-chelating siderophores, biosurfactants, antibiotics, and volatile organic compounds that were shown to elicit ISR in plants (Iavicoli et al. 2003; Raaijmakers et al. 2006; Bakker et al. 2007). Plants can also recognize microbes lacking one of these characters indicating that they can also recognize multiple MAMPs produced by microbes. This phenomenon is similar to PAMPs where this redundancy could still induce a strong and robust immune response in plants (Meziane et al. 2005; Bakker et al. 2007; Bittel and Robatzek 2007).

Among fungi, ISR is reported in some endophytic fungi such as *Trichoderma* (Martinez-Medina et al. 2013), *Sebacinales*, and some nonpathogenic strains of *Fusarium* spp. (Aime' et al. 2013). MAMP that produced immunity is

well studied in *Trichoderma* spp. (Vinale et al. 2008). Transcription factor gene MYB72 is involved in PGPR-specific responses. Analysis of this gene revealed that mutant *myb72* could not produce any response against necrotrophic and hemi-biotrophic pathogens (Van der Ent et al. 2008; Segarra et al. 2009). MYB72 is induced as part of ISR signaling pathway among different beneficial microbes including both PGPR and PGPF like *Trichoderma* sp. as evidenced from various studies in the recent past (Pieterse et al. 2014). Accumulation of transcripts and proteins which are involved in the plant defense in the roots of rice and tomato plants upon mycorrhizal colonization was reported by Pozo and Azcon-Aguilar (2007). Interestingly, the mechanism of induction of ISR in plants by diverse beneficial microorganisms relies on common pathways, for instance, JA signaling pathway (Song et al. 2013; Martinez-Medina et al. 2013).

An initial notion that rhizobacteria-mediated ISR is similar to that of pathogen-induced SAR was found to be not completely true by Hoffland et al. (1995). Different signaling pathways regulate rhizobacteria-mediated ISR and salicylic acid-dependent SAR. Support for this conclusion came from Ton et al. (2002) in which it was found that both rhizobacteria-mediated ISR and pathogen-mediated SAR are effective against a wide range of enemies, but they had partly divergent effectiveness range.

Colonization of beneficial microbes does not necessarily increase the production of JT/ET; rather, they increase the sensitivity to these hormones. This could be understood from the fact that these hormones are not generally associated with the activation of JA/ET-responsive genes in plants. In fact, transcriptional changes that occur due to the colonization of beneficial microbes are weaker than that of pathogen attack-induced massive transcriptional reprogramming. But the plants that are primed initially by the beneficial microbes display stronger defenses against the insect/pathogen (Fu et al. 2007; Liu et al. 2007; Van Wees et al. 2008). In general, beneficial microbes regulate ISR by SA-independent mechanisms. But many microbes that trigger ISR by a SA-dependent type of ISR are also reported. For example, PGPR *P. fluorescens* P3 overexpressing salicylic acid gene cluster of *P. aeruginosa* PAO1 was proved to induce SA-dependent SAR (Maurhofer et al. 1998). At the same time, rhizobacterially synthesized salicylic acid is usually not the reason for the systemic resistance observed in plants (Ran et al. 2005). The reason for this phenomenon is often the salicylic acid is not released into the rhizosphere, rather it is incorporated by SA moiety containing iron-chelating substances like siderophores that makes SA unavailable to trigger the SAR pathway (Bakker et al. 2014).

4.12 Benefits and Disadvantages of Priming

Priming of plants either by pathogens or by beneficial microbes is comparable to immunizing mammals and other higher organisms with specific modified microorganisms to elicit immunity against that particular microorganism when attacks in the future. Primed plants do not express defenses in the absence of an attacker but a stronger and quicker response at cellular level when the plant is challenged by its enemies compared to non-primed plants (Conrath et al. 2002, 2006; Frost et al. 2008). But the major difference between priming in plants and immunization is specificity in defense in the latter compared to more general defense response in the former. But, in natural situation, more general cellular defense response offers more benefit to plants that can be utilized against an array of plant pathogens or insect pests. The advantages of priming are first it is cost-effective as it does not require prior activation for primed state and second is its enhanced resistance against broad spectrum of attacker. It can be induced by beneficial microbes, pathogens, herbivore insects, chemical elicitors, and wounding (Conrath et al. 2002, 2006). It has been proved that primed defense can be transferred to the next generation through epigenetic changes to keep it in its memory (Slaughter et al. 2012; Rasmann et al. 2012; Luna et al. 2012). Moreover, numerous studies in the past show that PGPR enhances the plant growth, seed production, and other yield parameters apart from priming for defense, even though

these two are not related (Raupach and Kloepper 1998; Zehnder et al. 2001; Zamioudis et al. 2013). These results reveal that benefits against biotic stress without involvement of allocation costs associated with this. In *Arabidopsis*, Bur-0 is constitutively primed for increased defenses against biotic stresses like insect and pathogens, without an effect on growth (Ahmad et al. 2011). Zhang et al. (2015) showed recently that rice is capable of activating basal resistance against rice blast by perturbing OsDCL1-dependent miRNA biogenesis pathway.

The consortium of microbes for priming for disease resistance and plant growth is not always positive in its action, while some researchers found that the consortium resulted in increased performance than application of single organisms. For example, Walker et al. (2012) reported on par performance of microbial consortium consisting of *Glomus*, *Azospirillum*, and *Pseudomonas* in maize with that of *Glomus* alone with respect to secondary metabolites in maize roots. On the other hand, mycorrhizal inoculation resulted in reduced nodulation in common bean. But under abiotic stress conditions, the accumulation of trehalose was found increased in co-inoculation of mycorrhiza and rhizobia than individual inoculation (Ballesteros-Almanza et al. 2010). Several other researchers also reported a similar pattern in other crops. This could be attributed to the independent signaling pathways operating in the two different microbes in the consortia or the incompatibility between the consortia partners (Sarma et al. 2015). Even though priming using a chemical compound at a right concentration gives an immediate activation of robust defenses in plants (Roylawar et al. 2015), the priming by beneficial microbes outweighs the priming alone when compared to their holistic improvement in many plant growth-promoting attributes.

Over several decades, the role microorganisms in plant growth promotion have been well studied, and the potential of microbes in pest and disease management gained momentum for sustainable agriculture. Similar to case of immunization, beneficial microbes could offer an increased defense mechanism in crops when the pathogens are introduced. Such primed state has been well studied well in many crops. Even studies showed that defense priming state could be transgenerational (Pieterse et al. 2012; Luna et al. 2012; Slaughter et al. 2012; Rasmann et al. 2012; Sarma and Singh 2014). Microbial priming of plant defenses can overweigh the marginal costs in its environments where the disease or pest infestation occurs. Hence, microbial priming could be an environmentally safe, economically cheaper, and ecologically long lasting for the sustainable crop production.

4.13 Conclusion and Future Prospects

Increased demand for food crops poses great burden to agriculture, forcing it to have more intense monocropping with crops having improved genetic base for production whereas narrower genetic base for biotic and abiotic stresses. Such monocropping has increased incidence of pest and diseases after the advent of green revolution. But, the environment is always having solutions for such problems if it is properly explored. Despite having many studies on microbial interaction with plants individually, the complete understanding of microbial metabolism in microbial consortia is still lacking. This has resulted in failure of many microbial consortia under field condition when compared to individual microbes. Microbes live as a community in nature rather than isolation. Hence, it is of paramount importance to understand their interactions with other organisms in their immediate environment. More studies on molecular level impact of microbes with plants in priming defense against prominent crop pathogens need to be undertaken.

Priming offers a smart, effective, and natural means of capitalizing on the innate defense capacity when it is associated with conventional methods of plant protection. Complete understanding of physiological, molecular, and ecological aspects is essential to exploit the potential of priming as an integrated pest management strategy and stress amelioration technology in agriculture.

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Seed Priming-Mediated Induced Disease Resistance in Arid Zone Plants

5

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Abstract

Priming of seed provokes plants to activate defence responses more quickly and effectively against phytopathogens without alternating plant growth and has the potential to emerge as a strategic tool for modern plant protection. Seed priming is an attractive, simple and cost-effective strategy that induces systemic resistance to control the plant diseases. Seed primed through microorganisms reflects biochemical/physiological changes leading to the synthesis of proteins and chemicals involved in induced systemic resistance and increases the efficacy of the plant against several pathogens. The present chapter summarizes the current knowledge of the seed priming and its relevance for plant protection with special reference to bio-priming.

5.1 Introduction

Plant develops an enhanced resistance against vicious pathogens in the tissue distant from the site of infection. Several approaches have been developed to reveal the phenomenon of resistance wherein the most important is the induction of systemic resistance in the plant. Upon appropriate stimulation with certain agents, the plant defence mechanisms induced and elevated that enable the plant to combat further invasion by the pathogens. This response is termed induced systemic resistance (ISR) and/or immunization that is effective against a broad spectrum of pathogens (van Loon et al. 1998; Vallad and Goodman 2004; Conrath et al. 2006). In the recent years, ISR has received profound attention and achieved with plant growth-promoting rhizobacteria (PGPR) (Burdman et al. 2000; Ramamoorthy et al. 2001; Vallad and Goodman 2004; Kuc 2006). It has been reported that salicylic acid (SA), acetyl salicylic acid and bion have ISR characteristics against various diseases under controlled environments (Saikia et al. 2003; Sarwar et al. 2005). Various PGPRs lead ISR in many crop species wherein *Pseudomonas* spp. (*Pseudomonas fluorescens*) is the most important inducers.

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They enhanced plant growth and protect the plants from various plant pathogens in several crops (Burdman et al. 2000; Ongena et al. 2000; Ramamoorthy et al. 2001).

Priming is an ideal method for inducing resistance by biocontrol agents that augments the efficiency of ISR and has been identified to imply the defence secrete of plants for decades, but the phenomenon received much attention during the early 1990s (Kuć 1987). Seed priming presents many advantages and is reported to alleviate various physiological and pathological stresses that results in utilization, activation and enhancement of various cellular defence responses and resistance (Conrath et al. 2002). Seed treatment with PGPR promotes ISR in host plant to pathogen attack in many host-pathogen interactions and is associated with signalling proteins that remain inactive under normal conditions and activates with the exposure of plants to stresses (Mathre et al. 1999; Conrath et al. 2002, 2006). Microorganisms are incorporated into the seed during the priming process that allows rapid seed colonization and uniform coverage of the seed surface so that they either colonized before pathogen infection or induced disease resistance mechanisms (Sabalpara 2015).

By keeping views of induced resistance, the present chapter summarizes the current knowledge of the seed priming and its relevance for plant protection with special reference to bio-priming.

5.2 History of Seed Priming

The history of seed priming dates back to 60 A.D. Attempts to improve seed germination have been reported since Ancient Greeks (Everari 1984). Theophrastus (371–287 BC) investigated that the seeds soaked in water prior to sowing resulted into faster germination. Gaius (1949–1954) stated the relevance of pre-soaking of seeds in water to improve germination. Later, Olivier de Serres (1539–1619) revealed the effectiveness of the seed treatment on grains (*Triticum*, *Secale* and *Hordeum* spp.). Darwin (1855a, b) tested osmo-priming conditions by submerging the seeds of *Lepidium sativum* and lettuce in seawater and reported improved germination with the treatment. Later Ells (1963) revealed that 2 days of soaking in water at 23.6 °C was enough to cause premature germination in tomato.

Seeds have always been treated as one of the valuable resources, and our old people know to conserve them for future use. The primitive documents revealed that wine and crushed cypress leaves were used to maintain seed free from storage insects, and it is because of evolvement of hydrogen cyanide that kills insects and furthermore followed by soaking of seed with seawater showed significantly lesser infection of stinking smut than unsoaked seed (https://161www.apsnet.org/edcenter/advanced/topics/Pages/CerealSeedTreatment.aspx). The advantage of salt and lime in controlling common bunt in wheat has also been demonstrated, and hence the field of seed treatment has evolved into a more complex science, and after the 1920s, a new era in seed treatment has resulted in the many commercial fungicides (Goggi 2011).

5.3 Seed Priming

Priming of seed allows control of hydration level within the seeds to start the metabolic activity required for germination with the prevention of emergence of radical whereby the seed illustrates different physiological actions at different moisture levels. The radical emergence is the final physiological activity in the germination process that requires high seed water content. The seed priming plays an important role in the conservation of seed water content which improves phytochrome-induced dormancy in plants by reducing the time required for germination (Leopold and Vertucci 1989; Taylor 1997). The plant is able to respond more rapidly and strongly to the attack by pest without expression of any defences due to seed priming and acquires broad spectrum-enhanced resistance with minimal associated costs compared to direct activation of defence (Conrath et al. 2006; van Hulten et al. 2006). The number of studies suggests that priming is an effective mechanism for crop pro-

tection in the field (Beckers and Conrath 2007; Conrath 2011). Application of some hormonal and other chemical signal compounds along with some synthetic chemicals can activate priming response and influence future responses in tissue distinct from the site of attack (Conrath et al. 2006; Kathiria et al. 2010).

5.3.1 Seed Bio-priming

Bio-priming employs biological inoculation of seed with benign microorganisms to guard seeds and regulate seed hydration for biotic/abiotic stress management. It is the recent technique for controlling major seed and soilborne pathogens and to encourage more uniform seed germination/plant growth associated with fungi and bacteria coatings (Entesari et al. 2013). Seed priming alone and/or in combination with low dosage of fungicides and/or biocontrol agents has been used to improve the rate and uniformity of seed emergence and to diminish diseases. It has the potential to deliver the agents in the right amount and at the right place with right time and results in mobilization, activation and enhancement of various cellular defence responses for induction of resistance (McQuilken et al. 1998; Conrath et al. 2002). Seed priming with bio-inoculants helps in disease suppression by utilizing different mechanisms such as siderophore production, antimicrobial secondary metabolite and secretion of lytic enzymes (Keswani et al. 2014). Siderophores are small, high-affinity iron-chelating compounds secreted by bacteria and fungi and considered as one of the strongest soluble Fe^{3+}-binding agents mediated by several strains of *Pseudomonas* that control biologically soilborne pathogens when applied as seed inoculants to agricultural crops (Burr and Caesar 1984). The *Pseudomonas* siderophores are reported to suppress disease and enhance plant growth by the production of fluorescent siderophores that chelate molecular iron in rhizosphere (Singh et al. 2011, 2014; Jain et al. 2012).

Application of microorganisms to seed is an attractive proposition because of the combination of specific effect and limited environmental impact. It is a consensus among scientists and general public regarding serious health hazards upon the use of chemical in food supplies that has propelled research for eco-friendly alternative approaches for plant disease management with overall growth promotion (Wilson and Wisniewski 1994; Gerhardson 2002). Interestingly, seed bio-priming acts as a model system for the delivery of dense population of benign microorganisms to soil, where they can colonize with the emerging roots of crop plants. This practice is tremendously used for past decades effectively in the field and offers better/equal results over conventional fungicides (Callen and Mathre 2000; Niranjan et al. 2004).

5.3.2 Seed Bio-priming for Induced Systemic Resistance

Seed bio-priming is an ecological approach for the management of soil- and seed-borne disease by employing selected antagonists. It has been reported that seed bio-priming through benign and eco-friendly biological agents improved the physiology of seeds resulting into enhanced vigour of the seedlings (Ghassemi-Golezani et al. 2008). The pathogen-infected seeds contribute to the establishment of diseases and make the production strategy tedious (Reddy et al. 2011). The seed bio-priming not only dealt with plant disease control but also helps in improving various abiotic stress conditions. The frequent use of pesticides may lead to development of tolerance in the target organism, whereas the application of bio-primer substantially reduces the use of chemical fertilizers and pesticides which cause hazardous pollutions. The colonization of seedling roots with bio-primer microorganisms provokes broad spectrum ISR in plants and is fast emerging as a potential alternative to the use of chemical pesticides (Manjunatha et al. 2013). ISR in plants has been widely studied in selected strains of non-pathogenic, root-colonizing PGPR wherein strain comprises the strengthening of physical and mechanical strength of the host cell wall and changes biochemical or physiological make-up leading to synthesis of defence proteins with

increased activities of chitinase, β-1,3-glucanase, peroxidase, polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) enzymes and accumulation of phenolic contents (Burdman et al. 2000; Ramamoorthy et al. 2001; Heil and Bostock 2002; Dutta et al. 2008). Strains of *Pseudomonas*, *Bacillus*, *Trichoderma* and other microbes are outstanding bio-primers to ISR as they also promote growth and development of plants (Ongena et al. 2000; Ramamoorthy et al. 2001; Gnanamanickam et al. 2002).

5.3.3 Induction of Resistance by Chemicals

Various organic and inorganic compounds have been reported to activate the induced resistance in the plant (Kuć 2001, 2006). With the identification of SA as endogenous signal for the systemic acquired resistance response, a number of researches were started to identify other synthetic chemicals that able to induced resistance in the plants. Among them, 2,6-dichloroisonicotinic acid and its methyl ester were the first compounds reported to activate resistance response in the plants (Kessmann et al. 1994) followed by benzothiadiazole-7-carbothioic acid S-methyl ester (Görlach et al. 1996). It is assumed that these compounds activated the resistance via the signalling pathway (Ryals et al. 1996). Priming effects can be elicited by chemical ISR inducers, such as β-aminobutyric acid (BABA) (Jakab et al. 2001). The primed plants respond more effectively or rapidly with their re-exposer to the biotic and abiotic stress (Conrath et al. 2006). The treatment of tomato seeds with jasmonic acid (JA) and BABA provides long-lasting increase in herbivore and pathogen resistance in plants (Worrall et al. 2012)

5.4 Mechanisms of Induced Disease Resistance Through Seed Priming

The induction of default defence mechanisms of the plant is a novel plant protection approach (Reddy 2013). However, plants are also bestowed with various defence-related genes, but these genes are sleeping genes and require appropriate stimuli/signals for their activation (Greenberg et al. 2000). Several microorganisms are capable of producing SA and are involved for the induction of ISR in plants (Kloepper et al. 2004). Seed priming with PGPRs enhances the germination rate and improves seedling establishment that starts with the physiological process of germination and helps in the establishment and proliferation of PGPRs in the spermosphere (Taylor and Harman 1990). Bio-priming of seeds with bacterial antagonists increases the population load on the seeds whereby it protects the rhizosphere from plant pathogens (Callanet al. 1990). Furthermore, infection of a single leaf with a microbial pathogen can lead to direct activation of defence in the infected leaf and primed defence in other parts of the plant (Ton et al. 2007).

Microbial-mediated seed priming activates ISR against various fungal, bacterial and viral diseases (Liu et al. 1995a, b; Maurhofer et al. 1998), insect disease (Zehnder et al. 1997) and nematode pests (Sikora 1998). SA, a phenolic compound produced by seed primer microorganisms, is an important component in the signal transduction pathway and is essential for induction of resistance against pathogens to combat (Gaffney et al. 1993). Among microbial determinants, it was reported that lipopolysaccharides (LPSs) present in the outer membrane act as signal molecules and produce various defence compounds and the major elicitor of ISR. The O-antigen side chain of the LPS serves as a signalling agent to trigger the induction of defence mechanism (van Wees et al. 1999). JA and ethylene are also reported as signals for resistance against microbial pathogens that is established after treatment with various strains of root-colonizing bacteria (Dong 1998). In addition, *P. fluorescens* strain WCS417r could provoke systemic disease resistance in plants through a variety of signal translocation pathways like SA-independent signalling, JA-ethylene-dependent signalling, ISR-related gene expression and NPR1-dependent signalling (Pieterse et al. 1996). ISR in plants is also associated with increased activity of chitinase, β-1,3-glucanase, peroxidase (PO), polyphenol oxidase (PPO) and

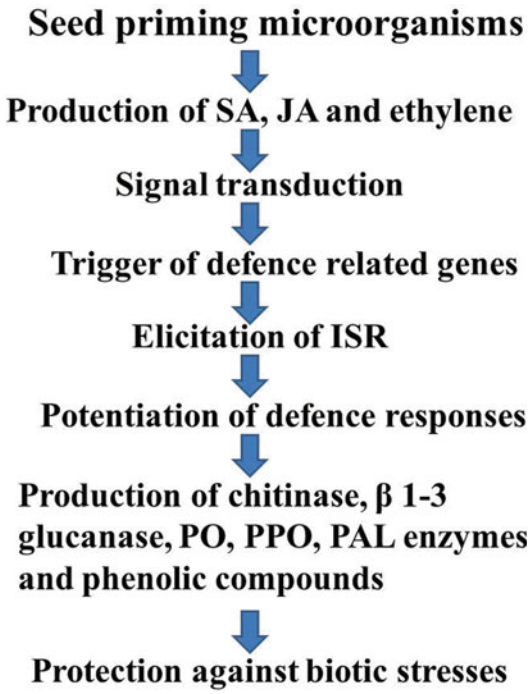

Fig. 5.1 Events associated with ISR in plants through seed priming

phenylalanine ammonia lyase (PAL) and accumulation of phenolic contents with other pathogenesis-related proteins (Dutta et al. 2008). Various events associated with ISR in plants during seed priming are depicted; vide Fig. 5.1.

The chitin has been considered as the major component of the cell walls of fungi, and the role of chitinase enzyme is to hydrolyse chitin that contributes to the defence of plants against phytopathogens (Jackson and Taylor 1996). Production of chitinase enzymes by seed primers for induction of ISR has been thoroughly studied. The role of chitinase and β-1,3-glucanase enzymes in defence against pathogens has been proposed, and their production degrades the chitin and glucan, respectively (Frindlender et al. 1993; Potgieter and Alexander 1996; Velazhahan et al. 1999). PAL is an enzyme involved in phenylpropanoid metabolism in plants and responsible for the biosynthesis of various chemical barriers, viz. phenolics, phytoalexins and lignins that are effective against pathogen. These compounds are responsible for disease resistance in the plants (Kloepper et al. 2004). PO is a key enzyme to oxidize phenolics to quinines and generate hydrogen peroxide that releases highly reactive free radicals and increases the rate of polymerization of phenolic compounds into lignin substances. These substances are deposited in cell walls and interfere with the further growth and development of the pathogens (Meena et al. 2000; Ramamoorthy et al. 2001). PPO is a copper-containing enzyme, oxidizes phenolic compound to quinines which are often more toxic to pathogenic microorganism than the original phenols and is involved in the terminal oxidation of diseased plant tissue simultaneously attributing its role in disease resistance (Meena et al. 2000; Ramamoorthy et al. 2001).

Phenolic compounds contain one or more benzene rings along with phenolic hydroxyl groups and are widely distributed in higher plants. Anthocyanins, leucoanthocyanins, anthoxanthins, hydroxybenzoic acids, glycosides, sugar esters of quinines, shikimic acid, esters or hydroxyl cinnamic acids and coumarin derivatives make wide class of phenolic compounds. These compounds act as hydrogen donors or acceptors in oxidation-reduction reactions and play an essential role in lignification and improve the resistance of plants to disease-causing pathogens (Ramamoorthy et al. 2001; Kloepper et al. 2004). Studies revealed that individual strain of PGPR produces ISR against multiple diseases on one plant host (Wei et al. 1996). The use of individual PGPR strains or mixtures of several strains may result in a more stable rhizosphere community, may provide several mechanisms of biological control and may suppress a broader range of pathogens. Raupach and Kloepper (1998) observed that mixtures of PGPR provided synergistic activity against a broader range of pathogens on one host. Seed treatment with *P. fluorescens* protected plants through induction of systemic resistance not only against the fungal root pathogen *F. oxysporum* f. sp. *raphani* but also against the bacterial leaf pathogen *P. syringae* pv. *tomato* and fungal leaf pathogens *Alternaria brassicicola* and *F. oxysporum* (Hofflands et al. 1996).

5.5 ISR in Arid Zone Crops

Agricultural productivity in arid zones faces great risk by the vagaries of weather, particularly uncertainty of monsoon rains, moisture and nutrient stress (Saxena et al. 2014). Besides physiological stresses crops also have to overcome the biotic stresses posed by diseases and pests. ISR against plant diseases by the means of biological control has been extensively studied under greenhouse and field conditions for arid zone crops (Kloepper et al. 1997; Ramamoorthy and Samiyappan 1999; Niranjan et al. 2004). Induction of ISR by *Pseudomonas putida* and *Serratia marcescens* against anthracnose of cucumber was established by Wei et al. (1991). Later studies showed that the same PGPR strains induced systemic protection against angular leaf spot caused by *P. syringae* pv. *lachrymans* (Liu et al. 1993a), *Fusarium* wilt incited by *F. oxysporum* f. sp. *cucumerinum* (Liu et al. 1993b) and cucurbit wilt caused by *Erwinia tracheiphila* (Kloepper et al. 1993). Seed treatment of *S. marcescens* has shown ISR in cucumber against anthracnose, cucumber mosaic virus, bacterial angular leaf spot and cucurbit wilt diseases (Kloepper et al. 1993; Liu et al. 1995a, b). In addition, the same bacterial strain has also been effective in controlling the striped cucumber beetle, *Acalymma vittatum*, and spotted cucumber beetle, *Diabrotica undecimpunctata howardi* (Zehnder et al. 1997). Application of *Pseudomonas* sp. protected plants systemically against *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *dianthi* (Van Peer et al. 1991).

PGPR strains applied as seed treatment resulted in a significant reduction in anthracnose disease caused by *Colletotrichum orbiculare* in cucumber (Wei et al. 1996). Similarly, induction of systemic resistance by *P. putida* and *S. marcescens* reduced *Fusarium* wilt of cucumber incited by *F. oxysporum* f. sp. *cucumerinum* (Liu et al. 1995a). PGPR as a seed treatment alone or as seed treatment plus soil drenching has protected cucumber plants against anthracnose disease (Wei et al. 1996). PGPR can also induce systemic protection against bacterial diseases. Seed treated with *P. fluorescens* protected beans against halo blight disease caused by *P. syringae* pv. *phaseolicola* (Jing et al. 2007). While treatment of cucumber seed with *P. putida* and *S. marcescens* decreased the incidence of bacterial wilt disease (Ahmed 2010). Similarly seed treatment of cucumber with *P. putida*, *Flavimonas oryzihabitans*, *S. marcescens* and *Bacillus pumilus* provided systemic protection against angular leaf spot caused by *P. syringae* pv. *lachrymans* by reducing total lesion diameter compared to nontreated plants (Liu et al. 1995b; Wei et al. 1996; Dey et al. 2014). The seed treatment with PGPR followed by soil application of its talc-based powder formulation successfully controlled wilt in chickpea and pigeon pea under field conditions and has increased the yield of the crop (Vidhyasekaran et al. 1997; Dey et al. 2014). Induction of systemic resistance by PGPR against viral diseases has been reported in cucumber plants. Seed treatment with *P. fluorescens* and *S. marcescens* has consistently reduced the number of cucumber mosaic virus-infected plants and delayed the development of symptoms in cucumber and tomato (Raupach et al. 1996). PGPR also induced systemic resistance against nematode pests (Oostendorp and Sikora 1990; Sikora 1992; Sikora and Hofmann-Hergarten 1992). *P. fluorescens* has induced systemic resistance and inhibited early root penetration of the cyst nematode, viz. *Heterodera schachtii*, in various economically important crops (Oostendorp and Sikora1990). Similarly, *B. subtilis* has induced protection against *Meloidogyne incognita* and *M. arenaria* in cotton (Sikora 1998). The level of infestation of root-knot nematode *M. incognita* on tomato was reduced with fewer galls and egg masses in the soil following root dipping with *P. fluorescens* (Ramamoorthy et al. 2001). Similarly, application of the bacterium, *P. chitinolytica*, reduced the root-knot nematode infection in tomato crop (Spiegel et al. 1991; Dey et al. 2014). In legume, seed treatment with *P. fluorescens* produced chitinases and β-1,3-glucanases. These lytic enzymes accumulate at the site of penetration of the fungus and degrade the fungal cell wall (Benhamou et al. 1996; Dey et al. 2014). Inoculation of tomato plants with the same strain

induced the production of plant chitinases when challenged with the wilt pathogen, *F. oxysporum* f. sp. *radicis-lycopersici* (M'Piga et al. 1997; Benhamou et al. 1998).

The role of defence enzymes and phenolic compounds in disease managements after seed priming of mung bean in semiarid area has been studied (Khan et al. 2008). Bio-priming of seeds, in the integrated management of *Alternaria* blight of sunflower, have been reviewed by (Rao et al. 2009). Umair et al. (2010) evaluated different seed priming technique in mung bean (*Vigna radiata*) and found that seedling vigour index was high as compared to seed without coating by seed primer microbes. Meena et al. (2000) studied *P. fluorescens*-mediated systemic resistance against leaf spot of groundnut and reported induced resistance against *Cercospora personata*. Seed treatment with *P. fluorescens* increased the activity of PAL, phenolic content and lytic enzymes. Ramamoorthy and Samiyappan (2001) studied induction of defence-related genes in *P. fluorescens*-treated chilli plants in response to infection of *C. capsici*. They observed that seed treatment with *P. fluorescens* increased the PAL, PO, PPO and β-1,3-glucanase and chitinase activity and accumulation of phenolics in chilli leaves due to expression of various defence-related genes. Colonization of bean root by fluorescent bacteria was correlated with induction of pathogenesis-related proteins, and increased activity of PAL, chalcone synthase, was reported against *Botrytis cinerea* (Zdor and Anderson 1992). In tomato, *P. fluorescens* induced accumulation of chitinase and prevented the infection of *F. oxysporum* f. sp. *radicis-lycopersici* (M'piga et al. 1997). De Mayer et al. (1999) reported that rhizosphere colonization by *P. aeruginosa* activated PAL in bean roots and increased the SA level in leaves. Elicitation of ISR by *B. mycoides* and *B. pumilus* was associated with enhanced PO activity and increased production of isozyme of chitinase and β-1,3-glucanase. Zhang et al. (2002) reported that plants treated with *B. pumilus* had greatly increased levels of SA, compared to that of non-treated plants (Bargabus et al. 2004).

Surekha et al. (2014) reported that *V. mungo* seeds treated with *T. viride* induced defence enzymes (PO, PPO, PAL) and total phenolic content in black gram exposed to pathogens *F. oxysporum* and *Alternaria alternata*. Jetiyanon and Kloepper (2002) studied the effect of mixtures of PGPR for induction of systemic resistance against multiple plant diseases including bacterial wilt of tomato caused by *Ralstonia solanacearum*, anthracnose of long cayenne pepper (*Capsicum annuum* var. *acuminatum*) caused by *C. gloeosporioides*, damping off of green kuang-futsoi (*Brassica chinensis* var. *parachinensis*) caused by *Rhizoctonia solani* and cucumber mosaic virus on cucumber (*Cucumis sativus*). Results indicated that four mixtures of PGPR and one individual strain treatment significantly reduced the severity of all four diseases compared to the non-bacterized control. Most of the mixtures of PGPR provided greater disease suppression than individual PGPR strains. Evaluation of *P. fluorescens* as a biocontrol agent and biofertilizer associated with chilli rhizosphere in arid zone areas was studied (Gehlot and Purohit 2002; Gehlot et al. 2005), and significant suppression of wilt disease with considerable increase of productivity by the application of *P. fluorescens* as seed primers was reported. Vanitha and Umesha (2011) studied *P. fluorescens*-mediated systemic resistance in tomato against bacterial wilt disease caused by *Ralstonia solanacearum* and observed increased activities of PAL, guaiacol peroxidase (POX), PPO and lipoxygenase (LOX) in the tomato seedlings. Reverse transcription polymerase chain reaction confirmed the maximum induction of all these enzymes in *P. fluorescens* pretreated seedlings. Sarwar et al. (2010) reported seed treatments induced systemic resistance in chickpea against *F. oxysporum* f. sp. *ciceri* wilt in wilt sick seed. The seed priming with *P. fluorescens* isolates resulted in improved growth of pearl millet and also induction of resistance against downy mildew disease caused by the fungus *Sclerospora graminicola* (Niranjan et al. 2004). All the test isolates reduced disease severity and promoted growth both under greenhouse and field conditions.

5.6 Constraints

- Maintenance of high numbers of microorganisms on the seed surface during seed treatment and storage.
- Bio-priming may not provide adequate seed protection under all conditions, and priming agents are sensitive to the temperature, pH or moisture of the soil.
- The expression of pathogen-induced transcription factors contributes to a long-lasting priming of defence genes after the invading pathogen has been constrained.

5.7 Opportunities

- ISR is often maintained for the lifetime of the plant and is effective against several pathogens.
- Priming allows plants to activate defence responses more quickly and effectively against biotic and abiotic stress and offers a robust, effective and realistic option for effective plant protection as compared to the traditional insecticides and pesticides.

5.8 Conclusion

Seed bio-priming may be the best alternate choice of biocontrol of soil and seed-borne diseases and creates a complimentary environment by increasing nutrient uptake from seed exudates and initial moisture of the seeds which can contribute to the proliferation of microbes on the seed surface. The technique is being used globally for the management of seed and soilborne phytopathogens of many economically important arid zone crops. The major constraint linked with seed bio-priming is the maintenance of high numbers of microorganisms on the seed surface during seed treatment and storage that can be overcome by the better understanding of the interactions between microorganisms, seed and formulation components.

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Trichoderma Secondary Metabolites: Their Biochemistry and Possible Role in Disease Management

6

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Abstract

The extensive use of pesticides affected soil quality, water quality and ecological balance and ultimately damaged the socio-economical scenario. The pesticide resistance is also one of the alarming problems of this century. Biological method of sustainable agriculture is the only way to overcome these problems. The current chapter focuses on the use of *Trichoderma* as biocontrol agent in present agriculture system and its advantages over traditional pesticides and fertilisers. *Trichoderma* spp. exhibited a wide range of secondary metabolites (volatile, nonvolatile, diffusable) responsible for the protection of plants from harmful pests, nutrient support, mineral solubilisation and pharmacological activities. *Trichoderma* showed mycoparasitism, antibiosis and competition mechanisms to combat major agricultural pests. The collective information of secondary metabolism, mechanism of action and applications would be useful to biologists, chemists and agriculturists for integrated pest and disease management.

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6.1 Introduction

Over the years, the widespread use of pesticides (herbicides, fungicides and insecticides) in agriculture had several benefits and also reported to cause deleterious effect due to dangerous chemicals. The agricultural pests destroy nearly 37 % of all crops produced in the USA every year, resulting in an economic loss of around $122 billion a year (Pimentel and Greiner 1997). The total loss occurring globally due to pest is impossible to estimate, as there are many factors involved in losses, although an approximate estimate suggests that about 15 %

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of crops worldwide are lost because of insects (Bebber et al. 2013).

In the past 40 years, pesticides have been widely used to improve the quantity of food production (Pittendrigh and Gaffney 2001; Gerhardson 2002) and to control crop pests ranging from weeds, insects and nematodes to fungi. Recently, the excess use of chemical fertilisers has been a matter of criticism due to increase in awareness for environmental protection and resistance of pathogens to these chemicals (Dekker and Georgpolous 1982). It has been proved that the pesticides had deleterious impacts on the environment and human health (Gerhardson 2002; Punja and Utkhede 2003; Bues et al. 2004). In general, the pests were used to control by curative measures such as physical methods (cultivation, handpicking, weeding, trapping), chemical methods (pesticides) and biological methods (crop rotation, antagonists, predators).

The ecosystem balance has been observed to be disrupted due to excessive use of pesticides, as it kills non-pest organisms supposed to be beneficial for soil health. The excess accumulation of pesticides results in leaching into ground water, which remains persistent in soil for decades and ultimately appears in the food chain (Stoate et al. 2001). Nowadays, an alarming concern of pesticides' negative impact on agriculture is the resistance of pests to the pesticides (Pittendrigh and Gaffney 2001; Gerhardson 2002; Hahn et al. 2002).

Pesticides have become less effective for many crop pests due to genetic mutations, which eventually encouraged farmers to increase the dosage or use more powerful and various combinations of chemicals in order to control pests. Crops improved by genetic engineering or biotechnology would be subjected to the same extensive testing as traditionally bred plants. In assessing the safety of human and animal foods derived from new plant varieties, the FDA's proposed policy addresses safety issues pertaining to the host plant being modified genetically and introduced (Kessler 1992; Federal Register 1992). The proposed policy recommends that the biotechnology community had considerable favourable experience with a decision-free approach to evaluating transgenic crop safety (International Food Biotechnology Council 1990).

In developing countries in an effort to increase food production and control pest-borne diseases, the use of pesticides has increased very steadily and resulted in negative side effects on human health and the biotic environment. The people are in high risk of becoming intoxicated when using pesticides in agriculture and other food products with high levels of pesticide residues (Hamilton et al. 2004; Maroni et al. 2006). Moreover the farmers, who are handling such pesticides, have no knowledge about its proper use or the precautions needed to be taken (Jørs et al. 2006). Prolonged consumption and chronic accumulation of heavy metals in the kidney and liver cause disruption of biochemical processes, leading to cardiovascular, nervous, kidney and bone diseases (WHO 1992). Due to these chronic health problems such as respiratory diseases, dermatitis and neurological disorders are also reported (McCauley et al. 2006; Maroni et al. 2006). Increased genetic damage has been shown among pesticide users (Sailaja et al. 2006; Castillo-Cadena et al. 2006). Furthermore, it is of concern because it might lead to miscarriages, birth defects and cancer (Sharma et al. 2009), which are the additional economic and social adverse effect on such countries. Several pesticides were restricted in India due to their harmful effects on the socio-economical paradigm. In this regard, the following pesticides, viz. aluminium phosphide, captafol, cypermethrin, dazomet, diazinon, dichlorodiphenyltrichloroethane (DDT), fenitrothion, fenthion, methoxyethyl mercuric chloride (MEMC), methyl bromide, methyl parathion, monocrotophos, sodium cyanide and benzene hexachloride (BHC) are banned in India (www.cibre.nic.in).

The best alternatives to overcome such an unwilling, uneconomic, costly, unhealthy and environmentally hazardous situation can be explained by few examples:

1. A least-toxic pesticide is in the form of botanicals, essential oils or derived from other plants or natural mineral sources.
2. Integrated pest management (IPM) is another alternative programme of prevention, monitoring and control. IPM does this by utilising a variety of methods and techniques, including cultural, biological and structural strategies to control a multitude of pest problems.
3. The biocompost prepared from waste natural products utilising microorganisms and earthworms consume and breakdown natural matter into simpler compounds, which can be available to agro-beneficial microorganisms.
4. Organic farming system is primarily aimed at cultivating the land and raising food crops in such a way to keep the soil in active and in good health by the use of organic wastes (crop, animal and farm wastes, aquatic wastes) and other biological materials along with beneficial microbes (biofertilisers) to release nutrients to crops for increased sustainable production in an eco-friendly pollution-free environment.

As per the definition of the US Department of Agriculture (USDA) study team on organic farming, 'organic farming is a system which avoids or largely excludes the use of synthetic inputs (such as fertilisers, pesticides, hormones, feed additives, etc.) and to the maximum extent feasible rely upon crop rotations, crop residues, animal manures, vermicompost, green leaf manure and biological system of nutrient mobilisation and plant protection'. A wide range of microorganisms and naturally produced substances like plant derived botanicals or antibiotic secondary metabolites of microbial origin are classified as biopesticides. Biopesticides share one percent of the total world pesticide market, organisms and compound with biopesticidal activity are increasingly recognised as a valuable component to the plant protection system (Copping and Menn 2000). Although biopesticides have several disadvantages compared to chemical pesticides, e.g. inconsistent field performance, limited shelf life and possibly higher economic cost, these aspects can be overcome by the number of advantages, which results from the usage of biological pesticides: (1) strongly reduced activity or toxicity towards non-targeted microorganism; (2) an optimised pesticide's resistance management due to a broader range of applicability of pesticidal agent; (3) feasibility of combining conventional and biological means of disease control, thereby reducing the output of chemical pesticides; and (4) an easier and less expensive registration process for biopesticides (Copping and Menn 2000).

6.1.1 Basic Approaches for Using the Microbial Antagonists

Biological control agents of plant diseases are termed as 'antagonists'. An antagonist is a microorganism that adversely affects other microorganisms by utilising different modes of action, viz. mycoparasitism, antibiosis and competition. All these mechanisms may operate independently or together, and their activities can result in suppression of plant pathogens (Singh and Faull 1988).

The use of biological control agents (BCAs) has been considered a more natural and environmentally acceptable alternative to the existing chemical treatment methods (Baker and Paulitz 1996) and to chemical products (Alabouvette et al. 2006; Mondejar et al. 2011). Antagonistic microorganisms are generally present naturally in the environment and can be isolated by selective screening methods (often based upon specific enzymatic activities/bioassay/replica plating). Therefore, exogenous and genetically modified microorganisms need not to be introduced, and crop pest management can be achieved through the enhancement of naturally present organisms. This is a cheap and renewable alternative than the use of chemicals (Gerhardson 2002). Many soil microorganisms have been identified as potential biological control agents (Killham 1994), and soil fungi have shown great capacity to control crop pathogens through mechanisms such as antibiotic secretion, mycoparasitism or nutrient competition (Gams and Bissett 1998; Paulitz 2000; Punja and Utkhede 2003).

In present scenario various BCAs, viz. *Bacillus thuringiensis* subsp. *israelensis*, are reported to be effective against mosquito larvae and some midges (Federici 1999; Lepe and Ramírez-Suero 2012). Viruses most frequently considered in the control of insects (usually sawflies and Lepidoptera) are the occluded viruses, namely, NPV, cytoplasmic polyhedrosis (CPV), granulosis (GV) and entomopox viruses (EPN) (Narayanan 2004). Fungi are pathogenic agents to various organisms, including the pests and the weeds. This feature is intensively used in biocontrol strategy. The entomopathogenic fungi, like *Metarhizium anisopliae* and *Beauveria bassiana*, cause death to the host by the secretion of toxins (Rossoni et al. 2014). A biological control being developed for use in the treatment of plant disease is the fungus *Trichoderma viride*. Fungi widely produce toxic compounds with varied biochemical structure and mode of action; it includes polypeptides, glycoproteins, amino acid derivatives, terpenoids, sterols and quinones (Kono et al. 1981; Stoessl 1981). Due to an array of such metabolites, it comprises the commercial applications in production of enzymes and production of biopesticides for biological control of plant disease (Samuels 1996). The mechanisms that *Trichoderma* uses to antagonise phytopathogenic fungi include competition, colonisation, antibiosis and direct mycoparasitism (Hjeljord and Tronsomo 1988; Howell 2003).

6.1.2 *Trichoderma*: A Potential Microbial Antagonist

Trichoderma is a genus of asexually reproducing filamentous fungi widely distributed over the world (Domsch et al. 1980) and found in all soils, including forest humus layers (Wardle et al. 1993) and natural habitat, containing or consisting of organic matter (Papavizas 1985). They are also found on root surfaces of various plants and on decaying bark, especially when it is damaged by other's fungi (Caldwell 1958). Locally isolated potential strains of *Trichoderma harzianum* and *Trichoderma viride* have been shown in Fig. 6.1.

During the last 70 years, a vast number of *Trichoderma* isolates from a diverse habitat were screened for the antagonistic potential (Monte 2001). The extensive screening was carried out in vitro and promising species were tested later in vivo for their performance. Initially Weindling (1932) reported the antagonistic properties of *Trichoderma* spp. He showed the parasitism and production of antifungal antibiotics of *T. lignorum* against *Rhizoctonia solani*. In extension to his work Weindling and Emerson (1936) extracted secondary metabolites responsible for antagonistic activity from cultural filtrate. Since then genus *Trichoderma* is well known for the biopesticidal activity of a large number of strains from several species (Harman and Bjorkman

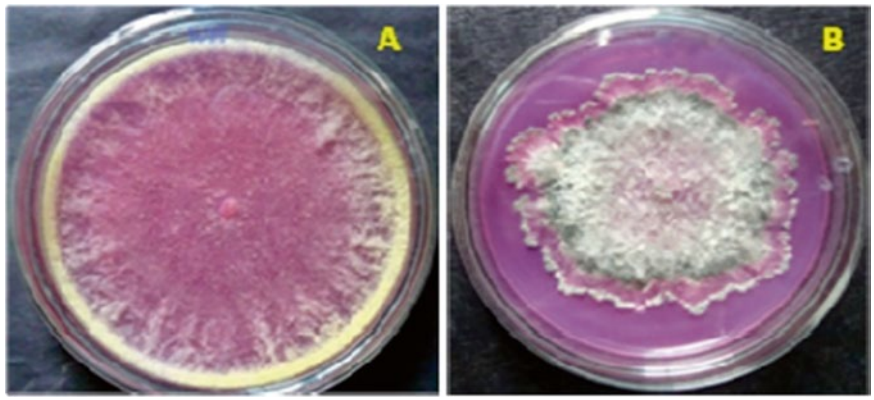


Fig. 6.1 Locally isolated potential *Trichoderma* species (**a**) *T. harzianum* (**b**) *T. viride* grown on rose bengal agar

1998), and the participation of secondary metabolites produced by these strains enhances their antagonistic potential (Sivasithamparam and Ghisalberti 1998; Szekeres et al. 2005).

Trichoderma species have been recognised for a long time as biological control agents (BCAs) for the control of plant disease and also for their ability to increase plant growth and development. They are widely used in agriculture and some of the most useful strains demonstrate a property known as 'rhizosphere competence', the ability to colonise and grow in association with plant roots (Harman 2000). The biology and applications of these fungi have been documented recently (Kubicek and Harman 1998; Harman and Kubicek 1998; Harman et al. 2004). The taxonomy of this fungal genus is being continuously revised and many new species are described (Overton et al. 2006; Samuels 2006; Komon-Zelazowska et al. 2007; Kubicek et al. 2008).

This antagonistic potential serves as the basis for effective biological control applications of different *Trichoderma* strains as an alternative method to chemicals for the control of a wide spectrum of plant pathogens (Chet 1987; Harman and Björkman 1998). Due to the ease of large-scale production, *Trichoderma* spp. have been developed into commercial products for biological control of numerous plant pathogens (John et al. 2008; Vinale et al. 2008a).

6.1.3 Coculture Techniques for Detecting Antagonistic Potential of *Trichoderma* spp.

Trichoderma is generally called as a secondary opportunistic invader, a fast-growing fungus, a strong spore producer, a source of cell-wall-degrading enzymes (CWDEs: cellulases, chitinases, glucanases) and an antibiotics producer (volatile and nonvolatile) (Vinale et al. 2008a). The mechanisms suggested to be involved in biocontrol by these fungi are antibiosis, mycoparasitism, lysis, competition and promotion of plant growth (Henis 1984; Papavizas 1985; Chet 1987; Baker 1988; Lynch 1990). It seems to assume that successful antagonism may rely on the combination of these modes of action (Ghisalberti and Sivasithamparam 1991).

Trichoderma spp. secrete a chemically diverse range of secondary metabolites, of which broad-spectrum antimicrobial properties have been demonstrated in in vitro assays. *Trichoderma* spp. produce both volatile and nonvolatile (diffusible) metabolites that adversely affect growth of different fungi (Dennis and Webster 1971a, b; Moss et al. 1975; Bruce et al. 1984; Corley et al. 1994; Horvarth et al. 1995).

The effect of the production of volatile organic compounds (VOCs) by *Trichoderma* isolates can be evaluated with the 'inverted plate technique' as described by Dennis and Webster (1971a, b). It has been reported that volatile metabolites also played a very important role in the control of pathogen (Dennis and Webster 1971a, b; Scarselletti and Faull 1994; Lopes et al. 2012; Patil and Lunge 2012; Keswani et al. 2014).

The diffusible metabolites can be assessed by various techniques such as confrontation test (also known as dual culture test) firstly introduced by Weindling (1932). The test is a comprehensive experiment that exhibits overall antagonistic potential of fungal biological control agent (Lopes et al. 2012; Patil and Lunge 2012; Pakdaman et al. 2013), competitive interaction by pathogen at centre technique (Rajput et al. 2013) and pathogen at periphery techniques (Asalmol and Awasthi 1990) as shown in Fig. 6.2.

The other modified techniques such as using the modified bilayer poison agar method (Rahman et al. 2009), direct assay method and slide interaction technique can be used to screen the antagonistic potential of *Trichoderma*. The differences between the two readings multiplied by 100 were taken as the percentages of inhibition of mycelial growth weight (PIWG) following the modified method of Skidmore and Dickinson (1976). Poisoned food technique (Nene and Thapliyal 1993) was followed to evaluate the effect of nonvolatile compounds/metabolites released by the *Trichoderma* spp. on the growth of pathogens (Nene and Thapliyal 1993).

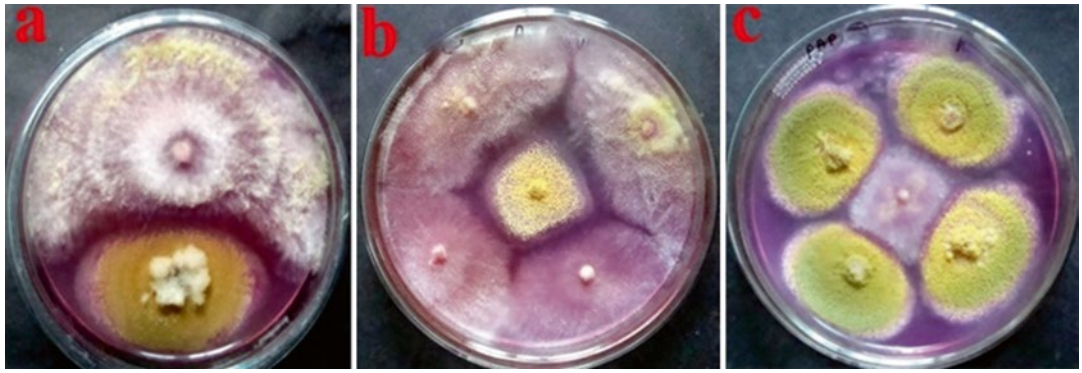


Fig. 6.2 Antagonism of *T. harzianum* against *Aspergillus flavus* isolated from rhizosphere of peanut (*Arachis hypogaea* L.) by three different methods: (**a**) dual culture technique, (**b**) pathogen at centre technique, (**c**) pathogen at periphery technique

6.1.4 Biocontrol Mechanisms of *Trichoderma* spp.

Nowadays various strains of *Trichoderma* are used as biocontrol agents owing to its broad-spectrum antagonistic activities against various soil-borne phytopathogens (Cook 1993; Jacobsen and Backman 1993; McSpadden Gardener and Fravel 2002; Singh et al. 2010, 2011). These mechanisms include mycoparasitism (Haran et al. 1996; Lorito et al. 1996) production of inhibitory compounds (Sivasithamparam and Ghisalberti 1998), competition for space and nutrients (Elad et al. 1999), inactivation of the pathogen's enzymes (Roco and Perez 2001) and induced resistance (Kapulnik and Chet 2000).

Antagonist potential of phytopathogenic fungi has been used to control agriculture-related plant diseases and 90 % of such applications have been carried out with different strains of the fungus *Trichoderma* (Mathivanan et al. 2000; Benitez et al. 2004). Various modes of action have been suggested for which include direct and indirect effects. Direct effects of the biocontrol agent over the pathogen include inhibition by secreting volatile and nonvolatile antimicrobial compounds (antibiosis), competition for colonisation sites and nutrients, degradation of pathogenicity factors and parasitism. A direct effect results in killing of a target pest, whereas indirect mechanisms effectively result into improvement of plant nutrition and damage compensation, changes in the root system anatomy, microbial changes in the rhizosphere and activation of plant defence mechanisms (Whipps 2001).

Trichoderma species antagonism is reflected in their capacity to secrete a spectrum of secondary metabolites (SMs), such as cell-wall-degrading enzymes, siderophores, chelating iron and volatile and nonvolatile metabolites (Reino et al. 2008; Vinale et al. 2008a; Druzhinina et al. 2011). SMs are not directly essential for growth yet have important roles in signalling, development, survival and interaction with other organisms (Osbourn 2010; Mukherjee et al. 2012a, b). *Trichoderma* spp. operate mainly through synchronisation between mycoparasitism and antibiosis against the fungal pathogens (Di Pietro et al. 1993; Schirmbock et al. 1994; Mumpuni et al. 1998; Vinale et al. 2008a). *Trichoderma* spp. constitutively secrete a variety of lytic enzymes to detect the presence of competent fungi by sensing their degraded cell wall components (Harman et al. 2004; Woo and Lorito 2007; Vinale et al. 2008a). Such mode of interaction was not reported in absence of a potential competitor. The major modes of *Trichoderma* action include:

6.1.4.1 Mycoparasitism

Direct confrontation with mycopathogens, secretion of cell-wall-degrading enzymes with lytic activity (Lorito et al. 1996; Lorito 1998), subsequent penetration and killing are the key

features of this mode (Ayers and Adams 1981; Chet et al. 1998; Woo and Lorito 2007). Generally the mycoparasitism can be regarded as the first-hand attack of one fungus on another and can be generally defined as straight antagonism (Dix and Webster 1995), which can be divided into four sequential steps. Chemotrophic growth is the first step, in which the secretion of a chemical stimulus by the target fungus attracts an antagonist fungus (Steyaert et al. 2003). Specific recognition is the second step, in which the antagonist fungus identifies the cell surface of the pathogen, whereas in the third step two distinct processes are involved: (1) coiling, where the *Trichoderma* hyphae surround its host, and (2) intimate hyphal interaction and contact where the *Trichoderma* hyphae simply grow along the host's hyphae. The last step involves the secretion of specific lytic enzymes, viz. β-glucanase, chitinase and proteinases, which degrade the host cell wall (Chet et al. 1998).

Trichoderma spp. were demonstrated to be very efficient producers of extracellular enzymes, with cellulases as the first example (Mandels 1975). Secretion of lytic enzymes has a major impact on the biological control potential of *Trichoderma* species (Viterbo et al. 2002; Mukherjee 2011). This was demonstrated with a mutant strain of *T. harzianum* where higher chitinase, β-1,3-glucanase and β-1,6-glucanase activity was expressed compared with the wild type (Rey et al. 2001). Synergistic action of lytic enzymes and antibiotics is another important factor that can enhance the ability of *Trichoderma* species to inhibit plant pathogens (Steyaert et al. 2003).

The complex mycoparasitic process has been extensively reviewed (Herrera-Estrella and Chet 1998). The final step is penetration of the host mycelium, which is enabled by partial degradation of its cell wall via secretion of mycolytic enzymes, mainly chitinases and glucanases (Viterbo et al. 2002). The roles of each protein in an enzymatic complex of *Trichoderma* appear to be different, and enzymes with different or complementary modes of action appear to be required for maximal antifungal effect on different pathogens (Lorito et al. 1993, 1994). Various enzymes, viz. chitinases-chitin-degrading enzymes (Sahai and Manocha 1993), N-acetylglucosaminidases (Lorito et al. 1994), chitobiosidases (Harman et al. 1993), endochitinases (Carsolio et al. 1994; Garcia et al. 1994; Lorito et al. 1998), glucanases (Thrane et al. 2001) and proteases (Delgado-Jarana et al. 2002; Antal et al. 2000), have shown their potential in controlling the plant pathogens.

Trichoderma genomes abound in genes for chitinases and glucanases and their role in mycoparasitism and biocontrol is well documented. In *Trichoderma* spp., particularly, *T. virens* and *T. atroviride*, contain the highest number of genes for chitinolytic enzymes. The two mycoparasitic strains of *Trichoderma* have expanded their secondary metabolism arsenal, harbouring more genes than *T. reesei*. For example, the *T. virens* genome contains 28 non-ribosomal peptide synthetases (including the gene for strongly antifungal compound gliotoxin), compared to 16 for *T. atroviride* and 10 for *T. reesei*. Many of the secondary metabolism gene clusters are unique to specific *Trichoderma* species whereas some are common in all the three species (Mukherjee 2011). The enzymes involved and their mode of action of selected *Trichoderma* spp. had been compiled in Table 6.1.

Lytic enzymes are essentially significant in mycoparasitism (Viterbo et al. 2002) which has been demonstrated by overexpression and deletion of the respective genes. During mycoparasitism, the transduction pathways of *T. atroviride* have led to the isolation of key components of the cAMP and MAP kinase signalling pathways, such as a-subunits of G proteins (G-a), which control extracellular enzyme, antibiotic production and coiling around host hypha (McIntyre et al. 2004). In *Trichoderma*, there is biochemical evidence for the participation of G-a in coiling, since an increase in coiling around nylon fibres was detected after the addition of activators of G-protein (mastoparan and fluoroaluminate) (Omero et al. 1999). G-a gene (tga1) has been expressed either under the control of its own promoter or under the control of the promoter of the basic proteinase *prb1* in *T. atroviride* (Rocha-Ramírez et al. 2002). The demonstration in Fig. 6.3 represents the *Trichoderma* hyphal interaction with the pathogen *Aspergillus* species, in

Table 6.1 Mechanism of antagonistic molecules produced by *Trichoderma* spp. against their target microorganisms

Trichoderma species	Antagonistic molecules	Mechanism of action	Target fungi	References
T. viride, *T. harzianum*	Hydrolases	Degradation of aflatoxin B1 (AFB1); ochratoxin A (OTA)	*Corynebacterium rubrum*, *Aspergillus niger*	Mann and Rehm (1976)
T. harzianum		Inhibition of sclerotia and rhizomorphs, inhibition of growth	*Sclerotium rolfsii*, *Phytophthora parasitica* f. *nicotianae*, *Ceratobasidium cornigerum*, *Pythium aphanidermatum*, *P. myriotylum*	Bell et al. (1982)
T. harzianum	Proteases	Inhibition of hydrolytic enzymes	*B. cinerea*	Elad (1996) and Elad and Kapat (1999)
T. polysporum, *T. viride*, *T. harzianum*	Amylase	Inhibition of growth and sporulation	*Cladosporium herbarum*	Barbosa et al. (2001)
T. harzianum	Endo-chitinase	Degradation of cell wall	*Alternaria alternata*	Roco and Perez (2001)
Trichoderma atroviride (T-15603.1)		Inhibition of growth and germination	*Ganoderma adspersum*, *Ganoderma lipsiense*, *Inonotus hispidus*, *Polyporus squamosus*	Schubert et al. (2008)
Trichoderma harzianum and *Trichoderma viride*	Chitinases and cellulases	Inhibition of mycelial growth	*Sclerotium rolfsii*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*	Joshi et al. (2010)
T. konigiopsis (*M32*, *M33*), *T. neokoningii* (*M6*), *T. harzianum*, *T. gamsii* (*M11*)	Chitinase, β-1, 3 glucanase	Inhibition of the mycelial growth and formation of sclerotial bodies	*S. rolfsii*	Ratnakumari et al. (2011)
T. viride, *T. harzianum*	Chitinase, β-1, 3 glucanase	Degradation of cell wall	*R. solani*, *S. rolfsii*	Kumar et al. (2012)
T. koningii, *T. harzianum*, *T. hamatum*, *T. viride*	Chitinase, β-1, 3 glucanase and protease	Degradation of cell wall	*Macrophomina phaseolina*	Gajera et al. (2012)
T. viride, *T. koningii and T. viridescens*		Inhibition of growth and germination	*Heterobasidion annosum*, *Heterobasidion parviporum*	Nikolajeva et al. 2012
Trichoderma asperellum TKD		Inhibition of aflatoxin biosynthesis	*Aspergillus flavus*	Darmayasa et al. (2014)
T. virens, *T. hamatum*	Viridiol	Inhibition of aflatoxin biosynthesis	*Aspergillus* spp.	Sakuno et al. (2000) and Wipf and Kerekes (2003)
Unidentified ascomycete	Flaviolin	Conidium germination inhibitor	*Magnaporthe grisea*	Thines et al. (2004)

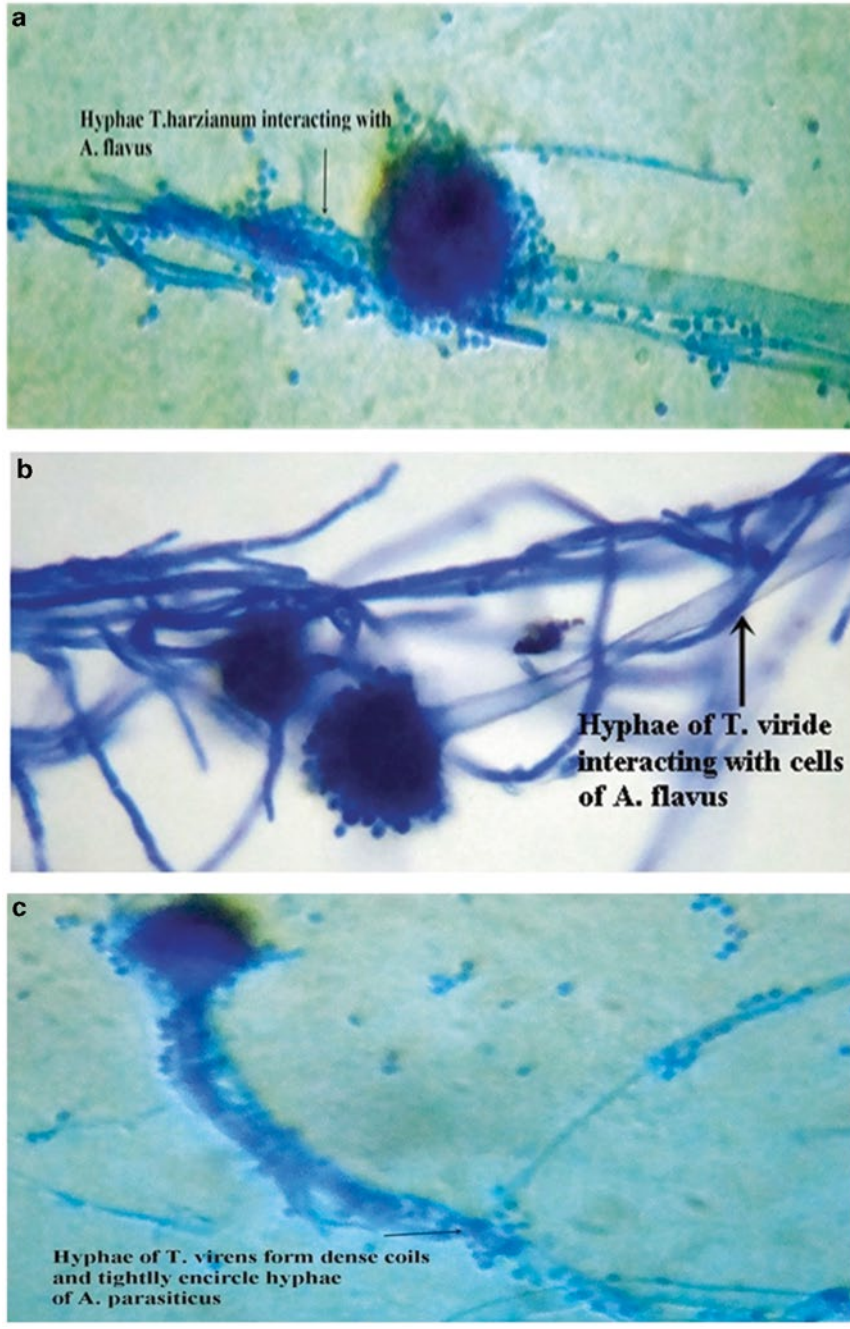


Fig. 6.3 Mycoparasitic interaction of *Trichoderma* species: (**a**) *T. harzianum* haustoria formation and penetration within the hyphae of *A. flavus*, (**b**) *T. viride* hyphal coiling and direct interaction with *A. flavus*, (**c**) *Trichoderma virens* established close contact with the pathogen by dense mycelium appearing to tightly encircle the hyphae of the pathogen

which hyphae entangle the pathogen mycelia and do not allow to grow further; this is a basic mechanism during mycoparasitism exhibited by *Trichoderma* spp.

During last decays genetic modification for the development of improved strains producing higher quantity of lytic enzymes had been reported (Mandel et al. 1971). Strain improvement for higher lytic enzyme production by UV irradiation (Faull et al. 1994; Graeme-Cook and Faull 1991; Rajappan et al. 1996; Singh et al. 2010; Patil and Lunge 2012; Shahbazi et al. 2014), EMS or physical mutagens, e.g. gamma rays (Youssef et al. 1999; Kredics et al. 2001; Afify et al. 2013) was reported. The salt-tolerant mutants were induced and isolated according to the methods of Gadgil et al. (1995), Migheli et al. (1998), Rey et al. (2000)and Mohamed-Benkada et al. (2006) with some modifications.

6.1.4.2 Antibiosis

Antibiosis refers to the production of antibiotics by fungi which had gained tremendous importance in the case of the *Trichoderma* genus. This type of interaction is generally defined as indirect antagonism since no hyphal contact between host and parasite is required (Dix and Webster 1995). Antibiosis generally occurs in synergy with mycoparasitism (Schirmbock et al. 1994), where the hydrolytic enzymes allow the antibiotics to penetrate the host cells. In turn, antibiotics can inhibit cell wall synthesis and, therefore, enhance the action of the hydrolytic enzymes (Lorito et al. 1996; Barakat et al. 2014).

Dennis and Webster (1971b) found that some *Trichoderma* isolates produced volatile components with a characteristic smell, in which acetaldehyde was identified as one of the tentative metabolites, which were inhibitory to the growth of other fungi. Dennis and Webster (1971a) reported production of nonvolatile antibiotics by the agar layer technique, viz. trichodermin and peptide antibiotic active against a range of fungi. It has been reported that *Trichoderma* spp. produce various types of SMs (natural products) and structures of more than 100 compounds (Reino et al. 2008); these include low-molecular-mass non-polar compounds such as pyrones, terpenoids, steroids and polyketides. *Trichoderma* spp. produce non-ribosomal peptides such as polythiodioxopiperazines (ETPs) and siderophores. Members of the genus are prominent producers of a subgroup of peptaibiotics known as peptaibols (short peptides of non-ribosomal origin characterised by the presence of high levels of non-standard amino acids).

Antibiotic production reported to be a species-specific phenomenon which affects the target pathogen by inhibition of growth, production of primary metabolites, uptake of nutrients and sporulation (Howell 1998; Ghisalberti et al. 1990; Howell et al. 1993). The production of secondary metabolites, which are natural compounds that aid the producing organism in survival and basic functions such as symbiosis, competition and differentiation (Shwab and Keller 2008) has been confirmed. The production of antibiotic secondary metabolites is often correlated to the biocontrol activity of *Trichoderma* strains (Ghisalberti et al. 1990; Worasatit et al. 1994; Vinale et al. 2006).

The *Trichoderma* secretes secondary metabolites having antimicrobial properties (Ghisalberti and Sivasithamparam 1991; Sivasithamparam and Ghisalberti 1998; Mathivanan et al. 2008). They produce volatile (e.g. ethylene, hydrogen cyanide, alcohols, aldehydes and ketones up to C4 chain length) and nonvolatile secondary metabolites (e.g. peptides) (Keszler et al. 2000). *Trichoderma* species seem to be an inexhaustible source of antibiotics, from the acetaldehyde's gliotoxin and viridin (Dennis and Webster 1971a, b) to alpha-pyrones (Keszler et al. 2000), terpenes, polyketides, isocyanide derivatives, piperacines and complex families of peptaibols (Sivasithamparam and Ghisalberti 1998). All these compounds produce synergistic effects in combination with CWDEs, with strong inhibitory activity to many fungal plant pathogens (Schirmböck et al. 1994; Lorito et al. 1996). *Trichoderma* isolates were found to produce fungal inhibitory volatile components proved to be dependent on the content of the growth medium (Wheatley et al. 1997; Polizzi et al. 2002).

Various reports on antibiotic production had been reported for several *Trichoderma* spp.

Trichodermin as sesquiterpenoid metabolites (Godtfredsen and Vangedak 1965; Dennis and Webster 1971b; Fedorinchik et al. 1975) and viridin as antibiotic substances (Weindling and Emerson 1936; Sivasithamparam and Ghisalberti 1998) inhibit fungi at low concentration. Ergokonin (Kumeda et al. 1994), viridin (Grove et al. 1996; Brian and McGowan 1945) and viridian fungin A, B and C (Harris et al. 1985) reported to be involved in the biocontrol of pathogens.

Among the volatile antifungal compounds produced by *Trichoderma* strains, the most important and well documented is 6-n-pentyl-2H-pyran-2-one (6-PAP), a polyketide with a characteristic sweet coconut-like aroma. 6-PAP and other α-pyrone analogues have been detected in cultures of several *Trichoderma* strains (Collins and Halim 1972; Claydon et al. 1987; Bonnarme et al. 1997; Simon et al.1988; Reithner et al. 2005, 2007) reported to have a role in plant growth regulation and activation of plant defence responses (Vinale et al. 2008a; El-Hassan and Buchennauer 2009). Volatile metabolites, 2- pentanone, α-pinene, β-pinene, p-xylene, 2-heptanol, ethyl octanoate, ethyl decanoate, methyl benzoate, α-curcumene and β-farnesene, were reported to be produced by using solid-phase microextraction (SPME) (Jeleń et al. 2014). The volatile metabolites, viz. 2-methoxy-1,3-dioxolane, methyl acetate, 2-*n*-heptyl-8-hydroxy-2*H*-1-benzopyran-5-one and 5,5-dimethyl-2*H*-pyran-2-one, were reported to be present in *T. atroviride* strain 11 (Keszler et al. 2000). Similarly 1-pentanol, 3-ethyl-5-methylphenol, 2-pentanone, 2-hexanone, cyclohept-3-en-1-one, geranyl acetone, methyl benzoate, α-pinene and β-pinene have been previously reported to be produced by *Trichoderma* species (Korpi et al. 2009; Polizzi et al. 2012; Müller et al. 2013).

Trichorzianines and a number of closely related peptaibols are produced by several *Trichoderma* spp. (Hajji et al. 1989; Ghisalberti and Sivasithamparam 1991); these antibiotics form voltage-gated ion channels in black lipid membranes (Molle et al. 1987) and modify the membrane permeability of liposomes in the absence of applied voltage (Hajji et al. 1989; Doan et al. 1986). Peptaibols induce leakage of compounds from *Rhizoctonia solani* and lysis of *Phytophthora cactorum* (Hajji et al. 1989).

Determination of volatile fungal metabolites usually is done by gas chromatography (GC). The known antagonistic growth system will be adapted for cultivation of the fungi in liquid (Pinches and Apps 2007) or solid growth media (Nemc ovic and Farkas 2008). The volatile metabolites can be extracted with organic solvents (Zeppa et al. 1990), solid-phase extraction using C18 or silica gel columns (Keszler et al. 2000), online gas enrichment on adsorption tubes (Wheatley et al. 1997) or various headspace techniques such as closed-loop stripping analysis (Meruva et al. 2004), dynamic headspace (purge and trap) (Deetae et al. 2007) and solid-phase microextraction (Van Lancker et al. 2008). Selected *Trichoderma* antibiotic metabolites with the target organisms were presented in Table 6.2. The chemical structures of major metabolites were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/), ChemSpider (http://www.chemspider.com/) and PDB (http://www.rcsb.org/pdb) database, visualised in MarvinView and presented in Figs. 6.4, 6.5 and 6.6.

The detection of GC-separated volatile compounds can be performed by flame ionisation detection (FID) (Elke et al. 1999; Siddiquee et al. 2012) mass spectrometry (MS), which is widely used (Hynes et al. 2007). Mass spectrometric detection offers the possibility of identifying individual volatiles from complex mixtures. Structure characterisation and confirmation of identity is usually achieved by comparing mass spectra with library spectra and determining chromatographic retention indices (Jelen 2003).

Based on the idea that the evolution of SM production was indeed driven by competition between species, many silent biosynthetic clusters could be activated by competition or by conditions that simulate the normal route of activation (Osbourn 2010; Brakhage and Schroeckh 2011). The production of SMs by the *Trichoderma* spp. is strain dependent and includes antifungal substances belonging to different classes of chemical compounds. These compounds have been classified by Ghisalberti and Sivasithamparam (1991) into three main categories:

Table 6.2 Toxic secondary metabolites (antibiotics) produced by *Trichoderma* spp. against their target microorganisms

Class	*Trichoderma* species	Toxic metabolites	Target organisms	References
Pyrones	*T. viride*, *T. atroviride*, *T. harzianum*, *T. koningii*	6-pentyl-2H-pyran-2-one (6-pentyl-α-pyrone) (**1**), cytosporone S (**2**)	*Fusarium moniliforme*	Scarselletti et al. (1994), Worasatit et al. (1994), and El-Hassan et al. (2007)
Lactones	*T. cerinum*	Cerinolactone	*P. ultimum*, *R. solani* and *B. cinerea*	Vinale et al. (2012)
Koninginins	*T. harzianum*, *T. koningii* and *T. aureoviride*	Koninginins A (**3**), B (**4**), D (**5**), E (**6**) and G (**7**)	*Gaeumannomyces graminis* var. *tritici*	Almassi et al. (1991) and Ghisalberti et al. (1993)
		Koninginin D (**5**)	*Rhizoctonia solani*, *Phytophthora cinnamomi*, *Pythium middletonii*, *Fusarium oxysporum* and *Bipolaris sorokiniana*	Dunlop et al. (1989)
Viridins	*T. koningii*, *T. viride*, *T. virens*	Viridin (**8**)	*Botrytis allii*, *Colletotrichum lini*, *Fusarium caeruleum*, *Penicillium expansum*, *Aspergillus niger* and *Stachybotrys atra*	Reino et al. (2008) and Brian and McGowan (1945)
	T. viride, *T. hamatum*	Viridiol (**9**)	*R. solani*	Moffatt et al. (1969) and Howell and Stipanovic (1994)
Heterocyclic compounds	*T. harzianum*	Harzianopyridone (**10**)	*Botrytis cinerea* and *R. solani*; *G. graminis* var. *tritici* and *Pythium ultimum*	Dickinson et al. (1989) and Vinale et al. (2006)
	T. harzianum	Harzianic acid (**11**)	*Pythium irregulare*, *Sclerotinia sclerotiorum* and *R. solani*	Vinale et al. (2009a)
Azapilones	*T. harzianum* T22	T22 azaphilone (**12**)	*R. solani*, *P. ultimum* and *G. graminis* var. *tritici*	Vinale et al. (2006)
Butenolides	*T. harzianum*	Harzianolide (**13-A**) T39 butenolide (13-B) and dehydro-harzianolide (13-C)	*P. ultimum*, *R. solani* and *B. cinerea*, *Gaeumannomyces graminis* var. *tritici*	Almassi et al. (1991), Vinale et al. (2006), Claydon et al. (1991), and Ordentlich et al. (1992)

Isocyano metabolites	*T. viride*, *T. koningii*, *T. hamatum*	Dermadin (**14**)	*Staphylococcus* species, *E. coli*	Pyke and Dietz (1966), Meyer (1966), Coats et al. (1971), Tamura et al. (1975), and Fujiwara et al. (1982)
	T. koningii, *T. viride*	Isonitrile trichoviridin (**15**), isonitrin A (**16**), isonitrin B (**17**)	*Staphylococcus* species, *E. coli*	Tamura et al. (1975)
	T. koningii	Homothallin I, homothallin II (**18**)	*Phytophthora* spp.	Pratt et al. (1972) and Edenborough and Herbert (1988)
	T. harzianum	Homothallin II (**18**)	*Phytophthora* spp.	Faull et al. (1994)
Diketopiperazines	*Trichoderma* (*Gliocladium*) *virens*	Gliotoxin (**19**)	*R. solani*	Howell (1991)
		Gliovirin (**20**)	*P. ultimum*	
Peptaibols	*Trichoderma harzianum*	Atroviridins, trichotoxins (**21**), alamethicins (**22**)	*Aspergillus fumigatus*, *A. niger*, and *Penicillium chrysogenum*	Daniel and Filho (2007), Lorito et al. (1996), and Fuji et al. (1978)
	T. harzianum	Trichorzins, trichokinidins, harzianins	Amoeba *Dictyostelium*	Hajji et al. (1987), Rebufatt et al. (1992), Goulard et al. (1995), Duval et al. (1998), and Leclerc et al. (1998)
	T. viride	Trichotoxin, paracelsin (**23**)	*S. aureus*, *E. coli*, *A. salina*	Kleinkauf and Rindfleisch (1975), Brewer et al. (1987), Irmscher and Jung (1977), Bruckner and Graf (1983), and Solfrizzo et al. (1994)
Miscellaneous metabolites observed	*Trichoderma viride*, *T. virens*, *T. harzianum*	6-nonylene alcohol, ketotriol (**24**), 3-methyl-heptadecanol (**25**), 2-methyl-heptadecanol	*Rhizoctonia bataticola*, *Fusarium oxysporum* f. sp. *ciceris*	Dubey et al. (2011)
		Massoilactone (**26**)		
	T. polysporum, *T. koningii*, *T. viride* and *T. harzianum*	Trichodermin (**27**), trichodermol (**28**), harzianum A (**29**)	*Cladosporium* species	Dickinson et al. (1995)
	T. harzianum, *T. longibrachiatum*	2-Phenylethanol, tyrosol (**30**), sorbicillin (**31**)	*Enterobacter dissolvens*, *Paecilomyces variotii*, *Armillaria mellea*,	Tarus et al. (2003)
	T. harzianum 25, *T. viride*	Anthraquinones	*Serpula lacrymans*	Score and Palfreyman (1994)

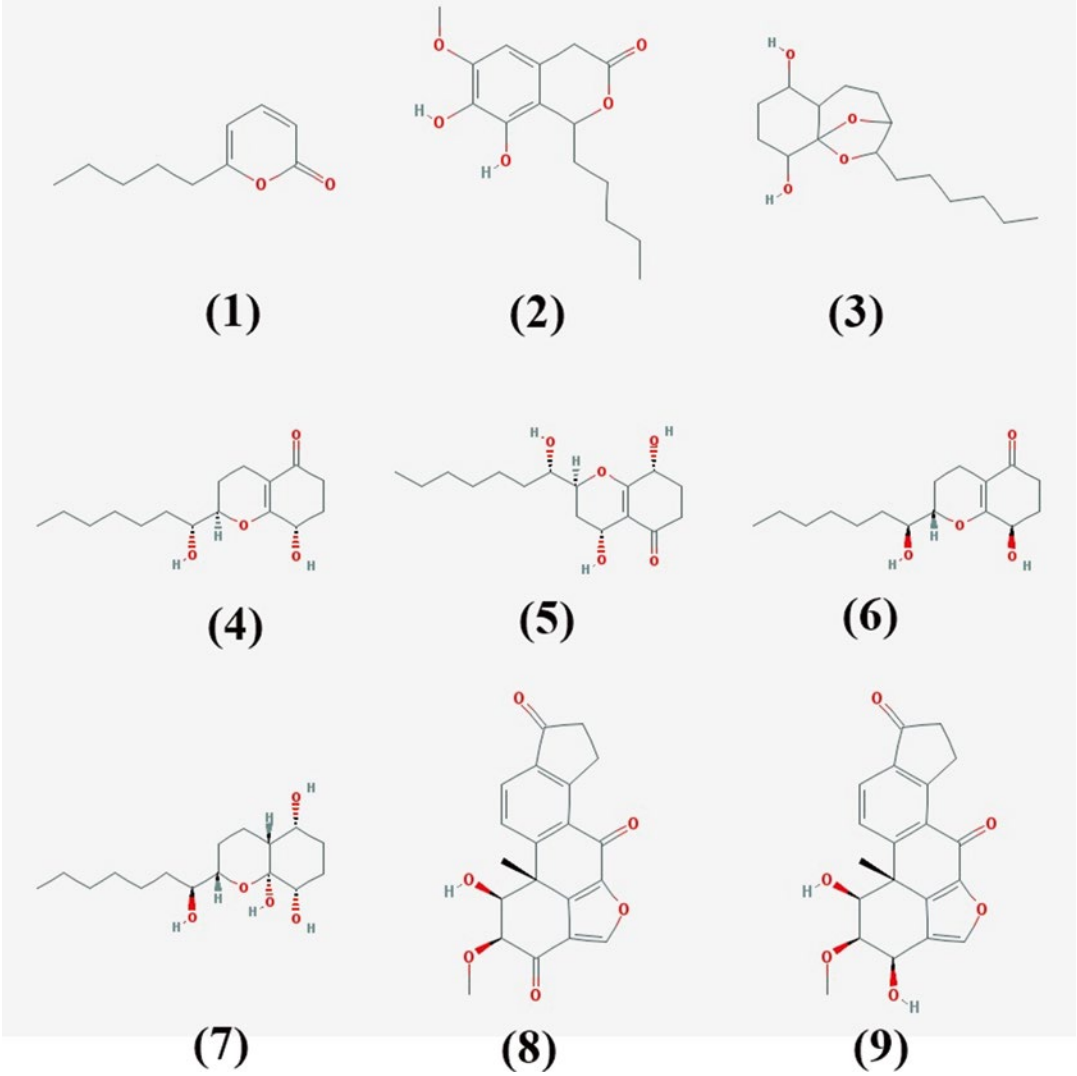


Fig. 6.4 Chemical structures of 6-pentyl-2H-pyran-2-one (*1*), cytosporone S (*2*), koninginins A (*3*), B (*4*), D (*5*), E (*6*) and G (*7*), viridin (*8*), viridiol (*9*)

1. Volatile antibiotics
2. Compounds soluble in water
3. Peptaibols

The different chemical structure of these substances suggests different mechanisms of action. The production of molecules of low molecular weight, non-polar and volatile (simple aromatic compounds, pyrones, butenolides, etc.) determines the presence of high concentrations of antibiotics in soil ranging influence on the microbial community even at a long distance. In contrast, the short distance may be associated with the production of antibiotics and polar peptaibols acting in the vicinity of the hyphae. Polar metabolites of high molecular weight could express their activity as a result of physical contact with the pathogen. As regards the peptaibols, given their amphiphilic nature, they associated with properties like detergents. They influence the permeability properties of phospholipid bilayer. Furthermore, it has been shown that the peptaibols inhibit the action of the enzyme β-glucan synthase and the enzyme chitin synthase of the

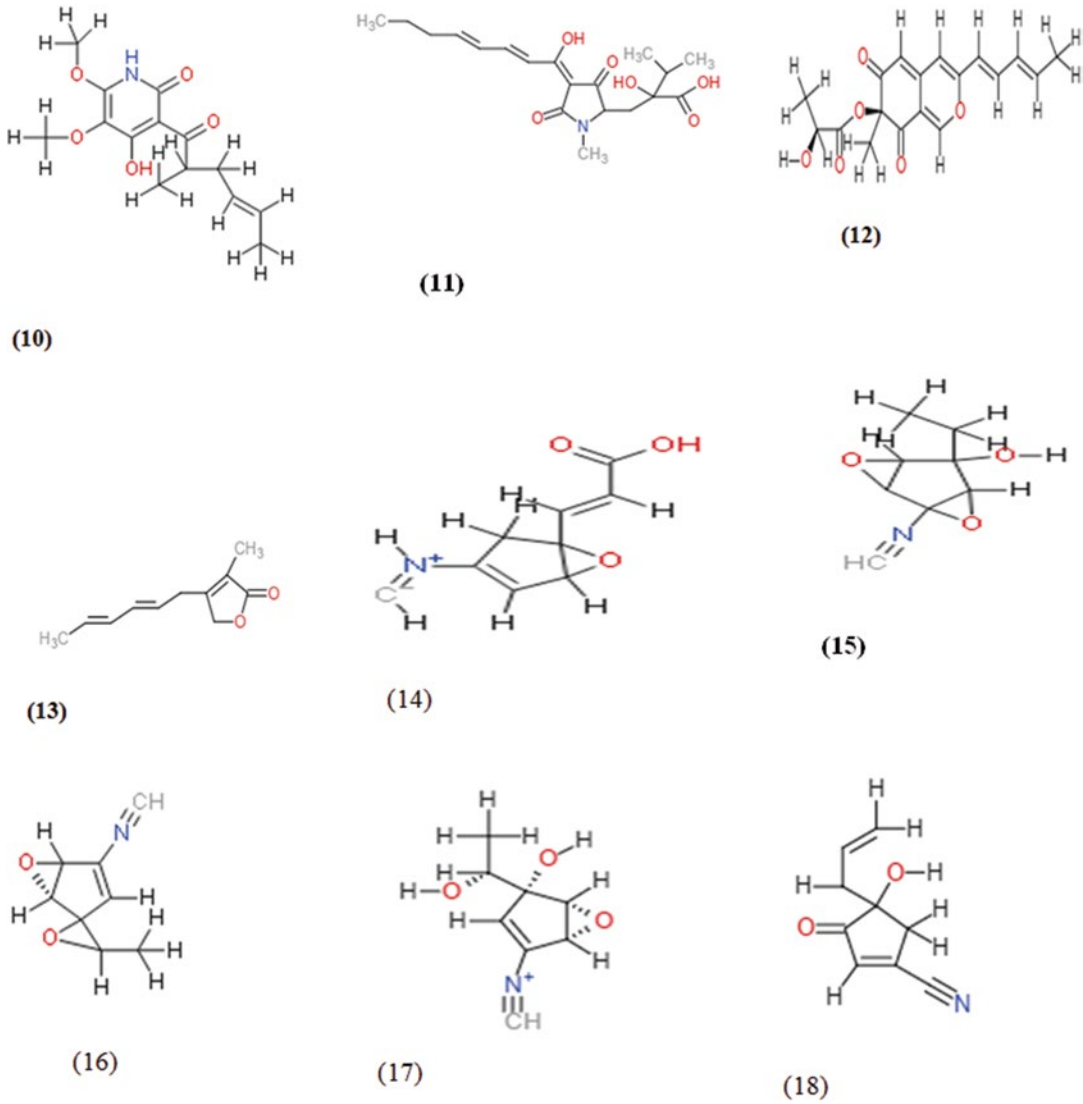


Fig. 6.5 Chemical structures of harzianopyridone (*10*), harzianic acid (*11*), T22 azaphilone (*12*), harzianolide (*13-A*) (R- $-CH_2CHOHCH_3$), T39 butenolide (*13-B*) (R- $-CH_2CHOHCH_3$), dehydro-harzianolide (*13-C*) (R- $-CHCHCH_3$)

fungus host, preventing the reconstruction of the cell wall of the pathogen and facilitating, at the same time, the destructive action of the chitinase.

Trichoderma strains seem to be an inexhaustible source of bioactive molecules (Sivasithamparam and Ghisalberti 1998). Some of these compounds produce synergistic effects in combination with CWDEs, with strong inhibitory activity on many fungal plant pathogens (Lorito et al. 1996; Schirmböck et al. 1994). The potential of genes involved in biosynthetic pathways of antibiotics, e.g. polyketides (Sherman 2002) and peptaibols (Wiest et al. 2002) with application in medicine still not explored. Based on the chemical properties, the *Trichoderma* secondary metabolites are classified into the following main categories.

Polyketides The microorganisms produce a number of secondary metabolites having the role of self-defence, aggregation, communication, competition, etc. The polyketides (PKs) also

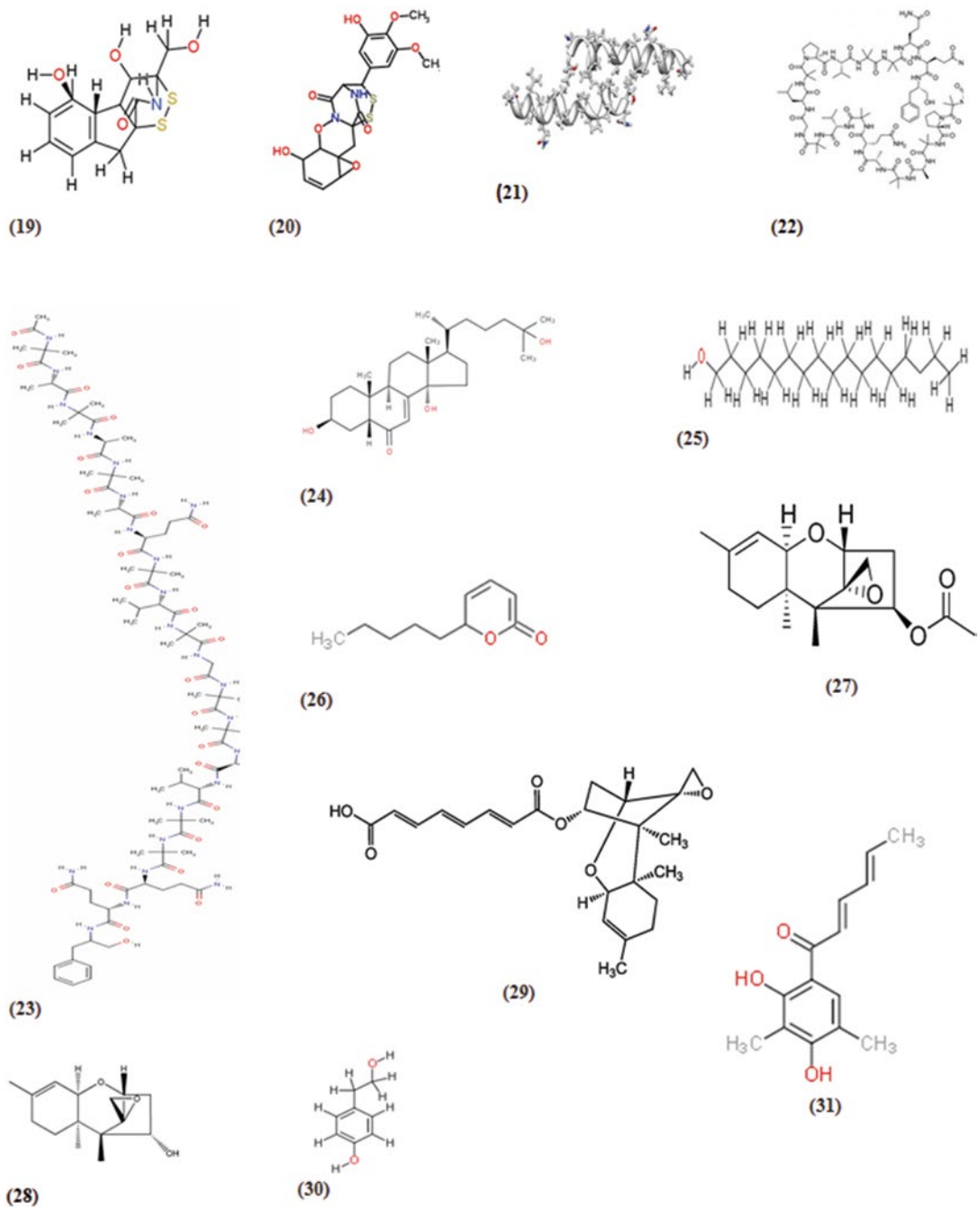


Fig. 6.6 Chemical structures of gliotoxin (*19*), gliovirin (*20*), trichotoxins (*21*), alamethicins (*22*), paracelsin (*23*), ketotriol (*24*), 3-methyl-heptadecanol (*25*), massoilactone (*26*), trichodermin (*27*), trichodermol (*28*), harzianum A (*29*), tyrosol (*30*), sorbicillin (*31*)

belong to the secondary metabolites group, with diverse structure and function variability. The major role of polyketides was observed in pharmacological and agricultural applications as reported to show antibacterial, antifungal, antiparasitic and antitumor activities. The source for polyketides varies from bacteria, fungi, plants, insects to marine organisms. The well-known PKs with potent biological activities are erythromycin-A from *Streptomyces erythreus*

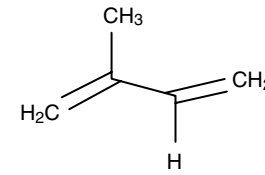
(US2653899 (A)), a broad-spectrum antibiotic; avermectin, an anthelmintic agent isolated from *Streptomyces avermitilis*; a soil actinomycete; and FK506, an immune suppression agent produced by *Streptomyces* species (Tanaka et al. 1987).

In agriculture *Trichoderma* spp. are the major producers of the PKs for beneficial purposes. The examples of PKs are trichoharzins (Kobayashi et al. 1993); trichodimerols (Zhang et al. 2014); trichodermatides A, B, C and D (Sun et al. 2008); koninginins A, B, D, E and G (Almassi et al. 1991; Ghisalberti 1993); and koninginins L and M (Lang et al. 2015). The polyketides produced by *Trichoderma* spp. are toxic to the plant pathogens such as *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia solani*, *Phytophthora cinnamomi*, *Pythium middletonii*, *Fusarium oxysporum*, *Bipolaris sorokiniana*, etc. The coculture of *Aspergillus nidulans* with actinomycetes that share the same niche triggers expression of fungal polyketide biosynthesis genes (Schroeckh et al. 2009).

The biosynthesis of polyketides takes place by repetitive joining of short-chain fatty acids, e.g. acetate or propionate by pathways similar to fatty acid biosynthesis. The reaction was mainly catalysed by the polyketide synthases enzyme (PKSs). The PKSs are multifunctional enzymes consisting of several active sites, with capacity of fusion of variable numbers of coenzyme A (CoA)-linked acyl monomers, such as acetyl-CoA and malonyl-CoA, into polymers known as polyketides (Kroken et al. 2003).

Terpenoids Terpenes are one of the important classes of secondary metabolites with a large number of active compounds. The terpenes are the natural hydrocarbons based on isoprene units. The terpenoids are similar to the terpenes, only they are differed by having additional functional groups. The term terpenes can be used interchangeably with terpenoids. The important biological activity of terpenes was observed in the defence system of plants or fungi. The terpenes are strong-smelling compounds. The terpenoids are the group of molecules based on various but definite number of isoprene units. Isoprene units are nothing but the methylbuta-1, 3-diene with five carbon atoms.

Isoprene unit



The terpenes can be categorised into different categories based on the number of isoprene units incorporated in the basic molecular skeleton. The terpenes with two isoprene units are called as monoterpenes. Similarly the categories evolved as sequiterpenes, diterpenes, sesteterpenes, triterpenes, carotenoids, rubber, etc.

The *Trichoderma* spp. showed a wide range of terpenoids with potent biological activates. The *T. koningii* and *T. harzianum* both showed presence of cyclonerodiol, a sesquiterpene with plant growth-promoting activity (Cutler et al. 1991; Ghisalberti and Rowland 1993). Trichodermic acids A and B were isolated from *T. virens* (Yamaguchi et al. 2010) responsible for anticancer activity. Two terpenoids, harzianone and trichoacorenol, were reported from *Trichoderma* spp. from mangrove plant *Xylocarpus granatum* (Zhang et al. 2014). The monoterpene compounds are not detected in *Trichoderma* spp. The volatile nature may be the reason, but detection of other volatile compounds such as 6-PP suggests that the monoterpenes may be produced in very small amounts (Sivasithamparam and Ghisalberti 1998).

Harziandione, a diterpene, was isolated from *T. harzianum*; although it lacks the antifungal activity, a similar compound was isolated from *T. viride* with antifungal potential, and the structures were identical to harziandione (Sivasithamparam and Ghisalberti 1998). *Trichoderma* spp. showed presence of a number of triterpenes and sterols as *T. pseudokoningii* produced lanostadiol, ergosterol, pyrocalciferol, etc. (Kamal et al. 1971). Viridiol is a steroid similar to viridin isolated from *T. virens* (formerly

known as *Gliocladium virens*). It shows plant growth inhibitory activity (Howell and Stipanovic 1994). Viridiol was also produced by *T. hamatum* which reduced the aflatoxin production in plant pathogens (Sakuno et al. 2000; Aloj et al. 2009).

The biosynthesis of terpenoids was proposed, as the origin is from mevalonic acid via isopentyl and dimethylallyl diphosphate as intermediates, and these two combine to produce a monoterpene, i.e. geranyl diphosphate. The further addition of isopentyl diphosphate units produces farnesyl (sesquterpene), geranylgeranyl diphosphate (diterpenes) and the chain may combine head to head of farnesyl diphosphate to produce squalane, a triterpene with 30 carbon atoms.

Pyrones The pyrones are the cyclic compounds containing unsaturated six-membered rings with one oxygen atom and a ketone functional group. The pyrones are observed in two isomeric forms 2-pyrones (α-Pyrone) and 4-Pyrone (γ-Pyrone).

The α-Pyrone is found to be abundant in nature. It has been isolated from natural sources such as fungi, plants, animals, marine organisms, bacteria and insects that show a wide range of biological activities, such as antifungal, antibiotic, cytotoxic, neurotoxic, phytotoxic, etc. (McGlacken and Fairlamb 2005).

The *Trichoderma* spp. showed a broad range of pyrones as their biological weapons to combat pathogens. The 6-pentyl-α-pyrone was a first volatile antifungal compound isolated from *T. viride* (Collins and Halim 1972). The coconut-like aroma of the *Trichoderma* cultures is due to the 6-PP pyrone; these metabolites also showed significant antifungal potential against *Rhizoctonia solani* Kühn (Dennis and Webster 1971a, b). The elucidation of biosynthetic pathways for 6-PP was attempted by Serrano-Carreon et al. (1993); they proposed the linolenic acid as the origin for 6-PP, although few other researchers prefer the polyketide synthesis of 6-PP (Sivasithamparam and Ghisalberti 1998). Moss et al. (1975) showed a similar compound to 6-PP, a dehydroderivative with the coconut aroma from *T. viride* and *T. koningii*. Hill et al. (1995) proposed *Trichoderma* as the biocontrol agent with patented lactones massoilactone and γ-decanolactone. Another compound, viridepyronone, showed promising antifungal activity against *Sclerotium rolfsii* with MIC at 196 μg/ml (Evidente et al. 2003). A hydroxyl lactone derivative, cerinolactone, was isolated from *Trichoderma cerinum* (Vinale et al. 2012). Recently trichodermaerin, a diterpene lactone, was isolated from *Trichoderma asperellum*; it showed potent antifungal activity (Chantrapromma et al. 2014).

The biosynthesis of 6-PP was mainly regulated by the G-protein Tga1 in *T. atroviride*, while similar transcription factor thctf1 was observed as regulation component for 6PP in *T. harzianum*. The mutant lines devoid of the thctf1 gene did not produce the two metabolites, i.e. 6-[(1´R,2´S)-dihydroxypentyl]-2H-pyran-2-one and 6-((1´S,2´R)-2´-propyloxiran-1-yl)-2H-pyran-2-one from 6-PP (Rubio et al. 2009; Daoubi et al. 2009).

Isocyano Metabolites The isocyano metabolites are the foul odour compounds with a characteristic five-membered ring. The xanthocillin was the first natural isocyano metabolite, reported in *Penicillium notatum* in 1956 (Scheuer 1992). The dermadin was isolated from *Trichoderma* spp. after 10 years. Liss et al. (1985) had isolated antibiotic metabolite 3-(3-isocyanocyclopent-2-enylidene) propionic acid from *Trichoderma hamatum* against rumen bacteria. The isonitrin C (trichoviridin) which inhibits melanin synthesis was reported first time by Tamura et al. (1975) from *T. koningii*. The other metabolites such as isonitrins A, B, C and D were isolated from *T. hamatum*, while isonitrinic acid E and F were also reported from *Trichoderma* spp. (Fujiwara et al. 1982). *Trichoderma koningii* also produced some cyclopentenes which showed inhibition against *Phytophthora* spp.; they are named as homothallin I (Pratt et al.1972) and homothallin II (Edenborough and Herbert 1988).

The isocyano metabolites had an effective mechanism of action in three different ways, (1) bacteriostasis induction (Brewer et al. 1982), (2) oospores induction of the A2 mating type of

Phytophthora spp. (Pratt et al. 1972; Reeves and Jackson 1972; Brasier 1975) and (3) melanin biosynthesis inhibition in mammals by tyrosinase inhibition (Le et al. 1997a, b).

The instable nature of the isocyano metabolites led to the ambiguities in structural characterisation of this group. The problem of misassignment of structures was overcome by exhaustive efforts in synthetic studies (Baldwin et al. 1991). The earlier studies suggest that the origin of isocyano metabolites is from the amino acid tyrosine (Baldwin et al. 1985b). An interesting result was observed by Faull et al. (1994) in *T. harzianum*; it normally shows the presence of 6-PP in culture conditions, although mutation by UV light converted *T. harzianum* into homothallin II producer. These results conclude that the isocyano metabolites can be induced in *Trichoderma* spp. by simple mutations.

Diketopiperazines The diketopiperazines (DKPs) are the smallest cyclic peptides produced by microorganisms. These compounds were discovered in 1880 and restudied by E. Fischer in 1906. DKPs were usually nonpreferred cyclopeptides due to its synthesis from protein hydrolysates and considered as the by-products of protein degradation. The potent biological activities exhibited by DKPs in past few years attracted chemists, microbiologists and agronomists towards the DKPs research. A number of DKPs were isolated and studied from microorganisms with noteworthy biological activities.

A phytotoxin thaxtomin A was isolated from *Streptomyces* species (Healy et al. 2000; King and Calhoun 2009). This DKP acts as the toxin by inhibiting cellulose synthesis in developing plant cells.

The first DKP isolated from *Trichoderma* was gliotoxin; it was firstly reported by Weindling and Emerson (1936) as a metabolic product of *Trichoderma lignorum* (Tode) Harz, whereas further it was described by Brian (1944) as the gliotoxin from *T. viride*. Gliotoxin and gliovirin are the members of the epipolythiodioxopiperazine class of toxins. Both showed characteristic disulphide bridges between cyclic dipeptides. Among these two DKPs, gliotoxin exhibits more activity against *Rhizoctonia solani* compared to gliovirin (Howell et al. 1993). The potent biological activity and extraordinary structure of gliotoxin became the subject of biologists. The biosynthetic pathway for the production of gliotoxin was proposed by Sivasithamparam and Ghisalberti (1998); they suggested that the gliotoxin was generated from L-phenylalanine and L-serine via the cyclic dipeptide. The enzyme involved in biosynthesis was reported as dioxopiperazine synthase (gliP) which generates the characteristic diketopiperazine ring (Dagenais and Keller 2009).

Peptaibols *Trichoderma* spp. show a class of secondary metabolites with unusual amino acids, commonly called as peptaibols. These are the linear hydrophobic peptides with high amount of α, α-dialkylated amino acids, α-amino isobutyric acid (Aib) and isovaline (Iva), an acetylated (or acylated) N-terminus and C-terminal amino alcohol. The unusual amino acids are mainly involved in intermediate synthesis of biomolecules. The peptaibols were first time isolated from *Trichoderma viride*, namely, alamethicin, although later it was found that the alamethicin is composed of at least 12 different compounds (Meyer and Reusser 1967; Brewer et al. 1987).

The genus *Trichoderma* was observed to be predominant in the production of peptaibols. The subclasses of the peptaibols are based on the chain lengths of peptides: 18–20 residues peptides are termed as the long-sequence peptaibols (Jung et al. 1976; Pandey et al. 1977; Bodo et al. 1985; Lida et al. 1993; Rebuffat et al. 1993), 11–16 residue peptides are short-sequence peptaibols (Rebuffat et al. 1995), while 7–11 residue peptides with N-terminal amino acids acylated by a short lipid chain are called as lipopeptaibols (Auvin-Guette et al. 1992).

The systematic classification of peptaibols was done into nine distinct subfamilies; these subfamilies were again differentiated according to the amino acid number, pattern and terminal

processing (Chugh and Wallace 2001; Neuhof et al. 2007).

Today about 317 sequences of peptaibols are compiled at the peptaibol database (http://peptaibol.cryst.bbk.ac.uk/home.shtml). The *Trichoderma* peptaibols were discussed extensively for structural characterisation by Daniel and Filho 2007.The examples of major peptaibols along with their sequences are showed in Table 6.3.

6.1.4.3 Competition

Competition is referred to as an indirect antagonistic method. Starvation is the most common cause of death for microorganisms during competition for limited nutrients and space. This unusual situation can be exploited for biocontrol of fungal phytopathogens (Chet et al. 1997). *Trichoderma* spp., being decomposers by nature, efficiently mobilise and uptake macro- and micronutrients from soil, which

Table 6.3 Major peptaibols isolated from *Trichoderma* species along with the peptide sequences

Trichoderma spp.	Peptaibols	Sequence	References
T. atroviride	Atroviridin A	Ac Aib Pro Aib Ala Aib Ala Gln Aib Val Aib Gly Leu Aib Pro Val Aib Aib Gln Gln Phe OH	Oh et al. (2000)
	Atroviridin B	Ac Aib Pro Aib Ala Aib Ala Gln Aib Val Aib Gly Leu Aib Pro Val Aib Iva Gln Gln Phe OH	
	Atroviridin C	Ac Aib Pro Aib Ala Aib Aib Gln Aib Val Aib Gly Leu Aib Pro Val Aib Iva Gln Gln Phe OH	
Trichoderma viride	Trichotoxin A 50 I	Ac Aib Gly Aib Leu Aib Gln Aib Aib Aib Ala Aib Aib Pro Leu Aib Iva Gln Val OH	Bruckner and Przybylski (1984)
	Trichodecenin I	(Z)-4-decenoyl Gly Gly Leu Aib Gly Ile Leu OH	Fujita et al. (1994)
	Suzukacillin	Ac Aib Ala Aib Ala Aib Ala Gln Aib Aib Aib Gly Leu Aib Pro Val Aib Aib Gln Gln Phe OH	Bruckner and Przybylski (1984)
	Alamethicin F50	Ac Aib Pro Aib Ala Aib Ala Gln Aib Val Aib Gly Leu Aib Pro Val Aib Aib Gln Gln Phe OH	Bruckner and Przybylski (1984)
T. harzianum	Trichokindin IIIa	Ac Aib Ser Ala Aib Aib Gln Iva Leu Aib Ala Iva Aib Pro Leu Aib Aib Gln Leu OH	Iida et al. (1994)
	Trichorzin HA I	Ac Aib Gly Ala Aib Aib Gln Aib Val Aib Gly Leu Aib Pro Leu Aib Aib Gln Leu OH	Goulard et al. (1995)
	Harzianin HB I	Ac Aib Asn Leu Ile Aib Pro Iva Leu Aib Pro Leu OH	Augeven-Bour et al. (1997)
T. koningii	Trichokonin Ib	Ac Aib Gly Aib Ala Aib Ala Gln Aib Val Aib Gly Leu Aib Pro Val Aib Aib Gln Gln Phe OH	Huang et al. (1995)
	Trikoningin KB I	Oc Aib Gly Val Aib Gly Gly Val Aib Gly Ile Leu OH	Auvin-Guette et al. (1993)

(continued)

Table 6.3 (continued)

Trichoderma spp.	Peptaibols	Sequence	References
T. saturnisporum	Paracelsin E	Ac Aib Ala Aib Ala Aib Ala Gln Aib Leu Aib Gly Aib Ala Pro Val Aib Aib Gln Gln Phe OH	Ritieni et al. (1995)
	Saturnisporin SA I	Ac Aib Ala Aib Ala Aib Ala Gln Aib Leu Aib Gly Aib Aib Pro Val Aib Aib Gln Gln Phe OH	Rebuffat et al. (1993)
	Saturnisporin SA II	Ac Aib Ala Aib Ala Aib Ala Gln Aib Leu Aib Gly Aib Aib Pro Val Aib Iva Gln Gln Phe OH	Rebuffat et al. (1993) and Goulard et al. (1995)
	Saturnisporin SA III	Ac Aib Ala Aib Ala Aib Aib Gln Aib Leu Aib Gly Aib Aib Pro Val Aib Aib Gln Gln Phe OH	Rebuffat et al. (1993)
	Saturnisporin SA IV	Ac Aib Ala Aib Ala Aib Aib Gln Aib Leu Aib Gly Aib Aib Pro Val Aib Iva Gln Gln Phe OH	Rebuffat et al. (1993)
T. longibrachiatum	Trichobranchin A-I	Ac Aib Asn Leu Leu Aib Pro Leu Aib Aib Pro Leu OH	Mohamed-Benkada et al. (2006)
	Tricholongin B-I	Ac Aib Gly Phe Aib Aib Gln Aib Aib Aib Ser Leu Aib Pro Val Aib Aib Gln Gln Leu OH	Rebuffat et al. (1991)
T. polysporum	Trichosporin TS-B-1a-1	Ac Aib Ala Gly Aib Ala Aib Gln Aib Lxx Ala Ala Vxx Ala Pro Val Aib Vxx Gln Gln Phe OH	Iida et al. (1993) and Sharman et al. (1996)
	Polysporin A	Ac Aib Pro Aib Ala Aib Aib Gln Aib Val Aib Gly Val Aib Pro Val Aib Aib Gln Gln Phe OH	New et al. (1996)
	Polysporin B	Ac Aib Pro Aib Ala Aib Aib Gln Aib Val Aib Gly Leu Aib Pro Val Aib Aib Gln Gln Phe OH	New et al. (1996)
T. pseudokoningii	Harzianin HK-VI	Ac Aib Asn Ile Ile Aib Pro Leu Leu Aib Pro Leu OH	Rebuffat et al (1996)
	Pseudokinin KL-VI	Ac Aib Asn Ile Ile Aib Pro Leu Val hydroxyketopiperazine	Rebuffat et al. (2000)
T. reesei	Paracelsin A	Ac Aib Ala Aib Ala Aib Ala Gln Aib Val Aib Gly Aib Aib Pro Val Aib Aib Gln Gln Phe OH	Bruckner and Przybylski (1984)
	Paracelsin B	Ac Aib Ala Aib Ala Aib Ala Gln Aib Leu Aib Gly Aib Aib Pro Val Aib Aib Gln Gln Phe OH	Bruckner and Przybylski (1984)

results in scarcity of nutrients for other microbes in its vicinity leading to decreased inter- and intraspecies competition (Chet 1987; Dix and Webster 1995; Benitez et al. 2004; Verma et al. 2007). *Trichoderma* species are generally considered to be aggressive competitors, grow very fast and rapidly colonise substrates to exclude pathogens such as *Fusarium* spp. (Papavizas 1985). Rhizosphere competence, following seed treatment, is an important strategy to create a zone of protection against pathogens (Howell 2003). Their colonising ability is greatly influenced by environmental factors, including soil pH, temperature and water potential (Danielson and Davey 1973; Domsch et al. 1980), and therefore, competition should be regarded as an effective means of biocontrol.

In filamentous fungi, iron uptake is essential for viability, and under iron starvation, most fungi excrete low-molecular-weight ferric iron-specific chelator, termed siderophores, to mobilise environmental iron (Eisendle et al. 2004). Subsequently, iron from the ferri-siderophore complexes is recovered via specific uptake mechanisms. Some *Trichoderma* isolates produce highly efficient siderophores that chelate iron and stop the growth of other fungi (Vinale et al. 2008a). Competition has proven to be extravagantly important for the biocontrol of phytopathogens such as *Botrytis cinerea*, the main pathogenic agent during the pre- and postharvest in many countries. The extraordinary genetic variability of this fungus makes it possible for new strains to become resistant to essentially any novel chemical fungicide on which it is exposed (Harman 2006).

Trichoderma species as BCAs, either directly added to the soil or applied as seed treatments, are reported to grow readily along with the developing root system of treated plants (Ahmad and Baker 1987). It has been confirmed that soil treatments with *T. harzianum* spores suppressed infestations of *Fusarium oxysporum* f. sp. *vasinfectum* and *F. oxysporum* f. sp. *melonis* by competing for both rhizosphere colonisation and nutrient uptake (Abdullah et al. 2005). *Trichoderma* metabolites may act by directly inhibiting the growth of pathogens, or by indirectly triggering the defence system in the host plant, thus increasing disease resistance and promoting plant growth (Vinale et al. 2012).

In an agro-environment, it is expected that microbial communities can produce secondary metabolites during processes of competition, symbiosis, parasitism or pathogenesis. During this, siderophores can be produced as one of the secondary metabolites, which are low-molecular-weight metabolites produced for scavenging iron from the environment and have a high affinity for iron (III) (Hider and Kong 2010). Such molecules help the plant to withstand pathogens by both promoting the growth and development of root and shoot systems and stimulating the defence mechanisms (Vinale et al. 2008b). Fe3+-chelating molecules can be beneficial to plants because they can solubilise the iron which is otherwise unavailable and can suppress the growth of pathogenic microorganisms by depriving them from necessary micronutrients (Leong 1986).

Trichoderma harzianum was reported to produce a nitrogen heterocyclic compound named harzianic acid (HA) which is novel siderophores (Vinale et al. 2013) with growth promotion effect (Vinale et al. 2009b). Microbial siderophores are used as iron-chelating agents which can regulate the availability of iron in the plant rhizosphere. It has been assumed that competition for iron in the rhizosphere is controlled by the affinity of the siderophore for iron. The significant factors, which participate, are concentration of various types of siderophores, kinetics of exchange and availability of Fe complexes to microorganisms as well as plants. Siderophores produced by beneficial agents may have important effects on both microbial and plant nutrition. Fe3+-siderophore complexes can be recognised and taken up by several plant species, and this process is considered crucial for plant iron uptake, particularly in calcareous soils (Sharma et al. 2003).

Trichoderma siderophores are mostly produced non-ribosomally by large multifunctional peptide synthetases, which are organised into repetitive synthase units and required to complete a different single amino acid elongation step for the synthesis of the peptide product (Wilhite et al. 2001). Anke et al. (1991) reported that siderophores, namely, coprogen, ferricrocin and a new coprogen derivative which carried a palmitoyl instead of an acetyl group, as palmitylcoprogen in the cell belongs to fusigen, ferrichrome and coprogen families (Mukherjee et al. 2012a, b) and their orthologous NRPS gene clusters involved in siderophore synthesis (SidD and NPS6) have been identified in *T. atroviride*, *T. reesei* and *T. virens* (Kubicek et al. 2011; Mukherjee et al. 2012a, b). The screening approach applied suggested a high diversity in siderophore production by *Trichoderma* spp. In total, 18 different siderophores were detected in the culture filtrates. Ferricrocin plays an important role in intracellular iron storage (Eisendle et al. 2006) and is usually described as an intracellular siderophore. Dimerum acid, fusarinine

A, in all samples, is due to hydrolysis of larger siderophores, which cannot be ruled out since they constitute subunits of many other larger siderophores. It has been reported that siderophores produced by *T. asperellum* Q1 under saline stress had the real potential to enhance cucumber growth by inducing physiological protection against sign of alleviating negative effect of salinity and available iron deficiency (Qi and Zhao 2013).

Harzianic Acid The *Trichoderma harzianum* strain, isolated from composted hardwood bark in Western Australia, was suppressive versus the phytopathogenic agent *Pythium irregulare*. The fungus produced mainly harzianic acid, a completely characterised tetramic acid derivative (Sawa et al. 1994). Harzianic acid has also been isolated from liquid cultures of a fungal strain obtained from a soil sample in Amagi, Japan (Kawada et al. 2004) along with N-demethyl analogue tetramic acids or pyrrolidinediones. In its biosynthetic perspective, naturally occurring tetramic acids can be regarded to arise from the assembly of an amino acid and an activated acyl entity (Sodeoka et al. 2001).

Harzianic acid and isoharzianic acid have both been reported to display antifungal activity and to increase seed germination and shoot and root growth in canola and tomato seedlings (Vinale et al. 2009a, 2014). The plant growth-promoting activity of harzianic acid is believed to be linked to its potent siderophoric properties (Vinale et al. 2013). Microbial siderophores are iron-chelating agents involved in iron solubilisation, which is a crucial mechanism in plant nutrient regulation (Hider et al. 2010). It has been reported that harzianic acid has increased seedling growth even under iron-deficient conditions and its uptake in plants (Vinale et al. 2013). The antifungal and plant growth-promoting activities of harzianic acid have highlighted it as a promising bioactive compound which could be used as an alternative to living antagonists. The antifungal potential is evident by the author's personal experiments, in which the ethyl acetate extract of *Trichoderma harzianum* can selectively extract terpenoids as shown in Fig. 6.7, whereas Fig. 6.8 shows dose-dependent inhibitory potential of *Trichoderma virens*. Similarly Fig. 6.8a showed comparative antifungal activity of pure and crude terpenoid

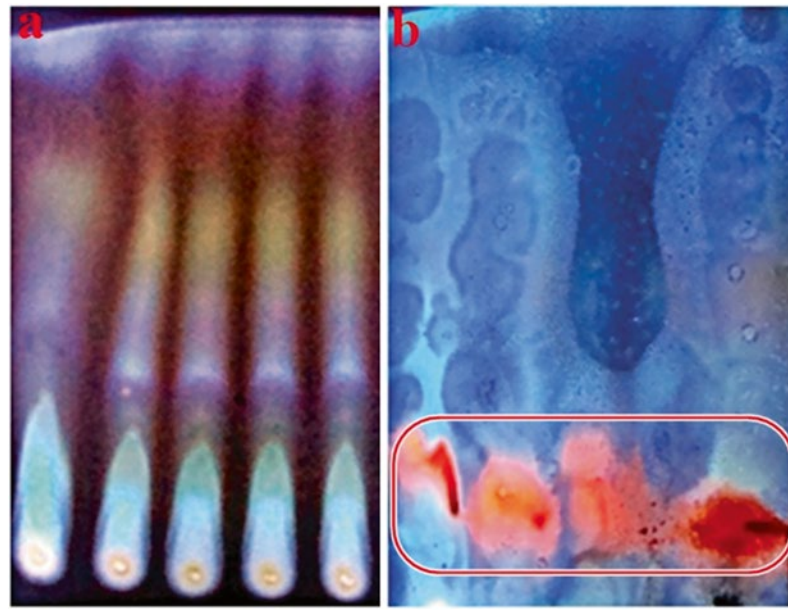


Fig. 6.7 TLC chromatogram of ethyl acetate-extracted fractions of *T. harzianum* metabolite detection for the presence of terpenoid (**a**) solvent-solvent fractionation before spraying, (**b**) after spraying with conc. H_2SO_4 (*red square* indicates the zone of active constituent)

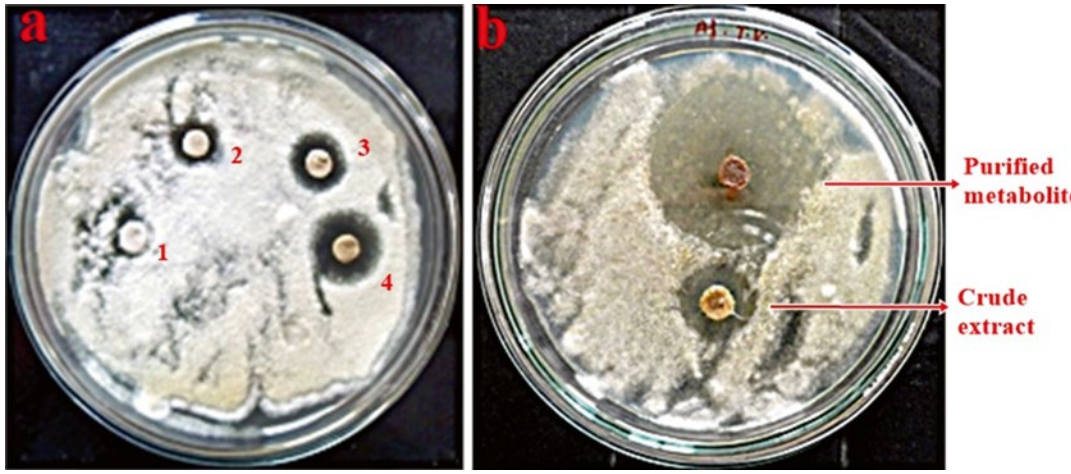


Fig. 6.8 (**a**) Inhibitory potential of *T. virens* against *A. flavus* in various concentrations (1, 25, 50 and 100 mg/ml) of ethyl acetate extract from *T. virens*, (**b**) comparative antifungal activity of crude and purified ethyl acetate extract of *T. harzianum* (50 mg/ml) against *A. flavus* isolated from rhizosphere of peanut (*Arachis hypogaea*)

extracts against pathogenic *Aspergillus flavus*, which confirms the antifungal nature of *Trichoderma* spp.

Harzianic acid (HA) and isoharzianic acid (iso-HA), stereoisomer of HA, and their structure and absolute configuration have been determined by spectroscopic methods, including UV-Vis, MS, 1D and 2D NMR analyses (Vinale et al. 2014). Recently the first total lab synthesis of harzianic acid (1a) and its three stereoisomers, including isoharzianic acid (1b), has been reported (Healy et al. 2015).

In conclusion, many *Trichoderma* SMs which are reported and characterised during the past were not fully investigated for a wide range of biological activity. The modern high-throughput techniques which are quite acute, sensitive and specific analytical methods would be especially fruitful in the bioscreening of newer *Trichoderma* SMs. This might well lead to the discovery of novel compounds or pathways, which in turn might reveal important new aspects for many human applications, including pharmaceutical and agri-biotechnological uses.

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Induced Systemic Resistance in Rice

7

Kalaivani K. Nadarajah

Abstract

Plants possess a plethora of defense mechanisms that respond to both biotic and abiotic stresses. The response of a plant to various pathogens and pests can vary dependent on factors such as host variety, strain, as well as environmental factors. How quickly a plant responds to these stresses will determine the level of resistance of a plant species. The SAR and ISR mechanisms in plants work together to provide the host with protection against pathogen and pest attacks. Unlike SAR, ISR is induced by nonpathogenic allies from belowground in the form of plant growth-promoting bacteria. ISR is induced in the root and in foliar tissue and is able to provide the host plant with systemically induced resistance against a broad spectrum of microorganisms. Selected strains of plant growth-promoting rhizobacteria suppress diseases and pest infestation by inhibition of pathogens/pests as well as resulting in the induction of systemic resistance *in planta*. In rice these organisms have been known to activate the JA/ETH and auxin pathways. Due to the higher levels of endogenous SA in rice, the SA-independent pathways are a preferred way of inducing resistance within the rice host. There are various types of determinants that have been implicated to play a role in ISR. These determinants can either work individually or in combination to induce ISR in plants. From in vitro, greenhouse and field studies on rice, several strains of bacteria such as *Pseudomonas* spp., *Bacillus* spp., *Serratia* spp., and *Azospirillum* spp. have been listed as organisms with potential to function as biofertilizers and biopesticides in rice.

7.1 Introduction

Rice (*Oryza sativa* L.) has been an important staple food for more than two billion Asians and millions of Africans and Latin Americans. Since a large portion of the dietary intake of Asians in

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general is made up of rice, hence great significance is placed on its farming and productivity. China, India, Indonesia, Thailand, Myanmar, Vietnam, and Malaysia are among the large rice producers worldwide. World rice production has evolved from the traditional farming methods to those adopting modern high-yielding varieties, adequate irrigation, use of fertilizers, and other complementary inputs (Akanbi et al. 2007). However in recent years, population growth has outpaced rice production worldwide, and this is especially true for the Asian region which is the largest consumer of rice. At present Malaysia produces 70 % of its rice requirements which necessitates import of rice from our neighboring countries. However the Ministry of Agriculture in Malaysia endeavors to achieve 100 % self-sufficiency by 2025. This increase in rice yield has to be obtained with the current land allocation and the increase of population in the years to come. Hence all contributing factors that affect the issue of yield have to be addressed to achieve this projected self-sufficiency in 10 years (Peng et al. 2008).

In Malaysia, rice is mostly cultivated in irrigated rainfed lowland and upland. The irrigated lowland ecology is the largest in terms of area and production, but with the least productivity due to biotic and abiotic constraints and low soil fertility. Disease incidences are a major constraint that eventually leads to the low productivity in lowland rice. For decades, rice disease management systems have relied primarily on the release of new resistant varieties and the application of pesticides. Resistant rice cultivars often do not withstand more than 1 or 2 years of cultivation before succumbing to diseases, due to either breakdown or gradual erosion in face of the high variability of the pathogen population. In addition to resistant cultivars, most rice fields also use chemical controls of pathogens. These chemicals have been used extensively resulting in toxic effect to nontarget microorganisms of the soil, contamination of underground water, and other detrimental effects to human, animals, and environment. In addition to the above factors, these chemicals increase the expense of production, and this is too costly for the poor farmers of Asia where 90 % of world's rice is grown (Dobermann et al. 2007). Another impeding problem is the emergence of pesticide-resistant phytopathogens hence resulting in the chemicals being rendered inefficient or the requirement for the application of higher concentration of chemicals. This therefore compounds the problem where crop rotation, breeding for resistance, and the application of pesticides are insufficient to control diseases and pest in rice.

PGPRs were selected as an alternative, effective, and environmentally friendly strategy against rice disease as opposed to the persistent use of synthetic chemicals (Suarez et al. 2005). This bioagent also has the potential in supplying "N" nutrition and suppressing phytopathogens which eventually leads to sustainable rice production. So what exactly are PGPRs? PGPR are beneficial rhizosphere/phyllosphere bound plant growth-promoting bacteria that promote plant growth and health through different mechanisms, including N-fixation, hormonal interaction, improvement in root growth, solubilization of essential nutrients, alleviation of soil salinity, and biocontrol against phytopathogens. Hence PGPR affect the plant by secreting exudates that compete for an ecological niche/substrate, production of inhibitory allelochemicals, and induction of systemic resistance in host plants (Kloepper et al. 1989; Lucy et al. 2004). The induction of systemic resistance by rhizobacteria is referred as ISR, whereas pathogen-induced resistance is called SAR (Hammerschmidt and Kuc 1995; Van Loon et al. 1998). Both SAR and ISR function as latent resistant mechanisms that are expressed upon subsequent or challenge inoculation with a pathogen (Van Loon 1997). Contrary to the attacker-specific primary immune response, induced resistance is typically effective against a broad spectrum of otherwise virulent pathogens. Induced resistance is also suspected to spread systemically throughout the plant, thereby protecting the entire plant against subsequent invasion. Unfortunately, compared to the wealth of information on inducible defense responses in dicot plant species, the information on Monocotyledoneae, plants are still largely inadequate and requires further investigation.

Proper understanding of ISR in plants is essential in the optimal deployment and commercial acceptance of induced resistance in an agricultural setting.

PGPR-induced systemic resistance results in the fortification of the physical and mechanical strength of the host leading to the synthesis of defense chemicals against the challenge pathogen. The utilization of natural PGPR strains as inducers of plant defense response may increase the chance of their applicability in delivering immunization to crops against pest and pathogens. Induced systemic resistance in rice seedling is augmented by increased activities of various defense-related enzymes and chemicals in response to infection by pathogen. Growing body of evidence reports that systemic protection against various rice pathogens resulting from ISR can be elicited by among others, *Pseudomonas* spp. (Nandakumar et al. 2001), *Bacillus* spp. (Jayaraj et al. 2005) and *Serratia* strains (Someya et al. 2005). In this chapter we will review the PGPRs that have been used by researchers that have been effective against pests and pathogens of rice. The determinants and the mode of action of these exudates in the host-pest-pathogen interaction will also be presented together with the implicated pathways, expression profiles, applications, future prospects, and constraints in PGPR utilization (Hafeez et al. 2006; Vidhyasekaran and Muthamilan 1995; Viswanathan and Samiyappan 1999).

7.2 Application of PGPR

Rice disease suppression by biocontrol agents is governed by a multitude of factors that varies with the type of biocontrol agent, plant cultivar, the nature of target pathogen, and the environment. There are different modes of action of biocontrol bacteria, such as competition for nutrients and space (Elad and Baker 1985; Elad and Chet 1987; Hafeez et al. 2006); antibiosis by antibiotics such as pyrrolnitrin, pyocyanin, and 2,4-diacetylphloroglucinol (Pierson and Thomashow 1992); and the production of siderophores that limit the availability of iron necessary for the pathogens (Kloepper et al. 1980; Lemanceau et al. 1992). Other mechanisms that have been implicated in the control of diseases are the production of lytic enzyme such as chitinases and β-1,3-glucanases (Frindlender et al. 1993), toxins (Défago et al. 1990) and the degradation of toxins produced by pathogen (Duffy and Défago 1997). A large number of bacterial antagonists belonging to the genera *Bacillus*, *Pseudomonas*, *Serratia*, and *Erwinia* have been found to inhibit mycelial growth of fungal pathogens such as *Sclerotium oryzae* (stem rot), *Bipolaris oryzae* (brown spot), *Pyricularia grisea* (blast), *Sarocladium oryzae* (sheath rot), *Rhizoctonia solani* (sheath blight), and *Fusarium fujikuroi* (bakanae). A number of these antagonistic bacteria associated with upland and lowland rice rhizosphere soils have been found effective in in vitro, greenhouse, and field inhibition of rice pathogens. Similarly certain *Bacillus* and *Pseudomonas* species have shown promise in the control of insect and nematode predators of rice. Table 7.1 shows various reports on the effectiveness of PGPRs on rice diseases, nematodes, and insects.

7.2.1 Against Disease

Contrary to the large body of information and research that has been conducted on ISR in dicotyledonous plants, very little is known about the mechanisms underlying rhizobacteria-induced resistance in monocots. Several researchers have worked on various PGPR strains and its effectiveness against rice diseases, and here are some of the strains that have shown promise. Pseudomonads have been one of the most important strains that induce ISR in rice as in other plant species. *Pseudomonas fluorescens* strains Pf1 and FP7, for instance, when used to treat rice seedling, showed higher induction of ISR against the fungal sheath blight pathogen, *Rhizoctonia solani* (Vidhyasekaran and Muthamilan 1995). *Pseudomonas fluorescens* have been reported to suppress soilborne plant pathogens by competition for iron, production of antibiotics, biosurfactants, and cell wall-degrading enzymes in addition to elicitation of ISR in host plants against

Table 7.1 PGPR and their application against control of disease, nematode, and insects in rice

Biotic stress	Causal organism	Biocontrol	References
Disease	*Magnaporthe oryzae*	*Pseudomonas fluorescens* (RB04)	Shyamala and Sivakumar (2012)
	Rhizoctonia solani	*P. fluorescens* FP7	Radjacommare et al. (2004); Radjacommare (2000)
		P. fluorescens PF1	
	Magnaporthe oryzae	*Pseudomonas EA105*	Spence et al. (2014)
	Magnaporthe oryzae	*P. aeruginosa* 7NSK2	De Vleesschauwer et al. (2006)
	Rhizoctonia solani		
	Magnaporthe oryzae	*P. fluorescens* WCS374r	De Vleesschauwer et al. (2008)
	Rhizoctonia solani	*P. fluorescens* isolate PFP-5 and PFR-5	Sivakamasundari and Usharani (2012)
	Magnaporthe oryzae	*P. fluorescens* Aur 6 *Chryseobacterium balustinum* Aur 9	Lucas et al. (2009)
	Magnaporthe oryzae	*Serratia plymuthica* IC1270	De Vleesschauwer et al. (2009)
	Cochliobolus myiabeanus		
	Rhizoctonia solani		
	Xanthomonas oryzae pv. oryzae	*B. pumilus* SE34, *B. subtilis* GB03	Chithrashree et al. (2011)
	Magnaporthe oryzae	*B. subtilis* UKM1	Ali and Nadarajah (2012, 2014)
	Rhizoctonia solani		
	Magnaporthe oryzae	*B. vallismortis* EXTN-1,	Chung et al. (2015)
	Rhizoctonia solani	*B. cereus*	
	R. solani	*B. subtilis* BBG111	Coutte et al. (2010)
		B. subtilis RFB104	
	P. oryzae	*B. mucilaginosus* isolates SSB-8, SSB-11, SSB-17	Vijayapriya and Muthukkaruppan (2012)
	Magnaporthe oryzae	*Bacillus* T4, SE34, 4-03, and 33	Ryu et al. (2003)
	Xanthomonas oryzae pv. oryzae	*Bacillus* strains YC7007 and YC7010	Chung et al. (2015)
	Burkholderia glumae		
	Xanthomonas oryzae pv. oryzae	*B. subtilis* NEB4, NEB5,	Bai et al. (2002)
		B. thuringiensis NEB17	
Nematode	*Meloidogyne graminicola*	*P. fluorescens* Pf1	Anita and Samiyappan (2012)
	Hirschmanniella gracilis, *Hirschmanniella oryzae*	*P. fluorescens*	Ramamoorthy et al. (2001)
	Meloidogyne graminicola	*P fluorescens* Pf1	Seenivasan (2011)
		Paecilomyces lilacinus	
	Meloidogyne graminicola	*B. subtilis*,	Sikora (1992)
		B. sphaericus,	Tian et al. (2007)
		B. pumilus (ToIr-FT and ToIr-MA), *Bacillus* sp. (ToIr-10)	
	Meloidogyne javanica	*Bacillus* spp.	Niu et al. (2006)
	Meloidogyne graminicola	*Rhizobium etli* G12	Reitz et al. (2002)
	Meloidogyne javanica		

(continued)

Table 7.1 (continued)

Biotic stress	Causal organism	Biocontrol	References
Insect	*Cnaphalocrocis medinalis*	*Pseudomonas* strains Pf1 + AH1 and *Beauveria bassiana* (B2)	Saravanakumar et al. (2007)
	Cnaphalocrocis medinalis	*Pseudomonas* Pf1, TDK1, PY15	Saravanakumar et al. (2007)
	Cnaphalocrocis medinalis	*P. fluorescens* PF1	Commarea et al. (2002)

the phytopathogens (Abbas-Zadeh et al. 2010). Similar findings were also reported by Sundaramoorthy et al. (2013) when a consortia of *Pseudomonas fluorescens* Pf1+TDK1+TV5 and *Bacillus subtilis* TH10 showed inhibition of sheath rot disease in rice through antagonistic activity. Shyamala and Sivakumar (2012) and De Vleesschauwer et al. (2006) in their study of *Pseudomonas fluorescens* RB04 and *Pseudomonas aeruginosa* 7NSK2, respectively, reported inhibition of the rice blast fungus *Pyricularia oryzae*, through the production of siderophores and hydrolytic enzymes. De Vleesschauwer and colleagues (2006) also reported that while *Pseudomonas aeruginosa* 7NSK2 was able to induce resistance against *Magnaporthe oryzae*, it was ineffective against *Rhizoctonia solani*. In this study, the pyocyanin mutants exhibited significant decrease in ISR to *Magnaporthe grisea*, but interestingly triggered ISR against *Rhizoctonia solani*. Another significant *Pseudomonas* strain, WCS374r, produced pseudobactin which was effective against *Magnaporthe oryzae* but like the 7NKS2 strain, this too was ineffective in inducing resistance against *Rhizoctonia solani* (De Vleesschauwer et al. 2008). Lucas et al. (2009) in their study in the rice fields of Spain found that isolates *Pseudomonas fluorescens* Aur 6 and *Chryseobacterium balustinum* Aur 9 were able to induce systemic resistance against blast disease as well as other leaf pathogens. These isolates were also effective against salt stress (Velagaleti and Marsh 1989). Sivakamasundari and Usharani (2012) concluded from their study that the active metabolism of the microbes is essential for the induction of systemic resistance to pathogens such as *Rhizoctonia solani* when log-phase culture of both *Pseudomonas fluorescens* isolates PFP-5 and PFR-5 showed a reduction in sheath blight disease incidence followed by lag- and stationary-phase cultures. In another study, *Pseudomonas* EA105 most effectively inhibited the growth and appressoria formation of *Magnaporthe oryzae* through a mechanism that is independent of cyanide production. In addition to direct antagonism, EA105 also appears to trigger ISR in rice plants through a mechanism that is dependent on JA and ET signaling, ultimately resulting in fewer blast lesions (Spence et al. 2014).

Another significant group of bacterial antagonists in rice are the *Bacillus* spp. Several species of *Bacillus* have been commercialized as biological agents due to their long persistence in adverse environments over long periods. Some of these were functional in many plants, by inducing systemic resistance (Chung et al. 2015). This group of organism has been used effectively in the control of diseases and pests in rice. Chitrashree and colleagues (2011) reported suppression of *Xanthomonas oryzae* by *Bacillus subtilis* GB03 and two strains of *Bacillus pumilus* (SE34 and T4). The SE34- and GB03-treated rice plants showed elevated levels of PAL, POX, and PPO after challenge inoculation with the pathogen which implied that these PGPRs may either directly or indirectly activate the defense pathway within the host and develop the host resistance against pathogenic microorganisms. Both PAL and POX play important roles in the biosynthesis of phenolics, phytoalexins, and lignin that are key factors in inducing disease resistance (Daayf et al. 1997; Ryals et al. 1996). These results are relatively similar to those obtained by Bai et al. (2002) that reported *Bacillus subtilis* NEB4 and NEB5 and *Bacillus thuringiensis* NEB17 reduced *Xanthomonas oryzae* infection through the activation of ISR. In Korea, two novel endophytic *Bacillus* strains YC7007 and YC7010 were reported to induce plant growth

and systemic resistance against *Xanthomonas oryzae* and *Burkholderia glumae* (Chung et al. 2015).

Meanwhile, Ryu et al. (2003) reported that *Bacillus* T4, SE34, 4-03, and 33 reduced leaf blast by 46 %. This is similar to the report by Vijayapriya and Muthukkaruppan (2012) who observed the inhibitory effect of *Bacillus mucilaginosus* isolates SSB-8, SSB-11, and SSB-17 on *Pyricularia oryzae* in lowland rice. Individual application of *Bacillus mucilaginosus* resulted in increasing levels of reducing and nonreducing sugars, total phenolic content, and induction of defense enzymes such as peroxidase and polyphenol oxidase which indirectly resulted in the induction of ISR (Young et al. 1995). In another report by Coutte and colleagues (2010), *Bacillus subtilis* BBG111 and *Bacillus subtilis* RFB104 triggered ISR against *Rhizoctonia solani*. This study also showed that *Bacillus subtilis* BBG111 acts as a positive regulator of resistance to the necrotroph *Rhizoctonia solani* while being ineffective against the hemibiotroph *Magnaporthe oryzae*. Other isolates that have shown significant induced resistance against rice blast, sheath blight, and bakanae are *Bacillus vallismortis* EXTN-1 and *Bacillus cereus* (Chung et al. 2015). In a study conducted in our laboratory, *Bacillus subtilis* UKM1 when used in combination with *Trichoderma* strains showed reduction in rice disease incidence and severity. Plants treated with these PGPRs were then challenged by phytopathogens, and qPCR analysis of the defense gene *PR-1b* showed elevated levels of the gene in PGPR-treated plants as opposed to the untreated rice plants (Nadarajah et al. 2014, 2015). *Trichoderma* and *Bacillus* affect plant pathogens by attacking and linking the causal agents through sugar linkages and release of extracellular enzymes such as cell wall-degrading enzymes (CDWEs), siderophores, and hydrogen cyanide that are toxic to microorganisms (Ali and Nadarajah 2012, 2014; Wang et al. 2009).

In a standardized soil-based assay, root treatment with *Serratia plymuthica* IC1270 rendered foliar rice tissues resistant to *Magnaporthe oryzae* and against various other rice pathogens with different modes of infection (De Vleesschauwer et al. 2009). However, IC1270-inducible ISR acts as a double-edged sword, and while it induces resistance to *Magnaporthe oryzae*, it increases vulnerability to *Rhizoctonia solani* and *Cochliobolus miyabeanus*. Similarly *Serratia marcescens* strain B2 controlled rice blast when applied to the phylloplane or rhizosphere soil of rice plants. This strain resulted in an early induction and accumulation of PPO, PAL, TAL, LOX, β-1,4-glucosidase, chitinase, and β-1,3-glucanase that have been associated with induced resistance in infected plants (Someya et al. 2000, 2001, 2002). The increase in LOX activity suggests the formation of unsaturated fatty acids that have antimicrobial activity and induce the formation of rice blade phytoalexins (Friedrich et al. 1996). LOX function has been closely correlated to jasmonate biosynthesis which is related to systemic resistance. In addition to the above products, this strain also produces lipopolysaccharides and siderophores which have also been implicated in the process of ISR in plants (De Vleesschauwer et al. 2009).

7.2.2 Against Nematodes

Nematode problems in rice are likely to increase mainly due to the increased use of aerobic rice that is currently cultivated in most rice-growing regions to help increase water-use efficiency in rice production (Mew et al. 2004). Although a large number of bacteria have shown antagonistic effects against nematodes, the most important strains are *Pseudomonas fluorescens* (Khan et al. 2005), *Paenibacillus* strains, *Pasteuria* spp. (Atibalentja et al. 2000), and *Bacillus* spp. (Niu et al. 2007; Terefe et al. 2009; Huang et al. 2010). Application of some of these bacteria has accorded promising results on the most damaging nematode attacking aerobic rice, the root-knot nematode (RKN), *Meloidogyne graminicola* (Bridge et al. 2005). To control RKN, Anita and Samiyappan (2012) had used *Pseudomonas fluorescens* Pf1 where they reported an accumulation of phenols and defense enzymes, viz., PO, POX, PAL, and chitinase in rice root tissues. They concluded that these factors collectively contributed to induce systemic resistance and decreased

nematode infection. *Pseudomonas fluorescens* was also reported as being able to act as a nematicidal agent against rice root nematodes, *Hirschmanniella gracilis* and *Hirschmanniella oryzae*. PGPR alone or in combination with chitin and neem cake was able to reduce the root and soil population of *Hirschmanniella oryzae* in the paddy fields (Ramamoorthy et al. 2001).

In a study conducted by Seenivasan (2011), *Pseudomonas fluorescens* Pf1 and *Paecilomyces lilacinus* were reported to antagonize *Meloidogyne graminicola* in rice roots grown under SRI cultivation system. The mechanism of action of the *Pseudomonas fluorescens* Pf1 against *Meloidogyne graminicola* was believed to be through the production of antibiotics such as 2,4 diacetylphloroglucinol (Cronin et al. 1997), phloroglucinol (Howell and Stipanovic 1984), pyrrolnitrin (Leyns et al. 1990), and phenazine (Gurusiddaiah et al. 1986) and hydrolytic enzymes that increased nematode egg mortality and premature hatch in vitro (Siddiqui et al. 2009). These products have been implicated as inducers of ISR in the host. Other frequently studied antagonistic rhizobacteria that affect RKN are *Bacillus subtilis*, *Bacillus sphaericus*, *Bacillus pumilus* (ToIr-FT and ToIr-MA), and *Bacillus* sp. (ToIr-10) (Tian et al. 2007). Niu et al. (2006) demonstrated that a serine protease in *Bacillus* spp. played an important pathogenic factor in the control of nematodes. The toxins produced by these strains inhibited egg hatching and increased juvenile mortality of *Meloidogyne javanica*. In another experiment, a root-nodulating bacterium *Rhizobium etli* G12 induced systemic resistance by cell surface LPS (Reitz et al. 2002). The resistance response decreases the penetration of *Meloidogyne graminicola* and *Meloidogyne javanica* but has no effect on nematode attraction and only slightly affects the development inside the roots.

7.2.3 Against Insects

Although PGPRs are not as efficient in the biocontrol of insect pest, there have been some reports of bacterial agents that have been successful. One such example is the combinatorial effect of *Pseudomonas* strains (Pf1 + AH1) and entomopathogenic fungus *Beauveria bassiana* (B2) against *Cnaphalocrocis medinalis*. *Beauveria* breaches insect cuticle and therefore is not dependent on ingestion of the organism. This is beneficial for a biocontrol agent as it is active against the nonfeeding stages of insects (Saravanakumar et al. 2007). The application of a Pf1 + AH1 + B2 mixture induced a higher activity of LOX and chitinase against *Cnaphalocrocis medinalis* insect in rice plants. Furthermore, these biocontrol strains stimulated the defense enzymes, PO and PPO, in plants that could be involved in the synthesis of phytoalexins. The most important enzyme involved in the synthesis of volatile compounds against insect pests is LOX. Certain PGPR strains activate octadecanoid, shikimate, and terpenoid pathways which in turn alter the production of volatiles in the host plant leading to the attraction of natural enemies (Bell and Mullet 1993). The dioxygenation of polyunsaturated fatty acid by LOX in response to insects leads to the formation of highly reactive LOX products (HPODE, hydroperoxy octadecatrienoic acid; HPOTE, hydroperoxy octadecadienoic acid). These products are subsequently transformed into jasmonates that regulate plant defense gene expression and synthesizes hydroperoxide lyase which behaves as a volatile phytoalexin.

Volatiles also have an indirect role in defense by attracting parasitoids of the pest or repelling females and thus reducing oviposition. *Pseudomonas* strains Pf1, TDK1, and PY15 were evaluated for their efficacy against leaf folder pest in rice plants under field conditions individually and in combinations. The results showed that when all three isolates where used in combination, the effect on *Cnaphalocrocis medinalis* was highest. Bioassays conducted on the treated plants indicated an increase in PPO and LOX activity under glasshouse conditions. This therefore indicates that these biocontrol agents are able to enhance natural enemy populations and resistance mechanisms against *Cnaphalocrocis medinalis* attack. In addition bacterial strain mixtures of PGPR also have the capability to induce

chitinase in plants. Chitinase plays an important role in hydrolyzing chitin, the structural component in gut linings of insects, and would lead to better control insect pest (Broadway et al. 1998).

7.3 Endophytic PGPRs

It is assumed that endophytic organisms are better biocontrol agents compared to rhizospheric bacteria as they do not compete for nutrition and/or niche in the apoplast and are also more adapted to environmental influences (Compant et al. 2005). Rosenblueth and Romero (2005) reported the ability of endophytic *Streptomyces* spp. (AB131-1, AB131-2, and LBR02) to reduce *Xanthomonas oryzae* infections *in planta*. The study showed that *Streptomyces* spp. AB131-2 when inoculated on *Xanthomonas oryzae*-infected rice plants exhibited reduced severity of bacterial leaf blight (BLB). Further, Compant et al. (2005) stated that the induced systemic resistance (ISR) exhibited by these treated plants is influenced by the secondary metabolites produced by these endophytic bacteria living on plant tissues. The plant disease suppression mechanism by endophytic actinomycetes is presumably caused by the production of bioactive compounds which can act as antibiotics and/or function as cell wall-degrading enzymes. These components have been listed as determinants of ISR and SAR in plant systems against pathogen infiltration (El-Tarabily and Sivasithamparan 2006).

Su and colleagues (2013) had looked into the ability of a fluorescent protein-expressing dark septate endophyte (DSE), *Harpophora oryzae*, to potentially inhibit the growth and spread of *Magnaporthe oryzae*. The bioantagonistic potential of *Harpophora oryzae* was visualized through inoculation with eGFP-tagged *Magnaporthe oryzae* in rice. *Harpophora oryzae* appeared to protect rice from *Magnaporthe oryzae* root invasion by the accumulation of H_2O_2, elevated antioxidative capacity, and induced systemic resistance against rice blast. This systemic resistance was mediated by the OsWRKY45-dependent salicylic acid (SA) signaling pathway, as indicated by the strongly upregulated expression of OsWRKY45. Lenin and Jayanthi (2012) identified an endophytic *Fusarium* isolate that demonstrated good antagonistic potential against *Meloidogyne graminicola* by reducing gall formation and juvenile penetration by up to 50 %. In vitro experiments showed that secondary metabolites produced by this endophytic fungus caused nematode mortality, while the in vivo experiments demonstrated that the fungus interfered with nematode reproduction, thus reducing nematode populations in the root over time.

7.4 ISR in Rice System

Induced systemic resistance can be defined as the phenomenon by which plants exhibit increased level of resistance to broad spectrum of phytopathogens by the prior activation of genetically programmed defense pathways. In the following segments, we will look into the determinants in rice and the key pathways activated in response to these determinants which result in ISR. Rice, like *Arabidopsis*, is an excellent crop to utilize in the functional analysis of host-pathogen-pest interactions. Various nonpathogenic strains of rhizobacteria such as *Pseudomonas* spp., *Serratia* spp., *Bacillus* spp., and others have been used to study the inducing effect of these microbes on the host system. Colonization of rice roots by ISR-inducing bacterium is believed to be able to protect the plants against different types of pathogens, nematodes, and insects.

7.4.1 Role of ISR

Unlike pathogens that result in necrosis and death of plant tissue thus resulting in the activation of systemic acquired resistance (Cameron et al. 1994) in host, beneficial rhizobacteria induce systemic resistance without necrosis but the induction of immune response within the host plants (Van Loon et al. 1998). The mechanism of elicitation shows several similarities to the generation of certain nonspecific defense reactions in plant cells that occur in response to general

microbe-associated molecular patterns (MAMPs) (Newman et al. 2013). These beneficial microbes trigger ISR through secretion of products which are likely to be distinct from those found in the elicitor molecules of pathogens. These determinants are what triggers ISR in the host and results in a mounted defense against pests and pathogens. The determinants induced resistance through the activation of jasmonate-, ethylene-, or salicylic acid-dependent pathways by either acting individually or in combination. In the following section, we have compiled a series of entries that report the role of various types of determinants in the induction of ISR in rice. However there is comparatively little information on the bacterial determinants that trigger ISR in rice.

7.5 Determinants of ISR

Lipopolysaccharides (LPS) (Leeman et al. 1996, 1995a), siderophores (Leeman et al. 1996; Audenaert et al. 2002), salicylic acid (van Loon 1997; Choudhary and Johri 2009), cyclic lipopeptides (De Vleesschauwer et al. 2008; Van Loon et al. 2008), lytic enzymes (Compant et al. 2005; Someya et al. 2000), and exopolysaccharides (Haggag 2007) are some of the bacterial determinants that have been implicated in the induction of systemic resistance in host plants (specifically in rice). In most organisms multiple determinants are produced in the rhizosphere/phylloplane. Table 7.2 lists the various determinants that have been reported to elicit ISR in rice in response to treatment with specific PGPR.

7.5.1 Lipopolysaccharides

Lipopolysaccharides (LPS) are outer membrane-bound molecules in the Gram-negative bacteria that are recognized as microbe-associated molecular patterns (MAMPs) which trigger immune response in plants and animals (Newman et al. 2013). Leeman et al. (1996), implicated LPS of *P. fluorescens* strains WCS374 as the determinant responsible for induced systemic resistance against rice blast which somehow resulted in increased disease incidence of sheath blight (De Vleesschauwer et al. 2008). In iron-replete conditions, WCS374 was able to induce resistance against Fusarium wilt; however the reverse was observed in a mutant lacking the O-antigenic side

Table 7.2 Determinants produced by PGPR in rice

Bacterial species	Determinants
P. fluorescens strain Pf1	CWDE, antibiotics, siderophore, defense enzymes (PO, PPO, POX)
P. fluorescens Pf1+AH1+B2	CWDE, defense enzymes
P. fluorescens Pf1+TDK1+PY15	CWDE, defense enzymes
P. fluorescens strain FP7	CWDE, antibiotics, siderophore
P. fluorescens WCS374	LPS, pseudobactin (siderophore), SA
P. fluorescens CHAO	Siderophore
Pseudomonas 7NKS2	SA, pyocyanin (siderophore), CWDE
Pseudomonas RB04	pyocyanin (siderophore), CWDE
Pseudomonas putida	LPS, Siderophore, HCN, exopolysaccharides
P. aeruginosa	LPS, Siderophore, HCN, EPS
Pseudomonas EA105	Defense enzymes
B. subtilis GB03	SA
B. subtilis BBG111	CLP (surfactin and fengycin)
B. subtilis RFB104	CLP (surfactin and mycosubtilin)
Bacillus SE34	SA and defense enzymes
B. mucilaginosus SSB-8, SSB-11, SSB-17	Defense enzymes
B. amyloliquefaciens	LPS
B. thuringiensis	CWDE and toxins
B. licheniformis, *B. cereus*, *B. circulans*	CWDE
Rhizobium etli G12	Siderophore, CLP
S. plymuthica IC1270	SA, siderophore, defense enzymes, CWDE, HCN
S. marcescens B2	Defense enzymes, CWDE
Azospirillum	CWDE
Streptomyces spp.	CWDE
Paenibacillus	Siderophore, CWDE
Enterobacter agglomerans	CWDE

chain of LPS. When iron concentration was low, the LPS mutants became as effective as WCS374, pointing to a role for iron-regulated metabolites. LPS induced the production of active oxygen species (AOS), extracellular medium alkalinization (MA), elevation of cytoplasmic Ca^{2+} ($[Ca^{2+}]$ cyt), and defense-related gene expression (PAL and GST) within the host in response to the microbial agent (Van Loon et al. 2008).

From previous studies, it is believed that the O-antigen side chain of the LPS triggers the induction of defense mechanism in plants and that this antigenic factor is host-microbe dependent. This differential effectiveness of ISR inducibility could be attributed to strain-specific differences in LPS (Leeman et al. 1995a). In order to experimentally proof this hypothesis, a homology study was conducted to identify genes of the WCS strains involved in the biosynthesis of the highly variable O-antigens of LPS which was followed by a search for putative O-antigen biosynthetic loci (OBL). This lack of homology between the OBLs of WCS strains is likely to result in variation in the O-chain composition, which may causally be related to the observed host-microbe specificity of LPS in eliciting ISR in host plants such as rice (Raymond et al. 2002).

7.5.2 Siderophores

Siderophores are low molecular weight compounds produced by PGPR under iron-limiting conditions that are involved in the suppression of plant pathogens. In a dicot system WCS374r- was reported to produce multiple bacterial determinants such as salicylic acid, siderophore and LPS to elicit ISR (Djavaheri et al. 2012; Leeman et al. 1995b, 1996). This was however not necessarily the case with monocots and may vary among different plant species. Leeman et al. (1996) reported that in iron-replete conditions WCS374 produced pyoverdine-type pseudobactin siderophore as an ISR determinant and that these pyoverdines differentially triggered ISR in different plant species suggesting a difference in structure and a possibility of pyoverdine being part of the host-microbe recognition system (Bakker et al. 2007). Although the purified pseudobactin (Psb) of WCS374 did induce ISR, a Psb-mutant was as effective as the wild-type strain due to the presence of LPS. Such redundancy in ISR-eliciting determinants can make the ISR-eliciting activity of beneficial rhizobacteria more robust (Leeman et al. 1995a; Bakker et al. 2007). However, when pyoverdine (PVD) knockout mutants WCS358-PVD–, WCS417-PVD– (Marugg et al. 1985; Duijff et al. 1993), and WCS374-PVD– were studied, only WCS374-PVD– retained its siderophore activity (Duijff et al. 1993). Mercado-Blanco et al. (2001) identified a second siderophore in WCS374 as pseudomonine (PSM) which is composed of salicylic acid (SA), cyclothreonine, and histamine. In this study the pseudobactin-negative mutants did not result in resistance to *Magnaporthe oryzae*; however, the pseudomonine-deficient mutant 4A1 inhibited *Magnaporthe oryzae* through pseudobactin production (Berendsen et al. 2015).

Pseudomonas aeruginosa 7NSK2 effectively protected rice leaves against challenge infection with rice blast disease while increasing susceptibility to *Rhizoctonia solani* (De Vleesschauwer et al. 2006, 2009). The pyocyanin-deficient strains, 7NSK2-phzM and KMPCH-phzM, were tested for their ability to induce resistance to blast and sheath blight where the pyocyanin-deficient mutants triggered resistance to *Rhizoctonia solani*, whereas the same mutants lost their ability to mount ISR to *Magnaporthe oryzae* suggesting that the secretion of pyocyanin may be responsible for the differential effectiveness of 7NSK2-mediated ISR in these pathogens (Höfte 1993). Hence pyocyanin appeared to be the main metabolite responsible for induced resistance to blast, while there was no role for SA or pyochelin as the SA-deficient mutants were more efficient in eliciting resistance. Therefore it was presumed that SA/pyochelin and pyocyanin act synergistically in the monocot rice and that the presence of either one component was sufficient to induce resistance. Pyocyanin inhibited *Magnaporthe oryzae* by triggering microbursts of H_2O_2 that resulted in HR-associated cell death and consequently lead to breakdown of the biotrophic infection phase of the *Magnaporthe oryzae*. The

oxidative burst and hypersensitive response (HR) may act as a double-edged sword in the interaction with the hemibiotrophic and necrotrophic pathogens in rice. The dual role of pyocyanin in *Pseudomonas aeruginosa* 7NSK2-mediated ISR suggests that rice requires distinct mechanisms for defense against *Magnaporthe oryzae* and *Rhizoctonia solani* (Höfte and Bakker 2007). Similarly *Serratia plymuthica* IC1270 elicited ROS accumulation that subsequently prompted a HR response in cells of an incompatible rice-*Magnaporthe oryzae* interaction, suggesting that IC1270-mediated ISR and R-gene-mediated ETI in rice. In addition IC1270-induced ISR seems to play an ambivalent role where rice plants treated with these bacteria rendered hypersusceptibility to *Rhizoctonia solani* and *Cochliobolus miyabeanus* (De Vleesschauwer et al. 2009).

7.5.3 Lipopeptides

Cyclic lipopeptides (CLPs) produced by *Bacillus* strains are known to be involved in plant immunity as elicitors of ISR (Ongena and Jacques 2008; Raaijmakers et al. 2010). CLPs may differ in their mode of recognition and/or action in rice (monocot) compared to dicot plant species (Chandler et al. 2015). Bacillus lipopeptides are novel compounds that are perceived by plant cells to trigger a defense response. Even if some *Bacillus* strains are well equipped genetically to produce a vast array of antibiotics (Chen et al. 2008), only limited components may be expressed readily in the rhizosphere (Nihorimbere et al. 2012). The reduced oxygen levels in the rhizosphere appear to act as a stimulant to the production of the cyclic lipopeptide (Dubern et al. 2006; Nihorimbere et al. 2009).

Coutte et al. (2010) reported that *Bacillus subtilis* BBG111 and RFB104 produced different types of CLPs with BBG111 synthesizing surfactin and fengycin and RFB104 producing surfactin and mycosubtilin. Soil application of *Bacillus subtilis* BBG111 protected rice against sheath blight and not rice blast. In addition, the CLPs fengycin and surfactin of *Bacillus subtilis* targeted JA, ET, and/or auxin pathways, indicating that there exist multiple cross-talking ISR-resistant pathways in rice. Considering that JA, ET, ABA, and auxin have been implicated in activation of basal rice defenses against *Rhizoctonia solani* (De Vleesschauwer et al. 2010; Helliwell et al. 2013), BBG111 seems to switch on all hormone pathways required for basal sheath blight resistance. Moreover, given the often reported redundancy in bacterial traits operative in ISR and considering the differences in gene expression between supernatant- and CLP-treated rice cells, it should be noted that CLPs may not be the only factor contributing to the onset and/or maintenance of induced resistance by the *Bacillus* spp. (De Vleesschauwer and Höfte 2009).

7.5.4 Salicylic Acid

One other determinant that is produced by Pseudomonads is the compound salicylic acid (Leeman et al. 1996). The efficacy of ISR in monocots against necrotrophic pathogens has been demonstrated repeatedly but only in a few cases the defense signaling pathway was investigated. Rice is unique in a sense as it has high basal level of endogenous SA and these levels are not significantly elevated by external stimuli. One such study that validates this finding was conducted by De Vleesschauwer and colleagues (2008) who reported their findings with regards to the ISR induced by *Pseudomonas fluorescens* WCS374r against *Magnaporthe oryzae* in rice. In their study they found that ISR post treatment with WCS374r resulted in the induction of a jasmonic acid (JA)/ethylene (ET)-modulated signaling which was independent from SA signaling. Similar results were also seen when EA105 was applied to rice plants, where ISR was triggered through a mechanism that is dependent on JA and ET signaling that ultimately resulted in fewer blast lesions. Isolate EA105 and *Pseudomonas chlororaphis*, inhibited mycelial growth of *Magnaporthe oryzae* and almost completely halted appressoria formation in *Magnaporthe oryzae*. D5, a HCN mutant of EA105, showed similar antagonistic abilities against *Magnaporthe oryzae*, indicating a mechanism of action which

is independent of HCN. This study reported that EA105 and *Pantoea agglomerans* EA106 reduced the number of blast lesions as a result of root pretreatment with the bacteria. Isolate EA105 was the only isolate which was effective both as a direct antagonist to *Magnaporthe oryzae* as well as an elicitor of the ISR response in rice (Spence et al. 2014).

In rice, the JA-dependent pathway induces resistance against pathogen and pest (Zhou et al. 2009). However, some PGPR or PGPF induced an SA-dependent pathway effective against biotrophic pathogens (Muyanga et al. 2005; Molitor et al. 2011). In a study conducted by Saikia et al. (2006), isolates of *Pseudomonas aeruginosa* showed plant growth-promoting activity and induced systemic resistance in rice against *Rhizoctonia solani* G5 through elevated levels of salicylic acid in host. *Pseudomonas aeruginosa*-pretreated rice plants produced increased levels of peroxidases with antifungal activities against three phytopathogenic fungi; *Rhizoctonia solani*, *Pyricularia oryzae* and *Helminthosporium oryzae* (Saikia et al. 2006).

7.5.5 Exopolysaccharides

Some PGPRs have been known to produce EPS in the rhizosphere/phylloplane. The application of *Pseudomonas fluorescens* and *Azospirillum* as co-flocculants in a rice-*Pyricularia oryzae* interaction altered the biochemical and physiological parameters in rice. The amount of reducing and nonreducing sugars, total phenol content, and defense enzyme activities such as PO and PPO were elevated to a significant level posttreatment. The application as co-flocculants resulted in growth promotion as well as the induction of defense enzymes such as PO and PPO, and a reduction in reducing and nonreducing sugar level was recorded. This ultimately led to a reduction of *Pyricularia oryzae* disease incidence in lowland rice. The EPS biosynthesis of PGPR cells during co-flocculation processes is required to enhance ISR in rice-*Pyricularia oryzae* pathosystem as the application of vegetative cells alone resulted in poor enhancement of ISR in the same pathosystem. The application of EPS collected from *Azospirillum* isolates augmented the height of rice plant and reduced the blast disease incidence in upland rice to a higher level when compared to the application of ISR-inducing chemicals alone (Kalaiarasu and Vivekanandhan 2014). Similar findings were reported by Umashankari and Shekar (2011) in their experimentation with vegetative, co-inoculated, and co-aggregated application of *Pseudomonas fluorescens* (PF-3) and *Paenibacillus polymyxa* (B-19). The co-aggregate preparation of these cultures resulted in altered biochemical and physiological processes within the rice-*Pyricularia oryzae* system resulting in elevated resistance within the host caused primarily by EPS.

7.5.6 Cell Wall-Degrading Enzymes

Cell wall-degrading enzymes are one of the major mechanisms that have been effective against soilborne pathogens (Chet et al. 1990). Enzymes such as β-1,3-glucanase, chitinase, cellulase, and protease that are secreted by PGPR are able to exert an inhibitory effect on the hyphal growth of fungal pathogens. Hydrolytic enzymes such as chitinases and β-1,3-glucanase have been effective in solubilizing the chitin component of fungal cell walls through the linearization of β-1,4-N-acetylglucosamine polymers. One such example is the ability to lyse fungal cell walls of *Fusarium oxysporum* by glucanases synthesized by *Paenibacillus* and *Streptomyces* spp. β-1,3-glucanase secreted by *Bacillus cepacia* was also responsible for the degradation of cell walls of soilborne pathogen such as *Rhizoctonia solani*, *Pythium ultimum*, and *Sclerotium rolfsii* (Compant et al. 2005). Sadfi and colleagues (2001) had identified isolates of *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus circulans*, and *Bacillus thuringiensis* that exhibited great chitinolytic potential. According to Nelson and Sorenson (1999), in addition to Bacilli, Gram-negative bacteria such as *Serratia marcescens*, *Enterobacter agglomerans*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens* have also been reported as PGPRs with chitinolytic

activities. Someya et al. (2000) reported that *Serratia marcescens* B2 secreted chitinolytic and antifungal substances that were able to inhibit soilborne pathogens such as *Rhizoctonia solani* and *Fusarium oxysporum*. In this study the mycelia of the fungal pathogens was co-inoculated with *Serratia marcescens* B2, and microscopic observations showed that the bacterial exudate had resulted in abnormal hyphal and tip formation as well as compromised the pathogens cell wall integrity. In our laboratory we have shown that the chitinolytic activity exhibited by fungal biocontrol agents was able to affect the structural integrity of the walls of the target phytopathogens. In addition to affecting the structural integrity, these biocontrol agents also induced the expression of *PR-1b* posttreatment. This therefore indicates the ability of these biocontrol agents to trigger the defense pathways in the host and thus induce resistance and protection against phytopathogens (Nadarajah et al. 2014, 2015).

7.6 Pathways Induced by PGPR in Rice

The high basal levels of endogenous SA with no further elevation in response to pathogen infection do seem to bring to question the role of SA in the regulation of the defense response in rice (Silverman et al. 1995; Durrant and Dong 2004). In the recent year however, some evidence has arisen to support an active role for a BTH-inducible and WRKY45- or NPR1-regulated SA signaling pathway in the rice defense response (Shimono et al. 2007; Yuan et al. 2007). This new evidence has provided us with a new and evolved view that in spite of the high constitutive SA levels in rice, this host has managed to evolve an SA-mediated SAR pathway that is not much different from which is exhibited in *Arabidopsis*. In 2008, De Vleesschauwer and colleagues through their study of *Pseudomonas fluorescens* WCS374r-*Magnaporthe oryzae* interaction showed that an SA-independent pathway was triggered in response to treatment. ISR bioassays with SA-non-accumulating NahG plants (Yang et al. 2004), the ET-insensitive OsEIN2 antisense line 471 (Jun et al. 2004), and the JA biosynthesis mutant habiba (Riemann et al. 2003) revealed that WCS374r mediated an SA-independent ISR that requires the intact responsiveness to ETH as well as a functional JA pathway. As such the JA-responsive genes, JAR1 and WRKY30, are crucial players in JA signaling and stimulation of ISR in rice (Peng et al. 2012). In studying these genes function, it was observed that both genes were highly expressed in EA105 and EA106 but not in CHAO treated plants. Similarly two ethylene-responsive genes, EIL1 and ERF1, were also highly expressed posttreatment. These genes have been implicated in ISR signaling and are important candidates in the reduction of disease susceptibility (Nakano et al. 2006). EA105 shows parallels in its ability to trigger ETH signaling while minimally impacting SA signaling (Krishnamurthy and Gnanamanickam 1998).

SA- and BTH-responsive PR genes, *PR1b* and *PBZ1*, showed differential expression when quantitated via RT-PCR indicating that WCS374r elicits an SA-independent signaling route (Agrawal et al. 2001). The differential expression of *PR1b* and *PBZ1* in pseudobactin-induced plants is most likely the consequence rather than the cause of pseudobactin-increased blast resistance, hence supporting the notion that ISR triggered by pseudobactin in WCS374r follows an SA-independent pathway. Contrary to the above, chemical induction of blast resistance by the SA analog benzothiadiazole is independent of JA/ET signaling and involves SA-responsive gene expression. Taken together, these reinforce the evidence that rice is endowed with a BTH-inducible SAR-like resistance pathway (Shimono et al. 2007; Yuan et al. 2007) but also hint at a conserved mechanism for ISR signaling in rice and *Arabidopsis*. Unlike WCS374r-ISR, ISR against *Magnaporthe oryzae* by *Pseudomonas aeruginosa* 7NSK2 was SA dependent indicating that the signal transduction pathway governing rhizobacteria-mediated ISR against *Magnaporthe oryzae* was at least in part determined by the bacterium. However, the similarities observed between WCS374r- and WCS417r-activated ISR signaling in rice and *Arabidopsis*, respectively, support and substantiate the possibility that there

may exist ancient inducible defense pathways that are shared between monocots and dicots (Morris et al. 1998). This concept does not by any means rule out the possibility that these pathways, though conserved, may vary in their fine-tuning in a species-specific manner. Hence, although rice and *Arabidopsis* appear to share a conserved ISR pathway, the modulation of this JA-dependent resistance conduit may be quite divergent (Mei et al. 2006).

7.7 Bioinoculants in Rice

Certain organisms such as *Azospirillum*, *Pseudomonas*, and *Methylobacterium* that have often been encountered in the rhizosphere or phyllosphere of lowland rice have been developed for utilization as agricultural bioinoculants worldwide. Bioinoculants are basically prepared in an easy-to-use formulation that will enable farmers to utilize them effectively. This is an important aspect as the bioinoculant formulation is crucial in determining the potential success of these bioagents in nature (Bashan 1986a, b). The study conducted by van Veen et al. (1997) on efficiency of bioinoculants led them to conclude that instead of trying single strain with single trait, a microbial consortia was more efficient and yielded multiple benefits which includes the ability to uniquely colonize ecological niches at an ideal proportion.

In developing bioinoculants for rice and any other crop, it is important to select for certain characteristics such as higher degree of stress tolerance, longer shelf life, enhanced survivability in soils and seeds, and consistent plant response to inoculation (Neyra et al. 1999; Velagaleti and Marsh 1989). The physiological status of microorganisms is critical in the preparation of a bioinoculant rather than their cell number to ensure survival in carriers, soil, seed, colonization in the rhizosphere, and positive plant response to bioinoculation (Okon 1985). Some of the most promising research topics on novel agricultural bioinoculant technology would include looking into aspects of concoction, carrier, and delivery system (Olubayi et al. 1998). Neyra et al. (1995) proposed the use of flocculated cultures of *Azospirillum* as a novel delivery system and a new generation of agricultural bioinoculant, to ensure the better establishment and interaction of the inoculated microbial cells in plant rhizosphere. Here they described the effectiveness of *Azospirillum* biofloc and its adhesion to plant roots and the production of EPS-rich network which provided higher degree of stress tolerance and longer shelf life to the bioinocula. The mechanism of EPS-mediated bioflocculation of *Azospirillum* and *Methylobacterium* cells has already been reported by many authors (Ntsaluba et al. 2013; Sadasivan and Neyra 1985). Rubiya (2006) reported the positive influence of the developed multigeneric diazotrophic co-flocs system that consists of *Azospirillum*, *Azotobacter*, and *Rhizobium* on augmentation, growth and yield parameters, and induction of resistance in lowland rice. Likewise, Vaidehi (2012) reported that the EPS-mediated bioflocs of *Methylobacterium* cells had a positive role in stimulating plant growth and inducing ISR-mediated biocontrol against *Pyricularia oryzae* in lowland rice.

7.8 Constraints and Future Prospect of PGPR Utilization on Rice in the Asian Region

PGPRs have been studied extensively, and their ability to fulfill diverse beneficial interactions in plants is a promising solution for sustainable and environment-friendly agriculture. While the initial focus on these organisms was for its ability to promote growth and yield, they have now been exploited as a means to induce resistance in plants to its natural pathogens and pest. These belowground allies through the colonization of the rhizosphere and interaction with the plant root system have resulted in the induction of defense pathways through the exudates secreted and their interactions. Many of these strains produce a variety of determinants such as lipopeptides, lipopolysaccharide, siderophores, lytic enzymes, and exopolysaccharides (Faltin et al. 2004) that enable them to colonize widely diverse ecological niches. Hence these determinants have

been instrumental in the induction of systemic resistance leading toward the provision of broad-spectrum control in integrated crop management (Kumar et al. 2011). Various examples of PGPRs that have shown promise in pathogen-pest control in rice have been discussed in the above segments.

Despite their potential as low-input practical agents of plant protection, widespread application of PGPR strains as commercial biocontrol products has been hampered by several reasons such as the limited number of field tests, the formulation of the bacteria, and the emergence of certain strains as facultative pathogens. One major drawback of practical application of these strains is the inconsistency in the performance of biocontrol agents in the field, due to poor rhizosphere competency (Weller 1988). Some of these PGPRs have also been reported to be opportunistic pathogens to humans, and therefore clarification on strains and their effect on environment, animal, and human needs to be elaborated (Nakkeeran et al. 2006). This raises concerns over the efficacy, feasibility, and safety of these cultures. Therefore while these agents show promise in control of biotic and abiotic, steps need to be taken not to ignore the biosafety factor while looking into their potential in rice farming. The process of lab testing and field testing taking into account the threat factors should be executed before these are marketed in a large scale.

In order for PGPRs to be used efficiently and effectively in agricultural practices, the following areas of concerns need to be addressed. While research is conducted to determine the ability to antagonize and elicit a defense response in a host, researchers also need to look into the reliability and the authenticity of the selected agents. It is not enough to just study the inhibitory and inductive ability of the organism, but attention needs to be given to their interaction in their ecological niche and their effect on the community, host, pest, and pathogens. Most often the lack of knowledge and awareness on the biological agents and the intended targets as well as the environment results in not so promising results in field and the lack of sustainable resistance to the biotic factors over a period of time. In applying these agents, one needs to be aware of the multiplication factor, the proper delivery system, the possibility of mutation and loss of desirable traits, and various other intrinsic factors that could contribute to the drop in efficiency of a biocontrol agent. Most often the lack of knowledge of ecology of the introduced PGPR strains results in a serious impediment to the establishment and multiplication of the PGPR strains and also on the ability of these isolates to induce resistance in the treated crops. The interaction of the introduced strains with the native flora and fauna within the rice rhizosphere will also be a deciding factor in the success of the biocontrol agent. This remains one of the major issues in ensuring the effectiveness of the PGPRs as the microbial community within the root system may impede the effectiveness of an in vitro tested potent biocontrol agent. This is why while much research is done, the marketability and the sustainability of its efficiency is wavering.

Hence for PGPRs to be used successfully in any farming environment (including rice), research has to be directed on the host, the target pathogen-pest, the environment, and the biocontrol agents. All facets will influence the success rate of the biocontrol agents. Therefore there is a great need to continuously look into the identification of new biocontrol agents. In order to optimally utilize these organisms, it would be necessary to understand the molecular basis of their beneficial effects and the way these traits influence numerous biotic and abiotic factors. Our lack of knowledge in this area hinders our attempts to optimize the biological activity by employing tailored application strategies. The advent of molecular biology and biotechnology allows for a better understanding of rhizobacteria and therefore to provide a clearer view of rhizosphere colonization. Thus, biotechnology can be applied to improve the efficacy of PGPR strains through genetically engineering them to overexpress one or more traits so that strains can act synergistically. This may involve genomic tinkering of naturally occurring PGPR strains with effective genes (Nakkeeran et al. 2006) which could lead to accentuated expression of genomic products that reduces incidences of pests and

disease through introduction of a single bacterium with multiple modes of action to benefit the growers. Further optimization is required for better fermentation and formulation processes of effective PGPR strains to introduce in agriculture. More detailed studies are needed on the composition of the rhizosphere population, the effect of cultivar on bacterial population dynamics, the influence of inoculum density on antagonistic activity, the survival of inoculum under adverse conditions, and the role of environmental conditions in altering the activity of rhizobacteria. An attempt to overcome problems of varying efficacy may be attained by strain mixing, improved inoculation techniques, or gene transfer of active genetic source of antagonists to the host plant (Oostendorp and Sikora 1986, 1989). Thus, future success of industries producing microbial inoculants, especially PGPRs, will depend on innovative business management, product marketing, extension education, and extensive research in improving the inoculum, understanding their targets, and also in engineering their environment.

7.9 Conclusions

In this chapter, the role of PGPR in ISR of rice has been presented. From the literature available, it was possible for us to conclude that PGPRs act as a green technology in addressing issues such as yield, growth, and biotic and abiotic stresses in crops. Through the review of research done on the application of PGPRs in inducing systemic resistance in rice, we observe that some great strides have been made in identifying isolates that have the potential of controlling rice diseases and nematodes, while very few isolates have shown success against insect predators. Hence, we concur with Tikhonovich and Provorov (2011) who believed that this group of organisms holds promise in maintaining agricultural productivity while reducing the inputs of inorganic fertilizers, herbicides, and pesticides and that plant beneficial microbes are the way forward in these issues. It is seen from the compilation of works done on PGPRs role in rice ISR that some of these microbes have the ability to elicit broad-spectrum resistance against multiple diseases and also provide protection against insects and nematodes, while there are some which are less effective. These microbes sometimes use a highly redundant system of determinants to elicit ISR in the host. The redundancy of these determinants may be the contributing factor to a more effective induction of resistance in the host. Due to the level of basal SA that is higher in rice than most other crops, it has been reported that in rice the induction of resistance is mostly dependent on JA/ETH pathways. In recent years however, researchers have also implied the presence of an SA-dependent mode of defense within the PGPR-induced system in rice. Nevertheless the JA/ETH pathways remain the dominant pathways for regulation of defense response in rice.

Though tall claims have been made by researchers over the past several decades about the potential applications of a plethora of PGPR biocontrol agents in managing a number of disease and pests in many crop species, we should be careful to include the points of contention with the efficacy of this method and use these issues to build and improve on its application so that this group of organisms will achieve greater, better, and sustainable outputs in the years to come. This will fuel a better success rate at the commercialization of the isolates for application in rice fields. Concerted efforts will be required to demonstrate the benefits of the PGPR biocontrol agents to the farmers so that the eco-friendly agents can be popularized not just as a means to increase growth and yield but also as an agent that provides plant protection. Research and building on the knowledge in this area of study is absolutely essential in ensuring the buy-in by end users.

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8 Plant Growth-Promoting Rhizobacteria-Mediated Acquired Systemic Resistance in Plants Against Pests and Diseases

S.K. Singh, Rakesh Pathak, and Vipin Choudhary

Abstract

Plant growth-promoting rhizobacteria (PGPR) are indispensable part of rhizosphere microbiota that grow in association with the host plants and stimulate the plant growth. PGPR microcosm establishes in soil ecosystem because of its adaptability in varied environments with faster growth rate and biochemical versatility. In recent years researches have emphasised the key role of PGPR in improving nutrition and productivity of important crops with therapeutic and industrial significance. Hence, therefore, the present chapter highlights PGPR-mediated acquired systemic resistance against phytopathogens and insect pests involving mechanisms of action. Field applications of PGPR-mediated results reflect their substantial role in inducing systemic resistance in crop plants.

8.1 Introduction

It is well known that plant roots provide an avenue for the proliferation of soil bacteria that thrive on the exudates and lysates of root. The area surrounding plant root, so-called rhizosphere, has 100-fold higher population densities of bacteria as compared to bulk soil. There are a number of microcolonies with diverse bacterial strains in the soil (Ferrari et al. 2005). The bacteria secrete metabolites in the rhizosphere that act as signalling compounds whereby they make an association with host root system and form symbiosis. The *Rhizobium*-legume symbiosis is a typical example of signal exchange wherein plant releases flavonoids that act as signal for the bacterium to secrete Nod factors. The Nod factor induces root nodules in which the *Rhizobium* can fix atmospheric nitrogen. The bacterium grows on the cost of carbohydrates of the host plant and provides in return nitrogen for amino acid biosynthesis in plants (Datta et al. 2015). Kloepper and Schroth (1978) introduced the term rhizobacteria to the soil bacterial community that colonise plant roots competitively and stimulate growth

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and reduce the incidence of plant diseases and later termed these benign rhizobacteria as plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth 1981). PGPR has been considered as an indispensable functional moiety of rhizosphere flora that stimulates the growth of the host and establishes in soil ecosystem per se due to higher adaptability in a wide variety of environments, faster growth rate and biochemical versatility. Presently, the bacterial strains that fulfil at least two of the three criteria, i.e. aggressive colonisation, plant growth stimulation and biocontrol, are considered as PGPRs (Vessey 2003). Based on occurrence, association with plant root and functional attributes, PGPRs can be classified into extracellular plant growth-promoting and intracellular plant growth-promoting rhizobacteria (Martinez-Viveros et al. 2010). The functionality of PGPRs relies on the release of enzymes (dehydrogenase, phosphatase, nitrogenase, etc.), metabolites (siderophores, antifungals, Hydrogen cyanide (HCN), etc.) and growth promoters (IAA, ethylene) and as inducers of systemic disease resistance has been widely studied (Teixeira et al. 2007; Singh et al. 2013). The PGPRs are grouped in two parts based on their involvement in the nutrient cycling and plant growth stimulation, i.e. bio-fertilisers (Vessey 2003), and on their involvement in the biological control of plant pathogens, i.e. biopesticides (Whipps 2001). Though the research on PGPR-mediated disease resistance originated several decades ago, its effectiveness has been demonstrated under field conditions only in the 1990s. The occurrence of ISR has been established in various plants through different species of rhizobacteria (Pineda et al. 2013). The present chapter highlights PGPR-mediated acquired systemic resistance against plant pathogens and insect pests involving mechanisms and determinants/traits of PGPR in determining the ISR in crop plants.

8.2 Disease Suppression by PGPRs

The PGPRs have shown capability to reduce the activity of pathogenic microorganisms and induce acquired defence in the host plant through induced systemic resistance (ISR)-mediated mechanisms (Gupta et al. 2015). Upon treatment with *Pseudomonas fluorescens*, carnation was systemically protected against *Fusarium oxysporum* f. sp. *dianthi* through ISR (Van Peer et al. 1991). The most reported strains of PGPRs are from *Pseudomonas* spp. that enhance plant growth and protect them from plant pathogens in various crops, viz. cucumber, radish, tomato, sugarcane and rice (Liu et al. 1995). The biological agent secretes effective enzymes that involved in biosynthetic pathways of secondary metabolites and are related to the defence responses of the plants against pathogenic agents (Kloepper 1993; Chen et al. 2000). PGPRs may influence the growth of plants directly/indirectly and produce antagonistic substances that eliminate specific harmful microbes from the vicinity of roots and provide protection against pathogens through ISR (Pierson and Thomashow 1992; Weller et al. 2002). Among mechanisms involved in ISR, antibiosis, parasitism and competition for nutrients play important role against phytopathogens (Podile and Kishore 2006). Elaborately ISR involves (i) the inhibition of microbial growth by diffusible antibiotics and volatile organic compounds (VOCs); (ii) toxins and antibiosis; (iii) competition for minerals, e.g. for iron through production of siderophores or efficient siderophore uptake systems; (iv) degradation of pathogenicity factors of the pathogen such as toxins; and (v) parasitism that may involve production of extracellular cell wall-degrading enzymes such as chitinases and β-1,3-glucanase (Raaijmakers et al. 2006; Kamal et al. 2009). ISR represent a state of enhanced persistence of the plant that depends on the signalling compounds jasmonic acid and salicylic acid (Conrath et al. 2001; Van Loon 2007).

8.3 Mechanisms of Acquired Disease Resistance by PGPR Mediation

The PGPRs induced changes in the physiological and biochemical parameters of the host plant that lead synthesis of various defence chemicals against the invading pathogen and fortify the physical and mechanical strength of the cell wall.

8.3.1 Structural Modifications in the Cell Wall

The cell wall of a plant builds a line of defence for protection against the spread of a pathogen, and PGPRs may induce structural modification in response to pathogenic attack (Benhamou et al. 1996, 1998; M'Piga et al. 1997). Seed priming with PGPR in bean induces the lignification of cell wall followed by thickening of cortical cell wall and accumulation of phenolic compounds at the site of pathogen attack (Anderson and Guerra 1985; M'Piga et al. 1997; Duijff et al. 1994). M'Piga et al. (1997) reported cell wall thickening, deposition of phenolic compounds and formation of callose restricted growth of *F. oxysporum* f. sp. *radicis-lycopersici* to the epidermal cell and outer cortex of the root system in the treated plants of tomato.

8.3.2 Biochemical and Physiological Changes

The PGPR-mediated ISR incurred in plants that lead biochemical/physiological changes with induced accumulation of pathogenesis-related proteins (PR-proteins) and synthesis of phytoalexin and other secondary metabolites (M'Piga et al. 1997; Park and Kloepper 2000; Chen et al. 2000). Among microbial determinants, rise in defence enzyme, namely, phenylalanine ammonia-lyase (PAL), peroxidase (POX) and polyphenol oxidase (PPO) activities, has been observed in plants treated with PGPRs (Dutta et al. 2008). An early induction of POX, PAL and chitinase has also been reported with the rice seeds treated with *P. fluorescens* (Nandakumar et al. 2001). Similarly, Sivakumar and Sharma (2003) reported that PAL, POX and PPO activities were higher in plants mediated through seed priming with *P. fluorescens* as compared to the increase in pathogen-inoculated plants. Upon challenge inoculation tomato, hot pepper and pigeon pea showed increased activities of POX and PPO that treated with fluorescent pseudomonads (Ramamoorthy et al. 2001; Dutta et al. 2008). *Bacillus* enhanced the levels of total phenols, PAL, POX and lipoxygenase in the bacterised seedlings, indicating the involvement of ISR in PGPR-mediated disease control (Sailaja et al. 1997). PGPRs are known to increase auxin in host plants (Vacheron et al. 2013) and in turn induce LOX (Xie et al. 2015). The volatile organic compounds secreted by *B. subtilis* and *B. amyloliquefaciens* induce ISR in *Arabidopsis* against *Erwinia carotovora* (Ryan et al. 2001) and play important role in ISR (Ping and Bolland 2004; Ryu et al. 2004). Upon interaction with PGPR, the systemic resistance acquired in plant is mainly associated with the cell wall modification followed by the biochemical changes. The defence mechanisms induced against insect pests are different from that of pathogens wherein PGPRs do not kill insects, but their application brings some physiological changes in the host plant that prevents the insects from feeding. Zehnder et al. (1997) reported a shift in the metabolic pathway in cucumber plants from the cucurbitacin synthesis due to PGPR treatment and observed fewer beetle attacks. In nematode control, PGPRs induce resistance by altering root exudates or inducing the host to produce repellents (Oostendorp and Sikora 1990) and altering the syncytial development or sex ratio in the root tissue (Wyss 1989).

8.4 Acquired Disease Resistance by PGPR-Mediated Plants

PGPR-mediated acquired disease resistance in plants against fungi, bacteria, viruses, insects, and nematodes has been reported by several groups of researches (Liu et al. 1995; Maurhofer et al. 1998; Sikora 1988; Zehnder et al. 1997). Several PGPRs have been shown to initiate ISR by *B. subtilis* (Ryan et al. 2001), salicylic acid (SA), jasmonate and ethylene (Pettersson and Baath 2004), thickening of cortical cell wall (Duijff et al. 1994), accumulation of phenolic compounds at the site of pathogen attack (M'Piga et al. 1997) and pathogenesis-related proteins (Park and Kloepper 2000).

8.4.1 Diseases

PGPRs show direct/indirect mechanisms for plant growth promotion and activate systemically the latent defence mechanisms and suppress the deleterious microflora (Hammerschmidt and Kuc 1995; Lugtenberg et al. 2001). Among mechanisms involved, PGPR produces different competition strategies for nutrients and space (Raaijmakers et al. 2009), antibiotics (Babalola 2010), siderophores (Pathma et al. 2011), lytic enzymes present in the cell wall of fungi (Mansoor et al. 2007), HCN (Josˇic' et al. 2012), degradation of toxin produced by pathogen (Compant et al. 2005) and secretion of VOCs (Ping and Bolland 2004). Besides this, developmental escape, physiological tolerance, microbial antagonisms in the rhizosphere, biochemical resistance, induction of phytoalexins, induction of pathogenesis-related proteins and priming of defence responses also play important role in the acquired systemic resistance. There are several reports wherein researches found role of different elicitors in conjunction with acquired resistance through production of siderophores (Burd et al. 2000), β-1,3-glucanase (Glick and Pasternak 2003), antibiotics (Chaiharn et al. 2008), chitinase (Budi et al. 2000) and hydrogen cyanide (Bhatia et al. 2005). This antagonism has widely been exploited towards the management of plant diseases (Haas and Defago 2005). PGPRs produce siderophore and may contribute to the disease suppression in the treated plants (Yeole and Dube 2000). Siderophores produced by *Pseudomonas* exert killing effect on the plant deleterious fungi *F. oxysporum* and *A. flavus* infecting wheat (Manwar et al. 2000). Fluorescent pseudomonads produce pseudobactin (PSB)-type siderophores (Jurkevitch et al. 1993) and are reported to produce siderophore pseudomonine in addition to the fluorescent pseudobactin type (Mercado-Banco et al. 2001). Bakker et al. (2003) found direct effects of antibiotics on plants and suggested the role of ISR through it. Several antibiotics have been identified to be produced by pseudomonads (Nielsen et al. 2002; de Souza et al. 2003). Rhizobacteria inhibited phytopathogens by the production of HCN and/or fungal cell wall-degrading enzymes, e.g. chitinase and β-1, 3-glucanase (Persello Cartieaux et al. 2003; Van Loon and Bakker 2005). HCN is produced by many rhizobacteria and is postulated to play a role in biological control of pathogens (Defago and Haas 1990). The *Pseudomonas* strain RRS1 isolated from Rajnigandha produced HCN. *P. fluorescens* strain suppresses the disease caused by *F. oxysporum* f. sp. *radicis-lycopersici* in tomato with the help of release of HCN (Duffy et al. 2003). In addition, de Werra et al. (2009) studied the role of gluconic acid production in the regulation of biocontrol traits of *P. fluorescens* and reported close association of gluconic acid metabolism with antagonistic activity against plant pathogens. Maksimov et al. (2011) reported that *Bacillus* and *Pseudomonas* sp. inhibited growth and development of filamentous fungi by secreting chitinases and glucanase and were considered as an alternative to chemical crop protectors. Van Peer et al. (1991) applied *Pseudomonas* sp. in carnation and protected plants systemically against *Fusarium* wilt caused by *F. oxysporum* f. sp. *dianthi*. PGPR strains applied as a seed treatment resulted in a significant reduction in anthracnose disease caused by *Colletotrichum orbiculare* (Wei et al. 1996) and *Fusarium* wilt of cucumber stimulated by *F. oxysporum* f. sp. *cucumerinum* (Liu et al. 1995). The induction of systemic resistance by the *Pseudomonas* strains was demonstrated in bean, carnation, rice and cucumber (Alstrom 1991; Wei et al. 1991; Nandakumar et al. 2001). These strains of *Pseudomonas* spp. were found to induce resistance against different pathogens in cucumber (Wei et al. 1991) and radish (Hoffland et al. 1996). The induction of defence genes against various pathogens in different hosts has been well documented (Anand et al. 2007; Ganeshmoorthi et al. 2008). Vidhyasekaran and Muthamilan (1999) reported higher induction of ISR against the sheath blight pathogen, *Rhizoctonia solani*, in rice seed treatment with *P. fluorescens* strains followed by root dipping and a foliar spray. Similarly, PGPR-mediated ISR against *Colletotrichum falcatum* causing red rot disease has been established in sugarcane (Viswanathan and Samiyappan 1999). Induction of systemic resistance by *P. putida* and

Serratia marcescens has been investigated against *Fusarium* wilt of cucumber (Kloepper et al. 1993; Liu et al. 1995). Similar investigations on the treatment of cucumber seeds against angular leaf spot disease caused by *P. syringae* pv. *lachrymans*, with a large number of PGPR strains such as *P. putida*, *Flavimonas oryzihabitans*, *S. marcescens* and *Bacillus pumilus*, have been reported (Wei et al. 1996). The enhanced defensive capacity in plants against broad-spectrum foliar pathogens by *P. fluorescens* has been studied (Pieterse et al. 2001).

PGPR can also induce systemic protection against bacterial diseases. Seed treated with *P. fluorescens* strain protected beans against halo blight disease caused by *P. syringae* pv. *phaseolicola* (Alstrom 1991), while treatment of cucumber seed with *P. putida* strain and *S. marcescens* strain decreased the incidence of bacterial wilt disease (Kloepper et al. 1993). Similarly seed treatment of cucumber with *P. putida* strain, *Flavimonas oryzihabitans* strain, *S. marcescens* strain and *Bacillus pumilus* strain provided systemic protection against angular leaf spot caused by *P. syringae* pv. *lachrymans* by reducing total lesion diameter compared with nontreated plants (Liu et al. 1995; Wei et al. 1996). Vanitha and Umesha (2011) used *P. fluorescens* as biological control agent against bacterial wilt disease caused by *Ralstonia solanacearum* and reported increased activities of phenylalanine ammonia-lyase (PAL), guaiacol peroxidase (POX), polyphenol oxidase (PPO) and lipoxygenase (LOX) in *P. fluorescens*-pretreated tomato seedlings. Alstrom (1991) observed induced systemic protection of PGPR against the halo blight disease caused by *P. syringae* pv. *phaseolicola* and reported that the bean seeds when treated with *P. fluorescens* protected the plant from the bacterial disease.

Induction of systemic resistance by PGPR against viral diseases has been reported in various plants. Seed treatment with *P. fluorescens* and *S. marcescens* strains has consistently reduced the number of cucumber mosaic virus-infected plants and delayed the development of symptoms in cucumber and tomato (Raupach et al. 1996). Soil application of *P. fluorescens* strain has induced systemic protection against inoculation with tobacco necrosis virus in tobacco (Maurhofer et al. 1998). Induction of systemic disease resistance in faba bean (*Vicia faba* L.) against bean yellow mosaic potyvirus via seed bacterisation with *P. fluorescens* and *Rhizobium leguminosarum* has been investigated by Elbadry et al. (2006). *P. fluorescens* could stimulate systemic disease resistance in plants through a variety of signal translocation pathways, i.e. SA-independent JA-ethylene-dependent signalling, ISR-related gene expression, NPR 1-dependent signalling, etc. (Pieterse et al. 2001). Stimulation of resistance by PGPR strains has been demonstrated on tomato, bell pepper, muskmelon, watermelon, sugar beet, tobacco and cucumber through the activation of various defence-related enzymes like chitinases, β-1,3-glucanase, PO, PAL and PPO (Bharathi 2004). The interactions between *Bacillus* spp. and plants have been studied (Choudhary and Johri 2009) with special reference to induced systemic disease resistance. Various strains of *Bacillus*, viz. *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides* and *B. sphaericus*, are presently recorded to reduce the disease incidence on diversity of hosts (Ryu et al. 2004).

8.4.2 Insect Pests

Reports on PGPR-mediated ISR against insects are restricted to very few crops. It has been described that fluorescent pseudomonads may influence the growth and development of insects at all stages of their growth wherein *P. maltophilia* affects the growth of larval stage of the corn earworm, *Helicoverpa zea*, leading to more than 60 % reduction in adult emergence (Bong and Sikorowski 1991). Similarly, the relative growth rate, consumption rate and digestibility of feed by *H. armigera* were affected when larvae fed on cotton plants treated with *P. gladioli* (Qingwen et al. 1998). Induction of systemic resistance by PGPR strains, viz. *P. putida*, *S. marcescens*, *Flavimonas oryzihabitans* and *Bacillus pumilus*, has significantly reduced populations of the striped cucumber beetle *Acalymma*

vittatum and the spotted cucumber beetle *Diabrotica undecimpunctata howardi* on cucumber (Zehnder et al. 1997). Attempts have been made to transfer the insecticidal crystal protein from *B. thuringiensis* to *P. fluorescens* against lepidopteran insect pests. Transgenic *P. cepacia* strain has consistently shown insecticidal activity with the crystal protein gene against tobacco hornworm (Stock et al. 1990).

8.4.3 Nematodes

PGPR also induces systemic resistance against nematode pests (Oostendorp and Sikora 1990; Sikora and Hoffmann-Hergarten 1992). Oostendorp and Sikora (1990) induced *P. fluorescens* in sugar beet and found inhibited early root penetration of the cyst nematode, i.e. *Heterodera schachtii*. Similarly, *B. subtilis* has induced protection against *Meloidogyne incognita* and *M. arenaria* in cotton (Sikora 1988). The use of PGPRs as biological control agents of plant parasitic nematodes especially for sugar beet and potato cyst nematode has been reported as a successful strategy in the management of these nematodes (Sikora 1992). Treatment of rice seed with PGPR alone or in combination with chitin and neem cake has reduced the root and soil population of the rice root nematode, *Hirschmanniella oryzae* (Swarnakumari and Lakshmanan 1999). The application of the bacterium *P. chitinolytica* reduced the root-knot nematode infection in tomato crop (Spiegel et al. 1991), and the level of infestation of root-knot nematode was reduced with fewer galls and egg masses in the soil following root dipping with *P. fluorescens* strain (Santhi and Sivakumar 1995). Thus PGPR-mediated ISR is effective both in dicotyledonous plants, viz. *Arabidopsis*, bean, carnation, cucumber, radish, tobacco and tomato, and certain monocotyledonous plants, viz. rice, maize and sugarcane.

8.5 Constraints

The constraints in using PGPRs can be summarised as follows:

- The interaction between associative PGPR and plant is not always stable.
- Registration and marketing of PGPR products are one of the major constraints.
- Different cultures and plant species produce different types of root exudates which may or may not support PGPRs.
- Lack of consistent response in different host cultivars is also a restriction with PGPR products.
- Dry powder-based commercial formulations often lack appropriate shelf life and cell viability.

8.6 Opportunities

There are opportunities to develop, explore and exploit PGPRs with the advent and access to modern biotechnological tools and techniques. Some of the opportunities are the following:

- Stable formulations of PGPRs in sustainable agricultural system as a substitute of chemical fertilisers
- Eco-friendly biopesticides
- Multi-strain of PGPR with several modes of action and pathogens to increase crop production and health
- The application of molecular tools to understand and manage the rhizosphere leading to new products and effectiveness of PGPRs
- Improvement of efficient PGPR strains by creating transgenic that combine multiple mechanisms of action
- Mixture of PGPR strains, i.e. bacteria with bacteria or bacteria with fungi, to suppress phytopathogens with broader spectrum of microbial weapons

8.7 Conclusion

PGPR is a group of naturally occurring soil bacteria dwelling on the root surface which are directly or indirectly involved in the plant growth and development. The beneficial effects of PGPR include growth promotion, biological control and inducing systemic resistance in the host plants. In

addition to disease suppression against multiple pathogens, PGPR reduces the insect and nematode damage. The PGPR strains alone or in combination can provide an effective, economical and practical way of plant protection against multiple pathogens and pests.

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Acyl Homoserine Lactone-Producing Rhizobacteria Elicit Systemic Resistance in Plants

9

Ganga Viswanath, Jegan Sekar, and Prabavathy V. R

Abstract

N-acyl homoserine lactone (AHLs) produced by bacteria play a unique role in altering the expression of plant defence genes. AHL signals are constitutively produced by the vast majority of the rhizosphere and other groups of bacteria; and also varied levels of plant response are elicited through different types of AHL signals. Moreover, the defence mechanism of AHL-induced ISR is distinct from other bacterial compound-mediated plant response. It was also evident that the response of plants to bacterial AHLs may depend on plant species and chemical structure of AHLs. However, the question of how plants perceive the AHLs and distinguish between those molecules remains open. To date, no information is available either about plant AHL receptors or how plant cells can incorporate AHL signal molecules. Even though plants produce compounds similar to AHL signals, the precise source, structure and biological significance of these AHL mimics from plants are currently unknown. The specificity of plant mimics to stimulate or inhibit different types of AHL signals needs to be addressed. A thorough understanding of how plants perceive and respond to AHLs needs to be investigated. Copious questions remain to be addressed for the better understanding of quorum sensing of bacteria and trans-kingdom interactions of AHLs with plant cells.

9.1 Introduction

Rhizosphere microbiome influence plant growth and development; therefore, a collaborative action is essential for establishment of an efficient plant-bacteria interaction. Bacteria employ a variety of chemical molecules as their signals for communication across interspecies, intraspecies

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and intra-kingdom (Atkinson and Williams 2009). The bacterial communication is mediated by the exchange of small extracellular chemical signals which influence bacterial gene expression and physiological behaviour in a density-dependent signalling mechanism termed quorum sensing (QS). Among them, the best-studied QS mechanisms are from Gram-negative Proteobacteria, which use distinct group of biologically active metabolites, namely, *N*-acyl homoserine lactone (AHL) autoinducers as signal molecules (Swift et al. 1999; Whitehead et al. 2001). The QS-controlled phenotypes play a vital role for successful bacteria-host interactions, whether symbiotic or pathogenic (Boyer and Wisniewski-Dye 2009). The ecological distribution of AHL producers in natural environments and their potential roles have attracted much attention, and hence the diversity and distribution of AHL producers have been explored in different eco-niches (Cha et al. 1998; Huang et al. 2013; Lv et al. 2013; Viswanath et al. 2015), especially the rhizosphere regions which were reported to harbour high AHL population (DeAngelis et al. 2008; Elasri et al. 2001; Viswanath et al. 2015). The rhizosphere-associated AHL producers play a crucial role in plant health and growth and influence phenotypes such as root colonization and induction of systemic resistance in plants (Hartmann et al. 2004, 2014; Pang et al. 2009). Despite the intense study of AHL signalling in biocontrol bacteria, namely, *Pseudomonas* spp. (Wood et al. 1997; Chin-A-Woeng et al. 2001; De Maeyer et al. 2011), *Rhizobium* spp. (Wisniewski-Dye and Downie 2002) and *Serratia* spp. (Van Houdt et al. 2007), there is only limited information on AHL-dependent regulation in other beneficial plant-associated rhizobacteria. The ability of plant growth-promoting rhizobacteria (PGPRs) such as *Pseudomonas*, *Serratia*, *Bacillus* and non-pathogenic *Fusarium oxysporum* has been reported to promote plant health mediated through induced systemic resistance (ISR) (Kloepper et al. 2004; De Vleesschauwer and Hofte 2009). Similarly AHL-producing PGPRs triggered induced systemic resistance which had profound effect on the modulation of plant development and defence activity (Hartmann et al. 2014; Schenk and Schikora 2015). The AHL signalling molecules elicit plant response by systemic induction of defence gene expression especially against biotic stress (Schuhegger et al. 2006). To date, reports on diversity of AHL producers among PGPR and AHL-elicited ISR response in multiple plant species are limited. This chapter discusses the current status on the diversity of AHL bacterial communities associated with plant rhizosphere and the mechanism of AHL-elicited ISR-mediated defence response during plant-microbe interaction.

9.2 Induced Systemic Resistance in Plants

Plants develop local defence response due to colonization of beneficial bacteria or infection by pathogenic bacteria, which triggers immunity by the recognition of microbe-associated molecular patterns (MAMP) or effector proteins, resulting in systemic resistance. The two best understood mechanisms of systemic resistance are systemic acquired resistance (SAR) and induced systemic resistance (ISR). The SAR-mediated induced resistance is acquired upon local induction by a pathogen, whereas ISR is triggered by plant-associated beneficial microbes (Berendsen et al. 2012; Pieterse et al. 2014; Schenk et al. 2014). In both the systems, plants activate an elaborate matrix of signal transduction pathways via phytohormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) which act as key signalling molecules.

Induced systemic resistance (ISR) of plants against pathogens has been intensively investigated with respect to the underlying signalling pathways involved in defence response as well as its potential use in plant protection (Choudhary et al. 2007; Heil and Bostock 2002). In plants, ISR defence response is elicited by diverse bacterial determinants including bacterial surface components (flagellin, lipopolysaccharides and exopolysaccharides), volatile organic compounds (acetoin and 2,3-butanediol) and bacterial secondary metabolites (2,4-diacetylphloroglucinol

(DAPG) and pyocyanin) (De Vleesschauwer and Hofte 2009; Kloepper et al. 2004; Ryu et al. 2004). The triggered ISR activates defence response through various mechanisms, viz. induction of pathogenesis-related proteins (PRP), phytoalexins, cell wall reinforcement and priming defence responses (Beckers et al. 2009; Ryu et al. 2003; Slaughter et al. 2012; Van Wees et al. 2008).

9.3 *N*-Acyl Homoserine Lactone (AHL)

Bacteria use small chemical molecules to synchronize gene regulation within a population in a process called quorum sensing (Bassler 1999). The AHLs are the most common signal molecules used exclusively by Gram-negative bacteria. These molecules are composed of a fatty acyl chain ligated to a lactonized homoserine through an amide band. The length of the acyl side chain ranges from 4 to 18 carbon atoms, and based on the length of the acyl groups, AHLs can be broadly classified as short- or long-chain molecules. Short-chain AHLs have 4–8 carbon atoms in the acyl moiety, while long-chain AHLs have 10–18 carbons. The acyl side group can be substituted with an oxo or hydroxyl group at position C3 which confers signal specificity (Fuqua and Greenberg 2002; Thiel et al. 2009; Waters et al. 2008; Waters and Bassler 2005). Short-chain AHLs are believed to freely diffuse across the cell membrane, whereas AHLs with longer acyl side chains require multidrug efflux pump for transportation (Kaplan and Greenberg 1985; Pearson et al. 1999; Whitehead et al. 2001).

The canonical AHL QS involves two regulatory genes, a *luxI* family of AHL synthase genes and a *luxR* family of AHL-responsive transcriptional regulatory genes. Homologous to *luxI/luxR* QS system have been described in several Gram-negative bacteria, although the AHLs produced by the LuxI homologues as well as the genes regulated by them vary at the species or strain level (Whitehead et al. 2001).

The genes encoding these two proteins are often located adjacent to one another on the chromosome in almost all the AHL-producing proteobacteria (Fuqua et al. 1996; Churchill and Chen 2011; Gelencser et al. 2012). The LuxI proteins synthesize AHL signal molecules using the substrate *S*-adenosyl methionine for the backbone lactone ring, and acylated carbon chain from fatty acid biosynthesis pathway (Schaefer et al. 1996). LuxR-like proteins are transcriptional regulators which recognize the cognate AHL signals and mediate either activation or repression of QS-dependent gene expression (Fuqua and Winans 1994; Fuqua et al.1996). Also, the activated LuxR proteins up regulate *luxI* transcription and enhance the rate of AHL synthesis (Fuqua et al. 1996, 2001; Case et al. 2008). The recognition of bacterial AHL receptors to their corresponding AHL signals is highly specific, and hence the AHLs are classified as intraspecies signals among the proteobacteria (Huse and Whiteley 2011; Taga and Bassler 2003). A generalized scheme for an AHL quorum-sensing circuit in a bacterial cell is shown in Fig. 9.1.

9.4 Diversity of AHL-Producing Rhizosphere Bacterial Communities

The rhizosphere habitat provides a favourable environment for QS signalling since it contains significantly higher densities of microorganisms. A wide range of plant-associated PGPR, symbiotic, endophytic, epiphytic and pathogenic bacteria regulate their physiological functions through AHL signals (Ortiz-Castro et al. 2009; Venturi and Fuqua 2013). Recent studies indicate that AHL-based QS is highly prevalent in rhizosphere and endophytic communities of plants (Schaefer et al. 2013). The diversity of AHL-producing bacteria in the rhizosphere-associated bacterial communities of different plant species has been extensively studied and was represented only by the proteobacteria. In general, the proteobacteria group constitutes an estimated two thirds of

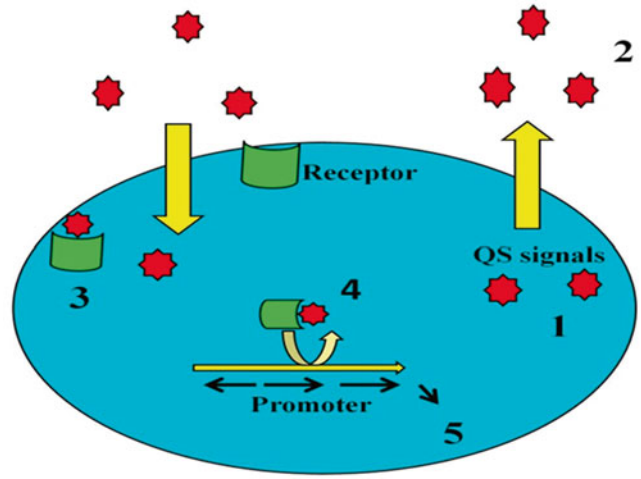

Fig. 9.1 Schematic representation of quorum-sensing mechanism. QS process: *1* synthesis of signal molecules, *2* diffusion of signal molecules, *3* signal recognition by receptor, *4* binding of signal receptor complex to promoter and *5* expression or repression of target genes (Source: Viswanath 2015)

many temperate plant rhizospheres (Hawkes et al. 2007). AHL-producing proteobacteria have been found to be more common in the rhizosphere than bulk soil (Cha et al. 1998; Elasri et al. 2001; d'Angelo-Picard et al. 2005).

In addition, several endophytic and epiphytic bacteria are also known to produce AHLs; however QS-dependent behaviours are poorly understood in these bacterial groups (Lv et al. 2013; Schaefer et al. 2013). The AHL-producing rhizobacteria were represented by α-, β- and γ-proteobacteria, isolated from the rhizospheres of tobacco (d'Angelo-Picard et al. 2005), potato, strawberry, oilseed rape (Berg et al. 2002), tomato (Steidle et al. 2001), wild oats (De Angelis et al. 2008), wheat (Pierson et al. 1998), cottonwoods (Schaefer et al. 2013), citrus (Trivedi et al. 2011), paddy (Steindler et al. 2008; Vial et al. 2006), cocoyam (De Maeyer et al. 2011), finger millet (Sekar and Prabavathy 2014), mangrove (Viswanath et al. 2015) and wetland plants (Zeng et al. 2014). The majority of the AHL-producing isolates from the plant rhizospheres belonged to the genera *Pseudomonas*, *Rhizobium*, *Serratia*, *Burkholderia*, *Erwinia* and *Pantoea* (Cha et al. 1998; d'Angelo-Picard 2005; Viswanath et al. 2015). The diversity of AHL-producing rhizobacteria was found to be ecological niche specific, e.g. majority of the AHL producers isolated from the mangrove rhizosphere were represented by the genera *Vibrio*, *Halomonas* and *Photobacterium* which were absent in the agriculture plant crops. Similarly, the genera *Rahnella*, *Pantoea*, *Enterobacter*, *Erwinia* and *Burkholderia* isolated from agriculture crops were not represented in the mangrove and other wetland rhizospheres (Viswanath 2015; Viswanath et al. 2015; Zeng et al. 2014).

The AHL signalling molecules produced by the rhizosphere bacteria varied from short to long chains and are reported to produce more than one type of AHL molecules, and its AHL profile was not strictly conserved at the genus or species levels. Even though rhizobacterial isolates produced similar group of AHL molecules, the role of AHLs involved in the regulation of phenotypes differed from strain to strain (Fuqua and Greenberg 2002; Gonzalez and Keshavan 2006; Venturi and Subramoni 2009). For example, in *Serratia marcescens* MG1, C6-HSL regulated swarming motility and biofilm formation, whereas in *S. marcescens* SS1, the same AHL regulated sliding motility and prodigiosin production (Eberl et al. 1996; Horng et al. 2002). The distribution of AHL molecules among the rhizobacteria was species or strain dependent. This could be due to the acquisition of AHL homologue genes through horizontal gene transfer (Gray and Garey 2001; Lerat and Moran 2004). The production of a similar type of AHL molecules in different genera might help interspecies communication in the natural environment where mixed communities are often represented (Atkinson and Williams 2009). Although the

diversity of AHL-producing rhizobacteria has been explored in recent times, still identifying a myriad of AHL signals from bacteria inhabiting diverse plant species is less studied. The predominant occurrence of AHL producers in rhizosphere suggests that AHL QS might be a trait of significant importance in bacterial growth and colonization in the rhizosphere. Therefore, research focus to understand the ecological roles of AHLs in plant-bacteria interaction is needed.

9.5 Interactions of AHL with Plants

In recent years, numerous studies have shown that plants also have evolved means to perceive and respond to AHL signal molecules produced by bacteria. Many of the AHL-regulated phenotypes in bacteria such as biofilm formation, motility and antibiotic and biosurfactant production have profound impact on plant health. Recent reports have revealed that plants have marked response to the AHL signals produced by its associated microbiome. The first indication of plant responses to bacterial AHLs was studied in the legume *Phaseolus vulgaris* (Joseph and Phillip 2003) and in *Medicago truncatula* (Mathesius et al. 2003). The exposure of AHLs from symbiotic *Sinorhizobium meliloti* or pathogenic *Pseudomonas aeruginosa* at nano to micromolar concentrations induced significant changes in defence and stress management genes and accumulation of over 150 proteins (Mathesius et al. 2003). The influence of AHL molecules in plant defence response was established during interaction of *Serratia liquefaciens* MG1 and tomato plants (Hartmann et al. 2004; Schuhegger et al. 2006). The rhizobacteria *S. liquefaciens* MG1 produced short-chain AHLs C4- and C6-HSL when colonizing the tomato root surface, which induced systemic resistance against the leaf-pathogenic fungus *Alternaria alternata*. The AHLs increased salicylic acid concentration and also induced the ethylene and salicylic acid-dependent defence genes. Similarly, 3-oxo-C6-HSL producing *Serratia plymuthica* HRO-C48 elicited defence response against damping-off disease caused by *Pythium aphanidermatum* in cucumber plants and grey mould-causing fungus *Botrytis cinerea* in tomato and bean plants (Liu et al. 2007; Pang et al. 2009). The production of 3-oxo-C14-HSL by *Ensifer meliloti* (*Sinorhizobium meliloti*) associated with *Arabidopsis* plant roots showed resistance against *Pseudomonas syringae* (Zarkani et al. 2013). Likewise, Hernandez-Reyes et al. (2014) described the induction of systemic resistance by 3-oxo-C14-HSL-producing *S. meliloti* in tomato, barley and wheat plants against diverse pathogens. In addition, constitutive expression of AHL genes in transgenic tobacco plants applied with rhizobacterium *S. marcescens* 90–166 showed increased induced systemic resistance against bacterial pathogens *Pectobacterium carotovorum* subsp. *carotovorum* and *P. syringae* pv. *tabaci* (Ryu et al. 2013).

Also, the application of synthetic AHLs at a concentration range of 1–10 μM to roots in an axenic system was shown to induce resistance in diverse plants. Tomato plants treated with C4 or C6-HSL directed a systemic induction of genes involved in defence (Schuhegger et al. 2006). Schikora et al. (2011) demonstrated increased systemic resistance against obligate biotrophic fungi *Golovinomyces orontii* in *Arabidopsis thaliana* and against *Blumeria graminis* f. sp. *hordei* in *H. vulgare* (barley) plants when treated with synthetic 3-oxo-C14-HSL and 3-oxo-C12-HSL. In addition, the molecule 3-oxo-C14-HSL treated *A. thaliana* plants showed more resistance towards the hemibiotrophic bacterial pathogen *P. syringae* pv. *tomato* DC3000 (Schikora et al. 2011). Likewise, 3-OH-C14-HSL and 3-oxo-C12-HSL showed similar level of defence response against biotic stress in *A. thaliana* but comparatively weaker than 3-oxo-C14-HSL (Schikora et al. 2011). The degraded product of AHL, namely, homoserine lactone, when added to legume *P. vulgaris* roots at a concentration of 10 nM increases stomatal conductance and transpiration (Joseph and Phillips 2003). In *Trifolium repens* (white clover), transcriptional analysis indicated that treatment with 3-oxo-C12-HSL increased transcription of elements associated with auxin-responsive promoters (Mathesius et al. 2003). In *Arabidopsis*, short-chain (C4- and C6-) AHLs increased the plant's hormone

auxin/cytokinin ratio, which resulted in root elongation (von Rad et al. 2008). Ortiz-Castro et al. (2008) demonstrated that C10-HSL elicited developmental changes in the root system in *Arabidopsis* plants by altering the expression of cell division and differentiation-related genes, and C12-HSL strongly induced root hair formation. Furthermore, treatment with 3-oxo-C6-HSL and 3-oxo-C8-HSL promoted root elongation in *Arabidopsis* at concentration range of 1–10 μM (Jin et al. 2012; Liu et al. 2012). In *Vigna radiata*, 3-oxo-C10-HSL induced the formation of adventitious roots (Bai et al. 2012). *H. vulgare* (L.) and *Pachyrhizus erosus* L. (yam bean) plants treated with C6-, C8- and C10-HSL triggered tissue- and compound-specific changes in the activity of important detoxification enzymes (Gotz-Rosch et al. 2015).

The plant response to bacterial AHL signals is dependent on the type of AHL molecules. The length of the AHL side chain is essential for its effect on plants; for example, C4-HSL, C6-HSL, 3-oxo-C6-HSL and 3-oxo-C8-HSL promoted growth in *Arabidopsis* and barley (Gotz et al. 2007; Liu et al. 2012; Schenk et al. 2012; von Rad et al. 2008), whereas 3-oxo-C10-HSL induced the formation of adventitious roots in mung beans (Bai et al. 2012). On the other hand, C6- and 3-oxo-C6-HSL induced systemic resistance in tomato, cucumber and barley (Pang et al. 2009; Schikora et al. 2011; Schuhegger et al. 2006), while 3-oxo-C12- and 3-oxo-C14-HSL were reported to have resistance-inducing attributes in *A. thaliana* and *M. truncatula* (Mathesius et al. 2003; Schikora et al. 2011). The apparent different reactions to long and short chain HSLs may suggest that different receptors or at least different signalling pathways are involved in these responses. Moreover, the reaction of plants to AHLs might also depend on the specific plant-AHL combination. Different acyl length chains of AHL that induce systemic resistance and growth promotion in plants are shown in Fig. 9.2.

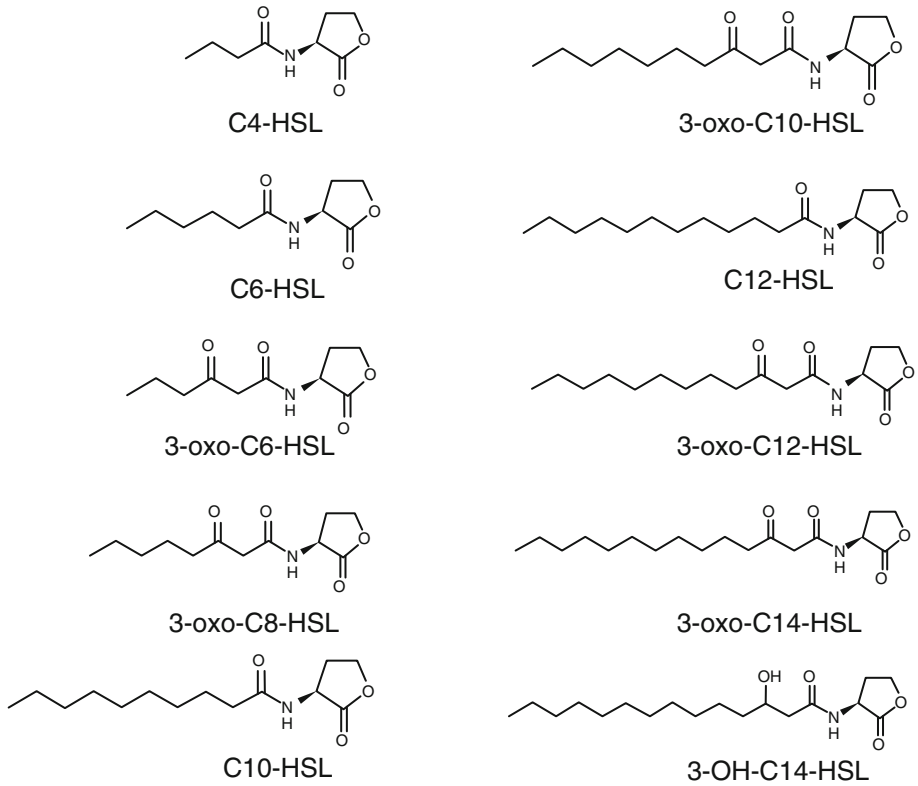

Fig. 9.2 Structures of AHL elicitors which induce ISR in plants (Source: Gera and Srivastava 2006; Ortiz-Castro et al. 2008)

Furthermore, plants also control the bacterial QS system by producing compounds that mimic AHL signals. Higher plants including *Pisum sativum* (pea), *Solanum lycopersicum* (tomato), *Oryza sativa* (rice) and *M. truncatula* secrete compounds that either stimulate or inhibit AHL responses (Bauer and Mathesius 2004; Degrassi et al. 2007; Gao et al. 2003; Perez-Montano et al. 2013; Teplitski et al. 2000).

9.6 Mechanisms of AHL Interaction in Plants

Although the response of plants to AHLs has been more extensively studied, understanding the molecular mechanisms of how plants perceive and respond to AHLs is still unclear. Very recently, possible mechanisms have been proposed to show how AHL signals influence plant defence and reinforce resistance in different plants against bacterial and fungal pathogens.

9.6.1 AHL in Plant Defence

The AHL signals use "priming" mechanisms for the induction of defence response in plants. The following possible mechanisms of plant defence response triggered by AHL molecules have been postulated:

1. Induction of SA-dependent pathway – The AHLs that triggered immune response in plants are activated through SA-mediated systemic resistance. The induction of systemic resistance in tomato against fungal leaf pathogen *A. alternata* is enhanced due to the increased levels of SA when treated with AHL-producing rhizobacterium *S. liquefaciens* MG1. Also, enhanced expression of pathogenesis-related 1a (*PR1a*) and two chitinase genes involved in SA-/ET-dependent pathways were identified in tomato leaves when C6-HSL or C4-HSL was applied to the roots of tomato plants. This strongly emphasizes that the systemic response mediated by short-chain AHL signals in tomato plant functions via an SA-dependent pathway (Schuhegger et al. 2006).
2. Induction via oxylipin-/salicylic acid (SA)-dependent pathway – The oxylipin *cis*-OPDA, a precursor of JA, and SA involved in plant defence response are elicited by AHL signal molecules (Schenk et al. 2014). The 3-oxo-C14-HSL-treated *Arabidopsis* plant showed increased accumulation of SA and *cis*-OPDA on leaves, which resulted in enhanced expression of heat shock proteins, GST6, GSTU19 encoding *HSP70* and *HSP17* genes and the cytochrome P450-encoding *CYP81D11* gene. The lack of enhanced expression of JA-dependent genes such as *MYC2* and *VSP2* and the ET-dependent genes *PR3*, *ERF5* and *ETR1* showed that AHL-treated *Arabidopsis* plants are independent of JA/ET pathway (Schenk et al. 2014).
3. Induction of stomatal defence response – The induction of SA/*cis*-OPDA pathway enhanced the stomatal defence response in 3-oxo-C14-HSL-treated *Arabidopsis* plants when encountered with the bacterial pathogen *P. syringae* DC3000 pathovar *tomato* (*Pst*). Stomatal responses such as an increased rate of stomatal closure and reduced open stomata were observed in AHL-pretreated plants (Schenk et al. 2014). The stomatal defence response in AHL-treated plants was independent of ABA pathway, which was revealed by the lack of *RD22*, *RD29* and *RAB18 gene* (Montillet et al. 2013; Schenk et al. 2014).
4. Induction via mitogen-activated protein kinase (MAPK) – The 3-oxo-C14-HSL-treated *Arabidopsis* plant roots induced systemic resistance through altered activation of MAPKs. AHL-treated plants inducted with pathogen-associated molecular pattern (PAMP) flg22 showed altered activation of MAPKs, AtMPK3 and AtMPK6. Further, the altered MAPKs induced high expression of the defence-related WRKY22 and WRKR29 transcription factors, as well as the pathogenesis-related 1 (PR1) gene (Schikora et al. 2011).
5. Induction via cell wall reinforcement – In 3-oxo-C14-HSL-treated *Arabidopsis* plants,

increased level of cell wall components such as callose, phenolic compounds and lignin was observed, which induced resistance through cell wall reinforcement (Schenk et al. 2014). When encountered with fungal plant pathogen *B. graminis* f. sp. *hordei*, 3-oxo-C14-HSL-treated barley plants showed induced resistance by the formation of papilla (cell wall apposition) structures, as a result of reactive oxygen species (ROS) accumulation (Schikora et al. 2011). Likewise, inoculation with 3-oxo-C14-HSL-producing *S. meliloti*, as well as pretreatment with the pure 3-oxo-C14-HSL molecule, primed barley and wheat plants for enhanced reactive oxygen species (ROS) production, which resulted in papilla formation and hence induced the defence response (Hernandez-Reyes et al. 2014).

9.6.2 Role of AHL in Plant Development

The growth promotion activity mediated by AHL signal molecules majorly depended on the induction of phytohormone auxin (von Rad et al. 2008; Bai et al. 2012; Liu et al. 2012). The proteome analysis in AHL-treated *M. truncatula* plants showed the accumulation of several auxin-induced proteins and enzymes involved in auxin metabolism (Mathesius et al. 2003). Furthermore, the exposure of 3-oxo-C12-HSL to the roots of transgenic *T. repens* plants with a β-*glucuronidase* (GUS) reporter gene under the promoters auxin-responsive *GH3* promoter and three chalcone synthases substantially increased the expression of auxin-responsive and flavonoid synthesis proteins (Mathesius et al. 2003).

The 3-oxo-C6- and 3-oxo-C8-HSL induced root elongation in *Arabidopsis*, eventually by the elevated expression of two G-protein receptors, namely, Cand2 and Cand7, which are involved in the activation of signal transduction pathways (Jin et al. 2012; Liu et al. 2012). The addition of 3-oxo-C10-HSL to the roots of mung bean actively accelerated the adventitious root formation by the induction of auxin metabolism via increased accumulation of H_2O_2, NO and cGMP (Bai et al. 2012).

The response to bacterial AHL QS molecules was very well understood in three different plant species, namely, *Arabidopsis*, barley and tomato. Two different response patterns, i.e. defence and growth stimulation, are induced by long-side-chain or short-side-chain HSLs, respectively. In accordance with plant response and interruption of AHL signals, it becomes clear that AHL signalling is an important factor in determining the outcome of plant-bacteria interaction. Moreover, production of AHL signal molecule is apparent in total microbiome of plants, including rhizobacteria, epiphytic and endophytic bacteria (Hosni et al. 2011; Kimura 2014; Lv et al. 2013). The AHL signals found in many species of legume-nodulating rhizobia are known to regulate phenotypes, including nodulation, nitrogen fixation, growth rate and polysaccharide production, which are all important for the establishment of a successful bacteria-plant symbiosis (Gonzalez and Marketon 2003). The current understanding of plant interaction with bacterial AHLs was limited to only AHL-producing PGPR strains; it is necessary to explore the role of other AHL-producing bacteria associated with plants. The plant growth is also influenced by AHL derivatives, which are obtained by the hydrolysis of AHL molecules through plant enzyme fatty acid amide hydrolase (FAAH). AHLs presented to the roots are taken up by the plants and hydrolyzed to L-homoserine by the enzyme FAAH. The accumulation of AHL derivative, L-homoserine, positively influenced the plant growth through increased level of nutrient uptake via transpiration and enhanced photosynthetic activity (Palmer et al. 2014). Also, bioengineered tobacco and tomato plants with AHL synthases promoted beneficial plant-bacteria interactions, thereby altering the plant growth and tolerance to biotic and abiotic stress (Barriuso et al. 2008; Mae et al. 2001; Scott et al. 2006).

So, future studies in plant-associated AHL-producing pathogenic, endophytic and epiphytic bacteria will reveal whether these groups have

similar effects of modulation in plant development as the rhizobacteria.

9.7 Conclusion

AHL defence response in plants may also have an impact on development of biocontrol or biological agents, which are useful in both integrated agriculture management and organic farming. To ensure food security, agriculture industry has to develop modern plant protection strategies, which provide sufficient yield and quality food and reduce impact of chemical pesticides on the environment. The development of biocontrol agents or biological products from beneficial, soil-borne microorganisms could be a competent approach to support agriculture. Moreover, the knowledge of microbe-plant interactions could contribute the success rate of products in natural environment. The bacterial QS molecules could be of use, since both purified AHL molecules and bacteria with increased production of AHLs have an impact on plant defence mechanisms and portrait the agricultural potential of homoserine lactones. Further, studies are needed to refine our understanding of AHL function in plant interactions under field conditions, where the AHL-producing bacteria or AHLs per se could be in a position to compete with other environmental factors.

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Biological Control of Chickpea *Fusarium* Wilts Using Rhizobacteria "PGPR"

10

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Abstract

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris* is one of the main threats to chickpea and affects sustainable food production. To combat the phytopathogens, successful measures the so-called "biocontrol" are developed over the years wherein numerous plant growth-promoting rhizobacteria (PGPRs) have been investigated for their capacities to protect plants from pathogens and stimulate plant growth. A putative PGPR qualifies as PGPR when it is able to produce a positive effect on the plant upon inoculation, hence demonstrating good competitive skills over the existing rhizosphere communities. This competence comprises the effective root colonization combined with the ability to survive and proliferate along growing plant roots over a considerable time period. In the present chapter, the author focused on PGPRs as biocontrol agents against *F. oxysporum* f. sp. *ciceris* (FOC).

10.1 Introduction

Chickpea (*Cicer arietinum* L.) is the world's fourth most important legume crop after soybean and contributes 3.1 % to the world grain legume production. In developing countries, chickpea is a rich complement to the cereal diet since it has a high nutritive value and is mostly grown for its highly proteinated edible seeds and may be treated for both seed and forage production (Yadav et al. 2011). From ancient period of time, chickpea has been grown in India together with the Middle East and parts of Africa (Upadhyaya et al. 2008). Despite its economic importance, the productivity is low owing to biotic stress wherein many soilborne as well as seed-borne pathogens of which the vascular wilt fungus *Fusarium oxysporum* f. sp. *ciceris* (FOC). *Fusarium* wilt of chickpea caused by FOC is one of the most important and destructive vascular diseases of

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chickpea whereby losses are estimated with the rate at 10 % in India and Spain, 40 % in Tunisia, and 17 % in Iran (Dileep kumar 1999; Jamali et al. 2004). The most effective control strategy for the containment of FOC is the use of resistant chickpea cultivar wherein numbers of chickpea lines have been reported as resistant to wilt from different countries of the world. A resistant cultivar to be deployed is made on the choice with a particular FOC race that is prevalent in the field. Control of *Fusarium* wilt through breeding lines has become a difficult struggle due to the existence of several physiological pathogenic races of FOC (Nene et al. 1981). If FOC inoculum establishes in the soil, it is difficult to find out the disease or eliminate the pathogen by employing crop rotation for more than 6 years, and because of this, developing new alternatives is required for more effective disease management (Haware and Nene 1982; Gupta 1991).

The application of chemicals helped in the increase of yields obtained, but two of the major problems with the constant use of chemicals are the resistance induced in target organisms and contamination of the environment with very toxic substances. It is extremely difficult to control soilborne fungi by employing conventional strategies that may include the use of synthetic fungicides. Upon the continuous use of chemicals, fungal spores survive for many years in the soil due to their resistance, and hence, biological control strategies are selected and handled in an eco-friendly way instead of using chemical fungicides (Okigbo 2004). Biological control of plant pathogens using antagonistic bacteria is a promising strategy for plant protection wherein plant growth-promoting bacteria (PGPB) specifically plant growth-promoting rhizobacteria (PGPRs) have been shown to improve plant health and increase yield (Kloepper et al. 1999; Maheshwari 2010).

This chapter examines biological control employing PGPRs and their mechanisms involved. In organization, the chapter opens with a discussion of concepts, viz., *Fusarium* wilt of chickpea, PGPR, and the use of PGPR for biocontrol of *Fusarium* wilt of chickpea including comprehensive trials of PGPR. A cohort effect of PGPR occurs by local antagonism to the pathogen or by induction of systemic resistance in chickpea. Several substances produced by antagonistic rhizobacteria have been related to pathogen control and indirect promotion of growth in many plants, such as HCN and antibiotics. The induced systemic resistance (ISR) has recently gained considerable importance in the control of *Fusarium* wilt of chickpea diseases; result of works presented in this chapter has shown the possibility of exploitation for greenhouses/fields. Throughout this chapter, the author uses data from long-studied research operated in the northwest of Algeria to support perspectives of the biocontrol of *Fusarium* wilt using native rhizobacteria.

10.2 *Fusarium* Wilt of Chickpea

It is our consensus that strains of *Fusarium* species are the major soilborne as well as seed-borne pathogens causing wilt and rot diseases in more than 80 plant species including chickpea that caused up to 100 % yield loss worldwide (Santos et al. 2002). The major limiting factor in chickpea production is *Fusarium* wilt that has been reported almost all over the world including India and defined by Butler in 1918, and further, its etiology was determined in 1940 by Padwick. McKerral (1923) described that *Fusarium* wilt is a soilborne disease that belongs to the genus *Fusarium*. An association of *Fusarium* sp. and *Rhizoctonia* sp. may also cause wilted plants (Narasimhan 1929). McRae (1932) as well as Prasad and Padwick (1939) reported FOC to be pathogenic to chickpea crop which is now accepted worldwide as the causal agent of *Cicer* spp. (Booth 1971; Kaiser et al. 1994). *Fusarium* wilt of chickpea is caused by *Fusarium oxysporum* (Schlechtend.:Fr.) f. sp. *ciceris* (Padwick) Matuo & K. Sato. The fungus was first named *Fusarium orthoceras* Appel & Wollenw. var. *ciceri* by Padwick, and later Chattopadhyay and Sen Gupta renamed the pathogen *F. oxysporum* Schl. f. sp. *ciceri* (Padwick) Snyder & Hansen. This was accepted as the correct name of the pathogen until revised by Holliday in 1980

(Jalali and Chand 1992; Nene and Reddy 1987). FOC is one of the few formae speciales of monophyletic origin in the *F. oxysporum* complex of the *Gibberella clade*, most of which are polyphyletic (O'Donnell et al. 1998; Baayen et al. 2000; Kistler 2001; Jiménez-Gasco et al. 2002; Demers et al. 2014; Jiménez-Díaz et al. 2015).

In the year 2000, around 33 countries of the world have been reported to be affected, causing 10–15 % yield losses annually depending upon the environmental conditions along with losses due to FOC. *Fusarium* wilt reduces chickpea production by decreasing both seed yield and seed weight (Singh and Dahiya 1973; Navas-Cortés et al. 2000; Nene et al. 1996). The FOC is more prevalent in the Indian subcontinent including the USA, Tunisia, Turkey, Ethiopia, Spain, and Mexico (Westerlund et al. 1974; Nene et al. 1989; Halila and Strange 1996). FOC is a primarily soilborne pathogen; however, it can be transmitted through seeds (Haware et al. 1978). Pathogens survive in soil and seed in the form of chlamydospores for many years. Mycelia enter the epidermal tissues invading through roots, extend to the vascular bundles, and form spores in plants (Chehri et al. 2010). The pathogen causes seed abortion and rot, necrosis, reduction or elimination of germination capacity, as well as plant damage at later stages of plant growth resulting in the development of the disease as systemic or local infection (Khanzada et al. 2002).

Upon pathogen attack, adult plants show typical wilt symptoms that involve drooping of petioles, rachis, and leaflets (Fig. 10.1). The roots of the wilting plants do not show any external rotting, but when split open vertically, dark brown discoloration of internal xylem is seen (Nene et al. 1991). Pods from the wilted plants look normal but seeds are generally smaller, wrinkled, and discolored. Though such seeds can be detected visually, a normal-looking seed harvested from wilted plants may also harbor the wilt pathogen.

Symptoms of the disease develop at any stage of plant growth, and affected plants may be grouped in patches or appear spread across a field (Trapero-Casas and Jimènez-Díaz 1985; Nene and Reddy 1987; Haware 1990). In molecular analysis, namely, random amplified polymorphic DNA (RAPD), DNA banding patterns allowed the identification of markers which differentiate among wilting and yellowing pathotypes (Kelly et al. 1994; Gupta et al. 2009). Upon occurrence, FOC exhibited two pathotypes and eight pathogenic races on chickpea; the yellowing pathotype induces progressive foliar yellowing and vascular discoloration with plant death within 40 days, whereas the wilt-causing pathotype induces severe and fast chlorosis, flaccidity, and vascular discoloration with plant death within 20 days after inoculation (Haware and Nene 1982; Jiménez-Diaz et al. 1993; Jorge et al. 2005). The eight races of FOC were identified as 0, 1A, 1B/1C, 2, 3, 4, 5, and 6, by reaction on a set of differential chickpea cultivars (Jiménez-Gasco and Jiménez-Diaz 2003). Besides, races 0 and 1B/1C cause yellowing, whereas races 1A, 2, 3, 4, 5, and 6 induce wilting (Jiménez-Gasco et al. 2001). Out of these, races 2, 3, and 4 have been reported only in India, while races 0, 1B/1C, and 5 have been found mainly in the Mediterranean region and in California, USA (Jimènez-Gasco and Jimènez-Diaz 2003). Race 1A has been reported from India, California, Morocco, and Spain, while race 6 has been found in California, Spain, Israel, and Morocco (Jimènez-Gasco et al. 2001).

10.3 Plant Growth-Promoting Rhizobacteria (PGPRs)

The German agronomist Hiltner first defined the rhizosphere, in 1904, wherein microbial activity was higher around the roots of legumes. This zone harbors a multitude of microorganisms that are affected by both abiotic and biotic stresses. Among these are the dominant bacteria that prefer living in close vicinity to the root or on its surface and play a crucial role in soil health and plant growth. These benign bacteria inhabiting the rhizosphere termed PGPR (Kloepper et al. 1989) were introduced in 1978 by the same author

Fig. 10.1 Typical *Fusarium* wilt symptoms of chickpea (Unpublished data)

in the Proceedings of the Fourth International Congress of Bacterial Plant Pathogens, conducted in France (Ramos Solano et al. 2008).

It is well established that only 1–2 % of bacteria promote plant growth in the rhizosphere, and among them, strains from genera *Pseudomonas*, *Azospirillum*, *Burkholderia*, *Bacillus*, *Enterobacter*, *Rhizobium*, *Erwinia*, *Serratia*, *Alcaligenes*, *Arthrobacter*, *Acinetobacter*, and *Flavobacterium* have reported to enhance plant growth (Glick 1995; Antoun and Kloepper 2001). The mechanism by which PGPRs promote growth of plants can be either direct mechanism (biofertilizer and biostimulator activity) or indirect mechanism (biocontrol activity). The direct promotion of plant growth by PGPR entails either providing the plant with a compound that is synthesized by the bacterium or facilitating the availability of a nutrient and its uptake from environment (Glick 1995). The rhizobacteria produce the secondary metabolites, which are directly utilized by the plants thus promoting plant growth (Glick et al. 1999).

There are several ways the PGPR may directly facilitate the proliferation of their plant hosts:

- Solubilize minerals like phosphates in a form that can be used by the plant
- Synthesize phytohormones like auxins that trigger plant cell growth and proliferation
- The ability to produce or change the concentration of plant growth regulators like indole acetic acid
- Synthesize enzymes that can modulate plant hormone levels
- Fix atmospheric nitrogen and supply it to the plant

The indirect promotion of plant growth occurs when PGPRs lessen/prevent the deleterious effects of phytopathogenic organisms through antibiosis and can be either due to the depletion of a scarce resource, required by the pathogen, or to the production and release of a compound that impedes the growth of the phytopathogenic organism (Glick 1995; Smitha et al. 2015).

The list of indirect mechanisms used by PGPR is substantial:

- Synthesis of enzymes able to hydrolyze fungal cell walls
- Synthesis of hydrocyanic acid (HCN) which suppresses growth of fungal pathogens
- Production of antibiotics that kill the phytopathogen fungus
- Induction of systemic resistance (ISR)
- Antagonism against phytopathogenic microorganisms by production of siderophores

A detailed discussion of the first four mechanisms listed above follows.

10.4 Comprehensive Trials of PGPR for Biocontrol of *Fusarium* Wilt of Chickpea

The first step in obtaining PGPR is the isolation of rhizospheric bacteria from the soil volume close to the roots. After isolation of the maximum number of bacteria to avoid the loss of bacterial variability, different tests were performed to reduce the various types of bacteria chosen, so that only the beneficial ones remain. For identification of successful PGPR, standard methodologies for isolation, screening, and mode of action have been well documented (Landa et al. 1997a, b; Swain and Ray 2007; Idris et al. 2007). Several protocols have been developed for the identification of this PGPR, which can be broadly classified as in vitro and greenhouse and field tests.

10.4.1 In Vitro Antagonistic Activity Trials

Antibiosis is an important mechanism used by biocontrol agents to suppress diseased plants by producing volatile and nonvolatile antibiotics which disrupt the cell contents of pathogenic microorganisms before coming in contact with the biocontrol agent. The in vitro trials have been successfully used with all groups of biocontrol agents such as PGPRs. These trials were performed in vitro to check biochemical activities that correspond with potential PGPR characteristic. Some of the frequently used methods are briefly described here.

10.4.1.1 Dual Culture Assay

This technique known as biculture/paired culture has been extensively used for preliminary screening of large populations of rhizobacteria. In principle, the pathogen and the rhizobacteria should be allowed to interact in a petri dish under optimum conditions for both the pathogen and the rhizobacteria. The inhibition is recorded in the form of the inhibition zone produced by the antagonistic rhizobacteria (Fig. 10.2). The antagonistic effects are scored and the interface region was observed under light microscope (Zaim et al. 2013). The antagonistic potential of *Bacillus* spp. is well documented (Johri et al. 2003; Saharan and Nehra 2011). Thus, this phenomenon has often been used as a means for in vitro screening of biocontrol agents (Zaim et al. 2013).

In our study, the *Bacillus* isolates Rb29, Rb6, Rb12, Rb4, and Rb15 caused a modification in the mycelium appearance (Fig. 10.3). These modifications were changes in mycelia color from white to red, reddish brown, or darker brown. With these isolates, a coagulation of fungal cytoplasm that can be observed up to the

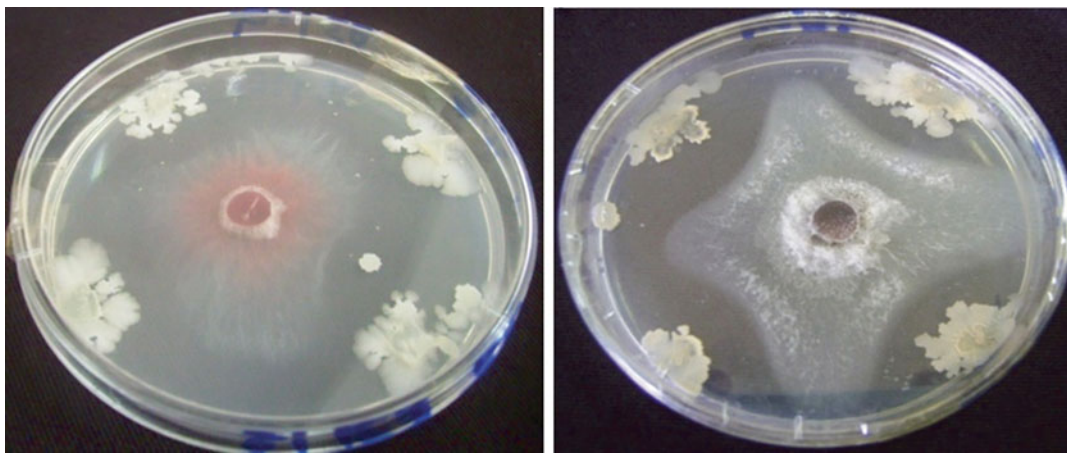

Fig. 10.2 Dual culture technique for evaluation of rhizobacteria isolates against *F. oxysporum* f.sp. *ciceris* causing *Fusarium* wilt of chickpea (Unpublished data)

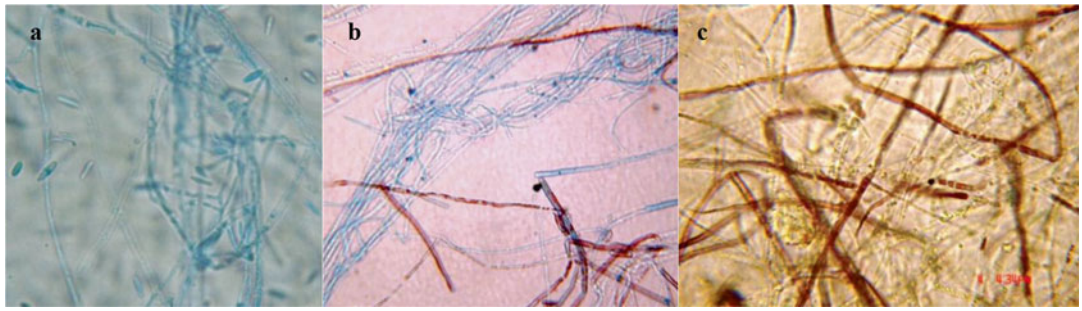

Fig. 10.3 Light microscopic images of mycelium color changing of FOC: (**a**) Control; (**b, c**) in the presence of *Bacillus* spp. (Unpublished data)

hyphae was detected, resulting in the presence of small vesicles and the appearance of big vacuoles. In this case, the destructive effect of FOC by rhizobacteria was high, resulting in serious damage of the hyphae, associated with a series of degradation events (Zaim et al. 2013).

10.4.1.2 Production of Volatile Inhibitory Compounds

Many biocontrol microorganisms produce chemicals that are inhibitory to the pathogens. These chemicals can either be volatile or released into the medium (nonvolatile). Dennis and Webster (Dennis and Webster 1971) have developed methods for studying the production of volatile inhibitory compounds by the biocontrol agents. While testing for the production of volatiles, the pathogen and the rhizobacteria are inoculated on individual petri dishes. Inoculated petri dish with the test fungus was inverted and placed over the rhizobacterial culture. The two plates were sealed together with Parafilm to prevent gas diffusion, and then they were incubated under optimum conditions. This incubation ensured that both organisms were growing in the same conditions though they were physically separated. Any radial growth increase of the test fungus was recorded. PGPR strains release a blend of volatile organic compounds (2, 3-butanediol and acetone) that promote growth and induce resistance against pathogen (Ryu et al. 2004). In our study, volatile metabolite activity was observed in all 29 isolates where the target pathogen FOC1 was inhibited from 14.11 to 44.68 % (Zaim et al. 2013). *Bacillus subtilis* G8 isolated from soil in China produced antifungal volatile organic compounds. These volatile organic compounds detected include alkyls, alcohols, esters, ketones, acids, amines, phenols, and heterocyclic compounds (Liu et al. 2008).

10.4.1.3 In Vitro Detection of Plant Growth-Promoting Traits

Among the functional tests used to find efficient PGPR traits, the most common are the following: (1) test for enzymes (chitinase and β-1, 3-glucanase) that can degrade pathogenic fungi cell walls preventing plant diseases, (2) test for antibiotic, (3) test for antifungal metabolites such as HCN which suppress growth of fungal pathogens, (4) phosphate solubilization test, (5) test for plant growth regulator production, and (6) test for bacteria capable of producing biochemical compounds associated with host defense. PGPR may use more than one of these mechanisms as experimental evidence suggests that biocontrol of plant pathogens is the net result of multiple mechanisms that may be activated simultaneously.

In addition to the above-described plant growth-promoting features, the PGPRs protect the chickpea from FOC by several mechanisms. The mechanisms include the production of antibiotic, production of lytic enzymes that can lyse the cell wall of pathogenic fungi, production of antifungal metabolites such as hydrogen cyanide which suppress growth of fungal pathogens, production of phytohormones like IAA (indole-3-acetic acid), production of antibiotic metabolites, and induction of systemic resistance in plants (Hammerschmidt 1999; Raju et al. 2008;

Moradi et al. 2012; Karimi et al. 2012; Kandoliya and Vakharia 2013; Patil et al. 2015; Smitha et al. 2015). Karimi et al. (2012) found that 232 bacteria isolated from the rhizosphere and root of chickpea showed substantial inhibition zones against FOC in vitro. Twelve out of 232 bacterial strains identified as *Pseudomonas* and *Bacillus* genera that exhibited high antifungal activity against pathogens were selected, and several biochemical activity indicators for putative PGPR abilities were tested. The indicators tested were the production of protease, siderophore, cyanide hydrogen, indole acetic acid, antifungal volatile, and extracellular compound. Moreover, *Bacillus* strains were tested for volatiles, cyanide production, and solubilization of phosphorus because of the potential implication of such traits in promoting plant growth (Bakker and Schippers 1987; Glick 1995).

Production of Lytic Enzymes That Can Lyse Fungal Cell Wall

The hydrolytic enzymes have received considerable attention because they play a role in controlling diseases by excreting cell wall hydrolases (Chernin and Chet 2002). Testing for production of hydrolases and antibiotics helps in the characterization of PGPR and thus deploys them in a systematic way.

Chitin and β-glucan are the main components of fungal cell wall of filamentous fungi. Chitin is a linear polysaccharide composed of β-1, 4-N-acetylglucosamine units and is found in nature as α- and β-chitin, whereas laminarin is a polymer of D-glucose in β-1, 3 configurations arranged in helical coils, from which minor polymers of β-1, 4 D-glucose branch. Fungal cell walls contain more than 60 % of laminarin which is hydrolyzed mainly by β-1, 3 glucanases (Cohen-Kupiec et al. 1999). Chitinases and glucanases have many roles in a wide range of different biological systems. These enzymes are usually extracellular, and they may be produced in multiple forms that differ in charge, size, regulation, stability, and ability to degrade cell walls (Koga et al. 1999). In in vitro trials, chitinases are inducible enzymes secreted only in the presence of chitin; hence, colloidal chitin was used as sole carbon source in the production medium. In the same context, glucanases are inducible enzymes secreted in the presence of cellulose. The fungal wall components such as chitin, β-1, 3-glucan, mannan, cellulose, and proteins may induce the lytic enzymes, thus showing antagonistic activities (Adams 2004).

Chitinolytic enzymes have been considered important in the biological control of plant pathogens because of their ability to degrade fungal cell walls (Hoster et al. 2005). Chitinases producing microorganisms have been reported as biocontrol agents for different kinds of fungal diseases of plants. There are effective tools for complete degradation of mycelia and conidial walls of phytopathogenic fungi (Kobayashi et al. 2002). Several rhizobacteria, including genera of *Bacillus* and *Pseudomonas*, are known to produce a battery of hydrolases such as chitinase and glucanase, which help in the maceration of cell walls of those plant pathogens (Lim et al. 1991; Singh et al. 1999; Huang et al. 2004; Bogas et al. 2007; Aktuganov et al. 2007). Singh et al. (2013) reported that chitinase-producing strain *Lysinibacillus fusiformis* B-CM18, isolated from chickpea rhizosphere, exhibited in vitro antifungal activity against a wide range of fungal plant pathogens, among them *F. oxysporum* f. sp. *ciceris*. This strain B-CM18 was also found to produce several PGPR activities that make these rhizobacteria an ideal candidate for biological control of chickpea pathogens. Patil et al. (2015) reported that two rhizobacterial strains isolated from chickpea, *Paenibacillus polymyxa* CTS-B19 and *Bacillus subtilis* CTS-G24, produced chitinase, and β-1, 3-glucanase may act synergistically in degrading fungal cell wall thus achieving biocontrol of pathogenic fungi *F. oxysporum* f. sp. *ciceris*.

Production of Antibiotics

One of the effective means of control of soilborne pathogens in a natural ecosystem is by means of production of antibiotics (Raaijmakers and Weller 1998). Production of antibiotics has been described as the potent mode of action in disease suppression by which development and/or activity of the pathogen is believed to be

directly inhibited. The antibiotics produced in vitro were generally assumed to be the compounds responsible for biocontrol in vivo (Leifert et al. 1995). The most common antibiotics produced by *Pseudomonas* are phenazines, 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (Prn), pyoluteorin (Plt), and others (Raaijmakers et al. 2002; Mavrodi et al. 2010). Handelsman and Stabb (1996) reported that a significant quantitative relationship existed between the disease suppression and the antibiotic production by the bacilli species. The beneficial rhizobacterium *Bacillus subtilis* is one of the best biocontrol agents that produced lipopeptides, viz., fengycin, iturin, and surfactin, which displayed multifaceted biocontrol activity against plant pathogens (Ongena and Jacques 2008). The antifungal activity of plant growth-promoting rhizobacterium *B. amyloliquefaciens* FZB42 has been attributed mainly to bacillomycin D production, and this has been shown to suppress the plant pathogenic fungus *F. oxysporum* (Koumoutsi et al. 2004). *B. cereus* strain UW85 is known to produce both zwittermicin (Silo-Suh et al. 1994) and kanosamine (Milner et al. 1996). Mycosubtilin is another variant of the iturin family and is produced by strains of *B. subtilis* (Leclere et al. 2005). Overproduction of mycosubtilin by a recombinant *B. subtilis* strain BBG100 has been found to show significant antagonistic properties against various fungal pathogens including *F. oxysporum* (Leclere et al. 2005).

Production of Antifungal Metabolites Such as HCN

PGPR produces a wide range of low-molecular-weight metabolites with antifungal activity wherein hydrocyanic acid (HCN) plays an important role that inhibits the electron transport and the energy supply to the cell leading to death of the organisms; it inhibits the proper functioning of enzymes and natural receptor's reversible mechanism of inhibition, and it is also known to inhibit the action of cytochrome oxidase (Dowling and O'Gara 1994). HCN is produced by many rhizobacteria which have antifungal properties and is postulated to play a role in biological control of pathogens. In in vitro trials, production of HCN is detected qualitatively using nutrient agar medium amended with 4.4 g glycine L^{1} (Lorck 2004). A Whatman filter paper no. 1 soaked in 2 % sodium carbonate solution and 0.5 % picric acid solution was placed on the top of the plates. Plates were sealed with Parafilm. Upon incubation of the rhizobacteria on the solid plates, color changes from yellow to pink/red color that indicated HCN production. Toyoda and Utsumi (1991) reported that *P. solanacearum* were able to produce HCN and hydrolyze the compound, fusaric acid. Fusaric acid is the causative agent of the damage to plant that occurs upon *Fusarium* infection. As a consequence of the ability to hydrolyze fusaric acid, the bacterial strains can prevent the damage that is caused by various species of the fungus *Fusarium* (Toyoda and Utsumi 1991).

Phosphate-Solubilizing PGPR

The use of rock phosphate as a phosphate fertilizer with its solubilization by microbes (Kang et al. 2002), through the production of organic acids (Maliha et al. 2004), has become a valid alternative to chemical fertilizers. Several studies have shown that phosphate-solubilizing microorganisms solubilize the fixed P in the soil resulting in higher crop yields (Gull et al. 2004). Most predominant phosphorus-solubilizing bacteria (PSB) belong to the genera *Bacillus* and *Pseudomonas* (Richardson 2001). Rhizobacteria solubilizing the phosphate can be isolated using serial dilutions or enrichment culture techniques on/in Pikovskaya medium supplemented with bromophenol (Pikovskaya 1948) from rhizosphere soils. Upon incubation of the organisms on the solid plates containing insoluble phosphate, phosphate-solubilizing PGPRs are detected by the formation of clear halos around their colonies. Finally, the selected efficient phosphate-solubilizing cultures are used for making the inoculants, and their performance under pot or field conditions is tested against various crops such as chickpea. Wani et al. (2007) showed that multiple inoculation with *Mesorhizobium ciceri* and phosphate-solubilizing rhizobacteria increased the nodule number and

biomass per plant. Similar results were obtained by Rokhzadi and Toashih (2011) which showed that inoculation treatments contain *Azospirillum* and *Azotobacter* strains in their combinations, suggesting that *Azospirillum* and *Azotobacter* jointly may have a role in promoting phosphorus uptake by chickpea. Similarly, PGPRs have been shown to solubilize precipitated phosphates and enhance phosphate availability to chickpea that represent a possible mechanism of plant growth promotion under field conditions (Verma et al. 2001, 2010). The use of PGPRs as inoculant biofertilizers is an efficient approach to replace chemical fertilizers and pesticides for sustainable chickpea cultivation.

Plant Growth Regulator Production Such as IAA (Indole-3-Acetic Acid)

The ability of bacteria to produce IAA in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by plant (Glick 1995). Plant growth regulators participate in the growth and development of cells, tissues, organs, and in fact the entire plant. These compounds are active in plants in very minute amounts and their synthesis is extremely regulated. Plants not only produce phytohormones but also numerous plant-associated bacteria that are both beneficial, and they produce one or more of these substances (Dobbelaere et al. 2003). Phytohormones that are produced by plant-associated bacteria, including indole-3-acetic acid (IAA), cytokinins, and gibberellins, can frequently stimulate germination, growth, and reproduction and protect plants against both biotic and abiotic stress (Taghavi et al. 2009). As the most studied phytohormones, IAA produced in the plant shoot and transported basipetally to the root tips associated with cell elongation and cell division (Rashotte et al. 2000) contributes to plant growth and plant defense system development (Navarro et al. 2006). In general, root elongation changes qualitatively based on the IAA level; therefore, the amount of released IAA could have an important role in modulating the plant–microbe interaction. Many rhizosphere bacteria produce IAA in culture media especially in the presence of tryptophan (Yadav et al. 2010; Patil et al. 2015). Yadav et al. (2010) reported that the bacterial strain *Pseudomonas putida* BHUPSB04 showed maximum significant concentration of IAA 25.65 μg ml^{-1} followed by *Pseudomonas aeruginosa* BHUPSB02 (21.35 μg ml^{-1}), *Bacillus subtilis* BHUPSB13 (16.23 μg ml^{-1}), *Paenibacillus polymyxa* BHUPSB17 (15.79 μg ml^{-1}), and *Bacillus boroniphilus* BHUPSB19 (11 μg ml^{-1}). In a similar study, the PGPR isolates significantly affected the length of chickpea seedlings. Results reveal that the shoot length increased in PGPR-treated plants over uninoculated control. The highest shoot length 15.6 cm $plant^{-1}$ was recorded in treatment of *P. putida* BHUPSB04 isolate followed by statistically at par values due to isolates *P. aeruginosa* BHUPSB02 (14.5 cm $plant^{-1}$). *B. subtilis* BHUPSB13, *P. polymyxa* BHUPSB17, and *B. boroniphilus* BHUPSB19 which showed significantly higher shoot length over control (Yadav et al. 2010).

Induction of Systemic Resistance

When physical contact of the pathogen and the protecting microorganism is required, the process is known as biocontrol (Bloemberg and Lugtenberg 2001; Compant et al. 2005). As already mentioned, the existence of microorganisms capable of preventing diseases in plants without the plants' participation is known. This occurs by systems such as niche exclusion or pathogen-inhibiting substance production. Apart from the direct action against plant pathogens, many PGPRs induce resistance in the plant system by signaling host defense mechanisms. The plant and bacterial interactions in the rhizosphere are important for plant health and resistance to disease. PGPRs are known to rapidly colonize the rhizosphere and enhance plant resistance, which is termed induced systemic resistance (ISR), while pathogen-induced resistance is called systemic acquired resistance (SAR) (Hammerschmidt 1999). Recently, several studies have reported the importance of strains of PGPR in enhancing plant resistance (Kloepper 1993; Martin and Loper 1998; Silva et al. 2004; Moradi et al. 2012; Altinok et al. 2013).

They are both related with the induction of pathogenesis-related (PR) proteins. Moradi et al. (2012) showed an increase in the induction of resistance to *Fusarium* wilt in chickpea by *B. subtilis*. They also demonstrated that PGPR resulted in the accumulation of PR proteins via increased synthesis of chitinase and β-1, 3-glucanase. In this case, Raju et al. (2008) claimed that induction of proteins and accumulation of phenolics might have contributed to restrict the invasion of FOC, in resistant cultivar ICCV10. Their investigation showed that Hashem cultivar contained higher levels of soluble protein content and β-1, 3-glucanase activity than Pirooz cultivar after inoculation with a biocontrol agent such as *Bacillus subtilis* which is apparently associated with the establishment of a higher level of resistance to *Fusarium* wilt of chickpea (Moradi et al. 2012).

Jiang et al. (2015) demonstrated that in the interactions with invading pathogens, plants frequently activate defense-related genes that lead to the expression of pathogenesis-related (PR) proteins. Among the studied PGPRs are some *Rhizobium* spp. which have been shown to induce a defense response in chickpea infected with FOC. Arfaoui et al. (2005) suggested that treatment of germinated seeds with *Rhizobium* induced the expression of compounds involved in plant defense such as peroxidases and polyphenol oxidases and increased levels of phenolic compounds. It has been reported that volatile organic compounds may play a key role in the induced systemic resistance. In this case, volatiles secreted by *B. subtilis* GBO3 were able to activate an ISR pathway in *Arabidopsis* seedlings challenged with the soft-rot pathogen *Erwinia carotovora* subsp. *carotovora* (Compant et al. 2005), and the same isolate was found to suppress *Fusarium oxysporum* f. sp. *ciceris* (Hervas et al. 1998).

Various studies reported the importance of the phytoalexins medicarpin and maackiain in the overall defense response of chickpea (Stevenson et al. 1997). Peroxidases and hydrolases, particularly chitinases and glucanases, also play a major role in the defense mechanisms of this plant. PGPRs also induce ISR by triggering jasmonic acid (JA) and ethylene synthesis (Pieterse et al. 1998). ISR is dependent on colonization of the root system by sufficient numbers of PGPR, and this has been achieved by coating seed with high numbers of bacteria or by adding bacterial suspensions to soil before sowing or at transplanting (Kloepper 1996).

10.4.1.4 Greenhouse and Field Testing

Root colonization is a necessary requirement for the bacteria to exert its effect (Germida and Walley 1996). Unfortunately, the PGPR inoculation in distinct plant species sometimes produces erratic results. The good results obtained in vitro cannot always be dependably reproduced under field conditions. The variability in the performance of PGPR may be due to various environmental factors that may affect their growth and exert their effects on plant (Joseph et al. 2007). Further, after the screening process, the PGPR potential shown in vitro should be tested to ensure that the same effect occurs in the plant and so the evaluation of the isolates exhibiting multiple plant growth-promoting traits on the soil–plant system is needed to uncover their efficacy as effective PGPR. Inoculant bacteria are often applied to seeds or root of the plant for rapid colonization. After sowing, the inoculant bacteria must be able to establish in the rhizosphere at population densities sufficient to produce a beneficial effect. Therefore, efficient inoculant bacteria should survive in the rhizosphere, make use of nutrients exuded by the plant root, be able to efficiently colonize the entire root system, and compete with indigenous microorganisms. After being implicit in the colonization process, these rhizobacteria have the ability to survive on seeds, can multiply in spermosphere in response to seed exudates, and can attach to the surface of the root system and colonize. The use of inoculation with a beneficial, biological control organism that will colonize the rhizosphere shows some promise as a means to suppress plant disease (Cook 1993).

The first successful application and commercial production of PGPR is by a *B. subtilis* strain A13. *B. subtilis* A13 was isolated more than 25 years ago in Australia based on in vitro inhibitory

activity to all of nine pathogens tested and was subsequently shown to promote plant growth. Since 1990, *Bacillus* spp. have been developed as fungal disease control agents in the form of a commercial product, namely, Serenade, EcoGuard, Kodiak, Yield Shield, and Bio Yield (Idris et al. 2008). *Pseudomonas* and *Bacillus* strains have great potential in control of *Fusarium* wilt disease of chickpea (Hervas et al. 1997; Landa et al. 1997a, b; Anjajah et al. 2003; Inam-ul-Haq et al. 2003). Some species of *Bacillus* were isolated from the rhizosphere of chickpea and demonstrated to inhibit conidial germination and hyphal growth of *F. oxysporum* f. sp. *ciceris* (Landa et al. 1997b) and suppress *Fusarium* wilt development (Landa et al. 1997a).

In our study, the test in pots showed that the susceptible cultivar ILC 482 reacts to FOC1 with a high incidence of *Fusarium* wilt. Nevertheless, 6 weeks after sowing, there was 100 % more disease on wilted plants. However, bacterized seeds with five rhizobacteria Rb29, Rb6, Rb12, Rb4, and Rb15 isolated from rhizosphere soils of healthy chickpea plants significantly reduced the percentage of wilted plants, from 99 to 60 % (Zaim et al. 2013). Karimi et al. (2012) used six isolates of *Pseudomonas* and six isolates of *Bacillus* genera that were tested for biocontrol of *Fusarium* wilt and promotion of chickpea growth. In the same study, the isolates of *P. aeruginosa* and *B. subtilis* protected chickpea against *Fusarium* wilt with 15.8–44.8 % in seed treatment and soil inoculation. Therefore, growth parameters (plant height, fresh and dry weight of plants) were significantly increased. The influence of PGPR on chickpea yield under field conditions has been thoroughly studied. Studies have shown that a combined inoculation of *Azospirillum* spp., *A. chroococcum* 5, *Mesorhizobium ciceri* SWR17, and *P. fluorescens* P21 improved nodulation and increased dry matter accumulation in roots and shoots, grain yields, biomass, and protein yield of chickpea by a significant margin. This can be attributed to the cumulative effects of an enhanced supply of nutrients, mainly nitrogen and phosphorus, and the production of growth-promoting substances (Rokhzadi et al. 2008).

10.5 Conclusion and Perspective

The rhizosphere is a highly dynamic system with a vast number of fungi and bacteria interacting simultaneously; the difficulty of excluding endemic PGPR may preclude clear conclusions from inoculation experiments in the field. In order to increase our understanding of the role of various root-associated organisms as PGPRs in plant growth and health as well as make use of their potential beneficial features in plant production, more information is urgently needed on the interactions among plants and rhizosphere microorganisms. Selection of biocontrol agents for controlling diseases such as *Fusarium* wilt of chickpea has emphasized the use of individual agents. However, it would seem logical that increasing the number of biological control agents as a mixture may result in treatments that could persist longer in the rhizosphere, provide a wider array of biocontrol mechanisms, and/or function under a broader range of environmental conditions, especially if these mixtures were of different species. The ability of rhizobacterial mechanisms to suppress *F. oxysporum* f. sp. *ciceris* could be of significant agronomic importance. These mechanisms have essential functions in the microbial antagonism, on the one hand, but also are able to elicit induced resistance, on the other hand. Resistance-inducing and antagonistic rhizobacteria might be useful in formulating new inoculants, offering an attractive alternative of environmentally friendly biological control of *Fusarium* wilt of chickpea and improving the cropping systems into which it can be most profitably applied.

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AM Fungal Effect on the Growth of Selective Dicot and Monocot Plants

11

B. Sadhana, P.K. Monica, and S. Siva Sankari

Abstract

A mycorrhiza (fungus root) is a symbiotic association of a fungus and the roots of a vascular plant. In this association, the fungus colonizes the host plant's roots, either intracellularly as in arbuscular mycorrhizal fungi or extracellularly as in ectomycorrhizal fungi. Arbuscular mycorrhizal (AM) fungi are ubiquitous in soil habitats and form beneficial symbiosis with the roots of angiosperms. The present work was focused on the arbuscular mycorrhizal status of selective dicot plants such as chickpea (*Cicer arietinum* L.), cowpea (*Vigna unguiculata* L.) and green pea (*Pisum sativum* L.) plants and selective monocot plants such as *Triticum aestivum* (L.) and *Pennisetum glaucum* and its beneficial effect on the efficiency of morphological and physiological changes in such plants grown under greenhouse condition. The investigation result reported that the fresh and dry weight, shoot and root length and chlorophyll and carotenoid content in the AM fungi-treated plants increased significantly compared to control plants.

11.1 Introduction

Mycorrhizal symbiosis refers to the association of fungi with plant roots. This relationship is predominantly mutualistic, in which both partners were benefiting from the association. The term 'mycorrhiza' was first used by Professor Frank in the 1880s. Frank was the first person to describe the symbiotic relationship between trees and fungi, which he named 'mykorhiza'. The word comes from the Greek *mykes* and *rhiza*, the combination meaning fungus root. In return, a fungus may confer increased nutrient supply, defence against pathogenic attack and drought resistance to its partner plant. More than 90 % of all plant families studied (80 % of species) in both agricultural and natural environments form mycorrhizal associations, and they can be essential for plant nutrition. Mycorrhizas are found in a wide range of habitats, including deserts, lowland tropical rainforests, high latitudes and altitudes and aquatic ecosystems. There are few exceptions to the rule that mycorrhizas are found in all plant species that are economically important to man.

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A mycorrhiza is a symbiotic association of a fungus and the roots of the vascular plant. There are two types, namely, ectomycorrhiza (fungi penetrate root cortical cells intracellularly and form spherical haustoria-like structures interfacing with the host cytoplasm) and endomycorrhiza (fungi form external fungal mantle and on intercellular hyphal network in the cortex, called 'Hartig net'). External structures of AM fungi are the hyphae which penetrate the soil, and they form individual resting spores. These are produced asexually on straight, subtended hyphae and are known as chlamydospores. Arbuscular mycorrhizal (AM) fungi are ubiquitous in soil habitats and form beneficial symbiosis with the roots of angiosperms and other plants (Gerdemann 1968). These AM fungi belong to the family *Endogonaceae*, of the order *Mucorales*, of the class *Zygomycetes* (Gerdemann and Trappe 1974; Trappe et al. 1984). The AM spore-forming genera of the family include *Acaulospora*, *Entrophospora*, *Gigaspora*, *Glomus*, *Sclerocystis* and *Scutellospora*. The AM association is endotrophic and has previously been referred to as vesicular-arbuscular mycorrhiza (VAM). This name has since been dropped in favour of AM, since not all of the fungi form vesicles.

AM fungi are known to colonize a number of tropical plants including vegetables. They are characterized by the formation of unique structures such as arbuscules and vesicles by AM fungi of the phylum *Glomeromycota*. This fungus (AMF) helps plants to capture nutrients such as phosphorus, sulphur, nitrogen and micronutrients from the soil. Arbuscular mycorrhizal (AM) fungi are ubiquitous in soil habitats and form beneficial symbiosis with the roots of angiosperms.

The majority of plants established mycorrhizal symbioses with fungi. Some of these symbiotic fungi are arbuscular mycorrhizal fungi (AMF) which are obligate biotrophs that require the host plant to complete their life cycle. Others, such as ectomycorrhizal fungi (ECM), are less dependent on the host plant. In these associations, the fungus supplies the plant with inorganic nutrients, such as nitrate and phosphate, and the plant provides the fungal partners with photosynthates.

Mycorrhizal fungal hyphae extend into the soil, penetrating into nutrient depletion zone and increasing the effectiveness of immobile elements by as much as 60 times. The diversity of AM fungi is also studied in the soils of cultivated cereal crops and medicinal plants in Tamil Nadu (Selvaraj 1989; Mahesh 2002; Murugan 2002; Sankar 2002; Suresh and Selvaraj 2006) and in the coastal regions of Kongan and Servarayan hills of Tamil Nadu (Gopinathan et al. 1991), southeast coast of Tamil Nadu (Nirmala and Selvaraj 2005), west coast of India (Beena et al. 2000) and Western Ghats of Goa (Khade and Rodrigues 2003). In many ecosystems, the major benefits of AMF to symbionts include enhanced nutrient uptake, increased tolerance to root pathogens, drought resistance, tolerance to aluminium and manganese toxicity and improved soil aggregation and structure (Cardoso and Kuyper 2006; Xavier and Germida 2000).

The present research work was focused with the arbuscular mycorrhizal status of selective dicot plants and selective monocot plants grown under greenhouse condition. It was carried out with the following objectives: soil study by determining soil pH and soil moisture, isolation and identification of arbuscular mycorrhizal spores, assessment of AM fungal infection in host roots and finding out the effect of AM fungi on morphological and physiological efficiency of chickpea (*Cicer arietinum* L.), cowpea (*Vigna unguiculata* L.), green pea (*Pisum sativum* L.), *Triticum aestivum* (L.) and *Pennisetum glaucum*.

11.2 Materials and Methods

11.2.1 Soil Study

The soil samples used for this study were air-dried, mixed thoroughly and analysed for pH and moisture.

11.2.2 Soil pH

Ten grams of air-dried soil were added to 100 ml of distilled water and made to a suspension of 1:10 (w/v) solution. Then the pH of the suspension was determined using pH meter.

11.2.3 Determination of Soil Moisture

Soil moisture was determined by taking soil samples between 11 a.m. and 12 noon and drying them in an oven at 120 °C. The values are expressed as percentage on a wet weight basis.

11.2.4 Plants Grown Under Greenhouse Condition

Seeds of chickpea (*Cicer arietinum* L.), cowpea (*Vigna unguiculata* L.), green pea (*Pisum sativum* L.), *Triticum aestivum* (L.) and *Pennisetum glaucum* were surface sterilized with 0.1 % mercuric chloride for 5 min and washed with sterile water repeatedly. Sterile garden soil was used to fill the earthen pots (20 cm height, 25 cm diameter). About 3 kg of sterile soil was taken in each earthen pot. Ten seeds were sown in each pot. After germination, the seedlings were thinned out to 6 in. each pot. All experimental plants were maintained in the greenhouse under conditions of broad daylight. Sterile tap water was used to water the plants. The pots were assigned for the following treatments in five different plants:

C – Control (without AM fungi treatment)
T – Test (AM fungi treated)

11.2.5 Inoculation with AM Fungi

The selective AM fungal inoculum was mass cultured in the maize plants in sterile soil under potted conditions. Five grams of soil inoculum with AM fungal spores and sporocarps and infected root bits were spread over the lower layer of soil (1 kg) in each AM-labelled pot. Then 2 kg of soil was layered over the inoculum before sowing.

11.2.6 Determination of Growth

The vegetative growth of plant was measured for the following growth parameters at a regular interval of 15 days.

11.2.7 Determination of Fresh and Dry Weight

The plant materials were cut into bits and weighed. Then they were dried in an oven at 90 °C until the weight became constant.

11.2.8 Determination of Shoot and Root Length

The shoot and root lengths of the plants were measured using a metre scale.

11.2.9 Chlorophyll Estimation

The chlorophyll content of leaf tissue was estimated following the method of Arnon (1949).

Leaf tissue weighing 50 mg was homogenized in 80 % pre-chilled acetone (80 ml acetone + 20 ml water) in diffused light using a mortar and pestle and centrifuged. The pellet was extracted again with acetone and centrifuged. This process was repeated till the pellet turned nongreen. The supernatants were pooled and the absorbance of the extract was read at 645 nm and 663 nm. The chlorophyll content (mg/g fr.wt) was calculated on a fresh weight basis using the following formula:

$$\text{Total chlorophyll(mg / g fr.wt)} = \frac{22.4 \times \text{A645} + 8.02 \times \text{A663}}{1 \times 1000 \times \text{W}} \times \text{V}$$

$$\text{Chlorophyll} - \text{a (mg / g fr.wt)} = \frac{22.9 \times \text{A663} - 2.69 \times \text{A645}}{1 \times 1000 \times \text{W}} \times \text{V}$$

$$\text{Chlorophyll} - \text{b(mg / g fr.wt)} = \frac{22.9 \times \text{A645} - 4.68 \times \text{A663}}{1 \times 1000 \times \text{W}} \times \text{V}$$

Where

l = Path of light length in cm (1 cm)
V = Volume of the extract in ml
W = Fresh weight of the sample in g

11.2.10 Carotenoid Estimation

The absorbance of the acetone extract of leaves was read at 480 nm, 645 nm and 663 nm, and the amount of carotenoids was estimated according to Ridley (1977) using the formula

$\text{A480} + (0.114 \times \text{A663}) - (0.638 \times \text{A645})$ and the extinction coefficient of 100 mM^{-1} cm^{-1}.

11.2.11 AM Fungi-Host Root Colonization Study

The root tissues (1–2 mm thickness) were washed well in water, cut into 1 cm long segments and fixed in formalin-acetic acid-alcohol (2:1:3) mixture. Soil samples collected from the root zones of plant species at a depth of 15 cm were kept in polythene bags and stored at 5 °C for wet sieving.

11.2.12 Assessment of Fungal Infection

The root materials were cleared and stained using the improved procedure of Phillips and Hayman (1970).

The root segments in 10 % potassium hydroxide were incubated at 90 °C in an oven for 2 h and washed well with distilled water. Then, the segments were immersed in 30 % hydrogen peroxide for 10–15 min for bleaching. They were thoroughly rinsed in water to remove hydrogen peroxide and acidified in 5N hydrochloric acid. They were stained by immersing for 30 min in 0.05 % trypan blue in lactophenol and mounted. Then the root segments were squashed gently on slides containing few drops of acetic acid-glycerol (1:1 w/v) mixture and sealed the coverslips with nail polish. The slides were observed under a microscope and recorded the arbuscular mycorrhizal-infected root samples.

The percent infection of AM for each plant species was measured by grid line intersect method (GioVannetti and Mosse 1980) which is based on the method of Newman (1966) and calculated by using the formula

$$\%\ \text{infection} = \frac{\text{Number of AM infected roots}}{\text{Total number of root bits examined}} \times 100$$

11.2.13 Isolation of AM Spores from Soil Samples

Spores and sporocarps present in the root zone soil were isolated following the decanting and wet-sieving technique of Gerdemann and Nicolson (1963). Five grams of soil samples were suspended in water and were allowed to settle down for some time. The suspension was passed through a series of sieves with 250, 206, 90 and 40 µm pore size. The spores in the soil suspension were collected.

The spores collected were placed on the filter paper and examined under a binocular microscope, transferred to a clean microscopic slide with the help of a fine needle and mounted in lactophenol. Semi-permanent slides were made by sealing the edges of the coverslip with nail pol-

ish. Microscopic observations were made under high magnification for qualitative and quantitative characters of spores.

11.2.14 Statistical Analysis

The data collected in this study was subjected to analysis of variance (ANOVA), and means comparison has been done using Duncan's multiple range test (DMRT) Duncan 1995).

11.3 Result and Discussion

Rhizosphere soil is an imperative one to assess the AMF diversity in the roots of host plant and also associated with a great variety of plants of different taxonomic groups (Jeffries 1987). The rhizosphere is a highly dynamic, plant-driven micro-environment, which is characterized by interaction between plant root processes, soil characteristics and associated microbial population (Wenzel et al. 1999). In general, the distribution of AM spores in rhizosphere soil is governed by edaphic and certain climatic factors. According to Khaliel (1988), pH is the only edaphic factor which determines the abundance of AM fungi. However, pH did not influence the mycorrhizal spore density and frequency (Bergan and Koske 1981).

Mycorrhizal fungi usually proliferate both in the root and in the soil. The soilborne or extrametrical hyphae take up nutrients from the soil solution and transport them to the root. By this mechanism, mycorrhizae increase the effective absorptive surface area of the plant roots. In nutrient-poor or moisture-deficient soils, nutrients taken up by the extrametrical hyphae can lead to improved plant growth and reproduction (Harley and Smith 1983). As a result, mycorrhizal plants are often more competitive and are able better to tolerate environmental stresses than non-mycorrhizal plants.

Mycorrhizae are non-pathogenic symbiotic soil fungi which invade the root system of plants. This association is not only restricted to the roots of plants, but it is also found in all those organs of plants which are concerned with the absorption of substances from the soil. Among the different types of mycorrhizae, arbuscular mycorrhizal fungi (AMF) have gained much importance in the field of agriculture. The main advantages of mycorrhiza are its greater soil exploration and increased uptake of P, N, K, Zn, Cu, S, Fe, Ca and Mn and supply of these nutrients to the host roots (Sundar et al. 2010; Javot et al. 2007).

Microorganisms are present in great number near the fine feeder roots of most of the plant species, and they play vital role in numerous physiological processes. Most widespread symbiosis of plant is the mycorrhizal association between root-inhabiting fungi and the feeder roots (Marx 1977; Cordell et al. 1987). Mycorrhiza refers to an association of symbiosis between plants and fungi that colonize the cortical tissue of roots during periods of active plant growth. These symbioses are characterized by directional movement of nutrients where carbon flows to the fungus and inorganic nutrients move to the plant, thereby providing a critical linkage between the plant root and soil.

Ubiquitous occurrence and importance of AM fungi for plant growth are now a well-established fact. The symbiotic arbuscular mycorrhizal (AM) fungi develop on an extensive hypha network and provide water and nutrients to plants. The distribution of AM spores in rhizosphere soil is governed by edaphic and certain climatic factors. The soil pH did not influence the mycorrhizal spore density and frequency (Bergan and Koske 1981). High soil phosphorus and nitrogen content caused a reduction in infection and number of AM spores (Mosse 1981; Azcon-Aquilar and Barea 1982) as well as a decrease of dependency of the plant on the fungal association (Ojala et al. 1983). Khaliel (1988) showed that the pH is the only edaphic factor which determines the abundance of AM fungi.

Naturally, soil provides the physical support needed for the anchorage of the root system of a plant and also serves as the reservoir of air, water and nutrients which are essential for plant growth. Generally, the pH of the soil determines the mineral contents as well as microbial composition. Fungi are predominant in the rhizosphere under

low-pH conditions (<5.5), and beneficial nitrogen-fixing microorganisms are favoured at neutral pH. High pH releases K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+} and Al^{3+} by weathering processes of soil, whereas low pH favours solubility of salts including carbonates, phosphates and sulphates.

The water holding capacity of a soil is governed by the porosity or soil moisture. The field capacity of a soil is the amount of water held in the soil after the excess gravitational water has drained away. This can be measured by saturating the soil with water followed by draining for 2–3 days under normal conditions which is then expressed as the percentage of water in the dry weight volume of the soil. Soil moisture plays a significant role on mycorrhizal development and colonization (Singh 2001). The consequences of soil organisms promoting a mutually beneficial relationship between plant roots and bacteria in the rhizosphere on root architecture, nutrient uptake and plant productivity are therefore of current research interest (Mantelin and Touraine 2004).

In this study, soil analysis of potted soil showed highly alkaline pH (9.58), and the soil moisture was 33.4 %. The soil moisture and pH initiated the AM fungal colonization in roots and spore production in rhizosphere regions of leguminous plants. Soil moisture plays a significant role on mycorrhizal development and colonization (Singh 2001). Santhaguru and Sadhana (2000) reported that the rhizosphere soil of *Acacia* species had pH ranged from 7.4 to 8.0. Thus, the soil pH (Ouimet et al. 1996; Sidhu and Behl 1997) and soil fertility (Abbott and Robson 1991) determined the abundance of AM fungal spores.

The intensive root colonization of the host resulted in better plant growth in terms of dry matter (Abbott and Robson 1982). It was also observed that colonization and spore population vary with time and increase with advancing growth stages of the plant (Johri and Mathew 1989). The dicot plant study showed that the colonization of AM fungi was highest (94 ± 0.24 %) in *Vigna unguiculata* roots and was 80 ± 0.27 % in *Pisum sativum* roots (Table 11.6 and Plate 11.2a, b). But the monocot study showed that the root colonization of AM fungi was highest (92 ± 1.24 %) in *Triticum aestivum* roots and was 85 ± 1.09 % in *Pennisetum glaucum* roots (Table 11.6 and Plate 11.3a, b). The AM fungi differ widely in the level of colonization they produce in a root system and in their impact on nutrient uptake and plant growth (Sambandan 1995). Krishna et al. 2000 found that root colonization by indigenous VAM fungi differed among 30 pearl millet genotypes. In another experiment, with two male-sterile lines, restorer lines and their derived crosses, Krishna et al. 2000) also found that root colonization differed significantly among pearl millet genotypes, suggesting that the trait for VAM colonization is heritable.

AM fungi have been shown to differentially colonize plant roots, causing a variety of effects on plant growth, biomass and photosynthesis. There have been many reports on the effect of vesicular-arbuscular mycorrhiza (*Glomus mosseae*) on the growth and productivity of legumes. Researchers observed that VAM has significant effect when compared with non-mycorrhizal plants (Fidelibus et al. 2000; Mathew and Hameed 2002; Lukiwat and Simanungkalit 2002; Niranjan et al. 2002; Zaidi et al. 2003; Rohyadi et al. 2004; Singh et al. 2004; Jalaluddin 2005; Rajasekaran and Nagarajan 2005; Satyawathi et al. 2005; Deshmukh et al. 2007; Avis et al. 2008).

The extensive colonization of AM fungi in soybean roots has been found in soils with high phosphorus (Khalil et al. 1992). Mycorrhiza was also known to improve soil structure and stability by forming aggregates (Jakobsen et al. 1992). It is evident that hyphae of AM fungi bind sand grains forming sand aggregates, which remain intact even after the death of the root and hyphae (Koske and Polson 1984).

Abbott and Robson (1991) have reported that VAM hyphae extend beyond the root hair zone and increase the absorptive surface area of the host. The external VAM hyphae reach beyond the depletion zone around the root hairs, absorb soil phosphorus and translocate it to the arbuscules (Sanders and Tinker 1971) where phosphorus is transferred to the plant cell in exchange for carbon.

Fifteen arbuscular mycorrhizal (AM) fungi are reported in the rhizosphere soil of three solanaceous vegetables, namely, tomato, chilli and brinjal, collected from five different locations (Reddy et al. 2006). The genus *Glomus* is the most dominant fungus followed by *Acaulospora*, *Sclerocystis*, *Gigaspora* and *Entrophospora*. Udaiyan and Sugavanam (1996) reported that inoculation of *Glomus fasciculatum* with plants of *Casuarina equisetifolia* results in higher growth and biomass. Mudalagiriyappan et al. (1997) analysed that AM fungal inoculation significantly increased dry matter production and improved the growth rate and net assimilation rate.

Fries et al. (1998) have shown that the success of a mycorrhizal symbiosis is influenced by the availability of phosphorus in the soil. They have studied the effect of VAM fungus *Glomus intraradices* on the growth of *Zea mays* grown under five different levels of soil phosphorus. They have reported in maize that under low phosphorus levels, VAM plants have greater shoot dry weight (13 %), root phosphorus concentration (15 %) and protein concentration (30 %) than non-VAM plants. At higher phosphorus levels, mycorrhizal roots have weighed less than control plants (10 %) without an alteration of growth or root phosphorus concentration.

The presence of oval, round or irregularly lobed vesicles occurring between or inside cortical cells, attached to hyphae and containing oil globule, was a sign of AM fungal infection in *Cicer arietinum*, *Vigna unguiculata*, *Triticum aestivum* and *Pennisetum glaucum* roots. These vesicles act as storage structures. The presence of arbuscules in the infected roots is intended to serve as two-way channels for transport of nutrients; more particularly, carbohydrate structures known as appressoria connect AM fungal ramifications inside roots with the mycelium of the fungus outside the root and serve as absorbing elements from soil to roots (Plate 11.3b).

The four AM fungal species were isolated (Plate 11.1a–f) from the rhizosphere soils of three leguminous species belonging to the genera *Glomus* and *Acaulospora*, and such fungal spores were identified based on the keys proposed by Gerdemann and Trappe (1974), Nicolson and Schenck (1979), Trappe (1982) and Schenck and Perez (1987, 1990). Natural soil offer a consortium of indigenous mycorrhizal fungi and is often used as source of inoculum. AM fungi can be produced on a large scale by pot culture technique. The beneficial use of AM inoculum in agriculture and raising nurseries has been reported (Muthukumar et al. 2001; Smith and Read 1997).

Al-Raddad (1995) has assessed mycorrhizal infection and sporulation in five crops inoculated with *Glomus mosseae* grown for 10 weeks. The effectiveness of each host has been assessed by measuring spore numbers. The highest spore numbers are observed in the rhizosphere of barley plants followed by chickpea and beans. The lower spore numbers are observed in the rhizosphere of corn and okra plant. Hyphae growing beyond the rhizosphere soil increase the absorptive surface of the root (George et al. 1995).

In *Vigna unguiculata*, the fresh weight and dry weight of the control and AM fungi-inoculated plants were gradually increased, and it was a function of age. The AM fungi-inoculated legume showed significant ($P \leq 0.05$) increase in the fresh and dry weight (2.92 ± 0.02 and 0.89 ± 0.02 g/plant) when compared to control (2.81 ± 0.02 and 0.83 ± 0.01 g/plant). In *Cicer arietinum*, the fresh weight and dry weight of the control and AM fungi-inoculated plants were gradually increased, and it was a function of age. The AM fungi-inoculated legume showed significant ($P \leq 0.05$) increase in the fresh and dry weight (3.20 ± 0.01 and 0.85 ± 0.02 g/plant) when compared to control (2.97 ± 0.02 and 0.77 ± 0.04 g/plant). In green pea, the fresh weight and dry weight of the control and AM fungi-inoculated plants were gradually increased, and it was a function of age. This AM fungi-inoculated legume showed significant ($P \leq 0.05$) increase in the fresh and dry weight (2.83 ± 0.01 and 0.91 ± 0.04 g/plant) when compared to control (2.54 ± 0.03 and 0.86 ± 0.01 g/plant) (Tables 11.1, 11.2, and 11.3 and Figs. 11.1, 11.2, and 11.3).

In the monocot plant study, the fresh weight and dry weight of the control and AM fungi-

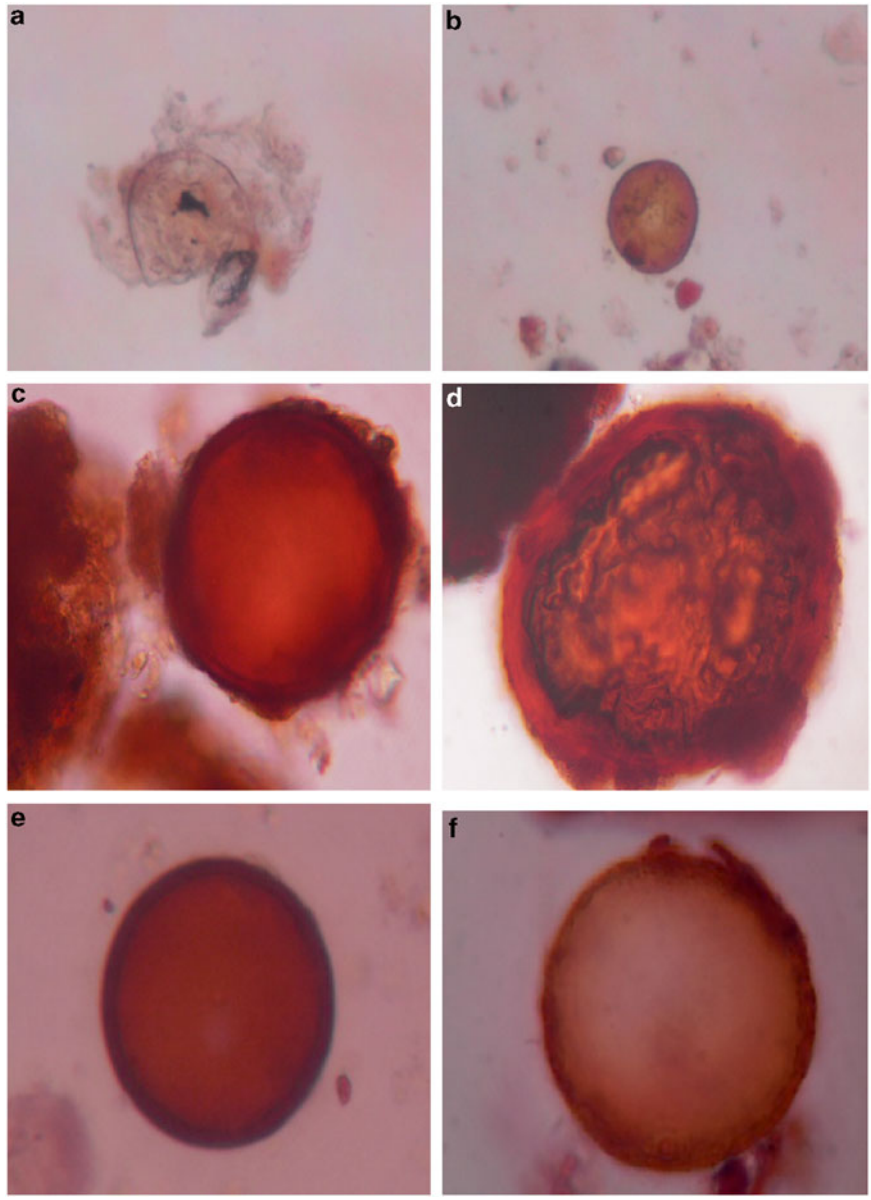

Plate 11.1 Isolated AM fungal spores. (**a**) *Glomus* sp. (**b**) *Acaulospora* sp. (**c**) *Glomus sporocarpia*. (**d**) *Glomus maculosum* (**e**) *Glomus invermatium*. (**f**) *Glomus versiformae*

inoculated *Triticum aestivum* and *Pennisetum glaucum* plants were gradually increased, and it was a function of age. The AM fungi-inoculated *Triticum aestivum* showed significant ($P \leq 0.05$) increase in the fresh and dry weight (0.75 ± 0.02 and 0.23 ± 0.01 g/plant) when compared to control (0.60 ± 0.02 and 0.18 ± 0.04 g/plant). In *Pennisetum glaucum*, the fresh weight and dry weight of the control and AM fungi-inoculated plants were gradually increased, and it was also a function of age. The AM fungi-inoculated *Pennisetum glaucum* showed significant ($P \leq 0.05$) increase in the fresh and dry weight (0.79 ± 0.02 and 0.26 ± 0.05 g/plant) when compared to control (0.58 ± 0.01 and 0.21 ± 0.03 g/plant) (Tables 11.4 and 11.5 and Figs. 11.4

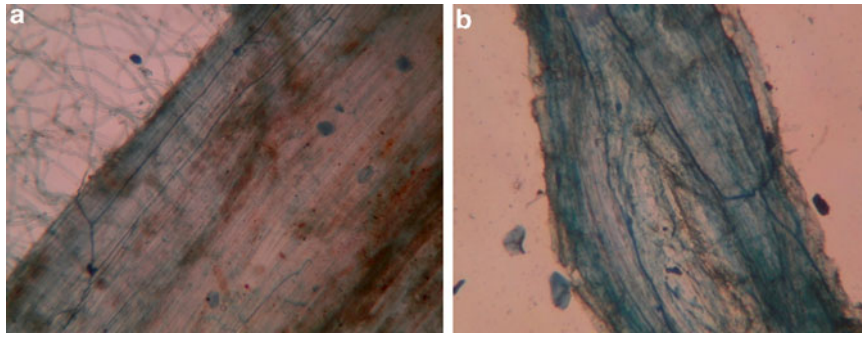

Plate 11.2 AM fungal mycelium infection in root tissues of *Vigna unguiculata* and *Cicer arietinum* (**a**, **b**)

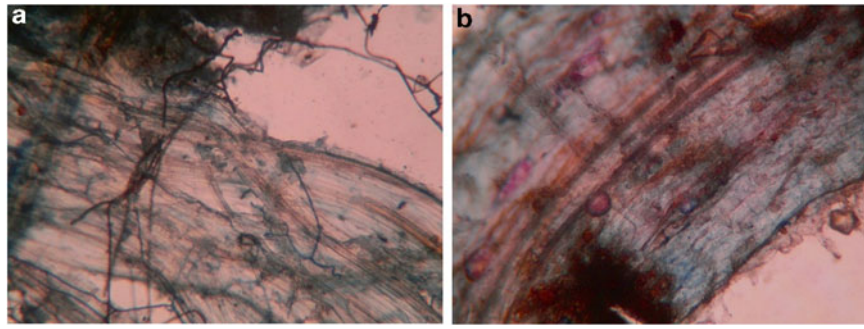

Plate 11.3 AM fungal mycelium and vesicular infection in root tissues of *Triticum aestivum* (**a**, **b**)

Table 11.1 Effect of AM fungi on the growth of cowpea (*Vigna unguiculata*)

Sl. no.	Growth parameters	Number of days					
		10th day		20th day		30th day	
		Control	Test	Control	Test	Control	Test
1	Root length (cm)	1.9 ± 0.03^{a}	2.8 ± 0.02^{a}	8.3 ± 0.02^{b}	9.8 ± 0.02^{b}	13.3 ± 0.02^{c}	14.2 ± 0.03^{c}
2	Shoot length (cm)	13.8 ± 0.02^{a}	15.4 ± 0.01^{a}	24.7 ± 0.02^{b}	25.3 ± 0.01^{b}	29.1 ± 0.04^{c}	31.5 ± 0.01^{c}
3	Fresh weight (g)	0.99 ± 0.04^{a}	1.08 ± 0.02^{a}	1.50 ± 0.03^{b}	1.75 ± 0.03^{b}	2.81 ± 0.02^{c}	2.92 ± 0.02^{c}
4	Dry weight (g)	0.24 ± 0.01^{a}	0.32 ± 0.02^{a}	0.58 ± 0.02^{b}	0.73 ± 0.01^{b}	0.83 ± 0.01^{c}	0.89 ± 0.02^{c}

Values are mean of five replicates ± SD
The mean difference is significant at P<0.05
Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using DMRT at $p < 0.05$

Table 11.2 Effect of AM fungi on the growth of chickpea (*Cicer arietinum*)

Sl. no.	Growth parameters	Number of days					
		10th day		20th day		30th day	
		Control	Test	Control	Test	Control	Test
1	Root length (cm)	3.6 ± 0.04^{a}	4.4 ± 0.02^{a}	7.3 ± 0.01^{b}	8.2 ± 0.01^{b}	10.4 ± 0.02^{c}	11.5 ± 0.03^{c}
2	Shoot length (cm)	9.1 ± 0.02^{a}	12.7 ± 0.01^{a}	18.6 ± 0.03^{b}	20.3 ± 0.04^{b}	25.3 ± 0.01^{c}	28.6 ± 0.02^{c}
3	Fresh weight (g)	1.16 ± 0.01^{a}	1.25 ± 0.02^{a}	2.26 ± 0.04^{b}	2.95 ± 0.01^{b}	2.97 ± 0.02^{c}	3.20 ± 0.01^{c}
4	Dry weight (g)	0.29 ± 0.02^{a}	0.31 ± 0.03^{a}	0.62 ± 0.02^{b}	0.65 ± 0.03^{b}	0.77 ± 0.04^{c}	0.85 ± 0.02^{c}

Values are mean of five replicates ± SD
The mean difference is significant at P<0.05
Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using DMRT at $p < 0.05$

Table 11.3 Effect of AM fungi on the growth of *Pisum sativum*

Sl. no.	Growth parameters	Number of days					
		10th day		20th day		30th day	
		Control	Test	Control	Test	Control	Test
1	Root length (cm)	3.5 ± 0.01^a	4.9 ± 0.04^a	9.7 ± 0.02^b	11.2 ± 0.03^b	12.9 ± 0.02^c	15.1 ± 0.01^c
2	Shoot length (cm)	7.3 ± 0.03^a	9.6 ± 0.02^a	20.5 ± 0.02^b	23.7 ± 0.01^b	26.7 ± 0.02^c	29.8 ± 0.04^c
3	Fresh weight (g)	0.98 ± 0.01^a	1.14 ± 0.03^a	1.84 ± 0.01^b	2.05 ± 0.02^b	2.54 ± 0.03^c	2.83 ± 0.01^c
4	Dry weight (g)	0.25 ± 0.02^a	0.31 ± 0.01^a	0.55 ± 0.02^b	0.64 ± 0.02^b	0.86 ± 0.01^c	0.91 ± 0.04^c

Values are mean of five replicates ± SD
The mean difference is significant at P<0.05
Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using DMRT at $p < 0.05$

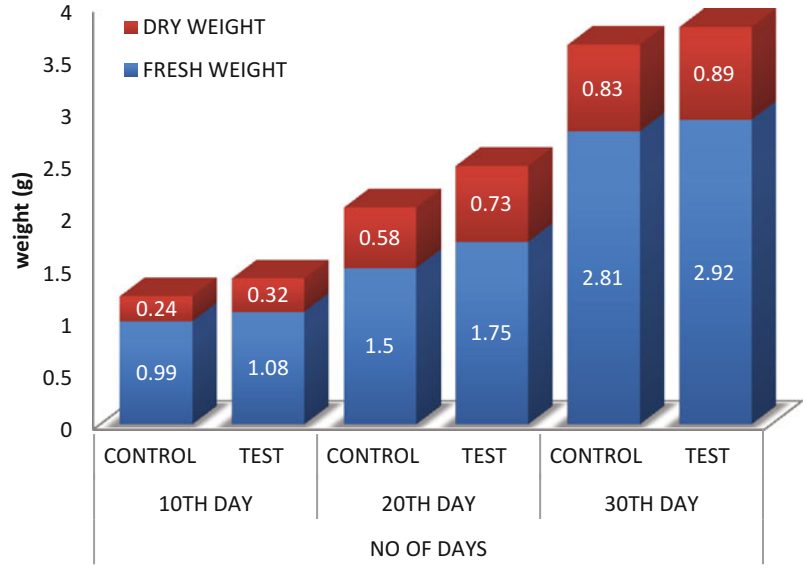

Fig. 11.1 Effect of AM fungi on the fresh and dry weight (g) of *Vigna unguiculata*

and 11.5). Abdulla and Fattah (2000) have reported that at all growth stages shoot and root dry weights of peanut plants inoculated with *Glomus mosseae* are significantly higher than those of non-inoculated plants.

Graminaceous plants establish strong symbiosis with arbuscular mycorrhizal fungi that can improve the uptake of phosphorus from soil. *Sorghum bicolor* is a sugar-rich multipurpose fodder crop well suited to dry farming. Deepadevi et al. (2010) studied the response of *Sorghum bicolor* (L.) Moench to dual inoculation with *Glomus fasciculatum* and *Herbaspirillum seropedicae*. Several host plants including sudan grass (*Sorghum bicolor var. sudanese*), bahia grass (*Paspalum notatum*), *Cenchrus* grass (*Cenchrus ciliaris*), clover (*Trifolium subterraneum*), strawberry (*Fragaria* sp.), sorghum (*Sorghum vulgare*), maize (*Zea mays*), onion (*Allium cepa*) and coleus (*Coleus* sp.) have been used for their suitability to multiply AM fungal inoculum. Sreenivasa and Bagyaraj (1988) reported that Rhodes grass (*Chloris gayana*) is the best host for mass multiplication of *Glomus fasciculatum*.

Thakur and Panwar (1995) reported that the AMF inoculation increased the root, shoot and total dry matter production in mung bean. Setua et al. (1999) have studied the effect of direct inoculation of VAM *Glomus fasciculatum* through sowing of maize seeds and observed that plant height, number of leaves/plant, leaf weight and leaf moisture are significantly greater than in control plants. Tabassum et al. (2011) found out the effect of arbuscular mycorrhizal inoculation on nutrient uptake, growth

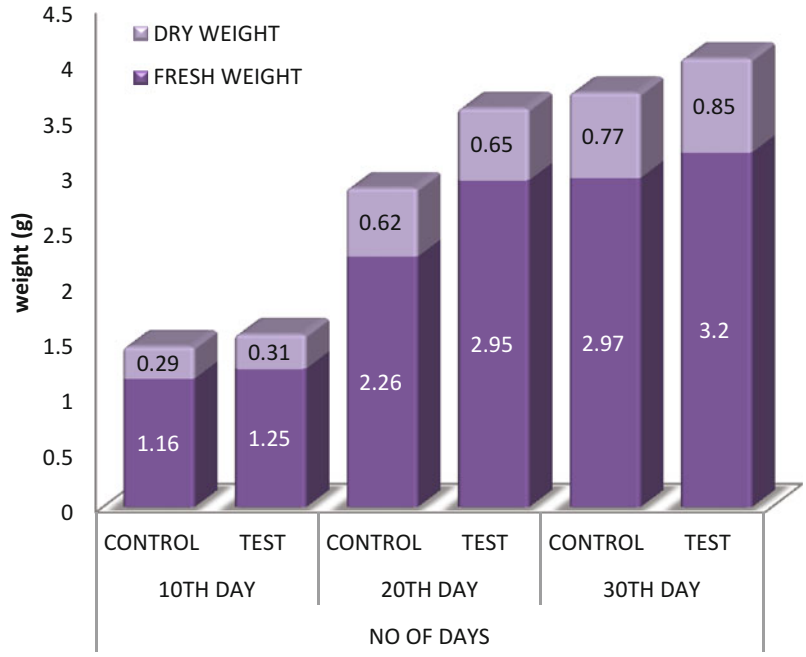

Fig. 11.2 Effect of AM fungi on the fresh and dry weight (g) of *Cicer arietinum*

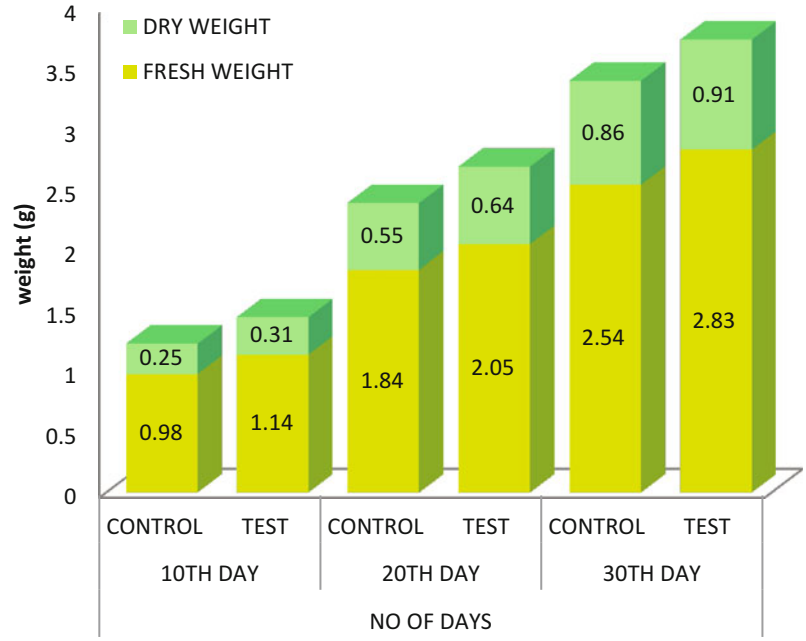

Fig. 11.3 Effect of AM fungi on the fresh and dry weight (g) of *Pisum sativum*

and productivity of cowpea (*Vigna unguiculata L.*) varieties. The AM fungi-inoculated plants showed significant increase in growth, plant height, number of nodules, mycorrhizal dependency and number of flowers per plant over non-inoculated plants. Manimegalai et al. (2011) studied the AM fungi isolation and identification in *Solanum viarum*, and they reported that AM fungi efficiently influenced the plant growth.

The shoot length of the control and all biofertilizer-treated *Vigna unguiculata*, *Cicer*

Table 11.4 Effect of AM fungi on the growth of *Triticum aestivum* (L.)

Sl. no.	Growth parameters	Number of days					
		10th day		20th day		30th day	
		Control	Test	Control	Test	Control	Test
1	Root length (cm)	1.6 ± 0.03^a	2.5 ± 0.07^a	3.4 ± 0.05^b	4.3 ± 0.08^b	4.9 ± 0.10^c	5.8 ± 0.12^c
2	Shoot length (cm)	4.3 ± 0.02^a	5.5 ± 0.04^a	9.7 ± 0.21^b	11.3 ± 0.08^b	14.5 ± 0.09^c	15.9 ± 0.12^c
3	Fresh weight (g)	0.15 ± 0.01^a	0.24 ± 0.02^a	0.49 ± 0.02^b	0.56 ± 0.03^b	0.60 ± 0.02^c	0.75 ± 0.02^c
4	Dry weight (g)	0.02 ± 0.01^a	0.05 ± 0.01^a	0.10 ± 0.01^b	0.15 ± 0.02^b	0.18 ± 0.04^c	0.23 ± 0.01^c

Values are mean of five replicates ± SD
The mean difference is significant at P<0.05
Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using DMRT at $p < 0.05$

Table 11.5 Effect of AM fungi on the growth of *Pennisetum glaucum*

Sl. no.	Growth parameters	Number of days					
		10th day		20th day		30th day	
		Control	Test	Control	Test	Control	Test
1	Root length (cm)	1.8 ± 0.03^a	2.8 ± 0.04^a	3.7 ± 0.01^b	4.7 ± 0.02^b	5.1 ± 0.01^c	5.9 ± 0.05^c
2	Shoot length (cm)	3.7 ± 0.01^a	4.8 ± 0.04^a	8.2 ± 0.03^b	9.7 ± 0.01^b	13.5 ± 0.04^c	14.2 ± 0.04^c
3	Fresh weight (g)	0.12 ± 0.02^a	0.23 ± 0.01^a	0.43 ± 0.04^b	0.57 ± 0.02^b	0.58 ± 0.01^c	0.79 ± 0.02^c
4	Dry weight (g)	0.02 ± 0.01^a	0.03 ± 0.02^a	0.11 ± 0.01^b	0.14 ± 0.01^b	0.21 ± 0.03^c	0.26 ± 0.05^c

Values are mean of five replicates ± SD
The mean difference is significant at P<0.05
Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using DMRT at $p < 0.05$

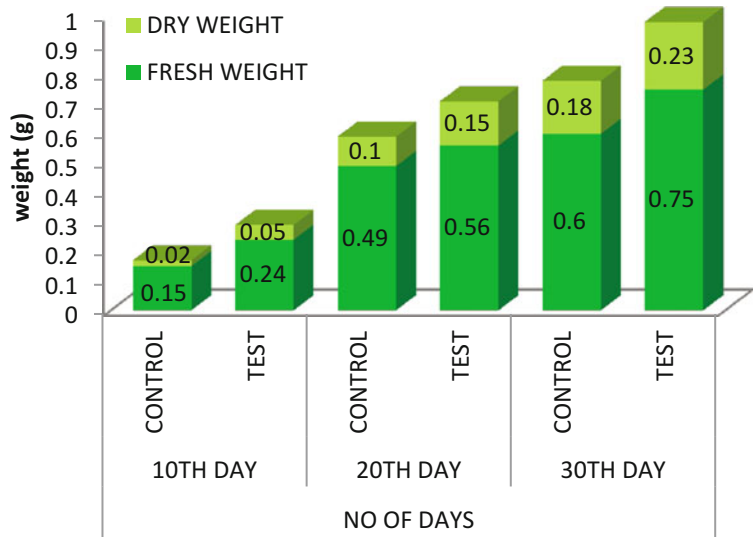

Fig. 11.4 Effect of AM fungi on the fresh and dry weight (g) of *Triticum aestivum*

arietinum and *Pisum sativum* plants increased progressively with age. There was a significant ($P \leq 0.05$) increase (31.5 ± 0.01, 28.6 ± 0.02 and 29.8 ± 0.04 cm/plant) in shoot length found in AM fungi-inoculated plants when compared to control plants (29.1 ± 0.04, 25.3 ± 0.01 and 26.7 ± 0.02 cm/plant). Significantly, the root length of *Vigna unguiculata*, *Cicer arietinum* and *Pisum sativum* plants was higher in AM fungi-inoculated plants (14.2 ± 0.03, 11.5 ± 0.03 and

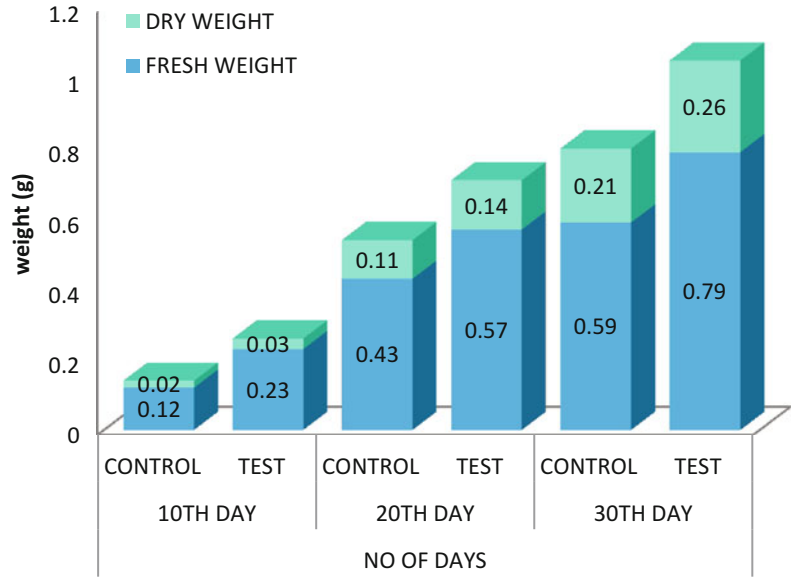

Fig. 11.5 Effect of AM fungi on the fresh and dry weight (g) of *Pennisetum glaucum*

15.1 ± 0.01 cm/plant) than the control (13.3 ± 0.02, 10.4 ± 0.02 and 12.9 ± 0.02 cm/plant) (Tables 11.1, 11.2 and 11.3 and Figs. 11.6, 11.7 and 11.8).

The shoot and root length of the control and AM fungi-treated *Triticum aestivum* and *Pennisetum glaucum* plants increased with age (Tables 11.4 and 11.5 and Figs. 11.9 and 11.10). There was a significant ($P \leq 0.05$) increase (15.9 ± 0.12 and 14.2 ± 0.04 cm/plant) in shoot length found in AM fungi-inoculated *Triticum aestivum* and *Pennisetum glaucum* plants when compared to control plants (14.5 ± 0.09 and 13.5 ± 0.04 cm/plant). The root length was significantly higher in AM fungi-inoculated monocot plants (5.8 ± 0.12 and 5.9 ± 0.05 cm/plant) than the control (4.9 ± 0.10 and 5.1 ± 0.01 cm/plant).

In the present study, the AM fungi-inoculated *Vigna unguiculata*, *Cicer arietinum* and *Pisum sativum* plants (0.1596 ± 0.02; 0.1161 ± 0.03; and 0.1071 ± 0.04 mg chl *a*; 0.1059 ± 0.01; 0.0860 ± 0.03 and 0.0587 ± 0.02 mg chl *b*; and 0.2006 ± 0.01; 0.1546 ± 0.04 and 0.1215 ± 0.02 mg total chl) showed a significant ($P \leq 0.05$) increase in chlorophyll *a* and *b* and total chlorophyll content when compared to control plants (0.0819 ± 0.04; 0.0988 ± 0.02 and 0.0828 ± 0.05 mg chl *a*; 0.0637 ± 0.02; 0.0714 ± 0.04 and 0.0367 ± 0.01 mg chl *b*; and 0.1167 ± 0.02; 0.1303 ± 0.03 and 0.1060 ± 0.02 mg total chl) noticed at the 30th day. And also, the carotenoid content of AM fungi-treated *Vigna unguiculata* plants showed maximum content (0.2882 ± 0.02 mg) when compared to other legumes and control plants (Table 11.7 and Fig. 11.11).

Sitaramaiah et al. (1998) reported that AM fungi-inoculated maize plants showed increased vegetative growth, total chlorophyll content and uptake of nutrients like nitrogen, phosphorus, potassium, calcium and magnesium. VAM-inoculated alfalfa plants were better adapted than non-mycorrhizal ones in coping with the water deficit and increased concentration of proline (Goicoechea et al. 1997). The beneficial use of AM inoculum in agriculture and raising nurseries has been reported (Muthukumar et al. 2001; Smith and Read 1997).

Significantly ($P \leq 0.05$), the AM fungi-inoculated *Triticum aestivum* and *Pennisetum glaucum* showed higher chlorophyll and carotenoid contents (0.18 ± 0.03 and 0.16 ± 0.04 mg chl *a*; 0.16 ± 0.02 and 0.77 ± 0.01 mg chl *b*; and mg 0.12 ± 0.04 and 0.10 ± 0.02 mg total chl) when compared to control plants (0.05 ± 0.02 and 0.04 ± 0.01 mg chl *a*; 0.08 ± 0.02 and 0.17 ± 0.05 mg chl *b*; and 0.10 ± 0.03 and 0.09 ± 0.01 mg total chl) noticed at the 30th day (Table 11.6 and Fig. 11.11). The carotenoid con-

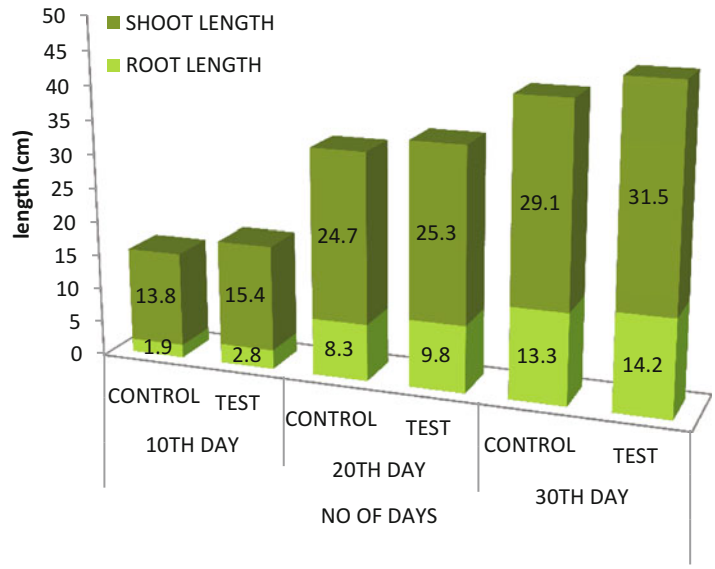

Fig. 11.6 Effect of AM fungi on the root and shoot length (cm) of *Vigna unguiculata*

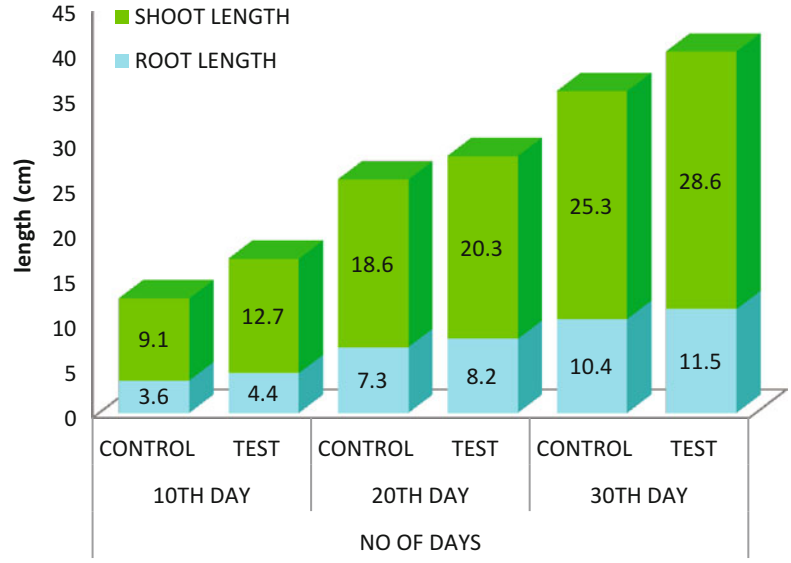

Fig. 11.7 Effect of AM fungi on the root and shoot length (cm) of *Cicer arietinum*

tent was also increased in AM fungi-inoculated monocot plants when compared to non-inoculated plants (Table 11.7 and Fig. 11.12).

The beneficial use of AM inoculum in agriculture and raising nurseries has been reported (Muthukumar et al. 2001; Smith and Read 1997). From the above discussion, the AM fungi play a key role in crop field management and are specifically applied as environment-friendly fertilizers in agriculture and forestry. Simpson and Daft (1990) have reported that the growth stage and physiology of host plants have been postulated to influence spore production of endomycorrhizal fungi. The age of the crop and the harvest date greatly influence the size of the spore population and extent of root colonization of *Glomus mosseae* (Al-Raddad 1991; Kapulnik and Koshnir 1991). Lin et al. (1993) have investigated that the mycorrhizal inoculation with *Rhizobium trifolii* on *Trifolium repens* significantly increases the dry weight of shoots and roots, nodulation, nitrogen fixation, total nutrient uptake, final dry matter and phosphorus absorption.

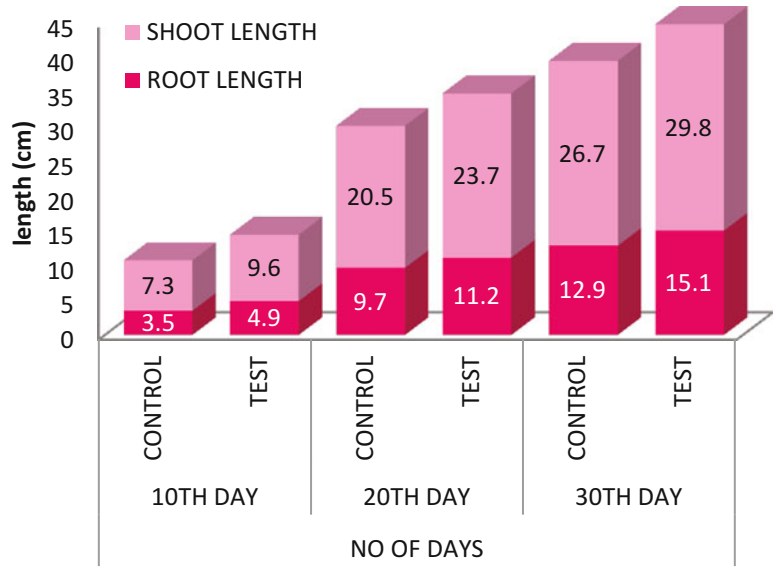

Fig. 11.8 Effect of AM fungi on the root and shoot length (cm) of *Pisum sativum*

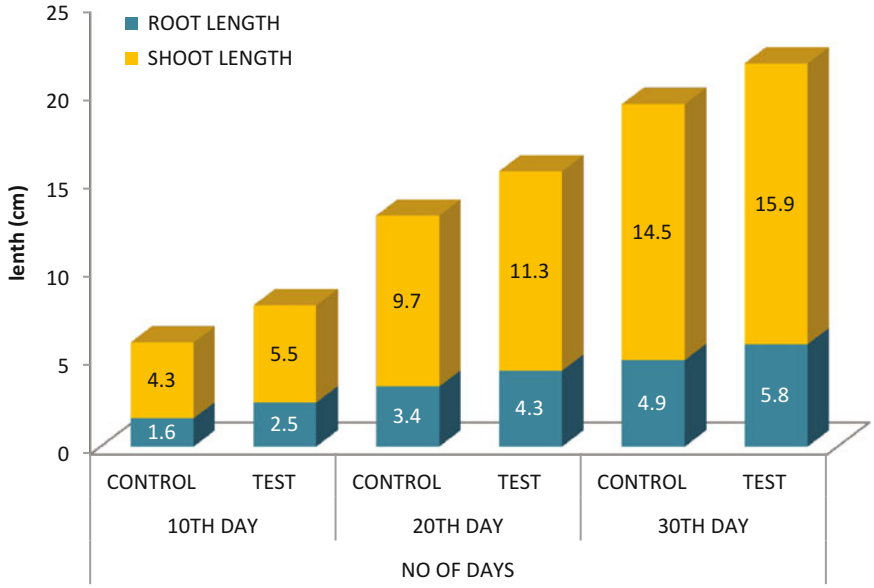

Fig. 11.9 Effect of AM fungi on the root and shoot length (cm) of *Triticum aestivum*

Chaurasia and Khare (2005) examined the four plant species, viz., *Hordeum vulgare*, *Triticum aestivum*, *Phaseolus vulgaris* and *Phaseolus mungo*, for mass production of a consortium of AM fungi present in the rhizosphere soil. Such mass production of AM fungi was observed in terms of (%) AM colonization; AM consortia were recorded in terms of height and dry weight of inoculated and non-inoculated plants. They observed that the *Hordeum vulgare* showed the highest colonization (92 %) at 74 spores per 25 g soil.

The present investigation reported that the AM fungi inoculation in both selective dicot and monocot plants stimulated the plant growth specifically root colonization by AM fungus in which such organism promoted the water and nutrient absorption. From the above discussion,

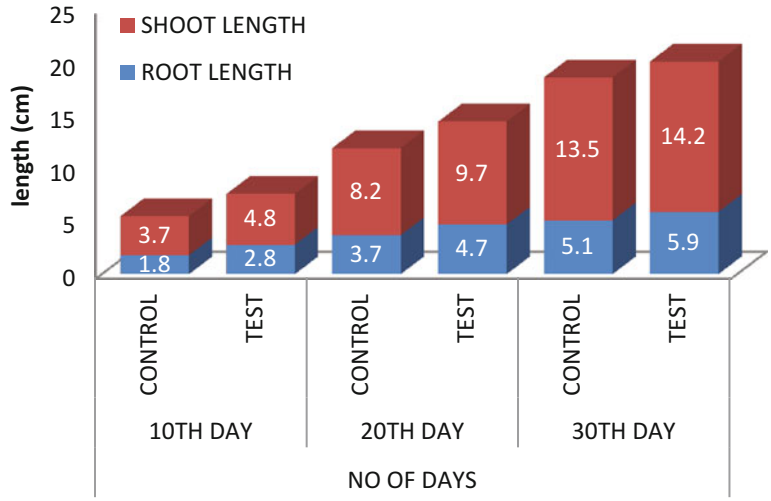

Fig. 11.10 Effect of AM fungi on the root and shoot length (cm) of *Pennisetum glaucum*

Table 11.6 Percent of AM fungal infection in the root tissues of selective dicot and monocot plants

Sl. no.	Plant name	Percentage of infection (%)	Vesicles	Arbuscules
1	*Vigna unguiculata*	94 ± 0.24	+	–
2	*Cicer arietinum*	92 ± 0.35	+	–
3	*Pisum sativum*	80 ± 0.27	+	–
4	*Triticum aestivum*	92 ± 1.24	+	–
5	*Pennisetum glaucum*	85 ± 1.09	–	–

Values are mean of five replicates ± SD + presence – absence

the AM fungi play a key role in crop field management and are specifically applied as environment-friendly fertilizers in agriculture and forestry. The present work is suggested that the AM fungi application in crop field does not cause pollution of any sort and that they are considered as eco-friendly fertilizers.

11.4 Conclusion

Cereals, pulses and millets are consumed as a source of human food and animal feed. Their importance as food lies primarily in their high carbohydrate, protein and fibre content. Legumes' grain protein is the natural supplement to cereal grain protein. They also provide fat and carbohydrates. The arbuscular mycorrhizal (AM) status of three leguminous plants and two monocot plants was studied under greenhouse condition. The alkaline pH of rhizosphere soil initiated the AM fungal colonization in selective dicot and monocot plants. The AM fungal spores were isolated from the rhizosphere soil of selective plants such as *Vigna unguiculata*, *Cicer arietinum*, *Pisum sativum*, *Triticum aestivum* and *Pennisetum glaucum*. The AM spores that belong to the genus *Glomus* and *Acaulospora* were isolated from the rhizosphere soil. The percent of AM fungal colonization was observed maximum (94 ± 0.24 %) in *Vigna unguiculata* roots and was minimum (80 ± 0.27) in *Pisum sativum* roots. The 92 ± 1.24 % of AM fungal colonization was observed in *Triticum aestivum* roots, and *Pennisetum glaucum* root tissues showed 85 ± 1.09 % of AM fungal colonization. The AM fungal inoculation on such legume plants and monocot plants enhanced the growth by increases in shoot and root length and fresh and dry weight and chlorophyll and carotenoid contents of such legume plants under greenhouse conditions. Thus, this study suggested that application of such AM fungal fertilizer in crop plant field promoted the crop growth without cause of any environmental pollution.

Table 11.7 Effect of AM fungi on the chlorophyll and carotenoid content (mg/g fresh weight) of selective dicot and monocot plants

Sl. no.	Plant name	Total chlorophyll		Chlorophyll *a*		Chlorophyll *b*		Carotenoid	
		Control	Test	Control	Test	Control	Test	Control	Test
1	*Vigna unguiculata*	0.1167 ± 0.02	0.2006 ± 0.01	0.0819 ± 0.04	0.1596 ± 0.02	0.0637 ± 0.02	0.1059 ± 0.01	0.0859 ± 0.04	0.2882 ± 0.02
2	*Cicer arietinum*	0.1303 ± 0.03	0.1546 ± 0.04	0.0988 ± 0.02	0.1161 ± 0.03	0.0714 ± 0.04	0.0860 ± 0.03	0.1741 ± 0.02	0.2757 ± 0.02
3	*Pisum sativum*	0.1060 ± 0.02	0.1215 ± 0.02	0.0828 ± 0.05	0.1071 ± 0.04	0.0367 ± 0.01	0.0587 ± 0.02	0.0813 ± 0.03	0.1184 ± 0.01
4	*Triticum aestivum*	0.10 ± 0.03	0.12 ± 0.04	0.05 ± 0.02	0.18 ± 0.03	0.08 ± 0.02	0.16 ± 0.02	0.15 ± 0.04	0.21 ± 0.03
5	*Pennisetum glaucum*	0.09 ± 0.01	0.10 ± 0.02	0.04 ± 0.01	0.16 ± 0.04	0.17 ± 0.05	0.77 ± 0.01	0.14 ± 0.02	0.15 ± 0.02

Values are mean of five replicates ± SD

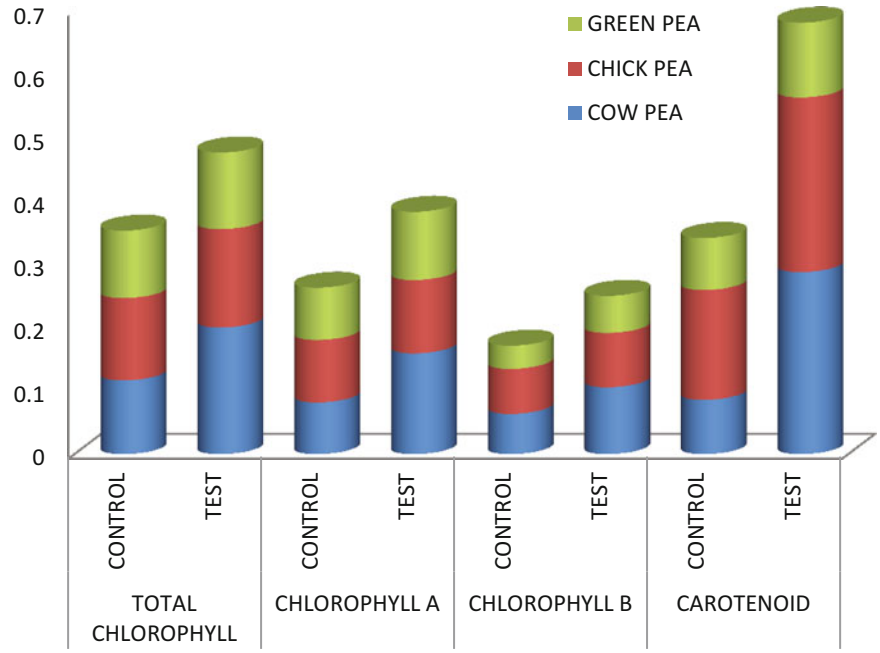

Fig. 11.11 Effect of AM fungi on chlorophyll (mg/g fresh weight) and carotenoid content (mg/g fresh weight) of selective dicot plants

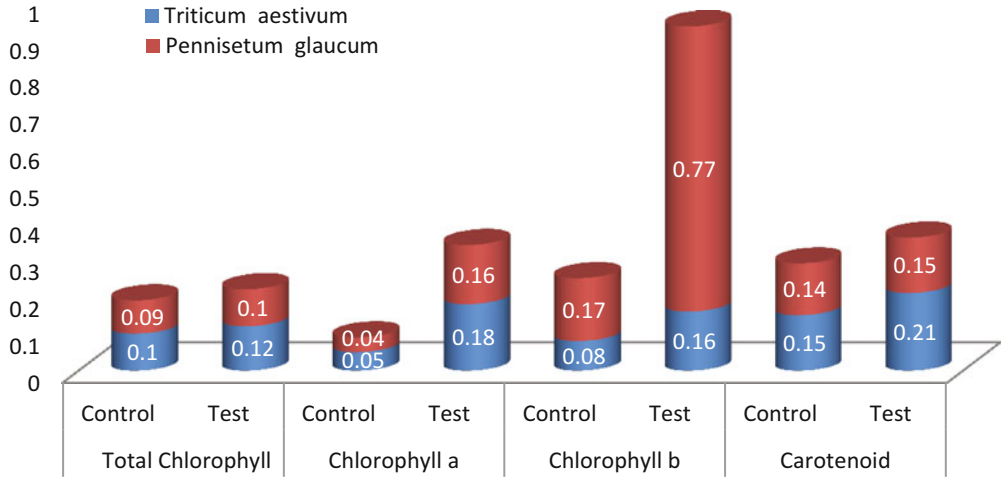

Fig. 11.12 Effect of AM fungi on chlorophyll (mg/g fresh weight) and carotenoid content (mg/g fresh weight) of selective monocot plants

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Trichoderma spp.: Efficient Inducers of Systemic Resistance in Plants

12

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Abstract

Defense response in plants, triggered by biocontrol agents (BCAs), is an intensively investigated area. In recent past, various agriculturally important microorganisms have been identified and described as efficient inducer of systemic resistance in plant. *Trichoderma* spp. are established plant root colonizers and their biocontrol nature is primarily due to mycoparasitism and antibiosis mechanisms against various pathogens. Progress in research in plant immunity induced by beneficial microorganisms suggests that other than mycoparasitism and antibiosis, *Trichoderma* spp. are potent inducers of ISR in plants. There is need for more intensive studies aimed at gaining insight into the signal transduction pathways and defense responses elicited by *Trichoderma*. Furthermore, quick progress in molecular studies will lead to gain deeper insight into the regulation of complex interaction between plant and biocontrol agents and increase the efficiency of currently existing biocontrol strategies and plant disease management modules.

12.1 Introduction

Prior art is loaded with reports of plant defense reprogramming at physiological and biochemical level by beneficial microorganisms against various biological stresses (Bisen et al. 2015; Choudhary et al. 2015; López-Mondéjar et al. 2011; Contreras-Cornejo et al. 2011; Salas-Marina et al. 2011; Perazzolli et al. 2008). Recognition of microbial elicitors by host receptors directly leads to the activation of various signaling pathways and triggers biochemical and physiological changes in plants resulting into strong reaction against pathogen attack (Harman et al. 2012; Contreras-Cornejo et al. 2011). Various mechanisms including direct antagonism to phytopathogens and induction of resistance responses in plants have been reported in disease suppression by rhizospheric microorganisms (Singh 2006; Keswani et al. 2013). The potential of these biocontrol agents to amplify plant defense responses has led to their wider applica-

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tion in management of plant disease (Carreras-Villasenor et al. 2012; Harman et al. 2012; Hermosa et al. 2012; Salas-Marina et al. 2011; Perazzolli et al. 2008).

Plant rhizosphere is a nutrient-rich habitat which harbors diverse communities of microorganisms (Lugtenberg et al. 2001; Walker et al. 2003). Beneficial and pathogenic microorganisms both are part of soil microbiome that either improve plant health or attack the plant. Plant growth-promoting fungi (PGPF) and plant growth-promoting rhizobacteria (PGPR) are nonparasitic, soilborne microbes which share a mutualistic relationship with the host. These beneficial microorganisms promote plant growth by stimulating the photosynthetic capacity of plants (Zhang et al. 2008), inducing systemic resistance responses against abiotic stresses (Yang et al. 2009; Ahn et al. 2007; Segarra et al. 2009), and suppressing plant pathogens both directly and indirectly by strengthening host immunity (Harman et al. 2004; Kloepper et al. 2004; Pozo and Azcon-Aguilar 2007; Van Loon et al. 1998). The disease suppression by biocontrol agents (BCAs) is put forth either through direct action against soilborne pathogens by mycoparasitism, antibiosis, and competition (Singh et al. 2012; Bakker et al. 2007; De Bruijn et al. 2007; Debode et al. 2007; Handelsman and Stabb 1996; Kamilova et al. 2008) or indirectly by triggering ISR response in plants (Kloepper et al. 2004; Van Loon et al. 1998; Van Wees et al. 2008). Local and systemic resistance elicited by BCAs is long-lasting and effective against a broad spectrum of pathogens.

12.1.1 Induced Systemic Resistance

Elevation of plant resistance against various diseases by nonpathogenic microorganisms has been well studied. It was initially demonstrated by experiments including colonization of roots by PGPR which led to the protection of aboveground plant parts from various pathogens (Van Loon et al. 1998). PGPR-ISR has been confirmed in many plants of diverse genera and shown to successfully protect against a wide range of pathogens (Kloepper et al. 2004; Van Loon and Bakker 2006; Van Loon et al. 1998; Van Wees et al. 2008). Nonpathogenic *Pseudomonas* spp. and *Bacillus* spp. are mostly documented PGPRs inducing ISR (Kloepper et al. 2004; Van Loon and Bakker 2006). Even though both ISR and SAR are effective against various pathogens, the range of their effectiveness is partially different. As in the case of *Arabidopsis thaliana*, it was demonstrated that ISR induced by PGPR *Pseudomonas fluorescens* WCS417r and SAR elicited by a weak strain of the bacterial pathogen *P. syringae* pv. *tomato* were equally effective against virulent pathogens *P. syringae*, *Hyaloperonospora arabidopsidis*, and *Fusarium oxysporum* (Pieterse et al. 1996; Ton et al. 2002). On the other hand, in *Arabidopsis*, SAR was shown to be ineffective against two phytopathogens including *Alternaria brassicicola* (Van der Ent et al. 2008) and *Botrytis cinerea* (Ton et al. 2002), whereas ISR was proved effective against these two pathogens.

Extensive research carried out during past decade in plant and fungal biocontrol agent interactions proved that PGPF is also able to elicit ISR in plants just like PGPR. Mycorrhizal fungi (Pozo and Azcon-Aguilar 2007), *Trichoderma* spp. (Harman et al. 2004; Vinale et al. 2008; Keswani et al. 2013; Singh 2014), *Penicillium* sp. GP16-2 (Hossain et al. 2008), nonpathogenic *F. oxysporum* (Duijff et al. 1998; Paparu et al. 2007), *Piriformospora indica* (Stein et al. 2008; Waller et al. 2005), *Pythium oligandrum* (Hase et al. 2008), and *Sebacinales* spp. (Waller et al. 2008) are among well-studied fungi that elicit immune responses in plant.

Trichoderma (teleomorph *Hypocrea*) is a saprophytic fungus commonly found in rhizospheric region of plants. *Trichoderma* spp. are plant symbiont and antagonists against a wide range of seed and soilborne phytopathogenic fungi. *Trichoderma* spp. also improves nutrient use efficiency and nutrient uptake in hosts. Prior art is loaded with biocontrol efficiency of *Trichoderma* spp. employing three major modes of action including mycoparasitism, antibiosis, and

Table 12.1 *Trichoderma*-mediated induced defense responses in various hosts

Biocontrol agent	Host	Pathogen	Reference
T. harzianum T39	Tomato, lettuce, tobacco, pepper	*Botrytis cinerea*	De Mayer et al. (1998)
T. harzianum	Cucumber	*Phytophthora capsici*	Yedidia et al. (1999)
T. harzianum	Pepper	*P. capsici*	Ahmed et al. (2000)
T. harzianum T-22	Corn	*Colletotrichum graminicola*	Harman et al. (2004)
T. harzianum rifai	Arabidopsis	*B. cinerea*	Korolev et al. (2008)
T. harzianum	Grapevine	*Plasmopara viticola*	Perazzolli et al. (2008)
T. harzianum RU01	Bean	*Uromyces appendiculatus*	Abeysinghe (2009)
T. harzianum MUCL 29707	Potato	*Rhizoctonia solani*	Gallou et al. (2009)
T. asperellum T-203	Cucumber	*Pseudomonas syringae* pv. *lachrymans*	Yedidia et al. (2003)
T. asperellum T-203	Cucumber	*P. syringae* pv. *lachrymans*	Segarra et al. (2007)
T. asperellum T-34	Cucumber	*P. syringae* pv. *lachrymans*	Shoresh et al. (2005)
T. asperellum SKT-1	*Arabidopsis*	*P. syringae* pv. *tomato*	Yoshioka et al. (2012)
T. virens	Maize	*C. graminicola*	Djonovic et al. (2007)
T. virens	Cotton	*R. solani*	Howell et al. (2000)
T. hamatum 382 *euvesicatoria*	Cucumber, Tomato	*P. capsici*, *Xanthomonas*	Khan et al. (2004) and Alfano et al. (2007)
T. atroviride	Tomato	*B. cinerea*	Tucci et al. (2011)
T. arundinaceum	Tomato	*B. cinerea*, *R. solani*	Malmierca et al. (2012)
Trichoderma spp.	Hot pepper	*P. capsici*	Bae et al. (2011)
Trichoderma spp.	Tomato	*X. euvesicatoria*, *Alternaria solani*	Fontenelle et al. (2011)
T. harzianum	Sunflower	*R. solani*	Singh et al. (2014a, b)
T. harzianum and *T. koningiopsis*	Chickpea	*Sclerotium rolfsii*	Saxena et al. (2015)

competition (Keswani 2015). Recently biocontrol potential of *Trichoderma* spp. was directly linked to the induction of defense responses in hosts (Table 12.1). It is difficult to categorize which mechanism is involved in the biological management of pathogens per se. Plant defense responses induced by *Trichoderma* spp. are the most viable strategy in case *Trichoderma* fails in direct encounter with pathogens locally. Moreover, this strategy also offers robust systemic resistance against foliar pathogens. *Trichoderma*-mediated induction of ISR responses in hosts is regulated by a network of various defense pathways. Their ability to positively influence host defense as well as direct antagonism against a wide range of soilborne pathogens has been extensively investigated under both in vitro and in vivo conditions (Singh 2014; Harman et al. 2012; Salas-Marina et al. 2011; Contreras-Cornejo et al. 2011).

12.2 *Trichoderma* spp.-Mediated Induction of ISR Response in Hosts

Calderón et al. (1993) first reported the evidence of *Trichoderma*-mediated induction of systemic resistance in grapevine. Later, in *Nicotiana tabacum*, induced resistance against *P. parasitica* was reported by *T. longibrachiatum*. A higher level of PR-1b and PR-5 was observed during induced resistance (Chang et al. 1997). *T. harzianum* T39, when inoculated in soil, triggered defense response in bean plant challenged with *C. lindemuthianum* and *B. cinerea* (Bigirimana et al. 1997). Application of *T. harzianum* in tomato provides local and systemic resistance against *Alternaria solani* (Howell 2003). *T. harzianum* T39 when applied to soil or leaves significantly reduced the disease incidence even in distant plant parts (De Meyer et al. 1998). Furthermore,

treatment of cucumber with T-203 led to cell wall strengthening due to the increased callose concentration (Woo et al. 1999). Increased terpenoid synthesis and expression of defense-related genes were reported in cotton and cucumber plants when treated with *Trichoderma* (Yedidia et al. 2000; Howell et al. 2000).

Trichoderma spp. are potential root colonizers and offer manifold benefits to their hosts. From monocots to dicots, *Trichoderma* spp. are found to induce resistance in an array of plant species (Table 12.1). In order to stimulate the defense response, *Trichoderma* must colonize the plant roots.

12.3 Root Colonization

The molecular mechanisms that direct the interaction between host root and *Trichoderma* are largely unknown. Advances in molecular techniques offered some insights into colonization of plant roots by *Trichoderma* spp. Signaling between *Trichoderma* and plant roots often depends on root-derived exudates (Bais et al. 2006). *Trichoderma* spp. have been isolated from nearly all climatic zones from various root ecosystems. Similar to mycorrhizae, *Trichoderma* growth in rhizosphere is facilitated by polysaccharides secreted by roots. It has been reported that the plant-derived sucrose is a key player in root colonization of *Trichoderma* (Gravel et al. 2007; Contreras-Cornejo et al. 2009; Vargas et al. 2009). Likewise a wide range of proteins are also utilized by *Trichoderma* spp. to facilitate host root colonization. TasHyd1 protein from *T. asperellum* is found to assist in root colonization (Viterbo and Chet 2006), whereas Qid74, a cell wall protein, was recognized as a central player in adherence and cellular protection of *T. harzianum* (Samolski et al. 2012). Swollenin TasSwo protein and the endopolygalacturonase ThPG1 from *T. harzianum* were also reported to facilitate root penetration (Brotman et al. 2008; Morán-Diez et al. 2009).

Advancement in root invasion, *Trichoderma* must tolerate or suppress the host defense mechanism. *Trichoderma* spp. are reported to endure plant-derived toxicants and antimicrobial secondary metabolites. The successful colonization of plant roots by *Trichoderma* leads to the modification at biochemical and physiological level, which assists in plant growth and disease resistance (Zhang et al. 2013; Mukherjee et al. 2012a, b).

12.4 Plant Defense Elicitors Secreted by *Trichoderma* spp.

Elicitation of ISR response in plants begins with the recognition of specific components from microbial cell surface known as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) (Schwessinger and Zipfel 2008) by plant receptors. Interaction between PAMP and corresponding plant receptor activates defense responses in the host which is referred to as PAMP-triggered immunity (PTI) (Jones and Dangl 2006). Similar to PAMPs, a variety of MAMPs from various BCAs have been linked with ISR (Bakker et al. 2007; Kloepper et al. 2004; Van Loon et al. 2008; Van Wees et al. 2008). MAMP responses start with the generation of ion fluxes, reactive oxygen species (ROS), nitric oxide, and ethylene (ET) and later involve the accumulation of callose and the biosynthesis of antimicrobial substances. In plants, various MAMPs have been identified for PGPRs, including lipopolysaccharides and flagellin. In addition to various volatile organic compounds (VOCs), antibiotics and biosurfactants have been unraveled to trigger a systemic resistance response in plants (Lorito et al. 2010).

So far, MAMPs involved in ISR have only been identified for *Trichoderma* spp. among various fungal BCAs (Table 12.2) (Vinale et al. 2008). The first MAMP identified from *Trichoderma* was an ET-inducing xylanase (Xyn2/Eix consisting five surface-exposed amino acids), eliciting plant defense responses in tomato and tobacco (Rotblat et al. 2002). *Trichoderma*-activated cellulases also trigger defense response by activation of the ET and SA pathways (Martınez et al. 2001). *Trichoderma* proteins involved in root colonization such as swollenin

Table 12.2 *Trichoderma* MAMP identified in various species

Trichoderma spp.	MAMPs	Action	Reference
T. viride	Xylanase Xyn2/Eix	Xylanase elicits biosynthesis of ethylene and hypersensitive response in leaf tissues of tobacco	Rotblat et al. (2002)
T. longibrachiatum	Cellulases	Cellulase elicits defenses by activating SA and ET signaling pathways	Martinez et al. (2001)
T. virens/*T. atroviride*	Cerato–platanins Sm1/Epl1	Hydrophobin-like SSCP induces defense responses in cotton	Djonovic et al. (2006), Seidl et al. (2006) and de Oliveria et al. (2011)
T. asperelloides	Swollenin TasSwo	Capable of stimulating local defense in cucumber against *B. cinerea* and *P. syringae*	Brotman et al. (2008)
T. harzianum	Endopolygalacturonase ThPG1	Colonization of tomato root and ISR defense	Mora'n–Diez et al. (2009)
T. viride	Alamethicin (20mer peptaibol)	Elicit JA and SA synthesis in bean	Engelberth et al. (2001)
T. pseudokoningii	Trichokonin (20mer peptaibol)	Production of ROS, accumulation of phenolic compounds, and virus resistance to virus in tobacco plants	Luo et al. (2010)
T. virens	18mer peptaibols	Elicit systemic defense in cucumber against *P. syringae*	Viterbo et al. (2007)
Trichoderma spp.	6-Pentyl-α-pyrone, harzianolide, and harzianopyridone	Activation of plant defense and plant growth regulation in pea, canola, and tomato	Vinale et al. (2008)

TasSwo also trigger defense responses in cucumber (Brotman et al. 2008). Another protein endopolygalacturonase ThPG1 stimulates resistance response in *Arabidopsis* (Moran-Diez et al. 2009). During colonization of maize and cotton roots by *T. atroviride* and *T. virens*, proteins Ep1l and Sm1 accumulated, respectively, in hyphae and act as MAMPs (Djonovic et al. 2006; Seidl et al. 2006).

Scavenging of chitin is essential for colonization of fungal pathogens as they act as defense elicitors in plant (de Jonge et al. 2010). As a mechanism for identifying chitin, plants secrete chitinases to free the polymers from the attacking fungi cell walls and triggering resistance responses. Accordingly, the mycoparisitic action of *Trichoderma* chitinases also releases chitooligosaccharides and indirectly helps in triggering a defense response. Various secondary metabolites from *Trichoderma* show antifungal effect at high concentration but act as MAMPs at low doses (Keswani et al. 2014). Harzianolide, 6-pentyl-α-pyrone, and harzianopyridone activate plant defense response and regulation in tomato, pea, and canola at 1 ppm concentration (Vinale et al. 2008). Alamethicin from *T. viride* triggered SA and JA biosynthesis in bean (Engelberth et al. 2001), whereas 18mer peptaibols elicit defenses against *Pseudomonas syringae* pv. *lachrymans* in cucumber (Viterbo et al. 2007). Peptaibols trigger manifold defense signaling pathways in tobacco plants against the tobacco mosaic virus (Luo et al. 2010).

Peptaibol secondary metabolites from *Trichoderma* spp. were shown to trigger a systemic defense response in plants (Leitgeb et al. 2007; Viterbo et al. 2007; Luo et al. 2010; Druzhinina et al. 2011). In maize, recently an enzyme hybrid was identified as PKS/NRPS which was involved in defense responses (Mukherjee et al. 2012a, b). Sm1/Epl1 elicitor from *Trichoderma* spp. is well studied (Djonović et al. 2006; Seidl et al. 2006), and knockdown of this gene resulted in weak induction of ISR responses in maize (Djonovic et al. 2007).

12.5 Defense Signaling Pathways Activated During ISR

Until the results published in 1998 suggesting the role of plant root colonization by *T. harzianum* and *T. asperellum* in induction of resistance responses in plant, ISR induced by *Trichoderma* was not well investigated (De Meyer et al. 1998; Yedidia et al. 1999). Root colonization by *Trichoderma* leads to the systemic alteration in proteome, transcriptome, and MAMP interaction in leaves (Shoresh et al. 2010). Plants react instantly to invasion by *Trichoderma* through rapid ion fluxes, oxidative burst, and callose deposition followed by polyphenol biosynthesis (Shoresh et al. 2010). Successive actions involve SA and JA/ET signaling resulting in induction of resistance responses of varied degrees in the entire plant against pathogen attack (Shoresh et al. 2010) (Fig. 12.1).

This action generally referred to as JA/ET-mediated ISR is similar to the PGPR-ISR. However it has been found that at higher concentration, *Trichoderma* triggers an SA-mediated SAR response that resembles to that elicited by necrotrophic phytopathogens (Segarra et al. 2007; Contreras-Cornejo et al. 2011; Salas-Marina et al. 2011; Yoshioka et al. 2012). Moreover, signaling events during induced resistance in plants are not meticulously studied. As in the case of PGPR-ISR, it has been confirmed that *Trichoderma*-mediated ISR includes JA/ET signaling pathways (Shoresh et al. 2005). JA/ET-deficient *Arabidopsis* plants when treated with *Trichoderma* showed enhanced susceptibility to *B. cinerea* (Korolev et al. 2008). It has also been reported that the induced plant responses is a time dependent and concentration dependent phenomenon. SAR-like response was observed in cucumber plant after 4 h of treatment with *Trichoderma* with a sharp increase in peroxidase and SA activity, and a systemic increase in JA and SA levels was recorded after high density of *Trichoderma* inoculation (Segarra et al. 2007). JA/SA-dependent defense response was observed after treatment of potato plant with *T. harzianum* challenged with *Rhizoctonia solani* (Gallou et al. 2009). Recent findings suggest that *Arabidopsis* root colonization by *T. asperellum* induces ISR response through SA signaling (Yoshioka et al. 2011). *T. atroviride* when colonizing the *Arabidopsis* root challenged with pathogens triggers local and systemic expression of SA and JA/ET pathways (Salas-Marina et al. 2011). When unchallenged by any pathogen, *Trichoderma* triggered a long-term upregulation of SA gene in plants; however, after the infection with *B. cinerea*, *Trichoderma* modulated the SA-dependent gene expression and, soon after the infection, JA signal transduction occurred, causing ISR to increase with time (Tucci et al. 2011). *A. thaliana* when treated with *T. hamatum* T382 showed a sharp activation of defense response after subsequent inoculation with *B. cinerea* (Mathys et al. 2012). Recent advances in gene expression studies have provided plentiful evidences in favor of JA and ET involvement in signal transmission. Genes related to JA/ET signaling pathways like *Pal*, *Lox*, *ETR1*, *Hpl*, and *CTR1* were found upregulated after *Trichoderma* inoculation (Yedidia et al. 2003; Shoresh et al. 2005; Gallou et al. 2009; Perazzolli et al. 2011) suggesting the key role of JA and ET in systemic resistance.

12.6 *Trichoderma*–Plant Cross-Talk

Plant growth and immunity are regulated through complex interconnected hormone signaling pathways (Pieterse et al. 2009). There is a cross-communication between vital defense pathways including JA/ET, SA, and the other signaling cascades associated with growth and abiotic stress including abscisic acid in plants. During interaction of plants with *Trichoderma*, the ACC deaminase (ACCD) action suppresses the level of 1-aminocyclopropane-1-carboxylic acid (ACC) which is necessary for the biosynthesis of ET. Reductions in ET level lead to plant growth promotion via gibberellin signaling through enhanced degradation of DELLA proteins. In addition, the gibberellin hormone may direct the launch of JA- and SA-dependent resistance responses in the plant by regulating DELLA pro-

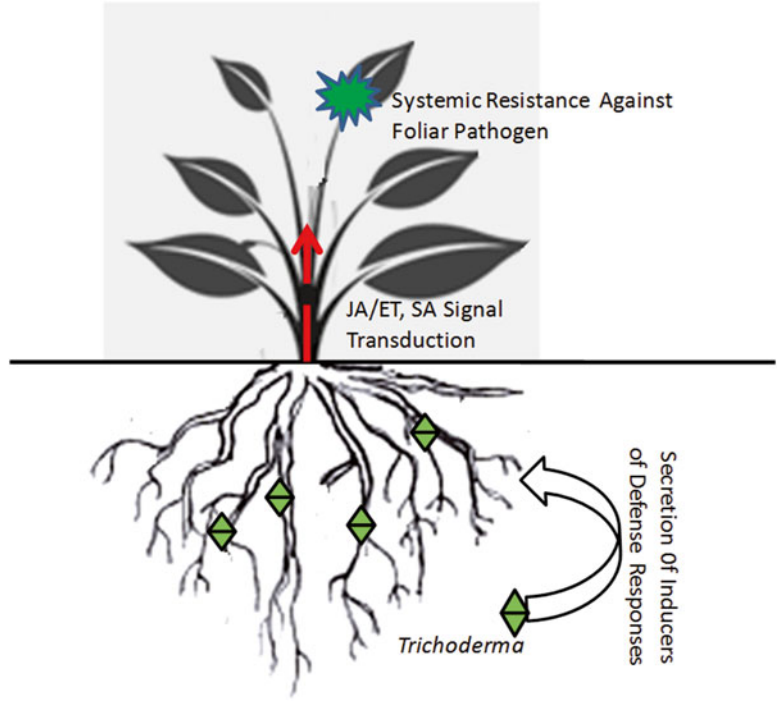

Fig. 12.1 Graphic representation of *Trichoderma*–plant signaling and induced resistance in plant

tein degradation. IAA and ET in plant roots can equally regulate each other's biosynthesis (Stepanova et al. 2007), and according to the reports, *Trichoderma* IAA contributes to ET biosynthesis via ACC synthase leading to the enhanced biosynthesis of abscisic acid.

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Induced Systemic Resistance by Rhizospheric Microbes

13

Manoj Kumar, Priyanku Teotia, Ajit Varma, Narendra Tuteja, and Vivek Kumar

Abstract

In a natural ecosystem, plants copiously form advantageous and constructive relations with soil microbiomes that are significant and vital for plant growth survival and, as such, influence plant biodiversity and overall ecosystem performance. Conventional and typical examples of symbiotic microbes are ecto- and endomycorrhizal fungi that assist in water and nutrients uptake and *Rhizobium* bacteria that fixes free atmospheric nitrogen for plant. Advantageous microorganisms in the overall microbiome of plant roots zone enhance the plant vigor. Induced systemic resistance (ISR) developed as a significant and imperative means and way by which the selected and potential plant growth-promoting microbes in the rhizosphere influence the whole plant structure for higher and better defense against the broad range of pathogens and insect herbivores. A plethora of root-associated mutualistic microbes, including mainly common microbes such as *Pseudomonas*, *Bacillus*, *Trichoderma*, and ecto- and endomycorrhizal species, trigger and induce the plant's immune system for boosted defense without precisely activating the expensive defenses. A lot of research work and evidences advocate that advantageous microorganisms are firstly established as possible plant invaders, after which the plant's immune system is triggered, while, at delayed stages of the plant-microbe interaction, the mutualists are able to trigger the plant defense mechanism to enable efficacious colonization of the plant roots.

13.1 Introduction

The plant fixes the solar energy that drives nearly all living processes on Earth. Subsequently, plants are fundamental and principle players in a multifaceted food web in which plentiful members profusely take benefit of the plant's

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resources. Besides many microbial pathogens and insect herbivores, plants also cultivate and encourage huge population of commensal and mutualistic microorganisms that provide plants with vital and indispensable amenities, such as higher mineral uptake, phosphate solubilization, nitrogen fixation, growth promotion, and also pathogen protection (Lugtenberg and Kamilova 2009; Shores et al. 2010). This plant microbiome is largely located on the plant root system, which deposits up to 40 % of the plant's photosynthetically fixed carbon sources into the rhizosphere zone. Therefore, this trivial region around the roots is one of the most energy-rich areas on the mother Earth (Bais et al. 2006).

Plant and pathogen interaction can lead to effective infection (a compatible response) or resistance (an incompatible response). The incompatible response or interactions, caused by viruses, bacteria, or fungi, will definitely provoke a set of localized responses in and around the infected plant cells or tissue. This response may include an oxidative burst, which could lead to cell death (Verhage et al. 2010). Consequently, the plant pathogen may be "trapped" in the dead plant cells and may be prevented from spreading from the site of original infection to the uninfected sites. Additional local responses in the nearby or surrounding plant cells also include modifications in cell wall conformation that may also constrain the intrusion by the pathogen and lead to the synthesis of de novo antimicrobial compounds such as phytoalexins (Thakur and Sohal 2013).

Numerous genera of the rhizosphere microbes, which are mentioned as plant growth-promoting rhizobacteria (PGPR), bacteria (PGPB), and fungi (PGPF), could augment plant growth and enhance overall health (Shores et al. 2010). Van Peer et al. (1991) demonstrated that after root system colonization by PGPB *Pseudomonas fluorescens* WCS417r, aboveground plant parts acquired a heightened level of resistance against infection by a fungal pathogen *Fusarium oxysporum*. Furthermore, *P. fluorescens* WCS417r-treated plants manufactured considerably extra antimicrobial phytoalexins at the site of infection by the intruding pathogen. Henceforth, the signals provided by *P. fluorescens* WCS417r to the root system sensitize aboveground plant parts for greater intruding pathogen protection. Wei et al. (1991) used a similar technique in cucumber crop and exhibited that root colonization by dissimilar favorable *Pseudomonas* and *Serratia* PGPB strains resulted in the noteworthy and substantial lessening of disease symptoms with the anthracnose pathogen *Colletotrichum orbiculare*. In both the important research work, PGPR and pathogen have been demonstrated to remain spatially separated during the experiments, which showed that improved level of disease resistance was caused by the plant-facilitated immune response known as microbial-induced systemic resistance (MISR) or popularly ISR. Experiment conducted by Alstrom (1991) did not lead to evidence for spatial separation between PGPR and the challenging pathogen *Pseudomonas syringae* pv. *phaseolicola*, while studies conducted by Wei et al. (1991) suggested that colonization of common bean roots by PGPR strain *P. fluorescens* S97 triggered ISR in foliar tissues. Since these publications on rhizobacteria-mediated ISR, thereafter hundreds of studies in dicots and monocots have reported on the ability of PGPR to promote plant health via ISR (Thakur and Sohal 2013). These research works largely involved microbes like *Pseudomonas*, *Serratia*, and *Bacillus* PGPR strains and nonpathogenic *F. oxysporum*, *Trichoderma*, and *Piriformospora indica* PGPF strains, but symbiotic arbuscular mycorrhizal fungi were also exhibited to elicit ISR. While the early review on ISR mediated by rhizobacteria was cited by Van Loon et al. (1998), noteworthy growth has been done in interpretation of molecular reason of ISR triggering, signaling, and expression, particularly in *Arabidopsis thaliana*, a model plant species.

13.2 Induced Resistance

The word "induced resistance" is a generic term for induced state of resistance in plants caused by biological or chemical inducers, which protects non-exposed plant parts against future attack by pathogenic microorganisms and herbivorous

insects (Ku'c 1982). Plants can develop induced resistance as a result of infection by a pathogen, in response to insect herbivory, upon colonization of the roots by specific beneficial microbes or after treatment with specific chemicals. The induced state of resistance is characterized by the activation of latent defense mechanisms that are expressed upon a subsequent challenge from a pathogen or insect herbivore. Induced resistance is expressed not only locally at the site of induction but also systemically in plant parts that are spatially separated from the inducer, hence the term ISR. Generally, induced resistance confers an enhanced level of protection against a broad spectrum of attackers (Walters et al. 2013). Induced resistance is regulated by a network of interconnected signaling pathways in which plant hormones play a major regulatory role (Pieterse et al. 2012). The signaling pathways that regulate induced resistance elicited by beneficial microbes, pathogens, and insects share signaling components. Therefore, we first highlight the important principles of pathogen- and insect-induced resistance before reviewing the current status of ISR mediated by beneficial soil-borne microbes.

13.3 Induced Resistance and Plant Immune System

In the past decade, groundbreaking conceptual advances in the understanding of the evolutionary development of the plant's immune system (Jones and Dangl 2006) placed our knowledge on induced resistance in a clear perspective. In the current concept of the plant's immune system, pattern-recognition receptors (PRRs) have evolved to recognize common microbial compounds, such as bacterial flagellin or fungal chitin, called pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) (Boller and Felix 2009; Zipfel 2009). Plants also respond to endogenous plant-derived signals that arise from damage caused by enemy invasion, called damage-associated molecular patterns (DAMPs) (Boller and Felix 2009). Pattern recognition is translated into a first line of defense called PAMP-triggered immunity (PTI), which keeps most potential invaders in check (Dodds and Rathjen 2010).

Successful pathogens have evolved to minimize host immune stimulation and utilize virulence effector molecules to bypass this first line of defense, by either suppressing PTI signaling or preventing detection by the host (Bardoel et al. 2011; De Jonge et al. 2010). In turn, plants acquired a second line of defense in which resistance (R) nucleotide-binding leucine-rich repeat (NB-LRR) receptor proteins mediate recognition of attacker-specific effector molecules, resulting in effector-triggered immunity (ETI) (Dodds and Rathjen 2010). ETI is a manifestation of gene-for-gene resistance, which is often accompanied by a programmed cell death at the site of infection that prevents further ingress of biotrophic pathogens that thrive on living host tissue. The onset of PTI and ETI often triggers an induced resistance 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 (Dempsey and Klessig 2012; Shah and Zeier 2013). This well-characterized form of pathogen-induced resistance is commonly known as systemic acquired resistance (SAR) (Vlot et al. 2009; Spoel and Dong 2012) and confers enhanced resistance against a broad spectrum of pathogens. As with the pathogen recognition system, plants also recognize herbivorous insects, most likely through a similar signaling concept (Howe and Jander 2008).

13.4 Immune Signaling in the Rhizosphere

Both symbiotic and nonsymbiotic beneficial microbes are initially recognized as alien organisms. Hence, active interference with the plant's immune system is fundamental for the establishment of intimate mutualistic relationships. Immune signaling in plants is initiated upon receptor-mediated perception of non-self-molecules that are often conserved among different classes of microbes, both pathogenic

and beneficial. These molecules are called microbe-associated molecular patterns (MAMPs), and MAMP-induced defense responses mounted in host plants are collectively referred to as MAMP-triggered immunity (MTI) (Boller and Felix 2009; Jones and Dangl 2006). Despite the fact that innate immune signaling in leaves has been extensively studied over the past years, very little is known about MTI in roots, where the majority of the plant beneficial microbes reside. Recently, Millet and associates (2010) demonstrated that *Arabidopsis* roots respond to different MAMPs in a tissue-specific manner and that MAMP-triggered immune signaling in the roots is very similar to that observed in the leaves. To establish a mutualistic interaction with the plant, beneficial microbes need to cope with host immune responses that are triggered locally in the roots upon MAMP perception.

13.5 Systemic Acquired Resistance Signaling Induced Pathogen

In the 1960s, Ross coined the term SAR for the phenomenon in which uninfected systemic plant parts become more resistant in response to a localized infection elsewhere in the plant (Ross 1961). Over the years, SAR has been extensively reviewed, and in the current concept of the plant's immune system, the onset of pathogen-induced SAR is triggered upon local activation of a PTI or ETI response (Mishina and Zeier 2007). In systemic tissues, SAR is characterized by increased levels of the hormone salicylic acid (SA) (Vlot et al. 2009). Early genetic studies in tobacco demonstrated that SA accumulation and signaling are essential for the establishment of SAR (Vernooij et al. 1994). In addition, SAR is accompanied by the coordinate activation of pathogenesis-related (PR) genes, many of which encode PR proteins with antimicrobial activity. Among the best-characterized PR genes is PR-1, which is often used as a marker for SAR (Van Loon et al. 2006).

For initiation of SAR in distal organs, a long-distance signaling cascade in the vascular tissues, in which the lipid-transfer protein Defective in Induced Resistance1 (DIR1) is likely to act as a chaperone for the mobile SAR signal(s), appears to be crucial (Maldonado et al. 2002). Despite the fact that SA accumulates in the phloem sap of SAR-expressing plants, grafting experiments with tobacco showed that SA itself is not the translocated SAR signal (Vernooij et al. 1994). Genetic and biochemical studies uncovered several metabolites putatively involved in long-distance SAR signaling, including the methyl ester of SA (MeSA), the diterpenoid dehydroabietinal (DA), a glycerol-3-phosphate (G3P)-dependent factor, azelaic acid (AzA), and pipecolic acid (Pip). In systemic tissues, the onset of SAR requires the function of flavin-dependent monooxygenase 1 (FMO1) (Mishina and Zeier 2006), possibly to transduce or amplify long-distance signals originating from primary leaves.

13.6 Induced Resistance Signaling by Herbivore

Green and Ryan (1972) demonstrated that herbivory and wounding of tomato leaves result in the systemic accumulation of proteinase inhibitors that inhibit digestive enzymes in the insect gut. It was proposed that long-distance signals produced at the site of tissue injury mediate a systemic resistance against herbivorous insects. Along with the production of anti-insecticidal toxins and feeding deterrents (direct defense), herbivory also triggers the production of volatiles that attract natural predators of the attacking herbivore (indirect defense). Herbivore-induced resistance signaling is initiated upon the release of plant-derived signals (e.g., DAMPs) and elicitors from insect oral secretions at the site of tissue injury, called herbivore-associated molecular patterns (HAMPs) (Mithofer and Boland 2008; Hogenhout and Bos 2011). Furthermore, insect-derived effector molecules have been reported that suppress host defenses (Hogenhout and Bos 2011). Hence, plants may have evolved R genes

against herbivore effectors, as they did for pathogen effectors. An example of this is the Mi gene that confers resistance against aphid feeding (Rossi et al. 1998).

Perception of herbivory-related elicitors results in rapid release of oxylipins from membrane lipids. The jasmonate (JA) family of oxylipins emerged as key signals, as JA biosynthesis and signaling mutants are impaired in herbivore-induced resistance (Wasternack and Hause 2013). Jasmonoyl-isoleucine (JA-Ile) was identified as the biologically active signal, which is perceived by a co-receptor complex consisting of the F-box protein CORONATINE INSENSITIVE1 (COI1) and JASMONATE ZIM-domain (JAZ) proteins. Perception of JA-Ile by the COI1-JAZ co-receptor results in proteasome-mediated degradation of the JAZ proteins that in un-induced cells suppress positive regulators of JA-mediated defense responses, such as the transcription factors MYC2, 3, and 4. In JA-stimulated cells, the JA signaling pathway becomes depressed, resulting in the activation of a large number of JA-responsive genes (Memelink 2009). The long-distance signal(s) for systemic expression of herbivore-induced resistance was obscure for a long time. In Arabidopsis, it was recently shown that wound-induced membrane depolarization by ion fluxes rapidly mediates JA biosynthesis and JA-responsive gene expression in distal leaves. Glutamate receptor-like proteins (GLRs) were shown to mediate these wound-induced surface potential changes, indicating that electric signaling is also important in wound-induced systemic signaling (Mousavi et al. 2013).

13.7 Host Immunity Modulation in the Legume-Rhizobia Symbiosis

Rhizobia have evolved to reduce stimulation of the host's immune system. In order to evade detection by the host's immune system, successful microbes evolved ways to minimize recognition of their MAMPs. Flagellin, the major structural protein of flagella, is one of the best-studied bacterial proteins that are recognized as a MAMP by the plant's immune system (Boller and Felix 2009). In *Arabidopsis*, flagellin is perceived by the FLAGELLIN-SENSING 2 (FLS2) receptor, after which an intracellular signaling cascade is initiated, leading to the activation of a defense program against the invading bacteria. The immunogenic properties of flagellin reside in the highly conserved N-terminus of the molecule. Flg22, a synthetic 22-amino-acid peptide that corresponds to the conserved N-terminus of flagellin, is a potent elicitor of defense responses in *Arabidopsis* and other plant species (Felix et al. 1999). Lopez-Gomez along with co-researchers (2011) demonstrated that flg22-triggered defense responses in the roots of *Lotus japonicus* negatively influence nodulation by inhibiting rhizobial infections and delaying the nodule organogenesis. However, flagellins from the legume symbiont *Sinorhizobium meliloti* are exceptionally divergent in the otherwise conserved flagellin epitope, and neither the crude flagellin extracts nor the corresponding flg22 synthetic peptide are able to elicit defense responses (Segonzac et al. 2012).

Large-scale gene expression profiling studies of early nodulation stages in the model legumes *L. japonicus* and *Medicago truncatula* revealed a significant induction of defense- and stress-related genes, indicating that the leguminous hosts initially recognize their symbiotic partners as a potential threat. However, the same cluster of genes was found to be downregulated at later stages of root nodule formation, indicating that the microsymbionts have evolved to actively suppress host defense responses (Moreau et al. 2011). Maunoury and researchers (2010) reported two waves of transcriptional reprogramming in *M. truncatula* involving repression of defense-related genes followed by the activation of a nodule-specific transcriptome. By using a collection of plant and bacterial mutants, the authors demonstrated that this transcriptome switch is dependent upon a molecular dialogue between both partners.

13.8 Host Immunity Modulation in Mycorrhizal Associations

Symbiotic mycorrhizal fungi reduce stimulation of the host's immune system. Other than through MAMPs, microbes can also be detected via damage-associated molecular patterns, which are endogenous plant-derived molecules that arise from damage or enzymatic degradation of cell walls, such as that caused by invading alien organisms. Interestingly, the genome of the ectomycorrhizal fungus (EMF) *Laccaria bicolor* lacks several gene families that encode for enzymes involved in the degradation of plant cell walls that could otherwise elicit immune responses (Zamioudis and Pieterse 2012). Likewise, the EMF *Tuber melanosporum* (black truffle) also carries a relatively small number of carbohydrate-cleaving enzymes (Chen et al. 2013). Early studies regarding the interaction between plants and arbuscular mycorrhizal fungi (AMF) revealed that expression of defense- and stress-related genes is prominent during early stages of the interaction and subsequently declines as the symbiosis develops (Kapulnik et al. 1996). Most research based on large-scale transcriptional profiling in various symbiotic plant-mycorrhiza interactions revealed that defense-related gene expression in host plants follows similar expression patterns (Heller et al. 2008; Liu et al. 2003). Thus, the transcriptional response activated in roots upon mycorrhization may be similar to the two-wave transcriptional reprogramming reported for *Rhizobium* sp. symbiosis.

Sequencing of the genomes of the EMF *Laccaria bicolor* and *T. melanosporum* provided the first important evidence that symbiotic fungi may use strategies similar to those of pathogenic fungi to evade host immunity. In *Laccaria bicolor*, whole-genome sequence analysis combined with genome-scale expression profiling revealed candidate molecules that may act as effectors in modulating plant innate immunity, as demonstrated for effectors of several pathogenic fungi and oomycetes (Pletta et al. 2014). Twelve predicted proteins of the symbiotic fungus share significant similarity with haustoria-expressed secreted proteins that are involved in pathogenesis of pathogenic basidiomycetes (Pletta et al. 2014). In addition, the genome of *Laccaria bicolor* encodes a number of small secreted proteins (SSP), many of which are induced during the symbiotic interaction. In contrast to the EMF, the genomes of the AMF are not assembled yet, and thus, predictions for putative effectors in the AM symbioses remain elusive. AMF, on the one hand, and certain biotrophic fungal and oomycete pathogens, on the other hand, employ similar invasion strategies to infect their hosts (Paszkowski 2006). The transcriptional responses mounted in host plants in response to biotrophic pathogens and AMF significantly overlap, pointing to the existence of conserved molecules that execute similar functions (Paszkowski 2006). Therefore, it is anticipated that certain molecules secreted by the AMF in the apoplastic or periarbuscular space during the interaction with the host act as either apoplastic or cytoplasmic effectors in order to short-circuit the plant defense program.

13.9 ISR in Nonsymbiotic Beneficial Interactions

Like rhizobia and mycorrhizal fungi, nonsymbiotic beneficial microbes such as PGPR, which often grow endophytically inside the roots, should also minimize stimulation of their host's immune system. Phenotypic variation or phase variation is an adaptive process by which bacteria can reversibly switch between colonies with different morphology (Davidson and Surette 2008). At the molecular level, phase variation is controlled by diverse genetic mechanisms, including site-specific DNA rearrangements and epigenetic modifications (Hallet 2001; Wisniewski-Dye and Vial 2008). Either of these mechanisms generates bacterial subpopulations within a clonal population that differentially express surface molecules (e.g., flagella or LPS) or express surface molecules with altered structure (Van der Woude and Baumler 2004). Phase variation provides bacteria

with a significant advantage of adaptation to different environments and has been extensively documented in several studies as a mechanism that animal pathogens employ to escape immune detection (Kingsley and Baumler 2000). Phenotypic variation is also common among rhizosphere pseudomonads and has been reported as a conserved strategy that bacteria have evolved in order to increase their overall fitness in the rhizosphere (Van den Broek et al. 2005).

Rhizosphere *Pseudomonas* bacteria may use antigen variation to reduce their antigenic potential and, therefore, minimize stimulation of the host's immune system. For instance, the PGPR *Pseudomonas brassicacearum* shows two distinct morphological variants designated as phase I and phase II (Achouak et al. 2004). Phase I cells are found on the basal parts of the root and produce significantly lower amounts of flagellin compared with phase II cells, which are predominantly found on secondary roots and root tips (Achouak et al. 2004). It is possible that, once colonization of new root niches is achieved, *P. brassicacearum* shifts into phase I cells in order to mask flagellin recognition by the host. Interestingly, *P. aeruginosa* was recently found to excrete an alkaline protease (AprA) that degrades flagellin monomers that serve as ligands for the immune receptors FLS2 in plants and TLR5 in mammals, thereby evading host immune activation in both plants and mammals (Bardoel et al. 2011). In *P. brassicacearum*, AprA was demonstrated to be expressed in phase I cells (Achouak et al. 2004), supporting the hypothesis that phase variation plays a role in immune evasion.

Immune responses to elicitor molecules derived from PGPR are best characterized for selected ISR-inducing strains of fluorescent pseudomonads (Bakker et al. 2007; Van Wees et al. 2008). Cell wall preparations of various ISR-inducing rhizobacteria all triggered typical immune responses in tobacco suspension cells, including a burst of reactive oxygen species, extracellular medium alkalization, rapid elevation of cytoplasmic Ca^{+2}, and defense-related gene expression (Van Loon et al. 2008). Furthermore, heat-killed *P. fluorescens* WCS417 bacteria were shown to activate the expression of MAMP-responsive reporters and trigger callose depositions in *Arabidopsis* roots (Millet et al. 2010). The PGPF *Piriformospora indica* is also recognized by the root's immune system through its MAMPs. Thus, both PGPR and PGPF possess a pallet of MAMPs able to elicit MTI in the roots of host plants (Jacobs et al. 2011).

In the *Piriformospora indica-Arabidopsis* interaction, the beneficial fungus recruits the jasmonic acid (JA) signaling pathway to suppress both early- and late-activated defense responses. Also, suppression of the flg22-mediated MTI in the roots by the plant pathogen *Pseudomonas syringae* was demonstrated to depend on a functional JA signaling pathway (Millet et al. 2010). In both cases, suppression of MTI was mediated via the JA signaling components JAR1 and MYC2, suggesting that activation of the JA pathway may be a common strategy to affect host immunity in the roots. Induction of SA-mediated responses has been demonstrated to reduce bacterial abundance in the plant rhizosphere (Doornbos et al. 2011; Kniskern et al. 2007). Also, colonization of *Arabidopsis* roots by the PGPF *Piriformospora indica* is affected by SA signaling (Jacobs et al. 2011). Many PGPR and PGPF are able to produce substantial amounts of phytohormone-like compounds, such as auxins and gibberellins (Lugtenberg and Kamilova 2009). Several phytohormones have been demonstrated to negatively cross-communicate with the SA signaling pathway and affect the outcome of the immune response (Verhage et al. 2010). Hence, it is tempting to speculate that nonsymbiotic microbes may produce phytohormones in order to attenuate the relative strength of the SA signaling via hormonal cross-talk mechanisms.

13.10 Conclusion

Since the discovery that selected beneficial soilborne microbes can stimulate plant immunity, now more than 20 years ago, a wealth of knowledge has accumulated on the mechanisms underlying ISR. The plant's immune system plays a

central role in the social network of plants that, on the one hand, can be activated to ward off enemies and, on the other hand, can be suppressed to accommodate mutualists. Both aspects of host immune modulation are operative in the ISR phenomenon, and their interplay will definitely be a subject of future studies. A major gap in our knowledge is how recognition of beneficial microbes at the root-soil interface drives the whole plant body toward enhanced growth and elevated stress resistance. The first steps toward unraveling the molecular dialogue between roots and ISR-eliciting microbes have been made, but major questions still need to be resolved. For instance, in the mechanism of signals from ISR-eliciting microbes perceived in the roots or leaves and translated into specific plant responses that mediate enhanced defense in foliar tissues, do plant roots produce one or more long-distance ISR signals, and if yes, what is their nature? Long-distance signaling molecules may be generated and/or modified in the outermost root cell layer, as indicated by the expression pattern of MYB72, which is required for the onset of ISR in the roots. As is the case with the establishment of SAR and microbe-herbivore-induced resistance, signaling cascades in the xylem parenchyma cells of the vascular bundle may also be critical for the establishment of ISR in foliar tissues. As plant roots respond to ISR-eliciting microbes in a cell type-specific manner, the analysis of root cell type-specific transcriptome and metabolome profiles in response to beneficial microbes will be highly informative. In natural ecosystems, plants have evolved in the context of complex microbial communities that fulfill important plant functions related to plant growth, vigor, and defense. However, these traits provided by the plant's second genome have not been major targets of classical plant-breeding programs. Hence, the continuous increase in our knowledge on the molecular and genetic basis of plant beneficial microbe communication in the context of its evolutionary and ecological relevance will be highly instrumental for the development of sustainable future crops that are better able to maximize profitable and protective functions from beneficial microbes in their root microbiome.

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14 Combinations of Plant Growth-Promoting Rhizobacteria (PGPR) for Initiation of Systemic Resistance Against Tree Diseases: A Glimpse

Vivek Kumar, Ajit Varma, Narendra Tuteja, Anfal Arshi, and Manoj Kumar

Abstract

The present chapter discusses the plant growth-promoting rhizobacteria (PGPR) which emphasizes the need to have unvarying significances for tree (i.e. *Eucalyptus*). The ecological diversity of PGPR will be exemplified by illustrations of genera and species selected from the available database and their action mechanisms for varied microbial groups. As PGPR are presented in an ecosystem where strong interactions are predicted, we define how flora, mycorrhiza and soil fauna impact the microbial diversity in the rhizosphere of *Eucalyptus*. Finally, the helpful interactions between PGPR and symbiotic microbiomes in the *Rhizobium*-eucalyptus tree symbiosis will be conferred. The combinatorial effect of PGPR will be discussed against the systemic diseases of eucalyptus tree caused by several pathogens. The chapter also describes the inducible systemic resistance (ISR) against any pathogenic attack in trees.

Keywords

PGPR • Rhizobacteria • *Eucalyptus* • Microbiome

14.1 Introduction

Tree is one of the planted timber resource in India owing to its high economical values and wide industrial and rural applicability. It has multiple significance due to its durability, elasticity and strength and rated as a valuable timber used in furniture, door and window and in gun butt (Bhattacharya et al. 2014; Chandra et al. 2014; Sharma et al. 2000). Due to overpouring pollutants around the tree system severe natural insults

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restricts the normal growth and develpoment and eventually trigger the abnormal fall, leads greater loss to ecosystem (Verrengia 1998). Tree rhizosphere is an unexplored area wherein studies of the microbial ecology of the rhizosphere may include the architecture of rhizoplane. In this chapter unless specified otherwise, the term rhizosphere will be used to refer to both zones. In the rhizosphere, very important and intensive interactions are taking place between the plant, soil, microorganisms and soil microfauna. Years back Chester (1933) proposed the term 'acquired physiological immunity' to express induced protection of plants against various biotic/abiotic elicitors. Furthermore, different terminologies were adopted, namely, systemic acquired resistance (Ross 1961), translocated resistance (Hurbert and Helton 1967) and plant immunization. Induced resistance is defined as an enhanced plant's defensive capacity against a broad spectrum of pathogens and pests upon appropriate stimulation.

The resultant elevated tolerance induced by specific agent through pathogenic infections is called induced systemic resistance (ISR) or systemic acquired resistance (SAR) (Hammerschmidt and Kuc 1995). The initiation of systemic resistance by rhizobacteria is referred to as ISR, while induction by other agents is called SAR (Van Loon et al. 1998). SAR is highly expressed in increased level while inducing organism causes necrosis (Cameron et al. 1994), whereas ISR by PGPR characteristically do not reflect any necrotic symptoms on the host plants (Van Loon et al. 1998).

14.2 Soil Bacteria

Interactions are frequently occurring among the soil bacteria, comprised of rhizobacteria, exist in rhizospheric soils, and the roots of plants have been studied intensively (Van Loon 1997; Van Loon et al. 1998; Pieterse et al. 1996, 1998, 2001; Ambrosini et al. 2012; Souza et al. 2013). Rhizobacteria are always meant for colonizing plant roots, capable of multiplying and occupying all the ecological niches that originate on the roots at every stage of plant growth and development (Antoun and Prévost 2006). Such bacteria may submissively interact with plants, openly by competing for nutrients. On the other hand, the interaction between rhizobacteria and the host plant can actively be accomplished. For instance, the bacteria may fight with sisters/co-pathogens for survival in the rhizosphere, or they may endorse mutualistic interactions with plants they were associated, allowing nutrient exchange and stimulating antibiotic production against phytopathogenic agents (Siddiqui 2006; Conrath et al. 2002). Root-colonizing plant-beneficial bacteria are commonly referred to as plant growth-promoting rhizobacteria (PGPR, Glick 2005).

14.3 PGPR Against Plant Diseases

There are multiple mechanisms by which PGPR controls plant diseases. The most frequently used approaches are antagonism and metabolite production. The metabolites comprise antibiotics, siderophores, HCN, cell wall-degrading enzymes, etc. (Enebak et al. 1998; Kloepper 1993). Multiple modes may in parallel act in a specific strain and provide biocontrol of diseases. Kloepper et al. (1980) elucidated two types of tolerance in plants. Induced systemic resistance (ISR) or systemic acquired resistance (SAR) is defined as the induction of chemical and physical ramparts of the plant host, leading to the control of several pathogens. There are numerous reports of antagonism of pathogenic fungi by PGPR. Pseudomonas strains MRS23 and CRP55b inhibited the growth of pathogenic fungi, i.e. *Aspergillus* sp., *Fusarium oxysporum* f. sp. *ciceri* and *Rhizoctonia solani* under culture condition (Goel et al. 2002). There are several reports of reduction of disease incidences by application of PGPR. *Bacillus* spp. isolated from healthy cabbage, kale and radish reduced black rot incidence in kale and cabbage caused by *Xanthomonas campestris* pv. *campestris* (XCC), in greenhouse and field experiments (Assis et al. 1996). In addition, Monteiro et al. (2005) reported that *Bacillus* strains produced lipopeptides and showed antagonism active against XCC during

delayed growth phase. Furthermore, ISR triggered in plants exhibited momentary variations in the plasma membrane which elevated plant defences (Ongena et al. 2009). Phenaminomethylacetic acid produced by strain, *Bacillus methylotrophicus* BC79, was reported to be a new kind of substance never reported in *Bacillus methylotrophicus* wherein culture filtrate of BC79 showed biocontrol efficiency against rice blast (Shan et al. 2013).

14.4 Effect of PGPR Toward Systemic Resistance

The deployment of PGPR for ISR against diseases has been demonstrated in field conditions against bacterial, fungal and viral diseases (Vidhyasekaran and Muthamilan 1997; Viswanathan 1999; Liu et al. 1995, Liu et al. 1995b; Maurhofer et al. 1998; Raj et al. 2003a, b; Halfeld-Vieira et al. 2006) and insect (Zehnder et al. 1997) and nematode pests (Sikora 1988). Upon ISR numerous advantages have been reported, viz. effectiveness against various pathogens; stability due to the action of different mechanisms of resistance, systemicity and energy economy; and metabolic utilization of genetic potential for resistance in all susceptible plants (Bonaldo et al. 2005). It has been reported that induced resistance was first analysed in 1961 by pre-inoculation of tobacco plants with TMV (Ross 1961) and helped against other viruses and resulted in the conception of 'systemic acquired resistance' (SAR). The induction of resistance to disease is an added advantage to the promotion of plant growth and yield by the application of PGPR. The presence of the PGPR in the rhizosphere makes the entire plant, including the shoot, more resistant to pathogens (Figueiredo et al. 2010).

14.5 Induced Systemic Resistance (ISR) in Tree

Upon pathogen attack resistance incurred in tree has been mediated by a wide range of biotic and abiotic agents (da Rocha and Hammerschmidt 2005; Lyon 2007). Probenazole, the first chemical resistance activator, was registered in Japan as Oryzemate in the year 1975, and since then, many other chemical and biological activators have been developed, including ASM, registered as Bion and Actigard (Syngenta), Milsana (Reynoutria sachalinensis extract; KHH BioScience), Elexa (chitosan; SafeScience) and Messenger (harpin protein; Plant Health Care). Induced resistance is defined as an enhancement of the plant's defensive capacity against a broad spectrum of pathogens and pests that is acquired after appropriate stimulation, so-called SAR (Hammerschmidt and Kuc 1995). The induction of systemic resistance by rhizobacteria is referred as ISR, whereas that by other agencies is called SAR (Van Loon et al. 1998). SAR is expressed to a maximum level when the inducing organism causes necrosis (Cameron et al. 1994), whereas ISR by PGPR typically do not cause any necrotic symptoms on the host plants (Van Loon et al. 1998). PGPR bring about ISR through fortifying the physical and mechanical strength of the cell wall as well as changing the physiological and biochemical reaction of the host leading to the synthesis of defence chemicals against the challenge pathogen.

14.5.1 Structural Cell Wall Modifications in the Host Plants

The success of a plant in warding off invading pathogens relies primarily on its ability to build a line of defence rapidly for protecting cell walls against the spread of a pathogen (Benhamou et al. 1996a). It is well reported that PGPR induces cell wall structural modification in response to pathogenic attack (Benhamou et al. 1996b, 1998; M'Piga et al. 1997). Seed priming with PGPR in bean induced the lignification of cell wall (Anderson and Guerra 1985). Treatment of pea seeds with *P. fluorescens* strain 63–28 has resulted in formation of structural barriers, viz. cell wall apposition (papillae) and deposition of newly formed callose and accumulation of phenolic compounds at the site of penetration of invading hyphae of *Pythium ultimum* and *F. oxy-*

sporum f. sp. *pisi* (Benhamou et al. 1996a). In tomato, cell wall thickening, deposition of phenolic compounds and formation of callose restricted growth of *F. oxysporum* f. sp. *radicis-lycopersici* to the epidermal cell and outer cortex in the root system in the treated plants (M'Piga et al. 1997). Similarly, seed treatment using *Bacillus pumilus* strain SE34 has also induced strengthening of cell walls in tomato against *F. oxysporum* f. sp. *radices-lycopersici* that promoted a rapid defence reaction at sites of fungal entry and allowed sufficient time for the host to build up other defence reactions to restrict pathogen growth to the outermost layers of root tissue (Benhamou et al. 1998).

14.5.2 PGPR Factors in ISR

There are several bacterial factors involved in the induction of systemic resistance by PGPR, the most important being lipopolysaccharides (LPS) present in the outer membrane of bacterial cells, siderophore and salicylic acid production (Van Loon et al. 1998). LPS present in the outer membrane of PGPR are the major determinants of ISR in certain PGPR strains. LPS of *P. fluorescens* strain WCS 417 induced systemic resistance in carnation against Fusarium wilt caused by *F. oxysporum* f. sp. *dianthi* (Van Peer and Schippers 1992). Similarly, LPS of *P. fluorescens* strains WCS 374 and WCS 417 have induced systemic resistance in radish against *F. oxysporum* f. sp. *raphani* (Leeman et al. 1995). They also established that mutant of *P. fluorescens* strain WCS 417, lacking the O-antigen side chain of the LPS, has not induced resistance in radish indicating the O-antigen side chain of the LPS might have served as a signal or trigger in the induction of defence mechanism in plants. In contrast, LPS of *P. putida* strain WCS 358 having O-antigen side chain do not induce systemic resistance in radish. In another study, LPS of WCS 417r and mutant of WCS 417r lacking O-antigen side chain of LPS elicit defence mechanism in *Arabidopsis* (Van Wees et al. 1997). This indicates that ISR by LPS of PGPR varies with different host plants, and lipopolysaccharide is not the only trait in determining the ISR.

14.6 Conclusion

The beneficial effects of PGPR include direct plant growth promotion, biological control and inducing systemic resistance in host plants. Specific PGPR strains bring about ISR against multiple pathogens attacking the same crop. In addition to disease suppression, application of PGPR also reduces the insect and nematode damage. The broad spectrum of control using PGPR strains can provide an effective, economical and practical way of tree protection. The endophytic nature of some PGPR makes them suitable for the use in vegetatively propagated trees because of their capability to colonize and persist in the intercellular space of epidermal cells thereby reducing the need for further application if the same vegetative parts are used as propagative material. Furthermore, certain PGPR strain mixtures have showed synergistic action in plant protection and growth promotion, indicating defined mechanism is involved in disease control. So, selecting such combinations of strains would be beneficial in tree production. Though the research on PGPR-mediated disease resistance originated several decades ago, its effectiveness has been demonstrated under field conditions only in the 1990s. It is concluded that instead of using single strain, it would be more effective to apply a mixture of strains showing synergistic action for broad spectrum activity against multiple pathogens and pests.

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15 Plant Growth-Promoting Microbial-Mediated Induced Systemic Resistance in Plants: Induction, Mechanism, and Expression

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Abstract

In the agroecosystem, plants are an attractive source of nutrients and life environment for many microbes. Pathogenic as well as nonpathogenic microbes get colonized to the plants resulting in various diseases and beneficial effects on plant growth or stress resistance, respectively. Plants are generally resistant to the majority of phytopathogens due to the presence of an efficient and complex immune system which is able to deal with most microbial invaders ubiquitously present in the environment. Plant growth-promoting microbes (PGPMs) elicit a higher level of resistance in addition to an indigenous immune system in the form of induced systemic resistance in plants and provide a heightened level of protection. Induced systemic resistance is a pre-activated induced resistance in plants leading to defense-related protein activation which is independent of salicylic acid and dependent on jasmonic acid and ethylene. Nonexpressor of pathogenesis-related protein 1 (NPR1) works as a master regulator of hormonal defense signaling pathway leading to activation of pathogenesis-related and defense-related protein that depends on the preceding signals. This chapter focuses on recent research study concerning interaction between PGPMs and plants under biotic stress condition.

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15.1 Introduction

Plants are members of complex communities which fix the solar energy that drives nearly all living processes on earth and function as a link between above- and belowground communities that consist of microbes, insects, and other vertebrate and invertebrate animals (Bezemer and van Dam 2005; Dicke and Baldwin 2010). The diverse microflora of soilborne bacteria and fungi which may have either beneficial or deleterious effects on the plant has the ability to quickly colonize with plant roots in a direct manner while indirect interactions occur via shared host plants between these different community members (Ohgushi 2005; Gehring and Bennett 2009; Pineda et al. 2010). Plants possess the ability to activate distinct defense responses against the invader microbial pathogens and herbivorous insects (Van Loon 2000). For survival, plants need to optimally allocate and use available resources for growth and defense (Herms and Mattson 1992). For example, in the presence of plant pathogens or insect herbivores, plants will allocate resources to the synthesis of defense compounds, and as a consequence, plant growth will decrease. Remarkably, plant growth-promoting microbes (PGPMs), including mycorrhizae, *Rhizobia*, and rhizobacteria including *Acinetobacter*, *Agrobacterium*, *Arthrobacter*, *Azospirillum*, *Bacillus*, *Bradyrhizobium*, *Frankia*, *Pseudomonas*, *Rhizobium*, *Serratia*, and *Thiobacillus*, form associations with plant roots and can promote plant growth by increasing their access to soil minerals and protect the plant against pathogens (Mendes et al. 2011; Berendsen et al. 2012; Bulgarelli et al. 2013; Kloepper et al. 1980). In addition, several species of PGPMs can trigger physiological changes and induction of defenses in the host plant leading to systemic effects on above- and belowground pathogenic communities involving organisms at several trophic levels (Leitner et al. 2010; Pineda et al. 2010, 2013; Katayama et al. 2011). This induced defense elicited by PGPMs in plants is known as induced systemic resistance (ISR) and often expressed not only locally but also in the distal parts from the site of primary infection, thereby protecting the plant systemically against subsequent attack. Induced resistance is regulated by a complex web of interconnecting signal transduction pathways leading to plant protection in which salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) function as key signaling molecules (Reymond and Farmer 1998; Pieterse and Van Loon 1999; Glazebrook 2001; Thomma et al. 2001). SA and JA accumulate in response to pathogen infection or herbivore damage/PGPMs, resulting in the activation of distinct sets of pathogenesis-related and defense-related genes. SA- and JA-dependent defense pathways have been shown to cross-communicate, providing the plant with a regulatory potential to fine-tune the defense reaction depending on the type of attacker encountered (Felton and Korth 2000; Feys and Parker 2000; Pieterse et al. 2001). By keeping view of plant growth promotion under biotic stresses, the present chapter will unravel the mystification of mechanisms involved in plant defense including ISR and SAR using sustainable development of plants.

15.2 Plant Immunity

Plants have an efficient and complex immune system that is able to deal with most microbial invaders, such as bacteria, fungi, or viruses, ubiquitously present in the environment. Besides the physical and chemical constitutive barriers, such as cuticle, cell walls, and antimicrobial phytoanticipins, plants possess a defense line that can be induced by the detection of microbial presence via immune receptors. Based on the types of molecules recognized by plant receptor as indicator of pathogen attack, they have two types of immune system, termed PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Vleesschauwer and Höfte 2009; Eulgem and Somssich 2007; Jones and Dangl 2006). In the former system, inducible immunity is based on the external recognition of "nonself" signals, notably microbe-/pathogen-associated molecular patterns (MAMPs/PAMPs). PAMPs/MAMPs are referred to as small molecular motifs/structures conserved within a class of microbes hence

characteristic of microbes and required for the overall fitness of microbes, such as flagellin from bacterial flagella or chitin or different glucans present in fungal/oomycete cell walls. Already many diverse MAMPs have been described; they can be (glyco)proteins, carbohydrates, or lipids (Newman et al. 2013). PAMPs/MAMPs are recognized by pattern recognition receptors (PRRs), located in the plasma membrane, leading to induction of a broad variety of defense responses commonly referred to as MAMP/PAMP/pattern-triggered immunity (PTI). PRRs activate a complex cascade of signaling events, including ion fluxes leading to plasma membrane depolarization, production of reactive oxygen species (ROS) and nitric oxide (NO), and activation of mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs) (Boller and Felix 2009; Boudsocq et al. 2010; Burketová et al. 2015) after binding with MAMP, leading to change in transcription factor (TF) activities to activate defense genes. Defense gene activation results in the accumulation of different enzymes and stress-specific metabolites which kept most of the potential invaders in check. Instead of it some pathogens possess virulence effector molecules and by its utilization can effectively minimize host immune stimulation either by suppressing PTI signaling or preventing detection by the host (Pel and Pieterse 2013; De Jonge et al. 2010; Bardoel et al. 2011; Borges and Sandalio 2015). In turn, for these types of pathogens, plants acquired a second line of defense in which it produces resistance (R) NB-LRR (nucleotide-binding leucine-rich repeat) receptor proteins which recognize attacker-specific effector molecules, resulting in effector-triggered immunity (ETI) (Dodds and Rathjen 2010). ETI is also known as gene-for-gene resistance which eventually leads to the programmed cell death at the site of infection to prevent further infection through biotrophic pathogens (Flors 1971). ETI is associated with hypersensitive response (HR), a strong local defense leading to programmed cell death at the site of infection (Dodds and Rathjen 2010). PTI and ETI also elicit induced resistance in undamaged distal parts of the plant from the site of infection through long-distance signals to provide enhanced defensive capacity (Dempsey and Klessig 2012; Shah and Zeier 2013). This well-characterized form of pathogen-induced resistance is referred to as systemic acquired resistance (SAR) (Spoel and Dong 2012; Vlot et al. 2009) and confers enhanced resistance against a broad spectrum of pathogens (Fig. 15.1). Through the similar signaling concept of the pathogen recognition system, plants also recognize herbivorous insects (Howe and Jander 2008).

15.3 Induced Resistance

An induced state of resistance in plants elicited by biological or chemical inducers to protect plants against future attacks by pathogenic microbes and herbivorous insects is known as induced resistance (Ku'c 1982). According to Agrios (1988), resistance is the ability of an organism to exclude or overcome completely or in some degree the effect of a pathogen or other damaging factors (Van Loon 1997). Induced resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated by specific environmental stimuli. By this enhanced defensive capacity, plants can get resistant against a broad range of pathogens and parasites. Plants can develop induced resistance upon getting elicitation from a pathogen/insect herbivory or colonization of the roots by specific beneficial microbes or after specific chemical treatments. In the process of induced resistance, activation of the latent defense mechanisms takes place in the plant upon a subsequent challenge from a pathogen/insect herbivore/beneficial microbes.

Induced resistance takes place not only locally at the site of induction but also systemically in the distal plant parts that are spatially separated from the inducer. Generally, induced resistance confers an enhanced level of protection against a broad spectrum of attackers (Walters et al. 2013) and is regulated by a network of interconnected signaling pathways in which plant hormones play a major regulatory role (Pieterse et al. 2012). The signaling pathways that regulate induced resistance elicited by

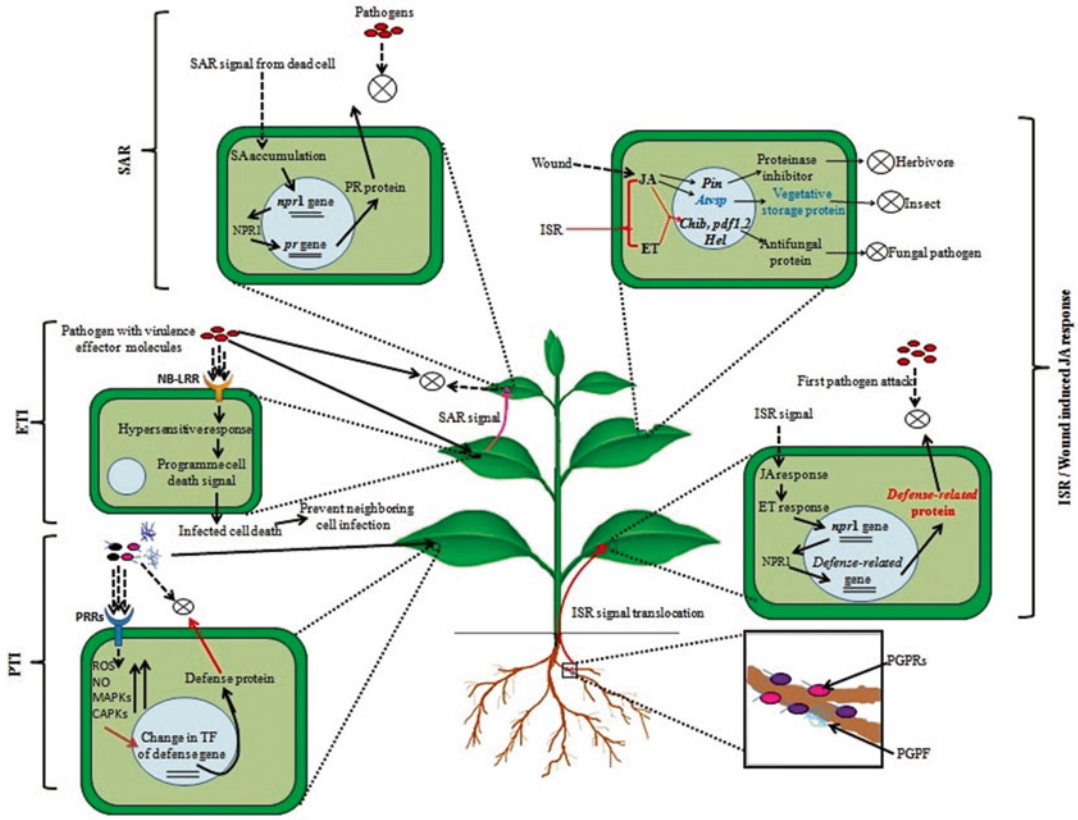

Fig. 15.1 Schematic representation of mechanism of overall plant defense system including ETI, PTI, SAR, and ISR

beneficial microbes, pathogens, and insects share signaling components.

15.4 Pathogen-Induced Systemic Acquired Resistance

According to Ross (1961) long-lasting and broad-spectrum disease resistance induced by limited primary infection with a pathogen is known as systemic acquired resistance that makes noninfected plant tissues more resistant against subsequent pathogen attack (Durrant and Dong 2004; Ross 1961; Wendehenne et al. 2014; Gao et al. 2015). In the onset of SAR, salicylic acid (SA) level increased to activate a specific set of *pathogenesis-related* (PR) genes, many of which encode PR proteins with antimicrobial activity (Van Loon et al. 2006). Priming, i.e., primary infection with pathogen, is a critical component of SAR, and primed tissues are in an alert state that enables them to more rapidly and efficiently deal with both biotic and abiotic stresses. In such potentiated cells, the defense compounds are only expressed upon the pathogen challenge. In SAR, the defense alert is amplified and transferred from the site of infection by a system of mobile signals into distal (systemic) plant parts (Conrath 2011).

Research studies with SA transgenic and mutant plants demonstrated a central role for this phytohormone in SAR (Loake and Grant 2007; Vlot et al. 2008a). Pieterse et al. (1998) reported the role of SA accumulation in SAR by experimentation on *Arabidopsis* SA-non-accumulating mutant plant NahG in which due to expression of bacterial salicylate hydroxylase (*nahG*) gene, SA gets converted into catechol and is unable to express SAR. SA is the primary molecule for SAR which activates further signaling cascade to

activate the gene responsible for resistance against pathogens, called the pathogenesis-related (PR) gene, which encodes different pathogenesis-related proteins (PRs) of families PR-2, PR-5, and PR-1, such as chitinases, β-1,3-glucanases, lipoxygenases, thaumatin-like proteins, antimicrobial peptides, etc. (van Wees et al. 1999; Uknes et al. 1992). All of these PRs have some antimicrobial properties primarily against fungal pathogens by which they establish SAR in plants (van Wees et al. 1999; Kombrink and Somssich 1997). Nonexpressor of PR genes-1 (NPR-1) protein encoded by *npr-1* gene plays an important role in SAR establishment as it acts as a transcriptional coactivator of PR gene expression after getting signal from SA accumulation (Pieterse et al. 1998).

Therefore, in contest to signaling event of SAR, first SA accumulation takes place against pathogen attack which activates *npr-1* gene that eventually leads to activation of PR genes (Fig. 15.1). According to Vleesschauwer and Höfte (2009), by some recent research study, the role of volatile molecule methyl salicylate (MeSA) in the form of long-distance mobile signal for SAR, to protect distal part of the plants, has been proven. MeSA itself appears to be biologically inactive, but due to MeSA-esterase activity of SA-binding protein-2, it gets converted into the SA through hydrolysis in the systemic tissue (Park et al. 2007; Vlot et al. 2008a, b). MeSA has the ability to travel by both air and vascular transport to mediate long-distance induction of resistance in distal leaves that lack a direct vascular connection to the attacked leaf and in neighboring plants (Heil and Ton 2008).

In addition to SA, other plant hormones are also implicated in SAR signaling. In tobacco, Verberne et al. (2003) demonstrated that ethylene (ET) perception is required for the onset of SA-dependent SAR that is triggered upon infection by the tobacco mosaic virus. In addition, Truman et al. (2007) showed that the JA-signaling mutants *sgt1b* (suppressor of g2 allele of SKP1 1b), *opr3* (12-oxo-phytodienoate reductase 3), and *jin1* (jasmonate insensitive 1) failed to develop SAR upon leaf infiltration with an avirulent strain of the pathogen *Pseudomonas syringae* pv. tomato, suggesting that JAs play a role in SAR as well. However, other JA-signaling mutants such as *jar1* (jasmonate resistant 1), *eds8* (enhanced disease susceptibility 8), and *coi1* (coronatine insensitive 1) were shown to develop normal levels of SAR (Attaran et al. 2009; Cui et al. 2005; Pieterse et al. 1998).

15.5 Microbial-Elicited Induced Systemic Resistance

In addition to pathogens, nonpathogenic microbes can also elevate the level of disease resistance in plants. Induced resistance triggered by these plant-associated microbes is referred to as induced systemic resistance (Pieterse et al. 2009). ISR is initiated in roots by PGPMs and leads to resistance priming not only in distant parts of roots but also aerial parts of plants. Unlike SAR, JA- and ET-dependent mechanisms are responsible for the ISR (Conrath 2011; Pieterse et al. 2014). Different studies show the role of JA in tandem with the ethylene hormone for defense against wounds, necrotrophs, and herbivore attacks (Glazebrook 2005; Robert-Seilaniantz et al. 2011). Root colonization with mycorrhizal fungi, *Trichoderma* fungi, or nonpathogenic *Fusarium* strains can also lead to the activation of ISR-like systemic resistance (Pieterse et al. 2014; Zamioudis and Pieterse 2012).

Van Loon et al. (1998) first evidenced by experiments that colonization of plant roots by plant growth-promoting rhizobacteria (PGPR) protects aboveground plant tissues against different types of pathogens. Like pathogen-induced SAR, this PGPR-mediated induced systemic resistance (ISR) has been demonstrated in many plant species and has a broad spectrum of effectiveness (Kloepper et al. 2004; Van Loon and Bakker 2006; Van Wees et al. 2008; Chen et al. 2014). Among the PGPR documented to date for ISR, nonpathogenic *Pseudomonas* spp. and *Bacillus* spp. were found to be most potent (Kloepper et al. 2004; Van Loon and Bakker 2006). Maize plants inoculated with *Pseudomonas putida* KT2440 are found to be resistance against the fungal pathogen *Colletotrichum graminicola*

(Planchamp et al. 2014). Both the mechanisms of resistance, i.e., SAR and ISR, are effective against different types of pathogens, but their range of effectiveness is to a certain extent different. For example, in *Arabidopsis thaliana*, it was shown that both SAR and ISR triggered by two different bacteria, i.e., an avirulent strain of the bacterial leaf pathogen *P. syringae* pv. tomato and PGPR *Pseudomonas fluorescens* WCS417r (WCS417r), respectively, have similar effects against diseases caused by the virulent *P. syringae*, the fungal root pathogen *Fusarium oxysporum*, and the downy mildew pathogen *Hyaloperonospora arabidopsidis* (Pieterse et al. 1996; Ton et al. 2002). However, in a different study, SAR was found to be effective against the turnip crinkle virus, whereas ISR was not (Ton et al. 2002). In a vice versa study, ISR was found to be effective against the necrotrophic pathogens *Alternaria brassicicola* (Ton et al. 2002), *Botrytis cinerea* (Van der Ent et al. 2008), and *Plectosphaerella cucumerina* (Segarra et al. 2009) to protect *Arabidopsis* plants, whereas SAR was not. Over the last decade, it has become clear that, like PGPR, many PGPFs such as mycorrhizal fungi (Pozo and Azcon-Aguilar 2007) and nonpathogenic strains of *F. oxysporum* (Duijff et al. 1998; Paparu et al. 2007), *Trichoderma* spp. (Vinale et al. 2008), *Penicillium* sp. GP16-2 (Hossain et al. 2008), *Pythium oligandrum* (Hase et al. 2008), *Piriformospora indica* (Waller et al. 2005), and related *Sebacinales* spp. (Waller et al. 2008) are able to trigger a similar broad-spectrum ISR.

15.5.1 ISR Signal Transduction

Unlike SAR, ISR takes place through a more diverse and complex route to establish a higher degree of prior resistance without any infection. Majorly, defense-related gene activation takes place in ISR through JA and ET accumulation instead of SA-activated PR gene (Pieterse et al. 1998; Boller 1991; Wasternack and Parthier 1997). ISR signal transduction pathway is independent of SA accumulation and totally dependent on JA and ET.

15.5.2 SA-Independent Signaling

Although phenotypically it seems similar as both, ISR and SAR confer a broad-spectrum disease resistance in systemic plant parts; they are regulated by different signal transduction pathways. First evidence for the differential regulation of SAR and ISR came from studies with the PGPR WCS417r. According to Hoffland et al. (1995), accumulation of PR proteins which are characteristic of SAR was not found in the radish plant treated with WCS417r upon challenge inoculation with *Fusarium* spp. causing *Fusarium* wilt disease. In addition, studies on transgenic mutant plants further clear the independency of ISR on SA. Pieterse et al. (1998) reported that SA is not required for ISR, as *Arabidopsis* SA mutant NahG plants which are unable to accumulate SA due to expression of the bacterial salicylate hydroxylase (*nahG*) gene responsible for conversion of SA into catechol also develop a normal level of ISR after treatment of the root with ISR-inducing rhizobacterial strain *Pseudomonas fluorescens* WCS417r against the challenge inoculation. Since then, many examples of SA-independent ISR have been demonstrated in *Arabidopsis* (Ahn et al. 2007; Iavicoli et al. 2003; Ryu et al. 2003; Segarra et al. 2009; Stein et al. 2008) and other plant species, such as tobacco (Press et al. 1997; Zhang et al. 2002), cucumber (Press et al. 1997), tomato (Hase et al. 2008; Tran et al. 2007; Yan et al. 2002), and rice (De Vleesschauwer et al. 2008). Hence, the ability to activate an SA-independent pathway controlling systemic disease resistance seems to be common for beneficial microorganisms and occurs in a broad range of plant species against different types of attackers.

15.5.3 JA- and ET-Dependent Signaling

Research on the microbial elicited ISR defense signaling pathway revealed that JA and SA are the central players in the regulation of ISR. Similar to that of SA mutant NahG plants, it was also reported that JA mutant jar1, jin1, eds8,

and coi1 and ET mutants such as *etr1* (ethylene response1) and *ein2* (ethylene insensitive 2) were unable to confer ISR upon against challenge inoculation and clear the dependency of ISR on JA and ET. Jasmonic acid and its different derivatives induce the expression of genes encoding defense-related proteins, such as thionins (Pieterse et al. 1998; Epple et al. 1995) and proteinase inhibitors (Pieterse et al. 1998; Farmer et al. 1992), whereas ethylene is involved in the expression of the pathogen-inducible genes (van Wees et al. 1999). Unlikely SAR, ISR is elicited by a nonpathogenic rhizobacteria or PGPR and there is no need for an initial infection as required in SAR. Root colonization by ISR-triggering bacteria leads to a heightened level of resistance against a diverse set of intruders. After getting elicitation from root-colonizing PGPR, the accumulation of JA and ET takes place and moves toward the distal part of the plant due to formation of a phloem-mobile signal. These signaling molecules further activate *npr-1* gene expression which encodes the NPR-1 protein followed by the activation of defense-related gene, upon any pathogen attack/challenge inoculation. NPR-1 proteins are known as master regulator of both defense pathways, as upon getting a preceding signal, it activates the expression of either a PR gene or defense-related gene for the establishment of SAR and ISR, respectively. Like MeSA, methyl jasmonate (MeJA) also works as a volatile signal for the distal part of the plant. Priming of tomato seeds with methyl jasmonate was found to induce the resistance to hemi-biotroph *Fusarium oxysporum* f.sp. *lycopersici* (Krol et al. 2015). Expression of different defense-related gene depends on the fact that NPR -1 is getting a signal from JA or ET or from both in concert. van Wees et al. (1999) have elaborately described about the different defense-related gene activation by JA and ET. Ethylene is involved in the expression of the pathogen-inducible genes *Hel* (encoding a hevein-like protein) (Potter et al. 1993), *ChiB* (encoding a basic chitinase) (Samac et al. 1990), and *Pdf1.2* (encoding a plant defensin) (Penninckx et al. 1996). Proteins encoded by all of these three genes have antifungal activity. JA was also found to be responsible for the activation of the *Hel*, *ChiB*, and *Pdf1.2* genes as well (Penninckx et al. 1996; Thomma et al. 1998). Plant defensin proteins possess antifungal activity, antibacterial activity, proteinase inhibitory activity, and insect amylase inhibitory activity, and for its full expression, both ethylene and jasmonate are required, indicating that these hormonal signals act in concert (Penninckx et al. 1998). The *Pal1* gene, which encodes phenylalanine ammonia-lyase, which plays an important regulatory role in the synthesis of phenylpropanoid such as lignin and of SA in *Arabidopsis* (Mauch-Mani and Slusarenko 1996), has been also found to be induced by JA (McConn et al. 1997). Besides this JA is also involved in the plant's protection from insect and herbivory (Fig. 15.1). In tomato plants, it is found that whenever plant tissues get wounded by any intruder, JA induces the expression of the *Pin* gene which encoded for the proteinase inhibitor proteins (Farmer and Ryan 1992). These proteins protect the plant against herbivory (Heitz et al. 1999). In addition to that, jasmonate also activates the expression of the *Atvsp* gene in *Arabidopsis* that encodes the vegetative storage protein (VSP), which possesses acid phosphate activity, and by using this activity, it retards the development of insects and increases the mortality rate (Berger et al. 1995). That is how, by activation of such a wide range of different defense-related genes, PGPR-elicited ISR helps protect plants against a broad range of pathogens, insects, and herbivores.

15.5.4 NPR 1: The Master Regulator of Defense Signaling Pathway

The defense regulatory protein NPR1 plays a key role not only in SA-dependent SAR but also in JA-/ET-dependent ISR (Dong 2004; Pieterse and Van Loon 2004). Both of the signaling pathways, i.e., SAR and ISR, are independent from each other but have an overlapping requirement for NPR1 protein (van Wees et al. 2000). Recent study on the mutant *Arabidopsis npr1* plants, which lacks NPR-1 protein synthesis activity, was found to be unable to express ISR upon colo-

nization of the roots by a broad range of plant growth-promoting microbes such as PGPR WCS417r (Pieterse et al. 1998), *P. fluorescens* CHAO (Iavicoli et al. 2003), *P. fluorescens* 89B61 (Ryu et al. 2003), *Pseudomonas putida* LSW17S (Ahn et al. 2007), *Serratia marcescens* 90–166, *B. pumilus* SE34 (Ryu et al. 2003), PGPF *Penicillium* sp. GP16-2 (Hossain et al. 2008), *P. indica* (Stein et al. 2008), and *T. asperellum* T34 (Segarra et al. 2009) upon challenge inoculation. Due to different initiation sites, that is, roots in the case of ISR and leaves in SAR, it was suggested that these two responses may not compete for NPR1, but these are not independent, however, and may compete for NPR1 in leaves. In SAR, NPR1 plays an important role as a transcriptional coactivator of SA-responsive PR gene expression (Kuai et al. 2015). However, SA-independent ISR is not accompanied by the activation of SA-responsive PR genes (Pieterse et al. 1996). This indicates a different role of NPR1 in ISR signaling pathway than in SAR. Van Wees et al. (2000) have shown that simultaneous activation of SAR and ISR can lead to an additively enhanced defensive capacity compared to that of SAR and ISR suggesting that the roles of NPR1 are not mutually exclusive. This suggests that NPR1 plays a master role to regulate and connect different hormone-dependent induced defense pathways (Dong 2004; Pieterse and Van Loon 2004; Pieterse et al. 2009; Yang et al. 2015). While the role of NPR1 in SA signaling is clearly connected to a function of this regulatory protein in the nucleus (Dong 2004), evidence is accumulating that the role of NPR1 in JA/ET signaling is connected to a cytosolic function of NPR1 (Leon-Reyes et al. 2009; Stein et al. 2008).

15.6 Defense Enzymes Induced by Microbial-Mediated ISR

Plants have endogenous defense mechanisms that can be induced in response to attack by insects and pathogens. It is well known that the defense genes are inducible genes and appropriate stimuli or signals are needed to activate them. Inducing the plant's own defense mechanisms by prior application of a biological inducer is thought to be a novel plant protection strategy. Prior treatment of plants with plant growth-promoting microbes elicited defense gene expression including lipoxygenase (LOX), phenylalanine ammonia-lyase (PAL), peroxidase (POD), polyphenol oxidase (PPO), chitinase, and b-glucanase against the biotic stress.

Peroxidases are expressed to limit cellular spreading of infection through the establishment of structural barriers such as lignin and suberin deposition or generation of highly toxic environments by massively producing ROS and reactive nitrogen species (RNS) (Passardi et al. 2005; Cavalcanti et al. 2004). A higher level of enzymatic activity of cell wall-bound PODs has been reported in different plants such as cucumber (Chen et al. 2000), soybean (Jain et al. 2013; Jain and Choudhary 2014), rice (Reimers et al. 1992), tomato (Mohan et al. 1993), and tobacco (Ahl Goy et al. 1992) upon challenge inoculation. The high POD activities detected in treatments are linked to lignification and generation of hydrogen peroxides that inhibit pathogens directly or generate other free radicals with antimicrobial effects (Hammerschmidt 1999). PPOs catalyze oxidation of hydroxy phenols present in the plants to their quinone derivatives, which have antimicrobial activity to combat against pathogens (Chunhua et al. 2001). PPO plays an important role in defense against plant pathogens due to its reaction products and wound inducibility property (Mayer and Harel 1979). Research study on different plants such as cucumber (Chen et al. 2000), banana (Thakker et al. 2007), tomato (Thipyapong and Steffens 1997), and poplar (Constabel et al. 2000) has been found with increased level of PPO upon pathogen infection. Constabel and Ryan (1998) showed that methyl jasmonate works as an inducer for the expression of PPO genes, a fairly general phenomenon.

Fungal phytopathogens, a major threat for the plant world, possess β-1,3 glucan and chitin, polymer of N-acetylglucosamine (NAG), as their cell wall components. Chitinase and β-1,3 glucanase are the major enzymes which play a direct role to ward off pathogens by directly degrading

the pathogen cell wall and in turn protecting the plant. PAL and LOX are the other defense enzymes elicited by bacteria in plants upon challenge inoculation. PAL is the first enzyme in phenylpropanoid metabolism and plays an important role in lignin production which is an inducible defense mechanism used for protection against pathogen attack (Liang et al. 1989), while LOX is requisite for the synthesis of antifungal oxylipins, such as jasmonic acid (JA) that may act as signal factor for eliciting ISR in the plant (Creelman and Mullet 1997; Pieterse et al. 1998). Several earlier studies on the plant-microbe interaction in the course of plant defense have found a significant role of PAL. Recently Ramamoorthy et al. (2002) found a higher level of PAL and LOX in the roots of tomato plants treated with *Pseudomonas fluorescens* Pf1 challenged inoculated with *F. oxysporum* f. sp. *lycopersici*. In cucumber, PAL was found to be a key enzyme in the production of phenolics and phytoalexins (Daayf et al. 1997). PAL activity could be induced in plant-pathogen interactions and fungal elicitor treatment (Ramanathan et al. 2000). In an experiment on the cucumber roots inoculated with *Pythium aphanidermatum*, Chen et al. (2000) reported elevated levels of PAL enzyme; however roots treated with *Pseudomonas corrugata* showed initially higher levels of PAL and levels were decreased after challenge inoculation with *P. aphanidermatum*. DeMeyer et al. (1999) reported induction of PAL in bean roots and increased level of salicylic acid (SA) in leaves upon colonization of rhizosphere by *P. aeruginosa* 7NSK2. Increase in mRNAs encoding for PAL and chalcone synthase could be recorded in the early stage of the interaction between bean roots and various rhizobacteria (Zdor and Anderson 1992). Jasmonates have been found to stimulate LOX gene expression, protein, and activity in plants (Rosahl 1996; Saravitz and Siedow 1996). Recently Jain and Choudhary (2014) have done comparative study of defense enzymes in the soybean plant upon challenge inoculation with *Fusarium oxysporum* and found a higher level of defense enzyme expression in different parts of the plant treated with bacterium. Phenolic acids also played an important role in plant defense by phytoalexin accumulation, biosynthesis of lignin, and formation of structural barriers. Marked accumulation of phenols leading to suppression of disease was observed in tomato (Ramanathan et al. 2000) and banana (Thakker et al. 2009).

15.7 Conclusion

In the present scenario of agro-world, the first priority of the cultivator is to produce a healthy plant, i.e., a plant without any infectious disease. Environmental stresses (biotic as well as abiotic) result in great yield losses. Stress alters the physiological and biochemical processes resulting in altered metabolism and thus retards growth. Plants employ different strategies to cope with stress, including excess production and accumulation of compatible organic osmolytes, selective uptake of ions, increased expression and activity of defense enzymes, and so on. The interaction of microbes with plants is a dynamic, sophisticated phenomenon wherein several external factors affect the structure and species composition of the bacterial communities. In the plant-microbe interaction, it has been observed that due to effective root priming and their interaction with plant and other microbial populations, PGPMs work like a bio-booster for the field of agriculture. It has great potential for improving crop yield quality as well as quantity in the form of enhanced root growth, enhancing biomass yield. In addition to normal growth-promoting traits, the PGPMs also protect plants from biotic stresses by activation of defense-related enzymes and phenolic production and elicit jasmonic and ethylene pathways. This chapter has focused on the role of PGPMs in the plant protection against biotic stresses ranging from microorganisms and parasites to nematodes and insects and explaining the mechanism of induced systemic resistance.

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Retraction Note to: Changes in Phytochemicals in Response to Rhizospheric Microorganism Infection

Mehrnaz Hatami and Mansour Ghorbanpour

Retraction Note to:
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The editors have retracted this chapter by Hatami and Ghorbanpour [1] because of overlap with a previously published article by Pedone-Bonfirm et al. [2].

Author Ghorbanpour does not agree with this retraction. Author Hatami did not respond to correspondence about this retraction.

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[2] Pedone-Bonfim MVL, da Silva FSB, Maia LC (2015) Acta Physiol Plant 37:27. https://doi.org/10.1007/s11738-015-1781-3

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