Naveen Kumar Arora
Editor

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Naveen Kumar Arora
Environmental Microbiology
School of Environmental Sciences
Babasaheb Bhimrao Ambedkar University
Lucknow, Uttar Pradesh, India

ISBN 978-81-322-1286-7 ISBN 978-81-322-1287-4 (eBook)
DOI 10.1007/978-81-322-1287-4
Springer New Delhi Heidelberg New York Dordrecht London

Library of Congress Control Number: 2013944060

Printed on acid-free paper

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

Preface

Symbiosis is a biological phenomenon that involves close association between two or more organisms. Plant microbe symbiosis is one of the most intriguing relationships in the living world which has to be exploited for feeding an ever increasing human population in a sustainable way, maintaining the balance, diversity and productivity of agroecosystems in an ecofriendly manner. It takes several millions of years for establishing an intimate relationship between as diverse organisms as those belonging to prokaryota, fungi and plantae. Plants and microbes communicate and understand each other by the help of molecular dialogues. It is essential to decode these dialogues so as to establish a successful symbiotic relationship for the enhancement of crop productivity. This book looks into the plant growth promoting (PGP) microbes that generally colonize the rhizosphere region and help the host plant in one way or the other. Understanding of how symbiotic associations are established between plants and microbes that can be of particular relevance to modern day agriculture is also provided in the book.

The book comprises 16 chapters contributed by researchers from around the globe that provide detailed review on current status of research related to plant microbe interactions for developing new and alternative ecofriendly agrotechnologies. The diversity of plant ecosphere is huge and we still know only a fraction of what is happening in this dynamic ecosystem. There are so many useful microorganisms residing in the rhizosphere region which form symbiotic relationships with plants. Some of the best known or studied PGP microorganisms like *Rhizobium*, *Pseudomonas*, mycorrhiza, endophytes etc. have helped in understanding the symbiotic relationships between plants and diverse microbes of the rhizosphere or soil. But still a lot has to be done so as to use these beneficial microbes as sustainable and successful agri-biotechnology. Overall, a comprehensive approach that merges the fundamentals with the advanced techniques in the fields of functional genomics, proteomics, metabolomics and bioinformatics is required to bioengineer the future formulations that are reliable and more effective in their action. The book on one hand covers the fundamentals of plant microbe symbiosis and on the other hand provides inputs for the future research in the field. It is now clear that the multifaceted and diverse mechanisms of plant associated microbes

participate and are involved in promoting plant growth, protecting plant health, sustaining the plant under stress, pollutant or contaminant affected conditions and protecting plants from the attack of phytopathogens.

Researchers working in the field of rhizosphere biology, PGPRs, plant-microbe interactions, bioformulation technology and related fields will find the compilation extremely useful. The book will be of great value to the teachers and graduate and postgraduate students of life sciences, specifically microbiology, biotechnology, biochemistry and agriculture sciences. Readers will find a feast of updated information as well as the future direction for research in the field.

Finally, I would like to thank all those who have in one way or other helped in compilation of this wonderful volume. I acknowledge the support of all the contributors to this tome. My sincere thanks to all the authors for their cooperation, providing latest information on the subject and despite their busy schedules sticking to the timelines of the project. Thanks to Dr. Mamta Kapila from Springer (India) for pushing me hard to initiate the project and once the initiation materialized, the product was also formed. My gratitude to Prof. D. K. Maheshwari, Department of Botany and Microbiology, GKVV, Haridwar, for time to time advice, ideas and support. I would like to thank my research scholars Mr. Sachin Singh, Ms. Sakshi Tewari, Mr. Jitendra Mishra and Ms. Rachna Singh for helping in compilation of manuscript. Last but never least, special thanks to my wife Ms. Preeti Arora for her tolerance and tireless support during the phase of compilation and my sons Pranay and Nav for their rejuvenating presence.

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About the Editor

Dr. Naveen Kumar Arora, Ph.D. Microbiology, Associate Professor in Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University (a central university), Lucknow, Uttar Pradesh, India, is a renowned researcher in the field of Environmental Microbiology and Biotechnology. His specific area of research is rhizosphere biology and PGPR. He has 35 research papers published in premium international journals and is a member of several national and international societies. He is also a reviewer of several international journals. He has delivered lectures in conferences and seminars around the globe. He has a long-standing interest in teaching at the PG level and is involved in taking courses in bacteriology, microbial physiology, environmental microbiology, agriculture microbiology and industrial microbiology. He has been advisor to 52 postgraduate and 2 doctoral students. Although an academician and researcher by profession, he has a huge obsession for wildlife and its conservation and has authored a book *Splendid Wilds*. He also has a dedicated website www.naveenarora.co.in for the cause of wildlife and environment conservation.

About the Book

Plant microbe interaction is a complex relationship that can have various beneficial impacts on both the communities. An urgent need of today's world is to get high crop yields in an ecofriendly manner. Utilization of beneficial and multifaceted plant growth-promoting (PGP) microorganisms can solve the problem of getting enhanced yields without disturbing the ecosystem thus leading to sustainability. For this to achieve, understanding of the intricate details of how the beneficial microbes form associations with the host plant and sustain that for millions of years must be known. A holistic approach is required wherein the diversity of microbes associated with plant and the network of mechanisms by which they benefit the host must be studied and utilized.

Plant Microbe Symbiosis: *Fundamentals and Advances* provides a comprehensive understanding of positive interactions that occur between plant and microorganisms and their utilization in the fields. The book reviews the enormous diversity of plant-associated microbes, the dialogue between plant–microbes–microbes and mechanisms of action of PGP microbes. Utilization of PGPR as nutrient providers in combating phytopathogens and ameliorating the stressed and polluted soils is also explained. Importantly, the book also throws light on the unanswered questions and future direction of research in the field. It illustrates how the basic knowledge can be amalgamated with advanced technology to design the future bioformulations.

Chapter 1
Transactions Among Microorganisms and Plant in the Composite Rhizosphere Habitat

Sakshi Tewari and Naveen Kumar Arora

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S. Tewari • N.K. Arora (✉)
Department of Environmental Microbiology, School of Environmental Science,
BBA University, Lucknow 226025, India
e-mail: nkarora_net@rediffmail.com

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_1, © Springer India 2013

Abstract Root exudates selectively influence the growth of microorganisms that colonize the rhizosphere by altering the chemistry of soil in the vicinity of the plant roots and by serving as signal molecules and selective growth substrates for soil microorganisms. Microbial signals to plants influence the cell metabolism and plant nutrition and growth. It is increasingly apparent that, in nature, microbes function less as individuals and more as coherent groups that are able to inhabit multiple ecological niches. Because of current public concerns about the side effects of agrochemicals, there is an increasing interest in improving the understanding of cooperative activities among plants and rhizosphere microbial populations. This review provides a better understanding of processes such as stimulation of microbial activity by root exudates, competition between microorganisms and roots for nutrients, and molecular talk between roots and microorganisms and among microorganisms in the rhizosphere. Various positive plant–microbe–microbe interactions along with their multifaceted communications are highlighted that should be studied in an integrated manner for the development of sustainable agriculture with global applicability.

Introduction

Communication is the strongest and most imperative gesture ever evolved by nature for every living creation. Mankind would not have evolved and progressed in the absence of communication. With the advancement of civilization, various means of communications have also improved tremendously and will keep on doing so in future. It is not only humans but plants, animals, and even the smallest living creatures—bacteria—also communicate with each other. The style of messaging between microbes and plant suggests that root exudates initiate and modulate dialogue between plant roots and soil microbes. In addition, root exudates maintain and support a highly specific diversity of microbes in the rhizosphere of a given particular plant species, thus suggesting the beginning of intimate woven evolutionary link (Badri and Vivanco 2009).

Every organism on earth relies on associations with its neighbors to sustain life. For example, plants form associations with neighboring plants, microflora, and

microfauna, while humans maintain symbiotic associations with intestinal microbial flora, which is indispensable for nutrient assimilation and development of the innate immune system. Most of these associations are facilitated by chemical cues exchanged between the host and the symbionts. In the rhizosphere (which includes plant roots and the surrounding area of soil influenced by the roots), plants exude chemicals to effectively communicate with their neighboring soil organisms. As autotrophic organisms, plants play a central role in sustaining all other life forms. Unlike animals, plants are sessile, thus releasing an array of chemical signals to interact with other organisms (Badri et al. 2009). The root system, which was traditionally thought to provide anchorage and uptake of nutrients and water, is a chemical factory that mediates numerous underground interactions. These include mutualistic associations with beneficial microbes, such as rhizobia, mycorrhizae, endophytes, and plant growth-promoting rhizobacteria (PGPR).

Plant growth and development involves a tight coordination of the spatial and temporal organization of cell division, cell expansion, and cell differentiation. Orchestration of these events requires the exchange of signaling molecules between the root and shoot, which can be affected by both biotic and abiotic factors. The interactions that occur between plants and their associated microorganisms have long been of interest, as knowledge of these processes could lead to the development of novel agricultural applications. Plants produce a wide range of organic compounds including sugars, organic acids, and vitamins, which can be used as nutrients or signals by microbial populations. On the other hand, microorganisms release phytohormones, small molecules, or volatile compounds, which may act directly or indirectly to activate plant immunity or regulate plant growth and morphogenesis (Castro et al. 2009).

The aim of the present review is to exemplify communications in the rhizosphere, especially between plant to microbes, microbes to plant, and microbe to microbe, and, more specifically, to describe signaling pathways that allow bacteria to sense a wide diversity of plant signals, plants to respond to bacterial signals, and bacteria to coordinate gene expression at population and community level. Apart from it, the dynamics of signal exchange and its biological significance is also elaborated. Study of these cooperative microbial interactions can be exploited as a low-input biotechnology and form basis for the strategies to develop sustainable, environmental-friendly practices fundamental to the stability and productivity of both agricultural and natural ecosystems.

Rhizosphere: Unique Habitat for Plant–Microbe Communication

According to the general outlook of the rhizosphere, it includes plant roots and the surrounding soil. This is a wide and wise definition, already coined more than 100 years ago by Lorenz Hiltner, as documented in detail by Hartmann et al. (2008). In the rhizosphere, biologically and chemically highly diverse, complex, and dynamic interactions occur between plant roots, soil (micro) biota, and physicochemical environment of the soil. The autotrophic partner (plant) provides substrate

and energy flow in the rhizosphere and gets in return essential nutrients for its development and growth. Heterotrophic soil biota usually is limited in the supply of carbon and energy, and, thus, a complex sequence of responses is initiated, which in due course also influence the plant. Soil biota (bacteria, fungi, microfauna, and the plant root) are themselves embedded in food webs, and, thus, interactions with consumers in the microbial as well as micro- and mesofaunal world are important to understand rhizosphere processes (Hartmann et al. 2009).

From the viewpoint of the plant, the rhizosphere is characterized by the investment of the plant into an effective development of the root architecture and the return of mineral nutrients and water from the soil. In the root system, sloughing off of root cells (in particular at the root tip), root death (root hair cells and epidermis cells in older root parts), and the exudation of carbon compounds are processes which support soil biota and according to their composition select a specific rhizosphere community. Already in the initial phases of the evolution of terrestrial plants, the necessity and opportunity appeared to integrate the abilities of soil microbes to explore the soil for nutrients and water for the development of plants. Vice versa a high number of soil microbes attained properties enabling them to interact more efficiently with roots and withstand the quite challenging conditions of rhizosphere life. This process can be regarded as an ongoing process of microevolution in low-nutrient environments, which are quite common in natural ecosystems (Schloter et al. 2000). Rhizosphere can only be successfully colonized with the appropriate tools of efficient substrate acquisition, resistance mechanisms, as well as competitive traits. Thus, evolution shaped soil biota to fit into these specific niche conditions which are also characterized by specificities based on the diversity of plants and soil environments. Furthermore, the colonization of the interior of plant roots by microbial endophytes and PGPR appears as the most attractive goal, because there plant nutrient resources can be explored even more effectively without the tough competition with the high number of other microbes colonizing the root surface and environment (Schulz and Boyle 2006). Plant is restricting or directing the development of the attracted organisms in a way to keep control of these guests by excreting quite selective mixtures of substances which provide selective conditions for rhizosphere organisms.

Rhizosphere is a heavily populated microhabitat which is characterized by mutualism, antagonism, competition, and even predation among the inhabitants. Therefore, soil organisms do experience the rhizosphere environment as microhabitat of great opportunities, but the big challenge is to understand and explore the diverse communications and ongoing interactive messaging among plant–microbe–microbe communities (Fig. 1.1).

Plant to Microbe Communication

Plants are sessile, multicellular organisms, which rely on developmental and metabolic changes for growth. The root system displays considerable plasticity in its morphology and physiology in response to variability within its environment.

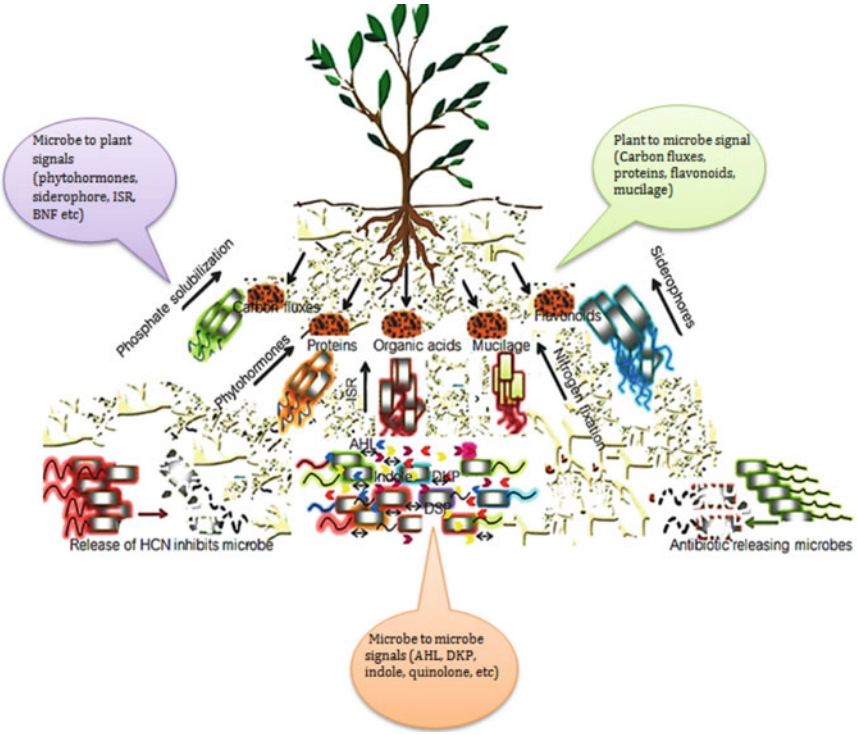


Fig. 1.1 Transactions among plant–microbes–microbes in rhizosphere

Extensive communication occurs between plants and microorganisms during different stages of plant development in which signaling molecules play an important role (Castro et al. 2009). Plants are able to recognize microbe-derived compounds and adjust their defense and growth responses according to the type of microorganism encountered (Bais et al. 2004). Plant–microbe interactions have been a central topic of interest which mainly includes the amalgamation of physiological, biochemical, and molecular interactions going on in the rhizosphere.

Messaging Through Biochemical Factors

Plants produce an array of biochemical compounds and signaling molecules to defend themselves against harmful organisms and to attract others that are beneficial. These compounds include root exudates that are used by soil bacteria for energy and biomass production (Haichar et al. 2008). Root exudates mainly belong to the following categories: low molecular weight, high molecular weight, and

volatile organic compounds (VOCs). Roots exude a variety of low molecular weight organic compounds including sugars and simple polysaccharides (such as arabinose, fructose, glucose, maltose, mannose, oligosaccharides), amino acids (such as arginine, asparagine, aspartic, cysteine, cystine, glutamine), organic acids (such as acetic, ascorbic, benzoic, ferulic, malic acids), and phenolic compounds. Some of these compounds, especially the phenolics, influence the growth and development of surrounding plants and soil microorganisms.

In addition, high molecular weight compounds consist of mucilage and proteins, while carbon dioxide, certain secondary metabolites, alcohols, and aldehydes constitute volatiles (Badri et al. 2009). Certain exudes like flavonoids, enzymes, fatty acids, growth regulators, nucleotides, tannins, carbohydrates, steroids, alkaloids, polyacetylenes, and vitamins may act as signals for microbial attraction or be used as carbon sources for microbial nutrition (Schulz and Dickschat 2007). Many of these compounds are involved in either primary or secondary plant metabolic processes and also in plant defense (Uren 2007).

VOCs are actively produced and used as a sophisticated "language" by plants to pursue communication with microorganisms. Plants release certain volatile metabolites like terpenes, the largest class of plant secondary metabolites, having many volatile representatives. The majority of hemiterpenes, monoterpenes, sesquiterpenes, and even some diterpenes have high enough vapor pressures at normal atmospheric conditions to allow significant release in soil (Dudareva et al. 2004). In fact, due to the volatile properties of these compounds, it is tempting to speculate that roots emit volatiles to be sensed quickly and effectively by other organisms such as microbes in order to establish a communication.

Several carbon and non-carbon sources are released as plant exudates which establish unique communications between rhizospheric microbes. A large percentage of the carbon in the root rhizosphere is a result of cuticle lysis or ruptured surface cells or by mechanical abrasions. The breaking of the cuticle allows the mucilage from the cells on the surface of the root to enter the soil matrix and enclose nearby soil colloids to form mucigel. A second important source of carbon in the rhizosphere is the organic material introduced as root exudate or secretion. There is a subtle difference between root exudation and secretion processes. Plant roots also secrete a battery of proteins to defend the plant against potential soilborne pathogens. The mechanism by which proteins are secreted is not completely understood, but it has been proposed that proteins are actively secreted from the root epidermal cells (Park et al. 2002).

The non-carbon-containing compounds present include the ubiquitous H^+, inorganic ions, water, and electrons. Even though their quantities are lower in root exudates than those of carbon-containing compounds, their presence is significant in facilitating internal metabolism and the latter external processes such as nutrient uptake (Uren 2007).

The physical, biochemical, and ecological characteristics of the rhizosphere are defined by the balance between different compounds released, timing of release, and any unique substances that are produced constitutively or in an inducible manner

(Bais et al. 2004). It is estimated that between 20 and 40 % of all photosynthetically fixed carbon is eventually transferred to the rhizosphere, significantly influencing the rhizosphere population by affecting processes such as nutrient and water uptake and establishment of beneficial interactions with soil microbial populations and increasing its number 10–100 fold (Campbell and Greaves 1990). Rhizosphere is thus a dynamic system in which interactions and communication between the root and microorganisms play an important role in continuing to maintain plant growth and productivity.

Messaging Through Physiological Factors

It has been reported that rhizosphere is an active environment whose distribution of resources varies along with age, space, and time (Yang and Crowley 2000). Exudation from the roots is plant specific and generally accepted to reflect the evolution and/or specific physiological adaptation to particular soil habitat conditions including nutrient stature, pH–redox-modulating factors, acidity–alkalinity, rapid and frequent changes in humidity, temperature, UV irradiation, and moisture (Hartmann et al. 2009). Moisture can influence nutrient concentrations and osmolarity and such environment is regarded suitable for microbial colonization.

The origin and adaptation to changing environmental conditions of root-mediated pH changes have recently been reviewed by Hinsinger et al. (2003). The reducing power is a long-observed property of plant roots and was demonstrated in several different approaches, such as the reduction of insoluble manganese oxide by roots (Uren 1981). Although other biogenic acids can affect soil acidification and weathering dissolution, root uptake of nutrient ions, organic acid production, redox cycling of electron-deficient metals, and the carbonic acid system are major contributors to rhizosphere acidification. Since oxygen is very actively consumed in the rhizosphere due to high rates of microbial decomposition and root respiration, steep redox gradients can develop between the root environment and the surrounding bulk soil. In contrast, roots are providing the rhizosphere with oxygen in waterlogged soils and sediments. As a consequence, iron-oxidizing bacteria precipitate Fe plaque as oxidized coatings at root surfaces (Uren 2007).

Bacterial communities associated with root tissues differed significantly from those of rhizosphere soil indicating specific recognition and nutritional selection of bacterial society on root, before diffusion of nutrients into the rhizosphere soil. On the basis of this specificity, recognization, and selection, Hayat et al. (2010) reported that bacteria closely related to *Burkholderia* and *Variovorax* species specifically colonized maize and rape roots and may be considered as selection specialists. Several bacteria like *Azospira*, *Dyadobacter*, *Kaistomonas*, *Sphingomonas*, and *Streptomyces* specifically colonize wheat rhizosphere. These bacteria are known to interact with plants and may exchange signaling molecules and utilize readily secreted compounds. Their proximity to the plant might also be beneficial for the plant, as bacterial-reactive molecules may act more efficiently in the vicinity of roots.

These results provide evidence that different plant species select different bacterial communities on their root tissues. Physiological factors play significant role in the release of exudates depending on the physiological state of the root cell and on the polarity of the compounds to be exuded. Lipophilic exudates are generally released under a typical cytosolic pH of approximately 7.1–7.4 (Marschner 1995). Polar intracellular low molecular weight compounds, including amino and carboxylic acids, exist as anions with low plasmalemma permeability. Root exudation of amino acids and sugars occurs mainly passively via diffusion and may be enhanced under stress. Factors that would affect the membrane integrity include nutrient deficiency (K, P, Zn), temperature extremes, or oxidative stress (Jones and Darrah 1995).

Chemicals contained in root exudates, when released in large quantities, generally enter the rhizosphere and are subjected to physical (sorption), chemical (metal oxidation), and biological (microbial degradation) processes in the soil (Huang et al. 1999). The biological activity of chemicals in the rhizosphere may be altered rapidly in terms of their efficacy because of chemical oxidation, microbial breakdown, or immobilization by irreversible binding to soil particles (Cheng 1995). The synthesis and exudation of allelochemicals along with increased overall production of root exudates are typically enhanced during stress conditions that the plant encounters, such as extreme temperature, drought, and UV exposure (Inderjit and Weston 2003). Growth stage of plant is another important factor that provides shape to the rhizobacterial community structure, and as reported in the case of potato rhizosphere, it could be the strongest one affecting the bacterial communities (van Overbeek and van Elsas 2008). All these specific communication and relations are maintained due to compatibility in the physiological reactions which reveals the intimate story between the two.

Messaging Through Molecular Interactions

Plant roots initiate cross talk with soil microbes by producing signals that are recognized by the microbes, which in turn produce signals that initiate colonization (Berg 2009). PGPR reach root surfaces by active motility facilitated by flagella and are guided by chemotactic responses (Pinton et al. 2007). This implies that PGPR competence highly depends either on their abilities to take advantage of a specific environment or on their abilities to adapt to changing conditions or plant species (Nihorimbere et al. 2011).

The population densities and the diversity of the root microflora may affect the number and activity of resistance-inducing rhizobacteria. Quorum sensing (QS) within and between bacterial populations is a major regulatory mechanism to adjust their metabolism to crowded conditions or other changes in the biotic and abiotic environment. Plants can produce and secrete various compounds that mimic QS signals of bacteria and, thereby, alter bacterial activities in the rhizosphere (Bauer and Mathesius 2004). Molecular mechanisms of plant–microbe coexistence presents studies on the complex and manifold interactions of plants–microbes at

the population, genomics, and proteomics level. Plants produce an array of chemical compounds and signaling molecules to attract beneficial microbes. The plant hormones jasmonic acid (JA), salicylic acid (SA), and ethylene are major regulators of plant innate immunity and draw focus of microbes. Plants respond with the production of a specific blend of these alarm signals (Pozo et al. 2005).

The production of these signals varies greatly in quantity, composition, and timing and results in the activation of differential sets of defense-related genes that eventually determine the nature of the defense response against the attacker encountered. Other plant hormones, such as abscisic acid (ABA), brassinosteroids, and auxins, have also been reported to play a role in plant defense against pathogens (Thaler and Bostock 2004). JA and its derivatives, collectively called jasmonates (JAs), are ubiquitous plant regulators. Their role in different aspects of plant biology has received considerable attention as they act as signals in plant cellular responses to different abiotic and biotic stresses and in plant–microbe interactions (Baldwin et al. 2002). Although the role of JAs in plant defense has been well documented, the importance of JAs in defense against pathogenic microorganisms has only been envisaged recently by the fact that JAs often accumulate in response to pathogen attack. Moreover, JA-dependent responses are associated with enhanced expression of several defense genes that encode antimicrobial proteins, such as plant defensins and thionins (Pieterse and Van Loon 1999).

Signaling molecules, such as SA and nitric oxide, induce the accumulation of a wide range of secondary metabolites including indole glucosinolates, phytoalexins, and alkamides, which may play a role in communication with microbial populations. The recent development of stable-isotope probing (SIP) (Radajewski et al. 2000) and its application to tracking plant-derived C into microbial nucleic (Neufeld et al. 2007) or other biochemical markers (Paterson et al. 2007) provide the opportunity to understand the functional diversity of plant-associated bacterial communities. It has been reported that root exudates of legume *Lotus japonicus* release signal compound strigolactone, 5-deoxystrigol (Akiyama et al. 2005). Strigolactones are group of sesquiterpene lactones and also appear as chemoattractants. It is clear that strigolactones exuded from host roots can trigger a cascade of molecular and cellular events leading to the formation of pre-hyphal branching structures in arbuscular mycorrhizae (AM). In the last few years, a growing number of studies have been conducted on the molecular changes occurring in AM fungi during pre-symbiotic stages (Breuninger and Requena 2004). For example, the flavone luteolin, secreted by alfalfa (*Medicago sativa*) seedlings and seed coats, provides one of the signals that induce the nodulation genes in *Rhizobium meliloti* (Hartwig et al. 1991).

Lectins and flavonoids are known as key signaling compounds in a number of plant–microbe interactions. Flavonoids act as chemoattractants for rhizobial bacteria and as specific inducers of rhizobial nodulation genes (*nod* genes), which are involved in the synthesis of lipo-chitooligosaccharide signals, called Nod factors (Perret et al. 2000). Flavonoids also act as signaling compounds in the mycorrhizae symbiosis and in different plant–soil pathogen interactions (Steinkellner et al. 2007). Root exudates often include phenylpropanoids and flavonoids, presumably synthesized in endoplasmic reticulum (ER) and released

into the soil. In the symbiotic legume–*Rhizobium* interaction, specific flavonoids produced by legume roots enhance the growth rate of bacterial cells, promote bacterial movement toward the plant, and induce transcription of rhizobial nodulation (*nod*) genes (Phillips and Tsai 1992). *Nod* gene induction is dependent on flavonoid concentration in root exudates. Besides acting as signaling substances for the establishment of symbiotic relationships between plant roots and microorganisms (Lerouge 1994; Stacey et al. 1995), other compounds in exudates play an important role in the determination of microbial community structure in the plant rhizosphere. Although the mechanisms by which these compounds are transported from the ER are still unknown, it is possible that they are transported by ER-originating vesicles that fuse to the cell membrane and release their contents (Travis et al. 2003). Similar messages by which plant interacts with microbes are reciprocated and delivered from microbes to plant.

Microbes to Plant Communication

The plant root–soil interface is an environment with high microbial inoculums, composed of both pathogenic and beneficial microbes (Rouatt et al. 1960). Thus, plant roots are constantly exposed to an array of microbes and must interact and defend according to the type of biotic stress (Bais et al. 2006). Plant beneficial interactions are roughly divided into three categories. First, those microorganisms that in association with plants are responsible for its nutrition (*i.e.*, microorganisms that can increase the supply of mineral nutrients to the plant). In this case, while most may not directly interact with the plant, their effects on soil biotic and abiotic parameters certainly have an impact on plant growth. Second, there is a group of microorganisms that stimulate plant growth indirectly by preventing the growth or activity of pathogens. Such microorganisms are referred to as biocontrol agents, and they have been well documented. A third group involves those microorganisms responsible for direct growth promotion, for example, by production of phytohormones. Many soil bacteria have the ability to promote the growth of plants and, therefore, are often designated PGPR (Kloepper and Schroth 1978). Different mechanisms are involved, of which the fixation of atmospheric nitrogen to ammonia by diazotrophs has been studied most (Dobbelaere et al. 2003). Besides fixing nitrogen, the *Azospirillum* secretes several plant hormones involved in the direct promotion of plant growth (Steenhoudt and Vanderleyden 2000). Another mechanism of plant growth stimulation by PGPR is the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase as reviewed by Saleem et al. (2007). Ryu et al. (2003) demonstrated that the volatiles 2,3-butanediol and acetoin produced by *Bacillus* also enhance growth of *Arabidopsis thaliana*, indicating a physical interaction between the PGPR and the plant.

The rhizosphere microflora can benefit plants by increasing tolerance to abiotic stresses such as drought, nutrient deficiency, and heavy metal toxicity as well as protection against pathogens through microbial antagonism and increasing plant

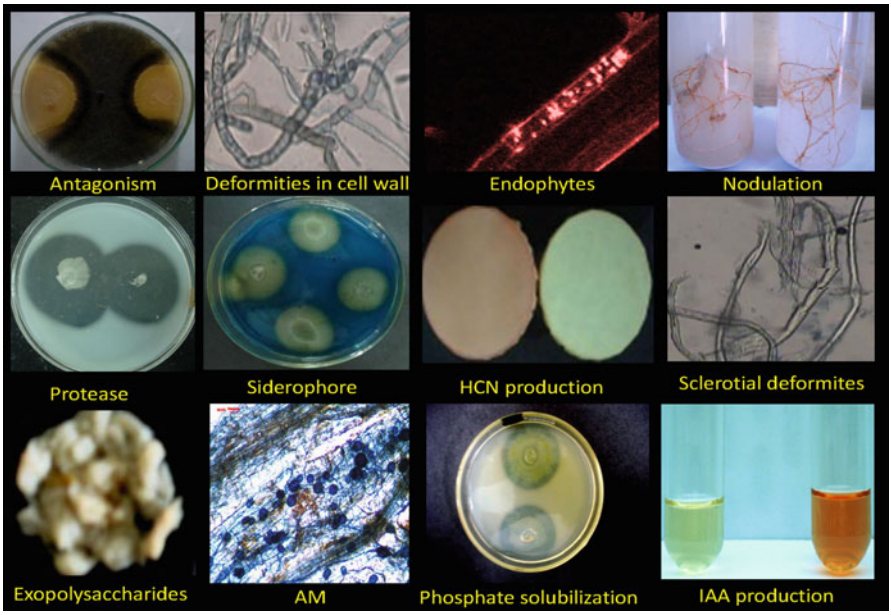


Fig. 1.2 Some attributes by which PGP microbes function in rhizosphere

defensive capacity (Bent 2005; Van Loon 2007) (Fig. 1.2). The following section includes certain PGP mechanisms by which soil microbes interact with plant and enhance plant growth.

Phytohormones

A wide range of microorganisms found in the rhizosphere are able to produce substances that regulate plant growth and development. Bacterial and fungal production of phytohormones such as auxins, cytokinins, and gibberellin can affect cell proliferation in the root architecture by overproduction of lateral roots and root hairs with a subsequent increase of nutrient and water uptake. Therefore, the balance between auxin to cytokinin and the site of hormone accumulation in the plant may determine whether a microbial interaction may be beneficial or detrimental. In the last 5 years, additional signals from microbes have been reported to play a role in plant morphogenetic processes. Plant signaling and physiology are affected by bacterial hormone synthesis and/or degradation in different ways, depending on the physiological role of the hormone, on the recalcitrance of plant tissue to changes in the hormone pool, and on the magnitude of the hormonal sink or source that these bacteria represent (Faure et al. 2009).

Indole Acetic Acid (IAA) Production

It is well known that auxins are quantitatively the most abundant phytohormones secreted by genus *Azospirillum*, *Pseudomonas*, and *Rhizobium* that are responsible for the stimulation of root system development and growth promotion. It is believed that approximately 80 % of rhizobacteria produce IAA (Parmar and Dufresne 2011). Khare and Arora (2010) reported that production of IAA, by *Pseudomonas*, has been associated with plant growth promotion, especially root initiation and elongation, and has an indirect role in disease suppression. The biosynthesis of IAA in rhizobacteria is affected by several environmental factors. In particular, IAA production increases in conditions of higher pH, limited carbon, and higher quantities of tryptophan (Spaepen et al. 2009). Thus far, six pathways for the biosynthesis of IAA have been identified in rhizobacteria, five of which are tryptophan dependant and one tryptophan independent. Instead of tryptophan, this pathway depends on the presence of indole-3-glycerolphosphate.

Recent findings about the role of fungal-produced IAA in different plant–fungus interacting systems open the possibility that fungi may use IAA and related compounds to interact with plants as part of their colonization strategy, leading to plant growth stimulation and modification of basal plant defense mechanisms. In maize (*Zea mays*) and *A. thaliana*, *Trichoderma* inoculation affected root system architecture, increased lateral root formation and root hair growth which was related to increased yield of plants. The signaling mechanisms by which *Trichoderma* promoted growth and development were further investigated in *A. thaliana* by Contreras-Cornejo et al. (2009). Genes involved in auxin transport or signaling, *AUX1*, *BIG*, *EIR1*, and *AXR1*, are reviewed by various workers (Terasaka et al. 2005; Yang et al. 2006; Wu et al. 2007) which assist in plant growth-promoting activities. Most rhizobial species produce IAA and several studies have suggested that changes in auxin levels in the host plant are necessary for nodule organogenesis (Mathesius 2008). Synthesis of IAA in bacterial cell is guided by the QS molecules. In *Pseudomonas chlororaphis*, the GacS/GacA (sensor kinase GacS and the response regulator GacA are members of a two-component system that controls the production of secondary metabolites) acts as a regulator of the tryptophan-dependent IAA biosynthesis (Kang et al. 2006). The involvement of RpoS and GacS in IAA production was further confirmed by overexpression of the *rpoS* and *gacS* genes of *Pseudomonas fluorescens* in two *Enterobacter cloacae* strains (Saleh and Glick 2001). In *Azospirillum* species RpoS is not present (RpoS is not detected in α-proteobacteria), and in this case alternative sigma factors, RpoN and possibly RpoH, regulate IAA expression (Gysegom 2005). Several workers reported that the majority of bacterial endophytes obtained from *Solanum nigrum* and *Echinacea* are efficient IAA producers and enhance plant growth (Lata et al. 2006; Long et al. 2008).

Cytokinins

Certain soil microbes have the potential to produce cytokinins. Cytokinins are purine derivatives that promote and maintain plant cell division and are also involved in various differentiation processes including shoot formation, primary root growth,

and callus formation. Their production by PGPR has been well documented and correlated with increased growth of plants. A recent report has provided important information on the role played by cytokinin receptors in PGP by *Bacillus megaterium*. Cytokinin producing *B. megaterium* was found to promote biomass production of *A. thaliana* and bean plants in vitro and in vivo conditions (Castro et al. 2009). Plants and plant-associated microorganisms have been found to contain over 30 growth-promoting compounds of the cytokinin group. These highly active hormones are usually present in very low concentrations. Various organisms are reported to produce cytokinins, although it is only in higher plants that cytokinins are unequivocally proven to have a hormonal role. The positive effect of cytokinins on growth at the whole-plant level has been demonstrated by the identification of genes involved in cytokinin perception and signaling. Three *A. thaliana* sensor histidine kinases, *CRE1/AHK4/WOL*, *AHK2*, *AHK3*, have been shown to act as cytokinin receptors. These receptors activate the expression of several response regulators in a cytokinin-dependent manner (Castro et al. 2009). Timmusk et al. (1999) reported that inoculation of cytokinins producing strain of *Paenibacillus polymyxa* in wheat rhizosphere promoted plant growth and development. It promoted seed germination, bud formation, release of buds from apical dominance, stimulation of leaf expansion, reproductive development, and retardation of senescence. Cytokinin production in *Azotobacter* is stimulated by various naturally occurring compounds (Nieto and Frankenberger 1990). It is also well known that other plant hormones, such as auxins, or other growth-regulating substances occur in the rhizosphere. In the vicinity of a plant root, such substances could modify the cytokinin effect to the plant in a synergistic way (Stenlid 1982). Cytokinins have also been reported to play an important role in the nodulation of legumes by rhizobia. Rhizobia produce Nod factors, molecules that trigger specific signaling in the roots, inducing expression of plant genes involved in symbiosis (nodulins) and cell division in the root cortex giving rise to the nodule primordium. A general nodulation model based on LHK1 (histidine kinase necessary for nodulation) signaling has been proposed where signals from symbiotic bacteria induce accumulation of cytokinins that bind to LHK1 in the root cortical cells triggering nodule organogenesis (Oldroyd 2007).

Gibberellins

Gibberellins are synthesized by higher plants, fungi, and bacteria; they are diterpenoid acids consisting of isoprene residues (generally with four rings); to date 136 different gibberellins have been identified and characterized (MacMillan 2002). They affect cell division and elongation and are involved in several plant's developmental processes, including seed germination, stem elongation, flowering, fruit setting, and delay of senescence in many organs of a range of plant species (MacMillan 2002). Gibberellins have also been implicated in the promotion of root growth since they regulate root hair abundance. However, in these processes gibberellins interact with other phytohormones and alter the plant hormonal balance thereby affecting plant growth (Bottini et al. 2004). The ability of bacteria to synthesize gibberellin-like substances was first described in

Azospirillum brasilense (Tien et al. 1979) and *Rhizobium*. It has since been detected in different bacterial genera that inhabit the plant root system including *Agrobacterium*, *Azotobacter*, *Arthrobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Clostridium*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*, and *Xanthomonas* (Joo et al. 2005). PGP by gibberellins producing plant growth-promoting bacteria (PGPB) has been reported by several workers, and this positive effect on plant biomass is frequently associated with an increased content of gibberellins in plant tissues (Gutierrez-Manero et al. 2001; Kang et al. 2009). Modification of the gibberellin concentration in plants is the result of either (a) gibberellin synthesis (Lucangeli and Bottini 1997; Piccoli et al. 1999), (b) deconjugation of glucosyl gibberellins, or (c) chemical activation of inactive gibberellins by PGPB. In *Azospirillum* several studies have characterized gibberellins by capillary gas chromatography–mass spectrometry (GC-MS), i.e., GA1, GA3, GA9, GA19, and GA20. Gibberellin production by *Azospirillum* and *Bacillus* has been implicated in the increased N uptake (Bottini et al. 2004).

ABA

ABA is involved in different physiological growth and developmental processes, such as bud formation and seed dormancy, fruit ripening, and homeostatic regulation under abiotic stress. This pathway is active in higher plants, especially under abiotic stress conditions such as water and saline stress. ABA confers the ability of higher plants to adapt under stress through a variety of physiological and molecular processes that include osmotic adjustment, stomatal closure, biosynthesis of stress-related proteins, and regulation of gene expression (Davies and Zhang 1991). One could say that ABA is considered the true root signal in water stress conditions. *A. brasilense* produced higher amounts of ABA when NaCl was incorporated in the culture medium. Inoculation of *A. thaliana* with *A. brasilense* resulted in twofold increase in the plant's ABA contents. PGPR synthesizing ABA have been described by Forchetti et al. (2007) which function to protect plant under stressful conditions and enhance plant growth. *Achromobacter xylosoxidans* and *Bacillus pumilus* isolated from sunflower (*Helianthus annuus*) roots produced significant amount of ABA in chemically defined medium. Sgroy et al. (2009) reported the production of ABA in chemically defined media for *Lysinibacillus fusiformis*, *Bacillus subtilis*, *Brevibacterium halotolerans*, *Bacillus licheniformis*, *Bacillus pumilus*, *Achromobacter xylosoxidans*, and *Pseudomonas putida* which suggested that ABA plays a significant role in enhancing PGP characters. In addition, ABA has also been detected by radioimmunoassay or TLC in supernatants of *Azospirillum* and *Rhizobium* cultures (Dobbelaere et al. 2003). Primary role of ABA in stomatal closure is well established as well as its uptake by and transport in plant; its presence in the rhizosphere could be extremely important for plant growth under a water-stressed environment, such as is found in arid and semiarid climates (Frankenberger and Arshad 1995).

Polyamines

A novel compound involved in promoting plant growth by *Azospirillum* is the polyamine cadaverine synthesized from the precursor L-lysine. Polyamines are low molecular weight organic compounds having two or more primary amino groups. Polyamines serve as growth-regulating compounds. One example is cadaverine, which has been correlated with root growth promotion in pine and soybean in response to osmotic stress (Aziz et al. 1997) and controlling stomatal activity in *Vicia faba* beans (Liu et al. 2000). *A. brasilense*, used as a wheat and maize inoculant in Argentina, is known to produce polyamines such as spermidine, spermine (Perrig et al. 2007), and putrescine (Thuler et al. 2003) in culture and also produces cadaverine in chemically defined medium supplemented with the precursor L-lysine and on inoculated rice plants.

Nutrient Availability

Soil microorganisms constitute a large dynamic source and sink of nutrients in all ecosystems and play a major role in nutrient cycling (Collins et al. 1992), soil structure, reduction in phytopathogens, and other alteration in soil properties influencing plant growth and development. A multiplicity of microorganisms and their functioning is required during formation of soil and maintain fertility through complex cycles and interactions. In fact, the smallest organisms are responsible for cycling nutrients such as N, P, K, and S and making these minerals available to plants. A gram of fertile agricultural soil may contain 2.5 billion bacteria besides other organisms playing diverse roles. Direct functional attributes including nitrogen fixation, phosphate solubilization, iron acquisition, zinc solubilization, and potassium mobilization are necessary for the uptake of insoluble nutrients from plants. Nutrients are important for the growth and development of plants and also microorganisms and are important factors in disease control (Agrios 2005). All the essential nutrients can affect disease severity (Huber and Graham 1999). However, there is no general rule, as a particular nutrient can decrease the severity of the disease but can also increase the severity of the disease or have a completely opposite effect in different environments (Graham and Webb 1991; Marschner 1995; Maheshwari et al. 2012).

Phosphate Solubilization

Phosphorus (P) is one of the major macronutrients for plant growth and development. It is present at levels of 400–1,200 mg kg^{-1} of soil. Phosphorus exists in two forms in soil, as organic and inorganic phosphates. To convert insoluble phosphates (both organic and inorganic) in a form accessible to the plant is an important trait for a PGPR in increasing plant yields (Rodríguez et al. 2006). The concentration of

soluble P in soil is usually very low, normally at levels of 1 ppm or less (Goldstein 1994). The plant takes up several P forms but major part is absorbed in the forms of HPO_4^{-2} or $H_2PO_4^{-1}$. The phenomenon of P fixation and precipitation in soil is generally highly dependent on pH and soil type. Several reports have documented microbial P release from organic sources (Hayat et al. 2010).

Several groups of fungi and bacteria, popularly called as phosphate-solubilizing microorganisms (PSMs), assist the plants in the mobilization of insoluble forms of phosphate. PSMs improve the solubilization of fixed soil phosphate, resulting in higher crop yields, and therefore are used as biofertilizers. A significant increase in the grain yield was observed for rice, chickpea, lentil, soybean, and cowpea, and also an increase in the phosphate uptake in the potato tubers was observed when phosphate-solubilizing strains of *Aspergillus awamori*, *Bacillus polymyxa*, and *Pseudomonas striata* were used either alone or in combination (Gaur and Ostwal 1972; Gilberto et al. 2013).

Microbial solubilization of inorganic phosphate compounds is of great economic importance in plant nutrition. Bacteria from genera such as *Achromobacter*, *Agrobacterium*, *Bacillus*, *Enterobacter*, *Erwinia*, *Escherichia*, *Flavobacterium*, *Mycobacterium*, *Pseudomonas*, and *Serratia* are highly efficient in solubilizing unavailable complexed phosphate into available inorganic phosphate ion. Among fungi *Penicillium* and *Aspergillus* are the most powerful phosphate solubilizers (Whitelaw 2000). A nematofungus *Arthrobotrys oligospora* also has the ability to solubilize the phosphate rocks (Duponnois et al. 2006). Many of the PSMs lower the pH of the medium either by H^+ extrusion (Illmer and Schinner 1995) or by secretion of organic acids or by release of enzymes phytase and phosphatase. Misra et al. (2012) checked the ability of PSM to solubilize phosphorus at high $ZnSO_4$ concentrations suggesting their potential as efficient biofertilizers. The other major group in phosphorus cycle and mutualists with plants are the mycorrhizal fungi. Among mycorrhizae AM fungi are the obligate symbionts of more than 80 % of terrestrial plants (Trépanier et al. 2005). In exchange for reduced carbon, AM fungi supply the plant with mineral nutrients, particularly phosphorus. These fungi are reported in almost every habitat in which plants are able to grow (Brundrett 2002). Several workers reported that the phosphate solubilized by the bacteria could be more efficiently taken up by the plant through a mycorrhizae-mediated bridge between roots and surrounding soil that allows nutrient translocation from soil to plants (Toro et al. 1997). In fact, Toro et al. (1997) using radioactive 32P labeling demonstrated that PSM associated with VAM improved mineral (P) accumulation in plant tissues. They suggested that the inoculated rhizobacteria could have released phosphate ions from insoluble rock phosphate and/or other P sources, which were then taken up by the external VAM mycelium.

Siderophore Production

Most of the iron in the soil is found in silicate minerals or iron oxides and hydroxides, forms that are not readily utilizable by microorganisms and plants or not in

bioavailable form. The bioavailable form of iron can be defined as the portion of the total iron that can be easily assimilated by living organisms. A large portion of iron in soils is present in highly insoluble form of ferric hydroxide; thus, iron acts as a limiting factor for plant growth even in iron-rich soils. Its availability to the organism is very limited due to the rapid oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) state. Ferric ion is highly insoluble under physiological conditions and makes its acquisition by microorganisms a considerable challenge (Neilands 1995). Microorganisms have evolved specialized mechanisms for the assimilation of iron, including the production of low molecular weight iron-chelating compounds known as siderophores, which transport this element into their cells. Siderophores have been implicated for both direct and indirect enhancement of plant growth by rhizospheric microorganisms (Neilands 1981). Hence, most microorganisms secrete siderophores that chelate iron which is subsequently acquired through membrane receptors (Loper and Buyer 1991; Neilands 1995).

Siderophores are usually produced by a large number of bacterial genera including *Aeromonas*, *Bacillus*, *Pseudomonas*, *Rhizobium*, and *Serratia*. The role of microbial siderophores dealing with enhanced PGP and biocontrol is reviewed by Saha et al. (2012). Examples of siderophores produced by various bacteria and fungi are hydroxamate siderophores (ferrichrome, deferoxamine, desferrioxamine E, fusarinine C, ornibactin), catecholate siderophores (enterobactin, bacillibactin, vibriobactin), and mixed ligands (azobactin, pyoverdin, pyochelin, yersiniabactin). Siderophores obtained from *Pseudomonas* display antifungal activity against the plant deleterious fungi, including *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus oryzae*, *Fusarium oxysporum*, *Macrophomina phaseolina*, and *Sclerotium rolfsii* (Arora et al. 2001; Taguchi et al. 2010). Currently, siderophore-producing microbes are being employed as bioinoculants and biocontrol agents for agricultural use. Recently new approach to isolate siderophore-producing microbe has been proposed by Nakouti et al. (2012) taking starch-casein agar media. It has been suggested that the ability to produce specific siderophores and/or to utilize a broad spectrum of siderophores may contribute to the root-colonization ability of biocontrol strains. In addition, siderophores also mediated the iron uptake by plant roots in iron-limiting conditions (Wang et al. 1993). *R. meliloti* and *Bradyrhizobium japonicum* bacterized seeds are known to have reduced *Macrophomina* infection, the mechanism involved being siderophore production which inhibits the growth of *M. phaseolina* by starving it for iron (Arora et al. 2001; Deshwal et al. 2003). Collaborative experiment conducted by Crowley et al. (1991) displayed that microbial population associated with plant root may artifactually affect the rates of Fe uptake and translocation from microbial siderophores and phytosiderophores. Uptake of Fe increased to 34-fold higher than axenically grown plants when supplied with 1 μM Fe as microbial siderophore, ferrioxamine. Acyl-L-homoserine lactone (AHL) also have been reported to have other roles besides their function as signal molecules. Kaufmann et al. (2005) demonstrated that *N*-(3-oxododecanoyl)-HSL and its nonenzymatically formed tetramic acid degradation product 3-(1-hydroxydecylidene)-5-(2-hydroxyethyl)pyrrolidine-2,4-dione function as antibacterial agents. The latter product was shown to bind iron with comparable

affinity to known bacterial siderophores, which might play a role in the observed bactericidal activity of the molecule. *Pseudomonas aeruginosa* produces 2-heptyl-3-hydroxy-4(1H)-quinolone, a QS signal that regulates numerous virulence genes including those involved in iron scavenging (Diggle et al. 2007).

Zinc Solubilization

Total Zn concentration in soil is generally adequate but the quantity that is readily available to plants is insufficient to meet the demand of the crops (Singh 2001). This is where the role of bacteria able to solubilize insoluble Zn compounds and increase their availability in the soil solution, similar to that of P nutrition, comes into play (Saravanan et al. 2007). In plants, more than 90 % of Zn is present in soluble forms. It plays major roles in carbohydrate metabolism, through photosynthesis, in sucrose and starch formation, protein metabolism, membrane integrity, auxin metabolism, and reproduction. In general, Zn solubility increases with a decrease in pH, and its activity declines upon precipitation as hydroxide, phosphate, carbonate, and silicate at slightly acid to alkaline pH (Baruah and Barthakur 1999). Zinc plays an important role in protein and starch synthesis, and, therefore, a low zinc concentration induces accumulation of amino acids and reducing sugars in plant tissue (Marschner 1995). Microbial Zn solubilization was previously focused on autotrophic bacteria-mediated solubilization, particularly by *Thiobacillus ferrooxidans* mainly in relation to leaching of metal ores (White et al. 1997). These studies can be grouped into three broad topics: Zn solubilization associated phytoextraction, Zn solubilization associated nutrient enhancement for crop system, and Zn mineral weathering by fungi. Several workers identified few bacteria that can solubilize Zn; these include *Microbacterium saperdae*, *Pseudomonas monteilii*, *Enterobacter cancerogenus* (Whiting et al. 2001), *Pseudomonas fluorescens*, and *Pseudomonas aeruginosa* (Fasim et al. 2002). Microbes have evolved several mechanisms for Zn resistance and detoxification, including (a) binding of the metal to the outer membrane, (b) efflux by antiport system, (c) efflux by P-type ATPase, (d) Zn-binding proteins (Choudhury and Srivastava 2001), and (e) complexation by organic acids (Appanna and Whitmore 1995).

Microbial metabolites could have an effect on the solubilization of these insoluble materials. Selection and inoculation of zinc-solubilizing bacteria either alone in soils inherently rich in native zinc or along with cheaper insoluble zinc compounds, like ZnO or $ZnCO_3$, will lead to lot of saving in crop husbandry, besides curtailing the expenditure on agro-input (Saravanan et al. 2003).

Biological Nitrogen Fixation (BNF)

Molecular nitrogen cannot be directly assimilated by plants, but it becomes available through BNF, a process that only prokaryotic cells have developed. BNF mainly occurs in nature through symbiotic nitrogen fixers including

legume–*Rhizobium*, nonlegumes–*Frankia* associations and the free-living soil bacteria including cyanobacteria, *Azotobacter*, *Azospirillum*, *Klebsiella*, and *Clostridium*. These associations cater to the nutritional needs of the biosphere and are responsible for generating almost two thirds of the fixed nitrogen annually. Flavonoids are suggested as actinorhizal plant signal molecules that influence *Frankia* growth and *Frankia* symbiotic factor production, the nature of which remains unknown except that it has some biochemical similarities to the *Rhizobium*. Other diazotrophic bacteria, which have been repeatedly isolated from plant roots, comprise the microaerobically nitrogen-fixing bacteria, *Acetobacter diazotrophicus*, *Herbaspirillum seropedicae*, and *Azoarcus*. So far, studies concerning these bacteria mainly dealt with establishing their endophytic nature (Steenhoudt and Vanderleyden 2000).

Species of *Azorhizobium*, *Allorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* form intimate symbiotic relationships with legumes by responding chemotactically to flavonoid molecules released as signals by the legume host. These plant compounds induce the expression of nodulation (*nod*) genes in rhizobia, which in turn produce lipo-chitooligosaccharide (LCO) signals that trigger mitotic cell division in roots, leading to nodule formation (Matiru and Dakora 2004). Nodules—the sites for symbiotic nitrogen fixation—are formed as a result of a series of interactions between rhizobia and leguminous plants. However, there are a number of factors which affect the nodulation on legume roots including host–microsymbiont compatibility, physicochemical conditions of the soil, and the presence of both known and unknown biomolecules such as flavonoids, polysaccharides, and hormones (Tisdale et al. 1990; Zafar-ul-Hye et al. 2007). It is a molecular dialogue between the host plant and a compatible strain of *Rhizobium* which serves as an initiate of the development of nodules (Murray et al. 2007). The genetics of nitrogen fixation was initially elucidated in *Klebsiella oxytoca* strain M5a1 (first identified as *K. pneumoniae*). In that strain, nif genes necessary for the synthesis of a functional nitrogenase are clustered in a 24-kb region (Arnold et al. 1988). Quorum sensing regulation in rhizobia and its role in symbiotic interactions with legumes have been clearly illustrated by Contreras et al. (2007). Most rhizobia tested were AHL producers. Different bacterial species can produce same AHLs with similar structures and properties, suggesting that cross talk between populations occurs, and it is evident that QS via AHL assists in nodule formation and to initiate the phenomena of BNF (d'Angelo-Picard et al. 2005). Recently Claudine et al. (2009) and Black et al. (2012) reviewed the genetics and diversity of nitrogen-fixing bacteria associated with leguminous and nonleguminous plants in diverse habitats.

ACC Deaminase

When plants are exposed to stress, they quickly respond with a small peak of ethylene that initiates a protective response by the plant, such as transcription of pathogenesis-related genes and induction of acquired resistance (Ciardi et al. 2000;

Van Loon and Glick 2004). If the stress is chronic or intense, a second much larger peak of ethylene occurs, often 1–3 days later. This second ethylene peak induces processes such as senescence, chlorosis, and abscission that may lead to a significant inhibition of plant growth and survival. In 1978, an enzyme capable of degrading the ethylene precursor, ACC, to ammonia and α-ketobutyrate was isolated from *Pseudomonas* (Honma and Shimomura 1978). Further studies demonstrated the presence of ACC deaminase activity in a wide range of soil microorganisms including the fungus *Penicillium citrinum* (Honma 1993) and various bacteria. ACC deaminase has been widely reported in numerous microbial species of Gram-negative and Gram-positive bacteria including endophytes, rhizosphere bacteria and fungi (Jia et al. 1999).

The ACC deaminase metabolizes the root's ACC into α-ketobutyrate and ammonia and checks the production of ethylene which otherwise inhibits plant growth through several mechanisms (Saleem et al. 2007). The overproduction of ethylene in response to abiotic and biotic stresses leads to the inhibition of root growth and consequently growth of the plant as a whole. Ethylene synthesis is stimulated by a variety of environmental factors/stresses, which hamper plant growth. These ACC deaminase PGPR boost plant growth particularly under stressed conditions by the regulation of accelerated ethylene production in response to a multitude of abiotic and biotic stresses like salinity, drought, waterlogging, temperature, pathogenicity, and contaminants (Arora et al. 2012). Bacterial ACC deaminase activity is relatively common. Duan et al. (2009) reported that 12 % of the isolated *Rhizobium* possessed this enzyme which helps in protecting plant from stress conditions. In addition, other processes such as the nodulation of legumes and mycorrhizal establishment in the host plant induce local increases in ethylene content. In this context, ACC deaminase-producing bacteria, lowering the ethylene content in the plants, can increase both nodulation and mycorrhizal colonization, respectively (Ma et al. 2003). The role of ACC deaminase has been reviewed in the management of stress by Arora et al. (2012).

Induced Systemic Resistance (ISR)

PGPR volatiles may play a key role in eliciting ISR, for example; volatiles secreted by *B. subtilis* and *Bacillus amyloliquefaciens* were able to activate an ISR pathway in *A. thaliana*. The majority of bacteria that activate ISR appear to do so via a SA-independent pathway involving jasmonate and ethylene signals. Elicitation of ISR in sugar beet by *B. mycoides* and *B. pumilus* was associated with enhanced peroxidase activity (Kloepper et al. 2004). ISR can be induced by many different rhizosphere bacteria in a variety of plant species (Bakker et al. 2007). However, successful elicitation is based on a specific interaction between the inducing strain and the host plant (Van Wees et al. 2008). It was demonstrated that ISR can be elicited in radish by *P. fluorescens* during ambient condition. Variation in the ability to express ISR is observed between different *A. thaliana* accessions (Doornbos et al. 2012).

ISR enhance plant innate immunity by mechanism designated priming, which enables the plant to react faster and more strongly to subsequent pathogen attack (Conrath et al. 2006). Primed plants do not exhibit augmented expression of defense-related genes in the absence of pathogen attack. Instead, an accelerated activation of plant defenses occurs upon pathogen recognition, providing a stronger and faster defense response. Possible mechanisms of priming in ISR involve the expression of signaling components such as transcription factors or the activation of protein kinases (Beckers et al. 2009), which stay inactive until pathogen recognition. Recent study by Jung et al. (2009) suggests that mobile signal molecule azelaic acid is required for the activation of systemic acquired resistance in *A. thaliana*.

Microbe–Microbe Communications

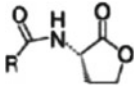
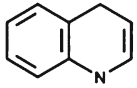
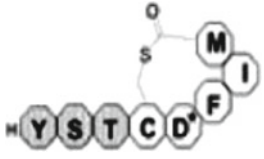
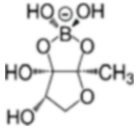
The extreme complexity of interactions that can occur in the rhizosphere involves multiple microbial interactive sessions. These open discussions in between microbes occur in the rhizosphere through various signal molecules which assist bacteria to sense their surroundings. The signaling pathways identified so far, from synthesis and recognition of the chemical signals to response, involve a remarkably small number of regulatory genes. The complexity of intra- and interspecies communication results in the eventual establishment of the rhizosphere population.

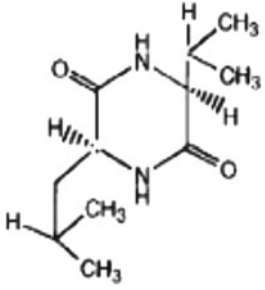
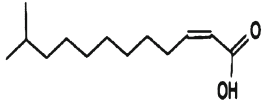
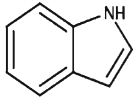
Bacteria have evolved sophisticated mechanisms to coordinate gene expression at population and community levels via the synthesis and perception of diffusible molecules. Because the concentration of the emitted signal in a confined environment reflects the bacterial cell number per volume unit (commonly cell density), such a regulatory pathway is termed QS (Fuqua et al. 1994). In an open environment, however, the concentration of the signal reflects both the bacterial cell number and the signal diffusion coefficient. In such open environments, the term diffusion sensing was proposed (Redfield 2002). A recent tentative to unify quorum and diffusion sensing states that the perception of a signal by a cell (efficiency sensing) is modulated by three essential factors: cell density (QS), mass-transfer properties (diffusion sensing), and spatial distribution of the cells (Faure et al. 2009).

In nature bacteria are more likely to grow in polymicrobial communities than in monoculture. Interactions between the community members are required for community development and maintenance and can involve interspecies signaling mediated by the same molecules as used in intraspecies signaling. In addition to signal exchange between partners that utilize the same or related signal molecules, bacteria can also eavesdrop on the communication of other organisms, modulating their behavior in response to cell–cell signals that they do not synthesize (Ryan and Dow 2008).

Bacterial QS mechanism is based on two groups of signal molecules: peptide derivatives typical for Gram-positive bacteria and fatty acid derivatives exploited by Gram-negative bacteria. The role of these autoinducer molecules or signal molecules is described in this section (Table 1.1).

Table 1.1 Quorum sensing-related signal molecules released by microbes and its associated functions

Signal molecules	Structure	Microbes	Associated functions	References
N-Acyl-L-homoserine lactones		*Pseudomonas*, *Vibrio fischeri*	Bioluminescence, plasmid conjugal transfer, biofilm formation, motility, antibiotic biosynthesis, and the production of virulence factors in plant	Ryan and Dow (2008)
Quinolone		*Pseudomonas*	Biofilm formation, virulence factor, biocontrol activity	Diggle et al. (2006)
Autoinducing peptides		*Xanthomonas*, *Pseudomonas*, *Lactobacilli*	Bacteriocin production, competence development, biological control	Kleerebezem and Quadri (2001), Ryan and Dow (2008)
Autoinducer-2 (AI-2)		*Bacillus*, *Escherichia*, *Enterococcus*, *Neisseria*, *Porphyromonas*, *Salmonella*, *Serratia*, *Staphylococcus*, *Vibrio*	Virulence factors, antibiotic production, biofilm formation, carbohydrate metabolism	McNab et al. (2003)

Diketopiperazines (DKP)		*Pseudomonas*, *Proteus*, *Citrobacter*, *Enterobacter*, *Micrococcus*	Antimicrobial activity, plant growth regulator	Huang et al. (2010)
DSF (diffusible signal factor)		*Xanthomonas*, *Burkholderia*	Including virulence factor synthesis, aggregative, behavior, biofilm formation	Ryan and Dow (2008)
Indole		*E. coli*, *Pseudomonas*, *Bacillus*, *Rhizobium*	Bacterial physiology, ecological balance, biofilm formation, virulence factor	Lee and Lee (2012)

N-*Acyl-L-homoserine Lactones (N-AHLs)*

The most intensively investigated signal molecules in Gram-negative bacteria especially *Pseudomonas* are N-AHLs. These QS systems are responsible for controlling various activities, e.g., antibiotic production, resistance, conjugation, replication, virulence determinant production, exoenzyme synthesis, swarming, biofilm formation, and bioluminescence, and similar homologous QS systems have been described in more than 70 different Gram-negative bacteria species (Pearson et al. 1994). The signal molecules of the AHL type contain a homoserine lactone moiety and a fatty acyl side chain depending on the type of the signal molecule. For synthesis of AHL, *S*-adenosylmethionine (SAM) and acyl-acyl carrier protein (acyl-ACP) are required (Hanzelka and Greenberg 1995).

The paradigm of QS, at the molecular level, consists of activity and cooperation of two components. The first is an AHL synthase (usually LuxI or LuxI homologue) which is responsible for the constitutive synthesis of signal molecules (Fuqua et al. 1994). The second one is a regulatory protein (LuxR and/or LuxR homologues) which promotes transcription of target genes, when bound with AHL (Fuqua et al. 1994). AHL binding requires three-dimensional changes of the regulatory protein and in turn allows its interactions with specific DNA regions enabling transcriptional activation of target genes. In most cases AHLs freely diffuse to the surrounding environment; however, AHL molecules with longer acyl side chains (over ten carbons) are transferred from cells to the environment by an active or carrier-assisted transport system (Pearson et al. 1999) and interact with nearby cells. The soilborne bacterium *Pseudomonas aureofaciens* competes with soil fungi of the genus *Fusarium* by QS-dependent and AHL-based production of an antifungal antibiotic phenazine which suppresses *Fusarium* growth. *P. aureofaciens* is used as a protective agent against *Fusarium* infections in plants (de Boer 2000).

Pierson et al. (1998) reported an example of positive communication between closely related bacteria using a *P. putida* AHL synthase mutant. Another example of AHL type of cross communication was demonstrated by Pierson et al. (1998) using *P. chlororaphis*. *P. chlororaphis* produces three broad-spectrum phenazine antibiotics, a yellow phenazine-1-carboxylic acid and two orange 2-hydroxy derivatives. The phenazine biosynthetic locus, composed of eight genes in a single operon (*phzXY-FABCDO*), is regulated directly by the PhzR/PhzI QS system. PhzI is an AHL synthase that produces hexanoyl homoserine lactone (HHL), and PhzR is the transcriptional regulator. Inactivation of *phzI* gene in strains resulted in loss of HHL production and orange pigmentation, which is a marker for phenazine production in these strains. Phenazine production can be restored by the addition of exogenous HHL.

Quinolone

P. aeruginosa produces another signal molecule, 2-heptyl-3-hydroxy-4-quinolone, which is designated as *Pseudomonas* quorum sensing (PQS) derived from anthranilate, an intermediate in the tryptophan biosynthetic pathway (Pesci et al. 1999). This

molecule belongs to the 4-quinolone family, which is best known for antibiotic activity. It is reported that PQS is produced maximally when cultures reach the late stationary phase of growth, long after the Las and Rhl systems (regulate virulence gene expression in *Pseudomonas*) have been activated (McKnight et al. 2000). Recently, the direct analysis of culture supernatants with liquid chromatography and mass spectroscopy revealed that PQS is produced essentially during the early stationary phase of growth (Le pine et al. 2003). The genes required for PQS synthesis include a cluster in the *phnAB* region: *phnA* and *phnB* (previously associated with phenazine biosynthesis) presumably synthesize the anthranilate precursor from chorismate, while *pqsA* may be involved in activating anthranilate for PQS synthesis. Furthermore, *pqsB*, *pqsC*, *pqsD*, and *pqsH* (final step addition of hydroxyl group) additionally play a role in PQS synthesis. Another gene, *pqsE*, may participate in the cellular response to PQS. Although the *pqsH* homologous *pqsL* gene could encode an enzyme that also acts on PQS, its exact function is not yet clear. *pqsR* encodes a member of the LysR family of transcriptional regulators. Transcription of *pqsH*, a gene required for PQS synthesis, was severely reduced in the lasR mutant background (Gallagher et al. 2002). Furthermore, it was shown that the phnAB operon is subject to QS regulation. In addition the microarray data obtained by Hentzer et al. (2003) showed that the entire pqs operon is controlled by the Las system. Interestingly, a Las-dependent upregulation of mvfR expression precedes AHL-induced expression of the pqs operon. PQS controls expression of LasB and causes a major induction of an rhlI lacZ fusion. PQS acts as a link between the Las and Rhl (autoinducing proteins present in *Pseudomonas*) QS systems by transcriptionally regulating RhlI and is probably not involved in sensing population density (Daniels et al. 2004). Heeb et al. (2011) reviewed the dual role of quinolones as antibiotics and autoinducer molecules.

Autoinducer-2

The only cell-to-cell signaling system identified to date that is shared both by Gram-positive and Gram-negative bacteria is mediated by autoinducer-2 (AI-2) (Schauder and Bassler 2001). Several bacterial genera producing AI-2 are *Bacillus*, *Escherichia*, *Enterococcus*, *Neisseria*, *Porphyromonas*, *Salmonella*, *Serratia*, *Staphylococcus*, and *Vibrio* (Winzer et al. 2002). Today, there is an extensive list of bacterial genera in which AI-2 plays a significant and regulatory role; it includes genes encoding virulence factors, antibiotic production, biofilm formation, and carbohydrate metabolism (McNab et al. 2003). A growing number of bacteria which produce AI-2 contain LuxS homologue suggesting that AI-2 is a universal language for interspecies communication language (Xavier and Bassler 2003). Biosynthesis of AI-2 requires the enzyme LuxS, whereas perception of AI-2 in *Vibrio harveyi* requires the periplasmic AI-2-binding protein LuxP and the sensor kinase LuxQ. LuxPQ is one of three signal transduction systems that converge to control bioluminescence.

However, LuxS fulfill a metabolic function as an integral component of the activated methyl cycle, which provides an alternative explanation for its widespread

conservation. This metabolic cycle provides activated methyl groups in the form of SAM generating *S*-adenosylhomocysteine (SAH), a toxic metabolite. SAH can be removed by one of two routes depending on the microorganism (Winzer et al. 2002), either in a one-step conversion to homocysteine by SAH hydrolase or by the production of *S*-ribosylhomocysteine (SRH) by Pfs nucleosidase (also known as methylthioadenosine/SAH nucleosidase). This SRH is subsequently cleaved to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) by LuxS. This biosynthetic pathway leading to DPD has been shown to be identical in numerous microorganisms (Ryan and Dow 2008). Next, spontaneous cyclization of DPD results in two epimeric furanones. It indicates that multiple derivatives of DPD are biologically active. Phenotypes linked to LuxS-dependent AI-2 production can therefore be considered either as true behavioral responses of a bacterial population by cell-to-cell signaling or as the result of pleiotropic effects of a disturbed activated methyl cycle on cellular metabolism (McNab et al. 2003). A growing number of bacteria which produce AI-2 contain LuxS homologue suggesting that AI-2 is a universal language for interspecies communication (Xavier and Bassler 2003).

Ryan and Dow (2008) reported direct relationship between LuxS and AI-2 suggesting that *P. aeruginosa* that does not have a *luxS* gene does not produce AI-2 and therefore do not participate in the microbial interactions with neighboring cells. Novel feature of AI-2 molecule has been described in *B. cereus*. The genome of *B. cereus* contains genes encoding an Lsr system (*lsrR*-like gene, which encodes the regulator of the *lsr* operon, and *lsrK* and *lsrF*-like genes), whose products are necessary for the synthesizing and processing of AI-2 which assist in ceasing biofilm formation (Auger et al. 2006).

Autoinducing Peptide

Many cell–cell signaling systems in Gram-positive bacteria use modified peptides as signals to regulate functions such as virulence and competence and antimicrobial compounds like bacteriocins. Most autoinducing peptide (AIP) signals are generated by cleavage from larger precursor peptides and subsequent modifications that include substitution with isoprenyl groups and formation of lactone and thiolactone rings and lanthionines (Ansaldi et al. 2002). Signal release from the cell requires dedicated oligopeptide exporters, whereas signal perception is mediated by sensor histidine kinases located in the cytoplasmic membrane that are part of a two-component regulatory systems. Many Gram-positive bacteria communicate with multiple peptides in combination with other types of QS signals. The specificity of signaling has been well studied for the agr (accessory gene regulator) system in the competence systems of *B. subtilis* (Dufour et al. 2002).

The classical example of peptide signal molecule is reported in *Xanthomonas oryzae* pv. *oryzae* (Xoo). Xoo strains producing the extracellular AvrXa21 elicitor trigger host defense responses in rice lines carrying the *Xa21* resistance gene. Although the Xoo molecule has not yet been isolated, it is established that the

activity is dependent on eight *rax* genes that provide clues to its regulation, secretion, and structure (Lee et al. 2008). Extracellular AvrXa21 activity depends upon the RaxRH two-component system, the RaxABC type I secretion system, and RaxPQST, which are required for the activation and transfer of sulfate. Furthermore, AvrXa21 activity is produced in a cell-density-dependent manner. These properties have led to the suggestion that AvrXa21 is a secreted peptide that acts as a QS molecule. Expression of the raxSTAB operon from Xoo in a related species, *X. campestris* pv. *campestris*, confers AvrXa21 activity. This suggests that the core AvrXa21 molecule is conserved (Lee et al. 2006), which may be important in the context of interspecies signaling within xanthomonads. Sturme et al. (2002) reported that biochemical, genetic, and genomic studies have shown that bacteria may contain multiple QS systems, underlining the importance of intercellular communication. Finally, in some cases different peptides may be recognized by the same receptor, while also hybrid receptors have been constructed that respond to new peptides or show novel responses. The role of autoinducing peptide signal molecules was reviewed by Sturme et al. (2002).

Indole

Indole is widespread in the natural environment. By far, at least 85 bacterial species have been shown to produce large quantities of this molecule, including both Gram-positive and Gram-negative bacteria (Hu et al. 2010). In the inquiry about bacterial communication, multiple chemical substances have been identified, and each has a biological story to tell. Indole is a direct product of amino acid catabolism, signals in multidrug exportation, cell division inhibition, stress resistance, and biofilm formation. Underlying the building of biofilms, there are essentially a network of signal molecules and proteins. The effects of indole are probably highly dependent on the status of the members in this network, and a slight change in one factor (either biotic or abiotic) can set off a chain of events that eventually result in the fluctuation of indole concentrations, which in turn nicely fits the bacterial response to the changed factor (Hu et al. 2010). In the perspective of evolution, this kind of network can help bacteria readily and precisely respond to various environmental/biological changes and properly modulate the cells to adapt to the changes, thus achieving the ultimate goal of all organisms' survival and interactions.

The molecule indole has been demonstrated to trigger the signaling cascade during biofilm formation; besides, it could still be further processed by bacteria to generate various derivates that might be involved in biofilm formations. For example, many bacterial oxygenases readily convert indole to oxidized compounds, like 2-hydroxyindole, 3-hydroxyindole, 4-hydroxyindole, isatin, indigo, isoindigo, and indirubin which play important role as inhibitor/inducer during biofilm synthesis (Fisherman et al. 2005).

Another promising indole derivative is IAA, which has been well known as a phytohormone. Diverse bacterial strains produce IAA, especially the plant-associated

endophytes. The interaction between IAA-producing endophytes and plants may lead to effects on their association as diverse as pathogenesis or phytostimulation (Spaepen et al. 2007). For example, IAA-producing endophyte *Pantoea agglomerans* isolated from rice can aggregate to form biofilm-like structure symplasmata and influence the physiology of its host (Feng et al. 2006). As IAA and indole are metabolically interconnected, it is possible that there is a crossover in their functions, too. This point has been confirmed by Bianco et al. (2006) reporting that IAA-treated cells increased the biofilm formation by promoting the production of biofilm-forming matrices trehalose, lipopolysaccharide (LPS), and exopolysaccharide (EPS). Moreover, IAA triggers an increased tolerance to stress conditions (heat and cold shock, UV irradiation, osmotic and acid shock, and oxidative stress) and toxic compounds (antibiotics, detergents, and dyes). A recent research on *Rhizobium etli* showed that IAA addition regulates genes involved in plant signal processing, motility, and attachment to plant roots, which clearly demonstrated a distinct role for IAA in legume–*Rhizobium* interactions (Spaepen et al. 2007). Arora et al. (2012) reported that the triggering of IAA by bacterial strains under stressful conditions is due to the release of QS molecules and it enhances PGP characters. Raut et al. (2012) demonstrated the multiple roles of indole as signal molecule, exhibiting inhibitory activity against pathogens and in developing biofilm.

Diketopiperazines

Diketopiperazines (DKPs) are the smallest cyclic peptides and commonly biosynthesized from amino acids in different macro- and microorganisms. Bacteria of the genus *Bacillus* are known for their prolific production of DKP. Elkahoui et al. (2013) reported that *B. subtilis* produces novel DKP *cis*-cyclo-(His,Leu) which displayed a wide range of antifungal activities against plant pathogenic fungi, probably assumed due to the production of lipopeptide antibiotics. In the biological screening, extracts of *B. subtilis* showed potent activity against bacterial pathogens *Staphylococcus aureus* and *P. aeruginosa* (Zhang et al. 2010).

DKPs, which were originally extracted from culture supernatants of *B. subtilis*, *Citrobacter freundii*, *Enterobacter agglomerans*, *P. aeruginosa*, and *Proteus mirabilis*, have been shown to influence QS in diverse fashions (Holden et al. 2000). DKP [cyclo(l-Pro-l-Met)] produced by *E. coli* stimulates the swarming motility of *P. mirabilis* as effectively as N-HSL. In contrast, cyclo(l-Pro-l-Tyr) (another DKP) antagonize the QS-regulated swarming of *Serratia liquefaciens* at a significantly lower concentration than those required to induce an *E. coli* biosensor (Holden et al. 1999).

DKPs may mimic the action of N-AHLs by interacting with LuxR proteins at, or near, the N-AHL binding site (Degrassi et al. 2002). It has also been demonstrated that DKPs influence the transcription of specific stationary-phase regulated genes in *E. coli* (Holden et al. 1999). In some cases however the concentrations of DKPs required to see effects in bacteria are considerably higher than the levels of N-AHL

required to activate the particular system under study. DKPs also have biological and pharmacological effects on cells of higher organisms, suggesting their potential role in communication with plant and animal cells. Huang et al. (2010) reviewed the structure, pathways, and biological activities of DKPs from marine organism displaying antimicrobial activity.

Diffusible Signal Molecules (DSM)

Bacteria communicate through the secretion and uptake of small diffusible molecules. These chemical cues, or signals, are often used by bacteria to coordinate phenotypic expression, and this mechanism of regulation presumably provides them with a competitive advantage in their natural environment. Examples of coordinated behaviors of marine bacteria which are regulated by signals include swarming and exoprotease production, which are important for niche colonization or nutrient acquisition (Rice et al. 1999). Synthesis and perception of the DSM require products of the Rpf cluster. The synthesis of DSM is dependent on RpfF, whereas the two-component system comprising the sensor kinase RpfC and regulator RpfG is implicated in DSM perception. The conservation of Rpf proteins and relatedness of DSM structures from different bacteria indicated cross species signaling between xanthomonads (Ryan and Dow 2008).

Two recent findings have extended the scope of DSM-mediated interspecies signaling (Boon et al. 2008). The first finding concerns the characterization of a signal molecule related to DSM from *Burkholderia cenocepacia*. Culture supernatants of *B. cenocepacia* contain a compound with DSM-like activity, able to restore the biofilm and extracellular polysaccharide production of Xcc (Boon et al. 2008). This signal molecule *Burkholderia* diffusible signal factor (BDSF) was identified by mass spectrometry and NMR analysis as *cis*-2-decenoic acid, which differs from DSF in the absence of the branched methyl moiety (Boon et al. 2008). Synthesis of BDSF is dependent on an rpfF homologue found in *B. cenocepacia*. In the second report, Ryan and Dow (2008) described the influence of DSF on the behavior of *P. aeruginosa*, an organism that does not carry an *rpf* gene cluster and does not encode any protein that is highly related to RpfF. When grown in coculture with *Stenotrophomona maltophilia*, *P. aeruginosa* develops biofilms with a filamentous architecture, different from the flat undifferentiated architecture seen with *P. aeruginosa* alone, where they control morphological differentiation and secondary metabolite production via QS (Chater and Horinouchi 2003).

Certain DSF including Ca^{2+} produced by fungi acts as potential signals to initiate mutualistic interactions in the rhizosphere. Mycorrhizal association allows a better understanding of the role of Ca^{2+} signaling in AM symbiosis and assists various rhizospheric microbes to experience new thrills. Navazio et al. (2007) reported that Ca^{2+} activated kinase may represent a key node in the Ca^{2+} signaling circuit, able to discriminate different Ca^{2+} signatures and decode the Ca^{2+}message into mycorrhization- and nodulation-distinct responses.

Role of Microbe–Microbe Signaling in Biological Control

Signal molecules discussed above are not only required for microbe–microbe interactions but apart from this also play certain role in triggering antimicrobial activities against certain phytopathogens. Utilization of microbial antagonists against plant pathogens in agricultural crops has been proposed as an alternate to chemical pesticides. These antagonists play an active role in the suppression of pathogenic microorganisms. Bacterial antagonists enforce suppression of plant pathogens by the secretion of extracellular metabolites that are inhibitory at low concentration.

Antibiotic Production

Antibiosis is commonly considered as one of the main characteristics of PGPR. One of the reasons may be that antibiotic production is one of the criteria for screening organisms for a study; antibiotic production has recently been recognized as an important feature in the biological control of plant diseases by rhizospheric bacteria. There are numerous reports of the production and importance of antimicrobial metabolites (Shilev et al. 2012). Certain PGP microbes are able to synthesize antifungal antibiotics and fungal cell wall-lysing enzymes to compete with other soil microorganisms during root colonization for an ecological niche or a substrate. Rhizobacteria are capable to induce systemic resistance against pathogens (Compant et al. 2005) and abiotic stresses in host plants.

DAPG

The polyketide antibiotic 2,4-diacetylphloroglucinol (DAPG) obtained from *P. fluorescens* has received particular attention because it plays a key role in suppressing a broad spectrum of crop diseases (Keel and Défago 1997; Lanteigne et al. 2012). Indigenous DAPG-producing populations have been identified as the driving force behind the development of natural disease suppressiveness in certain soils under long-term monoculture (Raaijmakers and Weller 1998). The DAPG biosynthetic locus has been identified in *P. fluorescens*. The locus comprises DAPG biosynthetic genes *phlACBD*, which are flanked upstream by the divergently transcribed *phlF* gene, encoding transcriptional regulator, and downstream by the *phlE* gene, coding for a putative export protein. Conservation of *phl* genes for biosynthesis of DAPG among ecologically and geographically diverse antagonistic pseudomonads further supports the global importance of DAPG production in biocontrol (Wang et al. 2001).

DAPG-producing biocontrol pseudomonads can interact synergistically in the rhizosphere by stimulating each other in the expression of their DAPG biosynthetic genes. The signal molecule is DAPG itself. DAPG also induces its own biosynthesis and acts as a diffusible signal at intra- and interpopulation levels. Interestingly, DAPG also appears to act on the expression of other biocontrol traits since it

strongly represses the expression of biosynthetic genes for pyoluteorin, another potent antifungal compound produced by some pseudomonads (Haas and Keel 2003). Dunne et al. (1998) applied a mixture of the DAPG producer *P. fluorescens* and a proteolytic rhizobacterium to enhance suppression of *Pythium*-mediated damping-off of sugar beet. Maurhofer et al. (2004) reported the natural diversity of different genotypes of DAPG-producing pseudomonads is exploited to design strain combinations that result not only in enhanced and consistent DAPG production in various soil environments but also in improved growth, activity, and competitiveness in the rhizosphere.

Pyoluteorin

Large number of bacterial strains including *P. fluorescens*, *Streptomyces aureofaciens*, and *Streptomyces pristinaespiralis* are reported to produce pyoluteorin, an antibiotic effective against phytopathogens. Pyoluteorin is an antibiotic that inhibits oomycete fungi, including the plant pathogen *Pythium ultimum*, and suppresses plant diseases caused by this fungus (Howell and Stipanovic 1980). Pyoluteorin is composed of a resorcinol ring, derived through polyketide biosynthesis which is linked to a bichlorinated pyrrole moiety whose biosynthesis remains uncharacterized. Because halogenation can increase the pharmacological effects of many compounds, considerable effort has been directed toward the isolation and characterization of haloperoxidases, enzymes that are capable of forming carbon–halogen bonds in the presence of halide ions and hydrogen peroxide. It has yet to be demonstrated, however, that any of the haloperoxidases thus far characterized are responsible for the in vivo halogenations of known natural products. Pyoluteorin production is affected by nutrient source, temperature, and cell density (Cuppels et al. 1986). Thompson et al. (1999) reported the characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* and its role in antagonism. Although the links between these extracellular factors and the intracellular regulatory pathways controlling pyoluteorin production are not yet understood, a complex picture is emerging that links regulation of production of pyoluteorin and other exoproducts to the physiological status of the cell.

Mupirocin

P. fluorescens produces several inhibitory substances with antimicrobial activities. Among the major metabolites, pseudomonic acid known as mupirocin is also responsible for its bactericidal activity (Fuller et al. 1971). Mupirocin inhibits isoleucyl-tRNA synthetase and prevents the incorporation of isoleucine into newly synthesized proteins (Hughes and Mellows 1980). Mupirocin producing strains of *P. fluorescens* overcome the inhibitory effects of antibiotic by altering the target sites, isoleucyl-tRNA synthetase. Mupirocin exhibits a high level of antibacterial activity against *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Staphylococci*, and *Streptococci*. But it is less sensitive against Gram-positive *Bacilli* and anaerobes (Sutherland et al. 1985). Derivatives

of monic acid A, the nucleus of mupirocin, are active against a range of mycoplasma species (Banks et al. 1998). Mupirocin has a unique chemical structure and contains C9 saturated fatty acid (9-hydroxynonanoic acid) linked to monic acid A by an ester linkage. Mupirocin is derived from acetate and acetate units are incorporated into monic acid A and 9-hydroxynonanoic acid via polyketide synthesis. Fernando et al. (2005) reviewed the biosynthesis antibiotic of mupirocin by several PGPR and its relationship in the biocontrol of phytopathogens causing plant pathogenesis.

Aminopolyols (Zwittermicin A)

Zwittermicin A is a novel bioactive molecule produced by *Bacillus* sp. It is an aminopolyol antibiotic having structural similarities to polyketide antibiotics with a broad spectrum of action against various microbes (Elizabeth et al. 1999). The diverse biological activity of this novel antibiotic includes the suppression of oomycete diseases of plants and is also responsible for the insecticidal activity of *B. thuringiensis* (Emmert et al. 2004). Every gram of soil contains a minimum of 10^4 cfu of Zwittermicin A producers worldwide (Raffel et al. 1996). Zwittermicin A is produced by *B. cereus* and *B. thuringiensis* and effective against oomycetes and other pathogenic fungi. The gene responsible for the synthesis of zwittermicin A production and resistance was identified in *B. cereus*. Genes that encode zwittermicin A biosynthetic enzymes are involved in the formation of aminomalonyl- and hydroxymalonyl-acyl carrier protein intermediate. In addition, the presence of homologues of nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) suggests that zwittermicin A is synthesized by a mixed NRPS/PKS pathway (Emmert et al. 2004). Kevany et al. (2009) characterized the complete zwittermicin A biosynthesis gene cluster from *B cereus*.

Phenazine

Phenazine is a low molecular weight, nitrogen-containing heterocyclic antimicrobial compound consisting of brightly colored pigment produced by the bacterial genera pertaining to *Brevibacterium*, *Burkholderia*, *Pseudomonas*, and *Streptomyces* (Tambong and Hofte 2001). Commonly identified derivatives of phenazine produced by *Pseudomonas* spp. are pyocyanin, 2-hydroxyphenazine-1-carboxylic acid (PCA), and hydroxy phenazines (Turner and Messenger 1986). The antimicrobial activity of phenazine depends on the rate of oxidative reductive transformation of the compound coupled with the accumulation of toxic superoxide radicals in the target cells (Hassett et al. 1993). Though phenazine plays a vital role in the management of soilborne pathogens, the chemotaxis and motility of the bacteria decide the antifungal action of the antibiotic producers. Thomas et al. (2003) have reviewed the role of phenazines in biological control of pathogens. The synthesis of phenazine compounds and shikimic acid pathway are closely related in several microorganisms (Turner and Messenger 1986). Shikimic acid is the basic precursor for the synthesis of phenazine and its derivatives (Ingledew and Campbell 1969). Shikimic

acid is converted to chorismic acid, which in turn branches out with amino-2-deoxy-isochorismic acid (ADIC); ADIC serves as the branch point compound of PCA formation. Later ADIC is converted to *trans*-2,3-dihydro-3-hydroxyanthranilic acid (DHHA). Ring assembly by dimerization of two DHHA moieties resulted in the formation of the first phenazine derivative PCA. *Pseudomonas* contains gene locus *phzABCDEFG* of 6.8 kb in which *phzC*, *phzD*, and *phz*E genes are similar to shikimic acid and chorismic acid metabolism. All these genes coupled with *phzF* are required for the production of PCA. *phzG* is similar to pyridoxamine-5′-phosphate oxidases and serves as a source of cofactor for the enzymes required for synthesizing PCA (Turner and Messenger 1986).

Hydrogen Cyanide (HCN)

Cyanide is a secondary metabolite produced by the members of the genus *Pseudomonas* and *Chromobacterium* (Askeland and Morrison 1983). HCN and CO_2 are formed from glycine catalyzed by HCN synthase (Castric 1994). PGPR produce HCN, which can have unfavorable effects on the growth of soil pathogens (Principe et al. 2007). HCN synthase of *Pseudomonas* oxidizes glycine in the presence of electron acceptors, e.g., phenazine methosulfate. *P. fluorescens* is an aerobic, root-colonizing biocontrol bacterium that protects several plants from root diseases caused by soilborne fungi (Voisard et al. 1994). HCN production by *P. fluorescens* suppresses black root rot of tobacco, caused by *Thielaviopsis basicola* (Sacherer et al. 1994). Mutants of *P. fluorescens*, defective in the synthesis of HCN, antibiotics, and exoenzymes, lost the ability to protect tobacco from black root rot (Voisard et al. 1989). However the, biocontrol activity was restored when HCN production was activated with plasmid mobilized functional *hcnABC* genes. Biosynthesis of HCN is regulated by the FNR-like transcriptional regulator ANR, which upregulates the expression of the *hcnABC* genes under oxygen-limited conditions, such as those in noncirculating hydroponic system. However, whereas these conditions may have favored HCN biosynthesis and a greater role of HCN in biocontrol, they did not preclude DAPG production. Under identical conditions, DAPG has been isolated from the rhizosphere of tomato plants inoculated with pseudomonads. Lanteigne et al. (2012) reported the production of DAPG and HCN by *Pseudomonas* contributing to the biological control of bacterial canker of tomato.

Biosurfactants

Biosurfactants are amphiphilic compounds that can damage cellular membranes, thereby causing leakage and cytolysis (Raaijmakers et al. 2006). They have antimicrobial activity against a variety of organisms, including the pathogenic oomycetes *Pythium* and *Phytophthora*, the fungus *Rhizoctonia*, as well as a number of

Gram-positive and Gram-negative bacteria such as *S. aureus* and *Proteus vulgaris* (Das and Mukherjee 2005). Recently, it has been reported that biosurfactants have been recognized as bioactive molecules with biocidal activity against bacteria, viruses, and fungi. Several workers reported antimicrobial activity of biosurfactant (rhamnolipid and lipopeptide) obtained from *P. aeruginosa* against fungal pathogens and several Gram-positive bacteria (Ballot 2009). General mechanisms by which these surfactants inhibit antagonist are through the disruption of plasma membrane of bacterial and yeast cells by accumulation of intramembranous particles in the cell and thus increasing the electrical conductance of the cell (Thimon et al. 1995). The synthesis of rhamnolipid in *P. aeruginosa* is carried out by rhl operon and few additional genes are required. Production of rhamnolipid occurs in bacteria during stationary phase and guided by QS molecules. The *rhl* gene cluster coding for rhamnolipid contains two additional genes coding for regulatory proteins (RhlR and Rhll). These proteins share similarity with bacterial autoinducer synthetase of Luxl type. RhlR is a putative transcriptional activator and Rhll protein directs the synthesis QS inducer *N*-butyryl-homoserine lactone. The RhlR–Rhll regulatory system is essential for the regulation of rhamnolipid production (Ron and Roserberg 2001). Amphisin, a biosurfactant produced by *Pseudomonas*, possesses PGP property (indirect antifungal properties) and brings about the inhibition of plant pathogenic fungi and promotes plant growth (Koch et al. 1991).

Enzymes

Soil microbes release extracellular enzymes for the initial degradation of high molecular weight substrates such as cellulose, chitin, pectin, and lignin and mineralize organic compounds to mineral N, P, S, and other elements. Microbial numbers and enzymatic activities are higher in the rhizosphere than in the bulk soil; the closer to the soil–root interface, the higher the numbers and the enzyme activities. It was hypothesized that bacteria are the main source of histidinase. Another mechanism by which rhizobacteria can inhibit phytopathogens is the production of enzymes phosphatase, β-gluconase, and dehydrogenase (Hayat et al. 2010). Chitinases, proteases, and other cell wall lytic enzymes are important antifungal factors produced by the microbes to kill phytopathogens in rhizosphere. Production of enzyme chitinase by the AHL signaling was described in *Pseudomonas*, *Chromobacterium*, and *Serratia* (Chernin et al. 1998). Microorganisms capable of lysing other organisms are widespread in natural ecosystems. Lysis of propagules in soil is a logically satisfying method of biological control since it could reduce inoculum density in soil. Species of *Pseudomonas* have been known to excrete chitinases and β-1,3-glucanases to digest the fungal cell wall chitin and glucan, respectively, and use these as a carbon and energy source and are also reported to produce a wide range of antifungal metabolites (Haas and Defago 2005; Arora et al. 2008). Glucanase-producing and PGP actinomycetes, when used in combination, could significantly promote plant growth and also inhibit the growth of *Pythium aphanidermatum* (El-Tarabily et al. 2009). *S. marcescens* produces at least

three chitinases (ChiA, ChiB, ChiC), a chitobiase, and a putative chitin-binding protein (CBP21) (Brurberg et al. 1996). It is conceivable, but not certain, that these five proteins represent the complete chitinolytic machinery of the bacterium. The chitinolytic machinery of *S. marcescens* is of great interest because it is one of the best characterized chitinolytic machineries known till date. Recently determined crystal structures of ChiA, ChiB, and the chitobiase provide detailed insight in how a natural set of chitinolytic enzymes may be built up (Tews et al. 1996). The genes encoding chitinases A, B, and C have been cloned and sequenced from four different strains of *S. marcescens*. Despite this molecular effort, little has been achieved in understanding the genetic regulation of chitinase production of *Serratia* (Brurberg et al. 1996).

Antagonism may be accomplished by competition, parasitism, and antibiotics or by a combination of these modes of action (Cook and Baker 1983). Parasitism involves the production of several hydrolytic enzymes that degrade cell walls of pathogenic fungi. The importance of β-1,3-glucanase and chitinase as key enzymes is responsible for fungal cell and sclerotial wall lysis and degradation. These enzymes have been shown to be produced by several fungi and bacteria and may be considered as an important factor in biological control. Mechanism by which *Trichoderma* inhibits the pathogen is by attaching to the host hyphae by coiling, hooks or appressorium-like structures, and penetrates the host cell walls by secreting hydrolytic enzymes such as a basic proteinase β-1,3-glucanase and chitinase *actinomycetes*, particularly *Streptomyces* chitinase, has been implicated against a variety of plant pathogenic fungi (Anitha and Rabeeth 2010). Lorito et al. (1993) tested antifungal activity of purified endochitinase and exochitinase (chitobiosidase) produced by *Trichoderma harzianum*. Combining the activities of the endochitinase and exochitinase (chitobiosidase) resulted in synergistic increase in antifungal activity. Shapira et al. (1989) demonstrated the involvement of chitinase in the control of *Sclerotium rolfsii* by genetic engineering techniques: the gene *chiA*, encoding the major chitinase produced by *Serratia*, was cloned into *E. coli*. The enzyme produced by the cloned gene caused rapid and extensive bursting of *S. rolfsii* hyphal tips. This chitinase preparation was also effective in reducing the incidence of diseases caused by *S. rolfsii* in bean and by *R. solani* in cotton under greenhouse conditions (Haran et al. 1996).

Root Colonization

Up till now the review has separately unfiled and unraveled the intimate ongoing interactions in between plant to microbes, microbes to plant, and microbes and microbes. What unify all plant–microbe–microbe interactions are the intricate cross talks that occur between the microbes and their hosts (plants), ultimately leading to the successful colonization of plant roots. These communication and interaction should be exploited concurrently in the natural environmental conditions. Dissection of these molecular conversations will be an integral part to utilize microbes in the rhizosphere. To completely understand the mechanism of biological control and plant growth

promotion, microbes introduced in the fields should be studied to understand long-term benign or beneficial interactions between them, host plant, and other microbes and how they affect each other's growth and development (Gamalero and Glick 2011).

As already discussed in the previous session, plant and microbe both select their compatible partners according to their will (nutrient niche), get engaged, and then establish an intermingled relation which last for millions of year. It takes several years to develop such a beautiful and intimate relationship, and all these things will only happen in nature if microbes will have the ability to form biofilm which will lead to effective root colonization. Furthermore, the success of the story depends on how intricate the root-colonizing ability of microbe is on the plant surface utilizing the signals from plants, quorum signals, and from other microorganisms in the vicinity. All the patterns and research clearly suggest an evolutionary link between biofilm formation and root colonization. Plants depend on the ability of roots to communicate with microbes. The converse is also true; many bacteria and fungi are dependent on associations with plants that are often regulated by root exudates.

Higher proportion of AHL-based QS molecules has a general role in biofilm formation and root colonization (de Kievit 2009). Signal molecules accumulate according to population density and are subsequently recognized by adjacent cells and ultimately affect gene transcription. Interestingly, the colonization strategy of microbes includes recognition, adherence, invasion (only endophytes and pathogens), colonization, growth, and several strategies to establish interaction through various signal molecules. Factors that contribute to recognition and adherence include the ability to sense and use root exudates composed of small organic molecules like carbonic acids, amino acids, or sugars (Berg and Smalla 2009). Chemotaxis plays especial role in biofilm formation and colonization of the rhizosphere in symbiotic plant-associated bacteria, e.g., *Ralstonia solanacearum* (Yao and Allen 2006) as well as *Rhizobium* (Miller et al. 2007). The formation of biofilms and root colonization is interlinked and regulated at different stages via diverse mechanisms (Waters and Bassler 2005). The most studied regulatory mechanism that has been found to control the production of EPS and biofilm formation and differentiation is QS regulation (Ruiz et al. 2008; Hooshangi and Bentley 2008). In general, the QS process involves the production, release, and detection of chemical signaling molecules, thus allowing microbial cells to regulate gene expression in a cell-density-dependent manner (Hooshangi and Bentley 2008). Khare and Arora (2011) reported that *Pseudomonas* through signal molecules influences biofilm formation and better root colonization by *Rhizobium*.

Several other bacteria like *Azotobacter*, *Bacillus*, *Pseudomonas*, *Rhizobium*, and *Serratia* are efficient root colonizers because of their ability of forming biofilms that results in better promotion of plant growth. Neal et al. (2012) reported the release of secondary metabolites benzoxazinoids, such as 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA), by the plant roots to attract several *Pseudomonas* isolates and initiate the phenomena of colonization. Efficient root colonization and establishment of PGPR bacteria are of key importance for effective suppression of deleterious organisms (Lugtenberg et al. 2001). Focus has been mainly on fluorescent pseudomonads because of their excellent root-colonizing capacity and ability to

produce antimicrobial compounds (Lugtenberg et al. 2001; Haas and Keel 2003; Haas and Defago 2005; Weller 2007). Several studies have demonstrated a correlation between inoculum density and efficacy of disease suppression. For example, Raaijmakers et al. (1995) demonstrated that effective biological control of *Fusarium* wilt in radish by *P. fluorescens* and *P. putida* required at least 10^5 cfu/g root. A small decline in population density below this threshold resulted in a rapid decrease of efficacy. Once biocontrol bacteria are established in the rhizosphere, a wide variety of mechanisms can result in the suppression of plant pathogens (Doornbos et al. 2012).

Conclusion

Several PGP attributes including antagonistic metabolites, signal-inducing molecules, and root colonization have been discussed in this review under separate sets. But in nature, all these phenomenon and machinery are engaged to occur simultaneously in a particular niche and habitat. The main focus here was to review the ongoing communications that occur between plant to microbe, microbe to plant, and microbe to microbe through signal cascade.

Up till now researchers have not explored the mutual, diverse, and intimate interactions going on between plant–microbe–microbe interfaces simultaneously. Several workers reported that all the mechanisms of PGP are triggered by several autoinducing signal molecules and they are highly specific. Eliciting of the signals induces and activates certain autoinducing molecules, and these mechanisms are interrelated, interwoven, and overlapped with each other and thus participate in enhancing plant growth and suppressing disease pathogenesis. Revelations about the multiple QS signal molecules eliciting different mechanisms of biocontrol and PGP actions can open new doors and avenues to understand combined interactive sessions going in between plant–microbe–microbe interface. Researchers should focus on principles and mechanisms of action that keep on working significantly and simultaneously and maintaining homeostasis balance in the nature.

Several examples are present in environment which explain the array of mechanisms working together under a common regulon and perform multifaceted functions. Same biomolecules can act in a distinct manner under a wide variety of conditions. The scenario is very convoluted in one of the sturdiest niches known as rhizosphere. Biosynthesis of PGP and antimicrobial compounds by rhizosphere microbes is closely regulated by molecules produced by the organism itself and by external environmental factors, including nutritional components, soil chemical and physical properties, host plant genotype, and nonpathogenic soil bacteria (Duffy et al. 2004). Because these factors and characters can determine the ability of particular strains to suppress disease, identifying them would facilitate the targeted application of strains that are more favorable for effective and consistent biocontrol activity.

The soil microbes are active elements for soil development and the basis of sustainable agriculture. From the point of sustainable agricultural development and good eco-environment establishment, a scientific fertilizer is required with

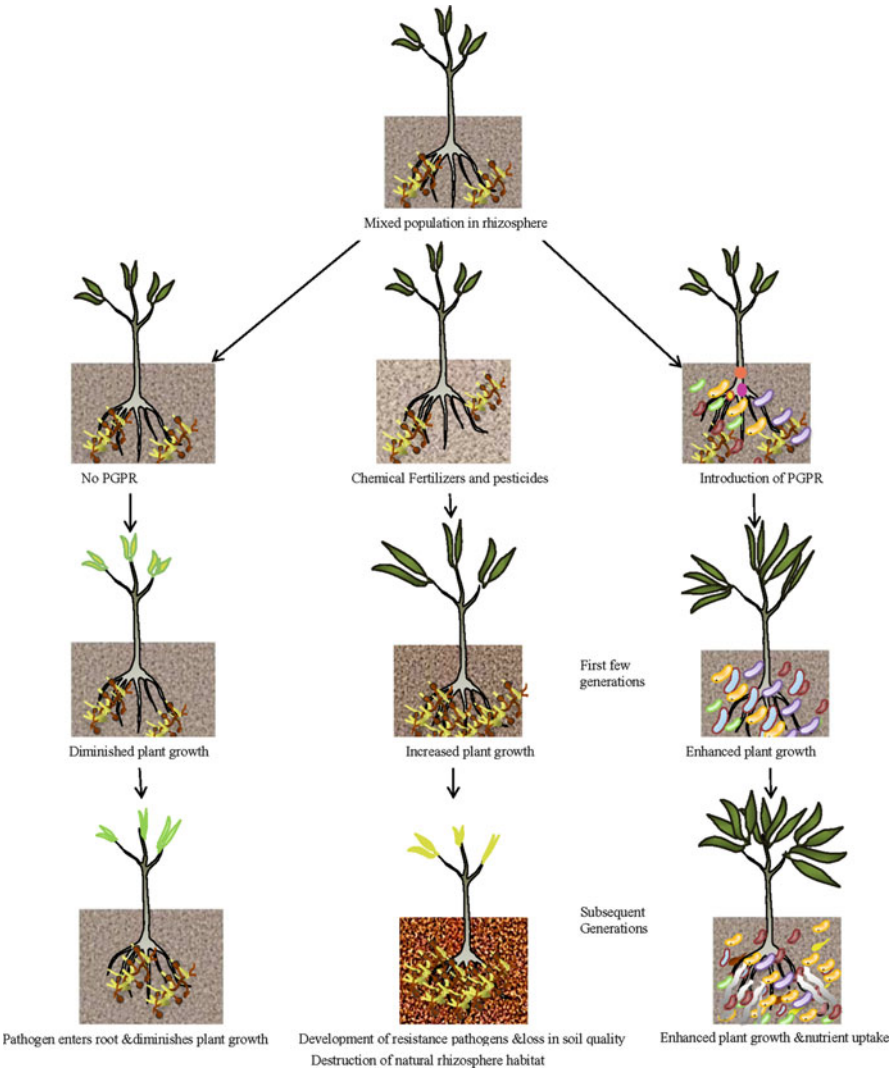


Fig. 1.3 Effect of PGPR on the sustainability of agriculture system

knowledge of the role of each and every component so as to balance in a rational way and achieve high and stable yields (Fig. 1.3). Multitrophic mechanisms which are responsible for PGP and antagonisms are yet not fully explored. There is an emergent need to study these mechanisms and interactions simultaneously under a common roof. The signal molecules responsible for triggering these direct PGP attributes should be studied in an integrated manner for the development of a holistic approach. Multiplicities of signals control the response of plant and their associated organism in the rhizosphere. The deciphering of

the interconnections between all these signals is a future challenge that will be supported by fine analytic tools including transcriptomics, proteomics, and metabolomics.

The need of today's world is to get in an eco-friendly manner high output yield and enhanced production of the crop, and that totally depends on the intimate talks prevailing in the rhizosphere. Hence, the research has to be focused on the new concept of rhizoengineering based on favorably partitioning of the exotic biomolecules, which create a unique setting for the interaction between plant and microbes. The diversity of organisms that communicate in the rhizosphere and the mechanisms implicated in this communication need to be explored more and more.

Acknowledgement Authors are grateful to Vice Chancellor Babasaheb Bhimrao Ambedkar University, Lucknow, India, for their support.

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Chapter 2
Plant–Microbe Interactions for Sustainable Agriculture: Fundamentals and Recent Advances

Sajid Mahmood Nadeem, Muhammad Naveed, Zahir A. Zahir, and Hafiz Naeem Asghar

Contents

S.M. Nadeem
College of Agriculture, DG Khan, Sub-campus, University of Agriculture, Faisalabad, Pakistan

M. Naveed
Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad 38040, Pakistan

Bioresources Unit, AIT Austrian Institute of Technology GmbH, Konrad-Lorenz-Strasse 24, 3430 Tulln, Austria

Z.A. Zahir (✉) • H.N. Asghar
Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad 38040, Pakistan
e-mail: zazahir@yahoo.com

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_2, © Springer India 2013

Abstract Coordinated interactions between plants and microbes have supreme importance for improving plant growth as well as maintaining proper soil conditions. Rhizosphere interactions that are based on complex exchange are more complicated than those occurring above soil surface or non-rhizosphere soil. Among diverse microbial population, plant growth promoting rhizobacteria (PGPR) gain special attention owing to their multifarious functional characters like effective root colonization, hormone production, solubilization of nutrients, and production of certain enzymes that are beneficial for sustainable agriculture. An understanding about their ecology, growth-promoting traits, mechanisms of action, and their application for plant growth stimulation has key importance for maximum utilization of this naturally occurring population. The present review highlights the importance of PGPR for enhancing crop production. The mechanisms of plant growth promotions as well as effectiveness of PGPR under different environments have been discussed. The effectiveness of multi-strain inocula over single strain has been explained with examples. Also, the limitations related to the use of bacterial inoculants under natural field conditions and some important basics related to their formulation and commercialization have been discussed.

Plant Growth Promoting Rhizobacteria: A Novel Source in Plant Growth Promotion

The zone surrounding the plant roots called as rhizosphere is a region of maximum microbial activity compared to surrounding soil (Hiltner 1904). This environment is a favorable habitat for microbial growth that exerts a potential impact on plant health as well as soil fertility (Podile and Kishore 2006). A number of beneficial microorganisms are associated with the root system of higher plants which depend on the exudates of these roots for their survival (Whipps 1990). In soil environment, particularly in rhizosphere, plants are mostly colonized by microbes (Berg et al. 2005). A variety of compounds present in root exudates including polysaccharides and proteins enable the bacteria to colonize plant roots (Somers et al. 2004; Rodriguez-Navarro et al. 2007). Due to competition for nutrients, those microbial populations having better ability to degrade complex compounds like chitin, cellulose, and seed exudates can survive better in such environment (Baker 1991). Among the diverse microbial population, bacteria are the most abundant microorganisms that competitively and progressively colonize the plant roots. Among this large bacterial population, a number of bacterial strains are considered as very important owing to their metabolically and functionally diverse characteristics. These are free-living plant growth promoting rhizobacteria (PGPR) that promote

plant growth by root colonization (Kloepper et al. 1989) and have been studied extensively due to their optimistic effect on plant growth and development. These PGPR belonging to some important genera include *Serratia*, *Bacillus*, *Pseudomonas*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Beijerinckia*, *Flavobacterium*, and *Gluconacetobacter* (Podile and Kishore 2006; Dardanelli et al. 2009; Nadeem et al. 2010b). These PGPR enhance plant growth through various mechanisms like synthesizing a compound essential for plant and facilitating the host in nutrient uptake and also through disease prevention (Glick 1995). The major mechanisms used by PGPR can be divided into two categories, i.e., direct and indirect mechanisms. Phosphate solubilization and phytohormone and siderophore production are some examples of direct growth promotion (Kloepper et al. 1989; Glick et al. 1995; Ayyadurai et al. 2007), while indirect growth promotion occurs by inhibiting the growth of plant pathogens (Glick and Bashan 1997; Persello-Cartieaux et al. 2003; Ravindra Naik et al. 2008). In addition to these general growth promotion mechanisms, PGPR also protect the plant from the deleterious effects of environmental stresses by some particular mechanisms. These include lowering of stress-induced ethylene, production of exopolysaccharides, regulating nutrient uptake, and enhancing the activity of antioxidant enzymes (Sandhya et al. 2009; Glick et al. 2007). There are a number of reports that show outstanding role of this natural microbial population for improving plant growth and development in normal as well as stress environment (Zahir et al. 2004; Glick et al. 2007; Jha et al. 2009; Tank and Saraf 2010; Nadeem et al. 2010b).

Better plant growth promotion depends upon positive plant–microbe interactions. Belowground plant–microbe interactions are more complex than those occurring above the soil surface (Bais et al. 2004), and understanding of these interactions is crucial for maintaining plant growth and health (Barea et al. 2005). The plant–microbe interactions as well as interactions between other rhizosphere microorganisms are still not much clear, and literature shows that most of these interactions are complex in nature. An understanding about microbial ecology, their growth-promoting traits, mechanisms of action, and their application for plant growth stimulation is of pivotal importance for maximum utilization of this naturally occurring population. The diverse study of PGPR is important not only for understanding their ecological role and interactions with plants but also for biotechnological applications (Berg et al. 2002).

Plant Growth Promotion Mechanisms

Plant growth promotion by PGPR is a well-known phenomenon, and this growth enhancement is due to certain traits of rhizobacteria. Some of these traits are very common among certain bacterial species; however, other traits might be specific with some particular species. There are a number of mechanisms used by PGPR for enhancing plant growth and development in diverse environmental conditions (Fig. 2.1). In general, PGPR work as phytostimulators, biofertilizers,

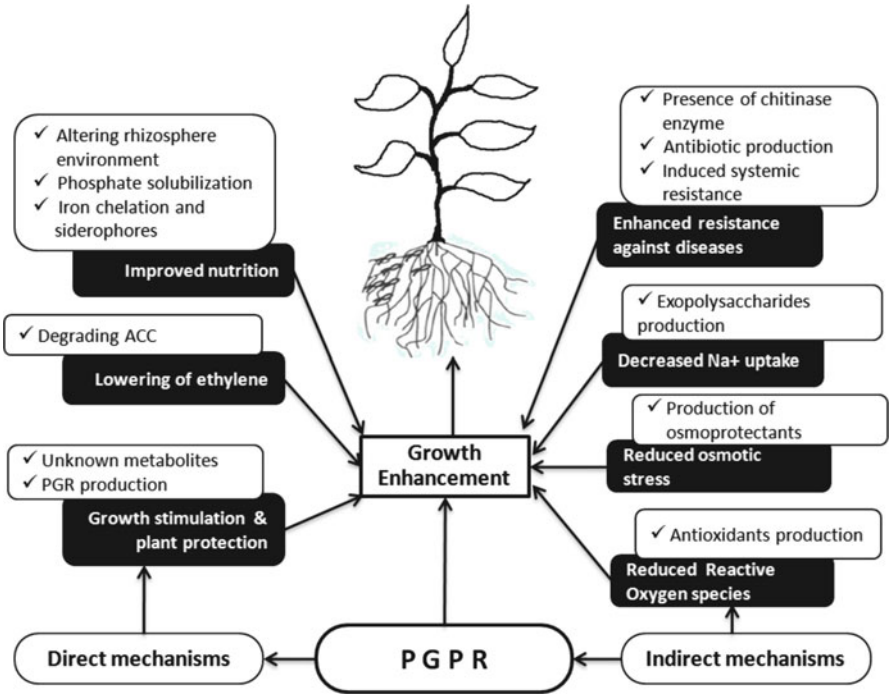

Fig. 2.1 Mechanisms used by PGPR for enhancing plant growth

biocontrol agent, root colonizers, and environmental protectors (Vessey 2003; Zahir et al. 2004). Some of the important and valuable mechanisms are discussed in the following sections.

Phytostimulation

One of the direct growth promotion mechanism used by PGPR is the production of phytohormones including indole acetic acid, abscisic acid, cytokinins, gibberellins, and ethylene. There are a number of reports which advocate the effectiveness of these growth regulators for enhancing plant growth and development (Zahir et al. 2004; Glick et al. 2007). These phytohormones enhance the plant growth by virtue of their positive effect on cell division, cell enlargement, seed germination, root formation, and stem elongation (Taiz and Zeiger 2000; Khalid et al. 2006). Phytohormones influence the physiological processes of plants and facilitate plant growth by altering the hormonal balance (Asghar et al. 2004; Kang et al. 2006). These phytohormones are equally effective in normal and stress conditions. For example, ABA abscisic acid (ABA) helps plant in stress conditions (Zhang et al. 2006) and plays an important role in the photoperiodic induction of flowering

(Wilmowicz et al. 2008). Patten and Glick (2002) observed 35–50 % longer roots in canola inoculated with wild-type GR12-2 compared to IAA-deficient mutant and uninoculated control. Fassler et al. (2010) demonstrated the importance of IAA in stress alleviation of sunflower. Seed inoculation with wild-type GR12-2 induced the formation of tap roots that were 35–50 % longer than the roots from seeds treated with the IAA-deficient mutant and the roots from uninoculated seeds. Similarly, many *Pseudomonas*, *Bacillus*, and *Azospirillum* spp. produce cytokinin and gibberellins (Gamalero and Glick 2011), and positive effects on plant biomass have been reported by these hormones (Gutierrez-Manero et al. 2001; Arkhipova et al. 2005; Spaepen et al. 2009). Steenhoudt and Vanderleyden (2000) demonstrated that the main mechanism used by *Azospirillum* for enhancing plant growth is the production of phytohormones. Although commercially available phytohormones are also used for promoting plant growth, however, microbially produced phytohormones are more effective due to the reason that the threshold between inhibitory and stimulatory levels of chemically produced hormones is low, while microbial hormones are more effective by virtue of their continuous slow release (Khalid et al. 2006).

Biofertilization

The potential of PGPR to enhance plant growth and their participation in carbon, nitrogen, sulfur, and phosphorous cycling increase the effectiveness of PGPR in sustainable agriculture. The application of PGPR for increasing nutrient availability for plants is an important and necessary practice (Freitas et al. 2007) and is very helpful for increasing the nutrient concentration of certain essential elements like N, P, K, Ca, Mg, Zn, Fe, and Mn (Dursun et al. 2010). Inoculation of cotton with PGPR showed enhanced uptake of N, P, K, and Ca (Yue et al. 2007), and similarly PGPR inoculation also enhanced the nutrient content of salinity-stressed maize (Nadeem et al. 2006).

The conversion of insoluble form of phosphorus to make them plant-available form is a common mechanism of various PGPR strains and plays important role to fulfill the phosphorus requirement of plant. Phosphate-solubilizing bacteria are common in the rhizosphere (Ravindra Naik et al. 2008; Jha et al. 2009) that solubilize inorganic phosphate by various mechanisms like production of organic and inorganic acids, release of H ions, and production of chelating substances and through enzymes like phosphatase (Rodriguez et al. 2004; Gamalero and Glick 2011). Also, the exopolysaccharides produced by these bacteria have indirect effect on phosphate solubilization by binding free phosphorus (Yi et al. 2008). It was also observed that cold-tolerant species were able to solubilize P at low temperature (Selvakumar et al. 2008). The application of P-solubilizing bacteria can solve the problem of P precipitation in the soil and therefore increase its availability to plants (Lin et al. 2006). The role of PGPR to improve the uptake of other macronutrients has also been established. Inoculation of *Pseudomonas* sp. having the ability to stimulate calcium (Ca) uptake caused significant improvement in tomato growth

and also reduced blossom-end rot of tomato fruits that generally occurs due to Ca deficiency (Lee et al. 2010). Similarly, the solubilization of biotite by silicate mineral-solubilizing bacteria like *Bacillus* sp. can enhance the availability of K^+ to plants (Sheng et al. 2008).

The production of low-molecular-weight ferric-chelating compound siderophores directly increases the iron availability for plant (Robin et al. 2008) and indirectly protects the plant from pathogenic organisms (Singh et al. 2010b). Siderophores play important role in iron nutrition of plants (Jin et al. 2006). Vansuyt et al. (2007) reported that Fe–pyoverdine complex synthesized by *Pseudomonas fluorescens* C7 was efficiently taken up by the *Arabidopsis thaliana* that resulted in enhanced iron content in plant tissue and better growth. Similarly, bacterial strains improved maize growth through biofertilization and phytostimulation mechanisms (Marques et al. 2010).

Certain bacteria can fix atmospheric nitrogen and make it available for plant. The symbiotic relationship between legumes and nitrogen-fixing bacteria and nitrogen fixation by free-living bacteria without forming association is a source of nitrogen for plant (Carvalho et al. 2010). Co-inoculation of PGPR with rhizobia caused positive effect on nitrogen fixation, plant biomass, and grain yield in various crops like alfalfa, soybean, and pea (Bolton et al. 1990; Dashti et al. 1998; Tilak et al. 2006). Similarly, *Azospirillum* sp. have the potential to increase nitrogen fixation (Rai and Hunt 1993) which can contribute about 70 % of the total nitrogen requirement of the host plant (Malik et al. 1997). The presence of such bacteria also enhances ability of plant to use nitrogen efficiently and minimizes its leaching and denitrification losses. Some important genera of such bacteria include *Enterobacter*, *Klebsiella*, *Pseudomonas*, and *Rhizobium* (James 2000).

Zinc is also an essential nutrient and in deficient soils the solubilization of Zn near the root zone can alleviate the deficiency for plants. The Zn solubilization by sugarcane-associated *Gluconacetobacter diazotrophicus* has been demonstrated by Saravanan et al. (2008). The inoculation with *Burkholderia cepacia* enhanced Zn uptake, its translocation from root to shoot, and improved plant growth (Li et al. 2007).

Due to high price and certain environmental concerns about the chemical fertilizers, the use of PGPR in the form of biofertilizers is an effective supportive strategy to provide crop nutrition (Cakmakci et al. 2006). The use of PGPR inoculants as biofertilizer provides a promising support to chemical fertilizers. Moreover the use of PGPR with inorganic fertilizer can increase the availability of nutrients to the crops (Kumar et al. 2009) and therefore could be useful for increasing efficiency of these fertilizers in one hand and also reducing their quantity on other.

Root Colonization and Rhizosphere Competence

Rhizosphere is a complex habitat with temporal and spatial changes where plant and microbial populations interact with each other and are affected by a number of biotic and abiotic factors. The success of bacteria to enhance plant growth

depends on its potential to colonize the plant root. The significant effects of microbial inoculation cannot be obtained unless the environment supports growth and survival of these introduced microorganisms (Devliegher et al. 1995). The ineffectiveness of PGPR, particularly in field conditions, is due to their inability to colonize plant root properly (Bloemberg and Lugtenberg 2001). One of the aspects of better root colonization is the ability of the bacteria to compete with the indigenous microbial populations. Being the most abundant microorganisms, it is very likely that bacteria can cause great effect on plant physiology owing to their better competitiveness for root colonization (Barriuso et al. 2008). Literature shows that certain PGPR strains have ability to tolerate unfavorable environment (Paul and Nair 2008; Malhotra and Srivastava 2009) and therefore can be considered as the best population for promoting crop production.

The microbes use different strategies for their survival in the environment. The success of these strategies depends upon their ability to adapt to the nutrient-limited conditions, efficient utilization of root exudations, as well as their interaction with plants (Devliegher et al. 1995; Van Overbeek and Van Elsas 1997). In soil environment, the survival of the inoculated bacteria depends on the availability of an empty niche, so that they can compete effectively with better adopted native microbial population (Rekha et al. 2007). It has been observed that PGPR which possess some particular traits like ACC-deaminase activity and the production of antioxidant enzymes, exopolysaccharides, and organic solutes have some selective advantages over other bacteria under stress environment (Mayak et al. 2004a, b; Saravanakumar and Samiyappan 2007; Sandhya et al. 2009). A variety of compounds, like surface proteins and polysaccharides, have a good role in adherence of bacteria to plant root (Dardanelli et al. 2003; Rodriguez-Navarro et al. 2007), and such bacteria have competitive advantages to colonize plant roots because these exopolysaccharides help them to attach and colonize the roots due to fibrillar material that permanently connects the bacteria to root surface (Sandhya et al. 2009).

Enzymatic Activity

Growth enhancement through enzymatic activity is another mechanism used by PGPR. Bacterial strains can produce certain enzymes such as cellulase, ACC-deaminase, and chitinase. Through the activity of these enzymes, bacteria play a very significant role in plant growth promotion particularly to protect them from biotic and abiotic stresses. For example, the reduction of elevated level of ethylene under stress by ACC-deaminase activity and disease suppression by chitinase activity are common mechanisms used by PGPR (Glick et al. 2007; Nadeem et al. 2010b). Similarly, the enhancement of nodule formation by rhizobia might be due to the production of hydrolytic enzymes such as cellulase which could make penetration of rhizobia into root hairs leading to increased numbers of nodules (Sindhu and Dadarwal 2001).

Growth Enhancement Through Vitamins

Vitamins are organic nutritional factors that influence the growth of living organisms. In addition to the vitamins present in root exudates as a source for bacterial growth (Mozafar and Oertli 1993), certain bacterial species also produce vitamins (Dahm et al. 1993). Like other growth promoting traits of PGPR, the production of vitamins also causes positive effect on plant growth and development (Derylo and Skorupska 1993; Azaizeh et al. 1996; Dakora 2003). More root colonization ability of vitamin-producing *Pseudomonas fluorescence* has been observed (Marek-Kozaczuk and Skorupska 2001). Similarly, co-inoculation of vitamin-producing *P. fluorescence* and *Rhizobium* stimulated the growth and symbiotic nitrogen fixation in clover plants (Marek-Kozaczuk et al. 1996).

Biocontrol Activity

Biocontrol mechanisms for diseases suppression are an important strategy against a number of plant pathogens that cause reduction in crop yield. PGPR also act as effective biocontrol agents by suppressing the effect of diseases (Kotan et al. 2009) and provide protection to the plants against harmful pathogens. The PGPR use certain mechanisms including competition, antibiotic production, degradation of fungal cell wall, and sequestering iron by the production of siderophores (Velazhahan et al. 1999; Siddiqui 2006; Ramyasmruthi et al. 2012).

Cell wall degrading enzymes are very important for controlling the phytopathogenic fungi (Picard et al. 2000). Chitinase, cellulase, and lyases are well-known fungal cell wall degrading enzymes (Inbar and Chet 1991; Lorito et al. 1996; Ayyadurai et al. 2007). These enzymes play very important role by suppressing the onset of diseases. The presence of chitinase enzyme in *Pseudomonas* sp. inhibits the growth of *Rhizoctonia solani* by degrading the cell wall (Nielsen et al. 2000). A volatile antibiotic hydrogen cyanide produced by certain bacterial strains also plays role in disease suppression. Suppression of black rot of tobacco by HCN producer *Pseudomonas* strain was observed by Voisard et al. (1981). The production of siderophores by the bacteria reduces the availability of iron to fungi (Sayyed et al. 2008), therefore causing negative impact on its growth (Arora et al. 2001). Matthijs et al. (2007) reported the suppression of disease caused by *Pythium* sp. owing to siderophores that decreased the availability of iron for fungal growth. It is also an evident fact that fungi are unable to absorb the iron–siderophore complex that causes unavailability of iron to pathogenic fungus (Solano et al. 2009). Bacterial siderophores are also suggested to be involved in inducing systemic resistance (ISR) that enhances plant's defensive capacity against pathogens. Enhanced ISR in tomato has been reported by siderophores, pyochelin, and pyocyanin (Audenaert et al. 2002). Similarly, a number of reports have shown the effectiveness of PGPR for enhancing ISR against various fungal and viral diseases (Radjacommare et al. 2002; Saravanakumar et al. 2007). Systemic resistance can

also be induced by a mechanism where inducing bacteria and pathogen remain separated without showing any direct interaction (Ryu et al. 2004).

The disease suppression by PGPR also occurs by the production of antibiotics. The antibiotics in addition to suppressing the pathogen also induce systemic resistance in the plant. The synergistic interaction between antibiotics and ISR further increases resistance against pathogens (Jha et al. 2011). The *Bacillus thuringiensis*, having the ability to produce insecticidal protein (Singh et al. 2010a), can be used as biocontrol agent.

In addition to above-discussed mechanisms, certain environmental factors like water, soil pH, temperature, nutrient contents, and competition for root exudates as well as indigenous microbial population affect the ability of an organism to colonize the plant root. The exclusion of pathogenic organisms from the rhizosphere is one of the significant mechanisms to protect the plant from deleterious effect of such disease-causing organisms. Above discussion shows that owing to their number of mechanisms, PGPR have great competitive advantages over pathogens and could be very effective for protecting the plant from their attack by suppressing their growth.

Removal/Detoxification of Organic and Inorganic Pollutants

Plant growth promotion by PGPR inoculation is also due to reduction and improving plant tolerance against heavy metals (Belimov et al. 2005; Sheng et al. 2008). Bacteria use different intra and extra mechanisms to detoxify the adverse effects of heavy metals in their tissues. These mechanisms include production of proteins which absorb heavy metals and detoxification by taking them in vacuoles (Gerhardt et al. 2009; Giller et al. 2009). The mechanisms used by PGPR for tolerating and detoxifying of heavy metals may also vary among bacterial species and also for different metals. For example, microbes can detoxify zinc (Zn) by binding it in the outer membrane, by producing Zn-binding protein, and/or by complexation of organic acids (Appanna and Whitmore 1995; Choudhury and Srivastava 2001). Bacterial inoculation resulted in degradation of chlorobenzoates and pesticides (Crowley et al. 1996; Siciliano and Germida 1997) and the enhancement of plant growth by PGPR inoculation in highly contaminated soils (Gurska et al. 2009).

The production of siderophores by metal-resistant bacteria plays an important role in the successful survival and growth of plants in contaminated soils by alleviating the heavy metal stress-imposed impact on plants (Belimov et al. 2005; Braud et al. 2006; Rajkumar et al. 2010). Also, the production of enzymes and certain hormones which mobilize heavy metals and plant–microbe interactions affects the process of bioremediation (Abbas-Zadeh et al. 2010). For example, the inoculation of *Lupinus luteus* with genetically engineered nickel-resistant *B. cepacia* showed high nickel concentration that was approximately 30 % more than uninoculated control (Lodewyckx et al. 2001). The application of such bacteria could be helpful for the removal of heavy metals from the environment.

Enhancement of Photosynthetic Activity

Photosynthesis is considered as one of the very important reactions in plant growth and development. Under stress environment, reduction in photosynthesis occurs that might be due to decrease in leaf expansion, premature leaf senescence, impaired photosynthetic machinery, and associated reduction in food production (Wahid and Rasul 2005). PGPR enable the plants to maintain their growth by causing positive effect on photosynthesis. Drew et al. (1990) reported that reduction in photosynthetic activity might be due to osmotic stress and closing of stomata; however, the application of PGPR minimized this negative impact and caused significant increase in photosynthesis (Golpayegani and Tilebeni 2011). Heidari and Golpayegani (2011) observed enhancement in chlorophyll contents in drought stress basil (*Ocimum basilicum* L.) by PGPR application. More improvement in chlorophyll content was observed where PGPR were applied in combination than alone. The increase in shoot length, chlorophyll content, and dry weight was observed when banana plants were inoculated with PGPR (Mia et al. 2010a). According to them, this growth enhancement in addition to other factors was likely to be due to the higher accumulation of nitrogen that contributed to chlorophyll formation which consequently increased the photosynthetic activity. While Xie et al. (2009) demonstrated that enhanced photosynthetic activity in *Arabidopsis* by volatile emission from *Bacillus subtilis* might be due to accumulation of iron, because iron is often a limiting ion in photosynthesis. They also observed that when bacterial volatile signal was withdrawn, the photosynthetic capacity and iron content returned to untreated levels. The importance of iron has already been documented by Spiller and Terry (1980) who demonstrated that biogenesis of the photosynthetic apparatus makes heavy demands of iron availability.

Stress Tolerance

Due to sophisticated signaling system, microbes develop high degree of adaptability to environmental stresses. Bacteria are well known for their ability to tolerate the stress conditions due to their exceptional genetic makeup. The PGPR strains have showed tolerance against stress conditions like salinity and drought (Sandhya et al. 2009; Tank and Saraf 2010). Andre's et al. (1998) demonstrated great resistance ability of *Bradyrhizobium japonicum* against high doses of thiram. Although the microbial adaptations to such situations are difficult to understand (Spaepen et al. 2009), however, it might be due to some of their particular traits which enable them to survive under unfavorable conditions. For example, production of exopolysaccharides (EPS) by the bacteria protects them against unfavorable conditions and enhances their survival (Sandhya et al. 2009; Upadhyay et al. 2011b). In an earlier study, Hartel and Alexander (1986) also showed a significant correlation between the amount of EPS produced by the bacteria and their desiccation tolerance. The accumulation of poly-β-hydroxybutyrate during saline conditions and other osmoprotectants like proline and ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine

carboxylic acid) are protective measures taken by bacteria to survive under stress conditions (Bernard et al. 1993; Arora et al. 2006). The occurrence of such stress-tolerant strains could be very effective for improving soil fertility and enhancing plant growth (Mayak et al. 2004a; Egamberdieva and Kucharova 2009), and application of such stress-resistant strains could also be very useful for enhancing plant growth under stress environment (Glick et al. 2007; Nabti et al. 2010). The above-discussed mechanisms not only show the abilities of bacterial strains to withstand in variable soil environmental conditions but also enable them to compete effectively with the other microbial population. These mechanisms could be very useful for maintaining proper soil conditions and promoting sustainable agriculture.

Application of Rhizobacteria for Plant Growth Promotion

Owing to their well-established growth promoting abilities, PGPR are being used effectively for enhancing crop production. The growth promoting abilities of PGPR have been observed in laboratory under control conditions as well as in natural greenhouse and filed conditions. The crop improvement by PGPR inoculation under normal and stress environment has been reviewed by various workers (Zahir et al. 2004; Glick et al. 2007; Nadeem et al. 2010b; Ahemad and Khan 2011).

Growth Promotion Under Normal Conditions

The use of PGPR is an effective biological approach to increase crop yield and is applied to a wide range of agricultural species. Inoculation with PGPR promotes plant growth through phytohormone production, phosphate solubilization, siderophore production, regulation of hormonal level, and certain other mechanisms which have been discussed in the previous section. The root length of canola, lettuce, tomato, barley, wheat, and oats increased when seeds of these crops were treated with PGPR (Hall et al. 1996). Qiaosi et al. (2005) also reported that the roots of inoculated plants were more in number and longer than untreated control. This growth enhancement is due to common and some particular trait of bacteria, as is evident from the work of Cattelan et al. (1999) who tested eight strains of PGPR for their growth-promoting activity in soybean. They examined that six strains promoted growth more as compared to other, and they observed that these strains contained ACC-deaminase activity in addition to other characteristics. The growth enhancement by the PGPR has also been reported under natural field conditions. Inoculation with PGPR increased the dry weight of leaf, stem, and grain of maize (Gholami et al. 2012). They observed that inoculation caused significant effects on leaf area index and crop growth index. A number of other studies have also shown the importance of PGPR for improving plant growth and development, and some selected examples have been mentioned in Table 2.1.

Table 2.1 Plant growth promotion by PGPR inoculation under normal conditions

Test crop	Bacteria	Experimental conditions	Proposed mechanism(s)	Specific comments	Reference
Arabidopsis thaliana	*Burkholderia pyrrocinia* Bcc171, *Chromobacterium violaceum* CV01	Petri plate assay	VOCs production	*B. pyrrocinia* showed growth-promoting effect with low dose (1 drop) on LB media while high dose (3 drops) on MR-VP media over control	Blom et al. (2011)
	Bacillus cereus L254, *Bacillus simplex* L266, *Bacillus* sp. L272a	Petri plate assay	VOCs production	Rhizobacterial inoculation stimulated plant biomass production by twofold compared to control	Gutierrez-Luna et al. (2010)
Medicago truncatula	*Arthrobacter agilis* UMCV2	Glass tube	VOCs production	Plants grown in the presence of UMCV2 also exhibited a 35 % increase in chlorophyll concentration compared to control plants	Orozco-Mosqueda et al. (2012)
Medicago sativa	*A. agilis* UMCV2	Axenic trial	VOCs production	*A. agilis* UMCV2 inoculation promoted plant biomass up to 40 % compared to control	Velazquez-Becerra et al. (2011)
Peppermint	*P. fluorescens*, *Bacillus subtilis*, *Azospirillum brasilense*	Petri dish assay	VOCs production	Production of essential oil (Eos) was increased twofold in *P. fluorescens-treated* plants compared to control	Santoro et al. (2011)

Pearl millet	*Pseudomonas*, *Citrobacter*, *Acinetobacter*, *Serratia*, *Enterobacter* spp.	Pot trial	P-solubilization	All the isolates significantly stimulated plant growth, i.e., increases of 45–75, 5–68, and 64–88 % in root, shoot length, and biomass, respectively, compared to control	Misra et al. (2012)
Wheat	*Bacillus* sp. AW1, *Providencia* sp. AW5, *Brevundimonas* sp. AW7	Pot experiment	N_2 fixation, nutrient solubilization	An enhancement of 14–34 % in plant biometric parameters and 28–60 % in micronutrient content was recorded by bacterial consortia compared to control	Rana et al. (2012)
	Pseudomonas, *Bacillus*, *Azospirillum*	Axenic, pot trials	Indole-3-acetic acid production	Maximum increase in spike length (33 %), number of tillers (71 %), and weight of seeds (39 %) was recorded at final harvest in plants inoculated with *Pseudomonas*	Hussain and Husnain (2011)
Cucumber	*Ochrobactrum haematophilum* H10	Pot trial	IAA production, P-solubilization, and ACC-deaminase	Strain H10 increased the growth of cucumber leaf and root length by 27 and 58 %, respectively, compared to control	Zhao et al. (2012)

(continued)

Table 2.1 (continued)

Test crop	Bacteria	Experimental conditions	Proposed mechanism(s)	Specific comments	Reference
Tomato	*Gluconacetobacter diazotrophicus* PAL 5 and UAP 5541	Greenhouse experiment	N_2 fixation	Inoculation of PAL 5 increased total fruit number and weight up to 18 and 14 % in 2nd year compared to control	Luna et al. (2012)
	Bacillus amyloliquefaciens IN937a and *Bacillus pumilus* T4	Greenhouse	N_2 fixation/uptake	PGPR inoculation led to increased nitrogen uptake compared to uninoculated control	Adesemoye et al. (2010)
Canola	*Achromobacter* sp., *Klebsiella* sp., *Pseudomonas* sp., *Klebsiella* sp., *Pantoea* sp., *Chryseobacterium* sp.	Greenhouse trial	IAA production, P- solubilization, and ACC-deaminase	The inoculation of canola with *Chryseobacterium* sp. increased plant dry matter 55 and 127 %, respectively, compared to N +ve and –ve control	Farina et al. (2012)
	Methylobacterium fujisawaense strains CBMB 20, CBMB 10	Gnotobiotic	ACC-deaminase activity	*M. fujisawaense* strains CBMB 20 inoculation increased root length up to 78 % compared to control	Madhaiyan et al. (2008)
Lentil	PGPR strains LCA-1, LCA-2, LCA-3, LCA-4, and LCA-5	Greenhouse experiment	IAA production and P-solubilization	Application of PGPR significantly increased shoot weight and root weight by 63 and 92 %, compared to control. Increases in root length, fresh weight, and dry weight were 74, 54, and 92 %, respectively, as compared to control	Zafar et al. (2012)

Cluster bean	*Bacillus coagulans*	Pot trial	P-solubilization	A significant improvement in plant biomass (25 %), root length (28 %), plant P concentration (22 %), and seed yield (19 %) resulted from inoculation when compared with control	Yadav and Tarafdar (2012)
Mammillaria fraileana	*Azotobacter vinelandii* M2Per, *Pseudomonas putida* M5TSA, *Enterobacter sakazakii* M2PFe	Greenhouse trial	Nutrient mobilization	Promotion of plant growth, manifested as an increase in dry weight, was greater in cacti inoculated with *Enterobacter sakazakii* M2PFe compared to control	Lopez et al. (2012)
Strawberry	*Paenibacillus polymyxa* RC05, *Bacillus* spp. RC23	Field trial	IAA production	Root inoculation increased yield, average fruit weight, and quality fruit ratio up to 21, 19, and 32 %, respectively, compared to control	Erturk et al. (2012)
Neem plant	*Streptomyces* strains AzR-010, 049, 051	Controlled	Indole-3-acetic acid	Bacterization improved germination %, root and shoot length by 39, 30, and 31 %, respectively, compared to control	Verma et al. (2011)
Black pepper	*Bacillus tequilensis* NII-0943	Pot trial	IAA production, P- solubilization, and ACC-deaminase	Black pepper cuttings showed 77 and 112.5 % more root and shoot length, respectively, compared to control	Dastager et al. (2011)

(continued)

Table 2.1 (continued)

Test crop	Bacteria	Experimental conditions	Proposed mechanism(s)	Specific comments	Reference
Sugar beet	*Acinetobacter johnsonii* strain 3-1	Pot trial	IAA production and P-solubilization	Inoculation increased plant dry weight and yield of beet by 69 and 37 %, respectively, compared with controls	Shi et al. (2011)
Muskmelon	*B. subtilis* Y-IVI	Pot trial	IAA production, siderophore production	The inoculation of *B. subtilis* significantly increased the shoot dry weight and length by 100 and 34 %, respectively, over control	Zhao et al. (2011)
Walnut	*Pseudomonas chlororaphis* W24, *P. fluorescens* W12, *B. cereus* W9	Pot trial	P-solubilization	Application of W24 or W12 remarkably improved plant height, shoot and root dry weight, and P and N uptake of walnut seedlings compared to control	Yu et al. (2011)
Groundnut	*Pseudomonas* spp. strains PGPR1, PGPR2, PGPR4, PGPR7	Axenic/pot/field trials	ACC-deaminase	Seed inoculation with PGPR containing ACC-deaminase significantly enhanced pod yield, haulm yield, and nodule dry weight (23–26, 24–28, and 18–24 %, respectively) over the control under field conditions	Dey et al. (2004)
Tobacco	*Pantoea agglomerans* strain PVM	Axenic trial	Indole-3-acetic acid production	In vitro root induction in *Nicotiana tobacum* was observed by inoculation over control	Apine and Jadhav (2011)

Rice	*Enterobacter cloacae* GS1	Hydroponics trial	IAA production and P-solubilization	Bacterization significantly improved the fresh weight, root length, shoot length, and nitrogen content as compared to control	Shankar et al. (2011)
	Pseudomonas sp. PAC, *Serratia* sp. CMR165, *A. brasilense* FT326	Glass tube assay	P-solubilization	Inoculation with PAC increased plant height and shoot P content compared to control	Nico et al. (2012)
	Bacillus sp. SVPR30, *P. polymyxa* ATCC 10343	Greenhouse	Indole-3-acetic acid production	*Inoculation with Bacillus* sp. SVPR30 produced 39 % increase in plant dry biomass compared to control	Beneduzia et al. (2008)
Maize	*Acinetobacter* CR 1.8, *Klebsiella* SN 1.1	Pot trials	IAA production and P-solubilization	The inoculation of maize seeds with the *Klebsiella* SN 1.1 showed nonsignificant response compared to control	Chaiharn and Lumyong (2011)
	Acinetobacter rhizosphaerae strain BIHB 723	Pot trial	P-solubilization	Inoculation increased shoot height, shoot biomass, and P uptake by 19, 32, and 83 %, respectively, compared to control	Gulati et al. (2010)
Banana	*A. brasilense* Sp7, *Bacillus sphaericus* UPMB10	Hydroponics	N_2 fixation/uptake	The PGPR inoculation increased the bunch yield up to 51 % compared to control	Mia et al. (2010b)

(continued)

Table 2.1 (continued)

Test crop	Bacteria	Experimental conditions	Proposed mechanism(s)	Specific comments	Reference
Sorghum	*A. brasilense* SM	Axenic	Indole-3-acetic acid production	Seed bacterization with *A. brasilense* improved shoot length and seedling dry weight up to 28 and 62 %, respectively, compared to control	Malhotra and Srivastava (2009)
Mung bean	*Acinetobacter* CR 1.8, *Klebsiella* SN 1.1	Pot trials	IAA production and P-solubilization	The inoculation of bean seeds with the *Klebsiella* SN 1.1 significantly increased the adventitious root length (7.6–7.8 cm^3) over control	Chaiharn and Lumyong (2011)
	Pseudomonas, *Escherichia*, *Micrococcus*, *Staphylococcus* sp.	Axenic conditions	Indole-3-acetic acid production	Bacterization of *V. radiata* seeds significantly enhanced shoot length and biomass up to 48 and 44 %, compared to control	Ali et al. (2010)
Apple	*Bacillus* OSU-142, *Bacillus* M-3, *Burkholderia* OSU-7, *Pseudomonas* BA-8	Field trial	IAA production, cytokinin production	Bacterial inoculation increased average shoot length by 59.2, 18.3, 7.0, and 14.3 % and fruit yield by 116.4, 88.2, 137.5, and 73.7 %, respectively, compared to control	Aslantas et al. (2007)

Considerable work conducted by different researchers shows that PGPR can be used as biofertilizers, and, thus, the use of chemical fertilizer can be reduced (De Freitas et al. 1997; Rabouille et al. 2006). Work of Godinho et al. (2010) showed that application of four PGPR strains having various growth-promoting traits enhanced biomass of eggplant due to balanced nutrient availability and uptake. This growth promotion was also associated with other growth-promoting traits especially indole acetic acid and siderophores. Similarly in a greenhouse study, the application of six bacterial strains on maize plant promoted root and shoot growth and the nutrient status of plant particularly nitrogen and phosphorus (Marques et al. 2010). Such findings have confirmed the perspectives of PGPR as phytostimulators and biofertilizer for agricultural crops. These microbes are also equally effective for promoting growth of fruit trees like apple, apricot, strawberry, plum, and mulberry (Sudhakar et al. 2000; Esitken et al. 2006, 2010; Karakurt and Aslantas 2010; Erturk et al. 2012). Early studies conducted by most of the workers show growth-promoting activity of the PGPR by some common direct and indirect mechanism; however, the production of volatile compound by the bacteria is another growth-promoting mechanism. Zou et al. (2010) found that volatile compounds produced by *Bacillus megaterium* had great growth promotion activity in *A. thaliana*. The fresh weight of inoculated plants was twofold more than uninoculated. They suggested that 2-pentylfuran is a compound that plays an important role in the plant growth promotion activity of this bacterial strain. Prior to this work, Ryu et al. (2003) showed the growth promotion of *A. thaliana* by the volatile compounds 2,3-butanediol and acetoin.

Effectiveness in Stress Agriculture

Environmental stresses are the most limiting factors for crop productivity. Both biotic and abiotic stresses including salinity, drought, extreme temperature, chilling, heavy metals, and insect and pathogen attack are the most detrimental and common stresses plants face in the natural environments. These stresses affect the normal plant processes in one or other way and therefore cause significant reduction in crop yield. PGPR inoculation also proved effective for alleviating the negative impact of these stresses. In addition to improved plant growth under normal conditions, PGPR have great potential for enhancing plant growth under adverse conditions. PGPR use various mechanisms to combat these stresses and enable the plant to maintain their growth under stress environment (Fig. 2.2). There are a number of reports elaborating the effectiveness of PGPR for improving plant growth under stress environment (Glick et al. 2007; Nadeem et al. 2010b; Nabti et al. 2010). The PGPR strains were found equally effective for this growth promotion in variable stress environment like salinity, drought, heavy metal, nutrient stress, and pathogen. Some of the selected examples have been discussed in this section and also listed in Table 2.2.

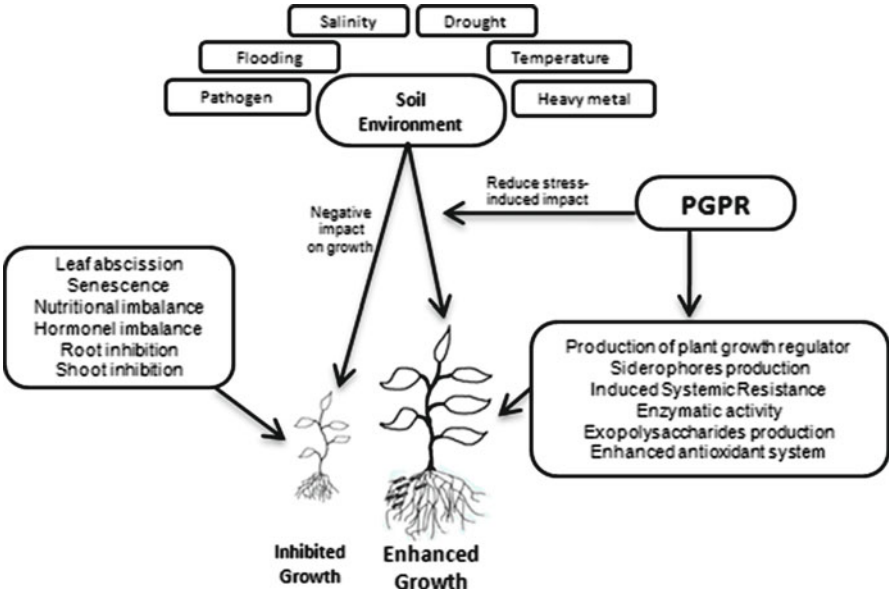

Fig. 2.2 Impact of environmental stresses on plant growth and effectiveness of PGPR for mitigating this negative impact

Abiotic Stress Tolerance

Among various stresses, salinity and drought are the most common that cause adverse effects on crop production in most of the arid and semiarid regions of the world. Salinity limits the production of nearly over 6 % of the world's land and 20 % of the irrigated land (Rhoades et al. 1992; Munns 2005). The changes in environmental scenario result in increasing aridity due to decrease in annual rainfall and because of agriculture under sustained pressure to feed an ever-increasing population. Water limitation in the growing medium reduces diffusion, nutrient uptake by roots, and transport of nutrients from roots to shoots due to restricted transpiration rate, impaired active transport, and altered membrane permeability (Sardans et al. 2008a, b). Similarly, under salinity stress, increasing Na^+ contents cause an increase in Na^+ uptake and, in general, decrease in K^+ and Ca^{2+} contents of plant. Moreover, under stress conditions, plants produce significant quantity of ethylene which can damage them due to negative impact on roots, and it can also cause epinasty, premature senescence, and abscission (Nadeem et al. 2010b). Many efforts have been made to understand the adaptive mechanisms of stress tolerance. These include the reduction of stress ethylene, reduction of toxic ion uptake such as Na^+, and formation of stress-specific protein in plants. Microbial inoculation to alleviate stresses in plants could be a more cost-effective and environment-friendly option which could be available in a shorter time frame.

Table 2.2 Plant growth promotion by PGPR inoculation under biotic and abiotic stress conditions

Test crop	Beneficial bacteria	Proposed mechanism(s)	Plant response	Reference
Drought stress/waterlogging				
Ocimum sanctum	*Achromobacter xylosoxidans* Fd2, *Serratia ureilytica* Bac5, *Herbaspirillum seropedicae* Oci9, *Ochrobactrum rhizos-phaerae* Oci13	ACC-deaminase activity	The Fd2 induced maximum waterlogging tolerance as treated waterlogged plants recorded maximum growth and herb yield (46.5 % higher) and stress ethylene levels (53 % lower ACC) compared to uninoculated waterlogged plants	Barnawal et al. (2012)
Capsicum annuum	*Achromobacter*, *Klebsiella*, *Citrobacter* sp.	ACC-deaminase	Root length and fresh weight in the inoculated plants showed up to 20 and 60 % increase depending on the bacterial strain, compared to the noninoculated stressed control plants	Marasco et al. (2012)
Vigna unguiculata	*Bacillus* sp. RM-2	ACC-deaminase, IAA production, P-solubilization	Seeds coated with RM-2 showed a significant increase in seed germination, shoot length and biomass, and pod yield over control	Minaxi et al. (2012)
Triticum aestivum	*Streptomyces coelicolor* DE07, *S. olivaceus* DE10, *S. geysiriensis* DE27	Production of phytohormones	The DE10 culture treatment improved seedling vigor and yield (up to 88 %), compared to control	Yandigeri et al. (2012)
Helianthus annuus	*Azospirillum lipoferum* 21, *Azospirillum brasilense* OF, *Azotobacter chroccoccum* 5	Not described	Inoculation increased grain yield up to 24, 29, and 27 %, respectively, under normal H_2O, mild, and severe stress	Jalilian et al. (2012)
	Pseudomonas sp. strain GAP-P45	Exopolysaccharide production	An increase in total dry biomass by 64.6 and 23 % due to strain GAP-P45 inoculation was observed under drought stress and no stress conditions, respectively	Sandhya et al. (2009)

(continued)

Table 2.2 (continued)

Test crop	Beneficial bacteria	Proposed mechanism(s)	Plant response	Reference
Vigna radiata	*P. fluorescens* Pf1, *B. subtilis* strains EPB5, EPB 22, EPB 31	Catalase/peroxidase enzyme	The greater activity of catalase and peroxidase was observed in green gram plants bacterized with *P. fluorescens* against water stress compared to untreated plants	Saravanakumar et al. (2012)
Cicer arietinum	*Paenibacillus lentimorbus* B-30488	Biofilm formation	The chickpea seed bacterization with B-30488 along with sodium alginate and $CaCl_2$ caused an increase of 30, 9, and 20 %, in shoot length, 100-seed weight, and plant dry weight, respectively, as compared to control	Khan et al. (2011)
Ornamental species	*Variovorax paradoxus* 5C-2	ACC-deaminase activity	Inoculation of growth media with *V. paradoxus* lowered ethylene emission from mature leaves of *Cytisus praecox* and consequently reduced abscission of the leaves under drought treatment	Sharp et al. (2011)
Saccharum officinarum cv. M 1176/77 and R 570	*Azospirillum* isolates, Azo 195, Azo 249, Azo 274	Auxin production	Inoculation increased shoot height and root dry mass by 15 and 75 % in cv. M 1176/77 when subjected to drought stress, whereas cv. R 570 responded negatively particularly in the absence of drought stress	Moutia et al. (2010)
Zea mays L.	*P. entomophila* BV-P13, *P. stutzeri* GRFHAP-P14, *P. putida* GAP-P45, *P. syringae* GRFHYTP52, *P. monteilli* WAPP53	Not described	Seed bacterization with *Pseudomonas* spp. strains improved plant biomass, proline, sugars, and free amino acids under drought stress. However, protein and starch content was reduced under drought stress conditions	Sandhya et al. (2010)
Trifolium repens	*Pseudomonas* sp., *P. putida*, *B. megaterium*	Indole-3-acetic acid production	Inoculation increased shoot and root biomass and water content under drought conditions	Marulanda et al. (2009)

Pisum sativum	*P. fluorescens* biotype G (ACC-5), *P. fluorescens* (ACC-14), *P. putida biotype* A (Q-7)	ACC-deaminase activity	Bacterization of ACC5 increased dry weight, root length, shoot length, number of leaves per plant, and water use efficiency on fresh and dry weight basis by 150, 92, 45, 140, 46, and 147 %, respectively, compared to respective controls	Zahir et al. (2008)
Catharanthus roseus	*P. fluorescens*	IAA/gibberellin production	*P. fluorescens* enhanced the growth parameters and partially ameliorated the drought-induced growth inhibition by increasing the fresh and dry weights significantly	Abdul Jaleel et al. (2007)
Salinity stress				
C. roseus	*A. xylosoxidans* strains AUM54, AUENR9, AUENRL3, AUENRL7	ACC-deaminase activity	*A. xylosoxidans* AUM54 inoculated plants increased germination % age (7 %), vigor index (48 %), plant height (14 %), root dry weight (13 %), and ajmalicine content (30 %) compared to uninoculated plants grown without NaCl	Karthikeyan et al. (2012)
Lactuca sativa L.	*A. brasilense* Sp245	Hormone (IAA) production	Inoculation increased leaf area, chlorophyll content, and dry weight up to 63, 24, and 102 %, respectively, at higher salinity. At 40 mol m^{-3}NaCl, 60 % and 73 % of plants remained alive in noninoculated and *Azospirillum*-inoculated plants, respectively	Fasciglione et al. (2012)
Oryza sativa L./*Abelmoschus esculentus* L.	*Agrobacterium* sp. SUND BDU1, *Bacillus* sp. strains SUND LM2, Can4, Can6	N_2 fixation, IAA production	The *Bacillus* sp. Can6 inoculation increased yield and N uptake by 7, 16, and 35, 42 % of rice and okra, respectively, compared to control	Barua et al. (2012)

(continued)

Table 2.2 (continued)

Test crop	Beneficial bacteria	Proposed mechanism(s)	Plant response	Reference
O. sativa L.	*A. brasilense* Az39	Cadaverine production	*A. brasilense* Az39 produced cadaverine in chemically defined medium and inoculated plants; this capacity correlated with root growth promotion or osmotic stress mitigation in hydroponics conditions	Cassan et al. (2009)
Gossypium hirsutum L.	*Raoultella planticola* Rs-2	ACC-deaminase activity, IAA production	Inoculation of Rs-2 increased germination % age, plant height, and dry biomass by 30, 15, and 33 %, respectively, compared to control	Wu et al. (2012)
	P. putida Rs-198	IAA production	The inoculation of Rs-198 increased the germination rate, plant height, and dry weight by 24, 13, and 10 %, respectively, as compared to control	Yao et al. (2010)
Z. mays L.	*Azotobacter* sp. C5, C7, C8, and C9	N_2 fixation, IAA production	*Azotobacter* sp. C9 increased shoot biomass and polyphenol content up to 122 and 27 %, respectively, at highest salinity level compared to respective control	Rojas-Tapias et al. (2012)
	Bacillus megaterium	Regulation of aquaporins	Inoculated plants showed higher root hydraulic conductance values; correlated with higher plasma membrane type two (PIP2) aquaporin amount in their roots under stressed conditions	Marulanda et al. (2010)

T. aestivum	*Streptomyces* isolates C	IAA production, siderophores	Inoculation increased germination % age and biomass up to 33 % compared to respective NaCl stressed control	Sadeghi et al. (2012)
	Bacillus sp. (SKU-13), *Paenibacillus* sp. (SKU11)	Exopolysaccharide production	Under saline condition, inoculation with SKU13 resulted in an increase of shoot weight by 34 % compared to stressed control	Upadhyay et al. (2011b)
	B. pumilus, *Pseudomonas mendocina*, *Arthrobacter* sp., *Halomonas* sp., *Nitrinicola lacisaponensis*	IAA production, siderophores, P-solubilization	Maximum shoot and root length (33.8 and 13.6 cm) and shoot and root biomass (2.73 and 4.48 g dry weight) were recorded in plants inoculated with *B. pumilus* compared to control	Tiwari et al. (2010)
	P. putida (N21), *P. aeruginosa* (N39), *Serratia proteamaculans* (M35)	ACC-deaminase activity	Inoculation increased the plant height, root length, grain yield, 100-grain weight, and straw yield up to 52, 60, 76, 19, and 67 %, respectively, over uninoculated control at 15 dS m^{-1}	Zahir et al. (2009)
Lycopersicon esculentum/*Cucumis sativus*	*P. chlororaphis* isolate TSAU13	Not described	The bacterial strain stimulated shoot growth (up to 32 %), dry matter (up to 43 %), and the fruit yield of tomato and cucumber (up to 16 %) compared to the uninoculated control plants under saline conditions	Egamberdieva (2012)
L. esculentum	*B. subtilis* FZB24 and FZB41	Peptides, auxin production	The application of *Bacillus* sp. metabolites increased root length and plant dry mass up to 23 and 36 %, respectively, compared to stressed control	Stavropoulou (2011)
	P. fluorescens NT1, *P. aeruginosa* T15, *P. stutzeri* C4	ACC-deaminase, siderophores	Inoculation with C4 strain increased plant height and biomass up to 25 % compared to control under stressed condition	Tank and Saraf (2010)

(continued)

Table 2.2 (continued)

Test crop	Beneficial bacteria	Proposed mechanism(s)	Plant response	Reference
H. annuus	*P. fluorescens* biotype F and *P. fluorescens* CECT 378^T	IAA production, siderophores	The isolate CECT 378T produced 66 % increment in leaves, 34 % in stems, and 16 % in roots, while the effect of isolate inoculation was (only) more evident in leaves and stems with 30 and 26 %, respectively	Shilev et al. (2012)
C. arietinum	*P. putida* MSC1, *P. pseudoalcaligenes* MSC4	IAA production, siderophores, P-solubilization	Plants inoculated with MSC1 or MSC4 isolates showed an increase in the parameters that evaluate plant growth when compared to uninoculated controls	Patel et al. (2012)
C. annuum L.	*Brevibacterium iodinum* RS656, *Bacillus licheniformis* RS111, *Zhihengliuela alba* RS16	ACC-deaminase activity	Inoculation of *B. licheniformis* RS656, *Z. alba* RS111, and *Br. iodinum* RS16 reduced ethylene production by 44, 53, and 57 %, respectively	Siddikee et al. (2011)
Temperature stress (heat and cold stress)				
Vitis vinifera L.	*Burkholderia phytofirmans* strain PsJN	Colonization/ metabolite production	The endophytic colonization of PsJN improved plant photosynthetic parameters and regulated carbohydrate metabolism compared to control	Fernandez et al. (2012a)
	B. phytofirmans strain PsJN	Metabolite production	At 4 °C, both stress-related gene transcripts and metabolite levels increased earlier and faster and reached higher levels in PsJN-bacterized plantlets than in nonbacterized counterparts	Theocharis et al. (2012)
	B. phytofirmans strain PsJN	Trehalose and trehalose 6-phosphate	Plants colonized by *B. phytofirmans* and cultivated at 26 °C accumulated T6P and trehalose in stems and leaves at concentrations similar to nonbacterized plants exposed to chilling temperatures	Fernandez et al. (2012b)

T. aestivum L.	*Pseudomonas* sp.	IAA production, siderophores, P-solubilization	Bacterization significantly improved the root/shoot length, biomass, and the level of cellular metabolites compared to control	Mishra et al. (2011)
	Pseudomonas lurida strain M2RH3	IAA production, siderophores, P-solubilization	Seed bacterization with the isolate positively influenced the growth and nutrient uptake parameters of wheat seedlings cv. VL 804 at controlled cold growing temperature	Selvakumar et al. (2011)
	Exiguobacterium acetylicum strain 1P (MTCC 8707)	IAA production, siderophores, P-solubilization	Seed bacterization with the isolate positively influenced the growth and nutrient uptake parameters of wheat seedlings at suboptimal cold growing temperatures	Selvakumar et al. (2010)
Sorghum bicolor L.	*Pseudomonas* sp. strain AKM-P6	IAA production, P-solubilization	Inoculation induced the biosynthesis of high-molecular-weight proteins in leaves under elevated temperature, reduced membrane injury, and improved the levels of cellular metabolites	Ali et al. (2009)
Cucurbita pepo	*S. marcescens* strain SRM (MTCC 8708)	IAA production, siderophores, P-solubilization	Seed bacterization with the isolate significantly enhanced plant biomass and nutrient uptake of wheat seedlings grown in cold temperatures	Selvakumar et al. (2007)

(continued)

Table 2.2 (continued)

Test crop	Beneficial bacteria	Proposed mechanism(s)	Plant response	Reference
Heavy metal stress				
Z. mays/*H. annuus*	*Pseudomonas* sp. DGS6	ACC-deaminase, IAA production/ phytoextraction	Inoculation with DGS6 increased the root–shoot dry weight of maize by 85, 49 % and sunflower by 45, 34 %, respectively, in Cu contamination	Yang et al. (2013)
	Pseudomonas strains 3–3.5-1, TLC 6–6.5-1, TLC 6–6.5	IAA production, P-solubilization/ metal solubilization	*Pseudomonas* sp. TLC 6–6.5-4 resulted in a significant increase in copper accumulation in maize and sunflower, and an increase in the total biomass of maize	Li and Ramakrishna (2011)
Solanum nigrum L.	*Serratia nematodiphila* LRE07	Endophytic colonization/ phytoimmobilization	The inoculation of bacterium alleviated the Cd-induced changes, resulting in more biomass production and higher photosynthetic pigments content of leaves compared with nonsymbiotic ones	Wan et al. (2012)
T. aestivum L.	*Staphylococcus arlettae* Strain Cr11	ACC-deaminase, IAA production/ phytoextraction	Bacterial inoculation in controlled Petri dish and soil environments showed significant increase in percent germination, root and shoot length, as well as dry and wet weight in Cr(VI)-treated and Cr(VI)-untreated samples	Sagar et al. (2012)
S. bicolor L.	*Bacillus* sp. SLS18	IAA production, ACC-deaminase, siderophores/ phytoextraction	Inoculation increased the dry weights of aerial part and root for sweet sorghum by 45.5, 81 % and 38.0, 80.3 % in Mn/ Cd-contaminated soil compared to noninoculated control, respectively	Luo et al. (2012)

Pteris vittata L.	*Rhodococcus* sp.TS1, *Delftia* sp.TS33, *Comamonas* sp.TS37, *Delftia* sp.TS41, *Streptomyces lividans* sp. PSQ22	Phytoaccumulation	Inoculation increased plant biomass by 53 % and As uptake by 44 % over control. Leaching was reduced by 29–71 % depending on the As-reducing bacterium	Yang et al. (2012)
Brassica napus	*Ralstonia* sp. J1-22-2, *P. agglomerans* Jp3-3, *Pseudomonas thivervalensis* Y1-3-9	ACC-deaminase activity/ phytoaccumulation	The aboveground tissue Cu contents of rape cultivated in 2.5 and 5 mg kg^{-1} of Cu-contaminated substrates varied from 9 to 31 % and from three- to four-fold, respectively, in inoculated rape plants compared to the uninoculated control	Zhang et al. (2011)
Brassica juncea	*A. xylosoxidans* Ax10	ACC-deaminase, IAA production/ phytoextraction	Inoculation of *A. xylosoxidans* Ax10 increased the root length, shoot length, fresh weight, and dry weight of *B. juncea* plants compared to the control	Ma et al. (2009)
Eucalyptus camaldulensis	*Microbacterium paraoxydans* BN-2, *Ochrobactrum intermedium* BN-3, *Bacillus fusiformis* BN-4	Fatty acid/ phytoextraction	Inoculation of *O. intermedium* BN-3 significantly increased the biomass and Pb accumulation by *E. camaldulensis* compared to the uninoculated control	Waranusantigul et al. (2011)
Sedum alfredii	*Burkholderia* sp.D54	IAA production, ACC-deaminase, siderophores/ phytoextraction	Bacterial inoculation significantly enhanced *S. alfredii* biomass production and increased both shoot and root Cd concentration, compared to control	Guo et al. (2011)
Pathogen stress				
A. thaliana	*P. fluorescens* strains Pf-5, Q2-87, Q8r1-96, HT5-1	ISR/2,4-diacetylphloroglucinol	Root colonization by 2,4-DAPG-producing *P. fluorescence* induced systemic resistance (ISR) against bacterial speck caused by *P. syringae* pv. tomato	Weller et al. (2012)

(continued)

Table 2.2 (continued)

Test crop	Beneficial bacteria	Proposed mechanism(s)	Plant response	Reference
Camellia sinensis	*Ochrobactrum anthropi* BMO-111	Antifungal metabolites	Foliar application of 36-h-old culture of BMO-111 significantly reduced the blister blight disease incidence compared to control	Sowndhararajan et al. (2012)
N. tobacum L.	*P. polymyxa* strain C5	Biofilm formation	In comparison with the control, the disease incidence was significantly reduced by 50 % with the application of *P. polymyxa* C5	Ren et al. (2012)
L. esculentum	*Bacillus amyloliquefacien* CM-2, T-5	IAA production, siderophore production	Both CM-2 and T-5 strains showed strong biocontrol and growth promotion effects on tomato seedlings. In comparison to the control, the disease incidence was reduced by 70 and 79 % for CM-2 and T-5, respectively	Tan et al. (2013)
	Pseudomonas strain, PCI2	Fungal cell wall-degrading enzymes (Protease)	In *Sclerotium rolfsii*, infested mixture, inoculation of tomato seeds with strain PCI2 improved seedling stand by 29 % and increased shoot and root dry weight of plants over the untreated pathogen controls	Pastor et al. (2012)
	B. cereus AR156	Induced systemic resistance (ISR)/ colonization	The AR156 inoculation elicited induced systemic resistance against *P. syringae*, reduced bacterial speck disease severity 1.6-fold. The tomato biomass increased up to 48 % by AR156 application over control	Niu et al. (2012)
G. hirsutum L.	*Streptomyces cyaneofuscatus* ZY-153, *S. kanamyceticus* B-49, *S. rochei*X-4, *S. flavotricini* Z-13	Production of fungal cell wall-degrading enzymes	The biocontrol efficacy of the four isolates against *Verticillium* wilt of cotton ranged from 18.7 to 65.8 % compared to uninoculated control	Xue et al. (2013)

Solanum tuberosum	*B. amyloliquefaciens* BAC03	Production of antimicrobial metabolites	The BAC03 applied in potting mix significantly reduced potato common scab severity and potentially increased the growth of potato plants compared to control	Meng et al. (2012)
C. sativus	*P. aeruginosa* PW09	Induced systemic resistance (ISR)/ colonization	The PW09 inoculation reduced seedling mortality by 60 % and increased biomass accumulation by 7 % under *Sclerotium rolfsii* stress	Pandey et al. (2012)
Pennisetum glaucum	*P. fluorescens* UOM ISR 17, *P. fluorescens*, UOM ISR 20, *P. fluorescens* UOM ISR 23, *Acetobacter* UOM Ab 9, *Acetobacter* UOM Ab 11, *Azospirillum* UOM Az 3	Induced systemic resistance (ISR)	*Pseudomonas* spp. UOM ISR 17 inoculation improved plant height, dry mass, leaf area, and plant protection of 44, 42, 47, and 73 %, respectively, against downy mildew disease stress compared to control	Jogaiah et al. (2010)
Citrullus lanatus	*P. aeruginosa* 231-1	Antibiosis and induced systemic resistance (ISR)	*P. aeruginosa* 231–1 treatment inhibited pathogen penetration and significantly reduced disease infection in plants against *Didymella bryoniae*	Nga et al. (2010)

Stress environment can also make physicochemical and biological properties of soil unsuitable for microbial and plant growth. However, particular characteristics of certain bacteria enable them to survive under such harsh environments. For example, certain bacterial strains have the ability to tolerate high salinity, and, similarly, the production of exopolysaccharides by the bacteria protects them from water stress. Besides developing mechanisms for stress tolerance, microorganisms can also impart some degree of tolerance to plants toward abiotic stresses like drought, salinity, metal toxicity, and high temperature (Grover et al. 2011). The exopolysaccharides released into soil can be adsorbed by clay particles and form a protective layer around soil aggregates (Tisdall and Oades 1982) and, therefore, protect the plant from desiccation. Moreover, exopolysaccharide production increases root colonization of microbes (Santaella et al. 2008), improves soil aggregation (Sandhya et al. 2009), channelizes water and nutrients to plant roots (Tisdall and Oades 1982; Roberson and Firestone 1992), and forms biofilm (Seneviratne et al. 2011) which is beneficial to plant growth and development. Alami et al. (2000) observed a significant increase in root-adhering soil per root tissue (RAS/RT) ratio in sunflower rhizosphere inoculated with the EPS-producing rhizobial strain YAS34 under drought conditions. The inoculation with ACC (1-aminocyclopropane-1-1carboxylic acid)-deaminase-containing bacteria can reduce negative impact of stress-induced ethylene (Mayak et al. 2004a, b). The elevated level of ethylene caused negative impact on plant growth by inhibiting the root growth particularly. These microorganisms secrete enzyme ACC-deaminase that hydrolyses ACC into ammonia and a-ketobutyrate. The rhizobacteria bound to plant roots act as sink for ACC (immediate precursor of ethylene) and thereby lower the level of ethylene in a developing seedling or stressed plant. Therefore, the inoculation of seeds with such strains containing ACC-deaminase would be very useful for enhancing plant growth under stress conditions by diluting the negative impact of stress-induced ethylene on root growth (Glick et al. 2007). As is evident from one of our greenhouse study conducted under salinity-stressed conditions, that application of PGPR strains having ACC-deaminase activity significantly enhanced the root length of maize compared to uninoculated control (Fig. 2.3). The work of Mayak et al. (2004a) shows that bacterial strain (*Achromobacter piechaudii*) containing ACC-deaminase conferred tolerance to water deficit in tomato and pepper. Ethylene production was reduced in inoculated plants, resulting in significant increase in fresh and dry weights compared to uninoculated controls. *Pseudomonas* spp. also improved the growth of pea (*Pisum sativum*) under drought stress in axenic conditions as well as in potted soil (Zahir et al. 2008). They concluded that inoculation might have reduced the ethylene synthesis, which resulted in better plant growth under drought stress. Similar results were also obtained by Arshad et al. (2008) while studying the effectiveness of *Pseudomonas* spp. for eliminating the drought effect on growth, yield, and ripening of pea. It has been observed that the presence of elevated levels of ethylene in the vicinity inhibits the nitrogen fixation by rhizobia. However, the co-inoculation of *Rhizobium* with PGPR having ACC-deaminase activity can minimize this negative impact of ethylene and enhance nodulation

Fig. 2.3 Effect of PGPR containing ACC-deaminase on root growth of maize in a pot trial at 12 dS m^{-1} salinity level

(Ahmad et al. 2011). Stimulation of root elongation and biomass production of different plant species by inoculation with PGPR having ACC-deaminase activity has been repeatedly documented, particularly when the plants were subjected to stressful growth conditions (Nadeem et al. 2007, 2010a; Saravanakumar and Samiyappan 2007; Tank and Saraf 2010; Siddikee et al. 2012). Similarly, the presence of other growth-promoting characteristics like indole acetic acid (IAA), siderophore production, phosphate solubilization, and phytohormone production may provide extra benefits for stress tolerance in plants and improve their growth. The production of antioxidant enzymes protects the plant from the harmful impact of reactive oxygen species. The reactive oxygen species (ROS) as singlet oxygen (O^{-}), hydrogen peroxide (H_2O_2), and hydroxide ions (OH^{-}) are developed in the photosystem of plants. These ROS denature cell membranes, proteins, and DNA through oxidation reaction. To combat/reduce the impact of these ROS, plant's immune system generates antioxidant enzymes such as superoxide dismutase, peroxide dismutase, catalase, and glutathione reductase (Arora et al. 2002). The PGPR inoculation also enhances the activity of these enzymes and helps them to reduce the negative impact of stress (Fu et al. 2010). Similarly, enhanced production of osmoprotectants by bacterial inoculation under stress enables the plant to maintain their internal water potential for better uptake of water and nutrients.

Rhizobacteria as Biocontrol Agent

In soil environment, there are a number of plant pathogens that reduce crop yield. Although these plant pathogens can be controlled by the application of chemicals and growing disease-resistant varieties, however, there are certain environmental concerns about the use of such chemicals like their persistent nature in the soil as well as accumulation of toxic residues of these chemicals in the food parts. Some of these toxic chemicals have been banned due to their persistent nature. Similarly in certain cases, the resistance of genetically resistant crops is often broken by the pathogen that results in reduction in crop yield (Fry 2008). An alternative strategy to overcome this problem is the use of PGPR that act as biocontrol agent by virtue of their certain biocontrol mechanisms like production of antibiotics, production of antifungal metabolites, decreasing availability of iron for pathogenic organisms, production of fungal cell wall-degrading enzymes, and through induced systemic resistance. Number of reports have shown the effectiveness of PGPR for enhancing plant growth by protecting them from pathogens (Siddiqui et al. 2005; Ayyadurai et al. 2007; Ravindra Naik et al. 2008; Srinivasan and Mathivanan 2009). PGPR have competitive advantage over fungi for iron uptake due to production of siderophores. These siderophores have very high affinity for iron, and bacteria can take up iron–siderophore complex. By using this mechanism, PGPR retard the pathogen growth by reducing the availability of iron and therefore providing protection to the plant against diseases (Penyalver et al. 2001).

The above-discussed review and number of examples mentioned in Tables 2.1 and 2.2 show the effectiveness of PGPR for enhancing plant growth and development under normal as well as stress environment. Such growth promotion was due to certain direct and indirect mechanisms used by PGPR. It was also evident from discussion that inoculation of plant seed or seedlings with most promising strains having best growth-promoting traits not only enables the plant to maintain their proper growth but also causes positive impact on soil health.

Role of Bacterial Consortium in Advance Agriculture: Effectiveness and Challenges

Although above-discussed review highlights the effectiveness of rhizobacteria for enhancing plant growth under stress environment, however, under certain cases, the results obtained in the laboratory could not be reproduced in the field (Zhender et al. 1999; Smyth et al. 2011). This might be due to the low quality of the inocula and/or the inability of the bacteria to compete with the indigenous population under adverse environmental conditions (Brockwell and Bottomley 1995; Catroux et al. 2001). Great variations in the plant response to PGPR in laboratory and field assays demonstrate that the full potential of rhizobacteria to promote plant growth should be more extensively investigated. It is necessary to develop efficient inocula that can perform better under field conditions (Ahmad et al. 2008). The application of multistrain

PGPR in combination could be more beneficial than a single strain. It has been reported that co-inoculation and coculture of microbes have better ability to fulfill the task in an efficient way than single-strain inoculation (Guetsky et al. 2002). Each strain in the multistrain consortium can compete effectively with the indigenous rhizosphere population and also enhance plant growth with its partners (Shenoy and Kalagudi 2003). The two strains used in a consortium showed that each strain not only competed successfully for rhizospheric establishment but also promoted plant growth (Shenoy and Kalagudi 2003). The co-inoculation of *Rhizobium* with PGPR proved useful for promoting growth and increasing nodulation (Tilak et al. 2006). The use of multistrain inoculants could be a good strategy that enables organisms to successfully survive and maintain themselves in communities (Andrews et al. 1991). Van Veen and others (1997) critically reviewed the reasons for the poor performance of agricultural bioinocula in natural environments and in the rhizosphere of host plants and suggested that instead of using a single strain for a single trait, multiple microbial consortia could be used for multiple benefits. Microbial studies performed without plants indicated that some combinations allow the bacteria to interact with each other synergistically, provide nutrients, remove inhibitory products, and stimulate growth of each other through physical and biochemical activities that may have beneficial impacts on their physiology (Bashan 1998). Rajasekar and Elango (2011) studied the effectiveness of *Azospirillum*, *Azotobacter*, *Pseudomonas*, and *Bacillus* sp. separately and in combination on *Withania somnifera* for two consecutive years. They observed that PGPR consortia significantly increased plant height, root length, and alkaloid content in *W. somnifera* when compared to the uninoculated control and single inoculation. Similarly, dual inoculation with *Azotobacter* and *Azospirillum* significantly increased total dry weight, leaf area index, and crop growth index (Gholami et al. 2012). Jha and Saraf (2012) observed that growth of Jatropha (*Jatropha curcas*) plant improved maximally in greenhouse and field experiments when three strains were applied together. Co-inoculation provided the largest and most consistent increases in shoot weight, root weight, total biomass, shoot and root length, total chlorophyll, shoot width, and grain yield. Similarly, the consortia of three strains gave the best performance in terms of growth parameters of *Lycopersicum esculentum* (Ibiene et al. 2012). They demonstrated that the use of combined biofertilizers containing consortia of bacteria is an excellent inoculant for growth performance of plants.

As far as growth under stress environment is concerned, Annapurna et al. (2011) studied the effectiveness of PGPR separately and in combination for reducing the impact of salinity on wheat growth. They found that single and dual inoculations of PGPR strains showed variations in their effect to enhance the crop tolerance to salts. The bacterial consortium was more effective for inducing salinity tolerance in wheat plants. They considered it as an acceptable and environment-friendly technology to improve plant performance and development under stress environment. In another study, Upadhyay et al. (2011a) evaluated the growth-enhancing potential of single and dual inoculation of *B. subtilis* SU47 and *Arthrobacter* sp. SU18 on wheat under saline conditions. They observed that in addition to enhancing dry biomass, soluble sugars, and proline content, wheat sodium content was reduced under co-inoculated

conditions but not after single inoculation with either strain or in the control. The results indicate that co-inoculation with *B. subtilis* and *Arthrobacter* sp. could alleviate the adverse effects of soil salinity on wheat growth. The bacterial consortium is also effective for protecting the plant from disease under field condition. It is evident from the work of Srinivasan and Mathivanan (2009) that effective control of necrosis virus in sunflower can be obtained by the application of powder and liquid formulations of PGPR consortia. They applied two bacterial consortia consisting of *Pseudomonas*, *Bacillus*, and *Streptomyces* spp. along with farmer's practice, i.e., imidacloprid + mancozeb. They observed a significant reduction in disease with an increase in seed germination, plant height, and crop yield. They demonstrated that PGPR consortia show high benefit–cost ratio compared to farmer's practice and untreated control.

Inoculant Technology: Formulation and Commercialization

The application of PGPR for improving crop production is becoming an emerging technology owing to their environmental friendly traits. For that purpose various microbial inoculants have been formulated and are being marketed. A number of strains having ability to protect plant from pathogens belonging to genera *Bacillus*, *Pseudomonas*, and *Agrobacterium* are being used as biopesticides (Fravel 2005).

Formulation of Microbial Inoculants

A number of PGPR strains have great potential to be formulated as biofertilizer for improving plant growth and development under normal and stress environment. Successful inoculation of PGPR can result in better plant growth and therefore higher economic return to the farmers. For effective transfer of research findings from laboratory to field, an excellent formulation technology has great advantages. Various microbial inoculants have been formulated, marketed, and applied successfully (Reed and Glick 2004). Commercial bioinoculants prepared from *Bacillus* spp. are used widely as biocontrol agents (Ongena and Jacques 2007). *B. thuringiensis*, which is used to control insect pest, is estimated having sale of >70 % (Ongena and Jacques 2007; Sanchis and Bourguet 2008). *Pseudomonas putida*, *Paenibacillus*, and *Bacillus* sp. are formulated and have successfully enhanced the growth and yield of wheat (Cakmakci et al. 2007). Similarly, field application of salt-tolerant bioformulation of certain bacteria enhanced plant growth under salinity stress (Paul et al. 2006).

The major bottleneck to the commercial use of PGPR as biofertilizers is their inconsistent performance in the field. In certain cases, plant growth promotion due to microbial inoculation is not so effective in terms of investment applied and net

return when compared with chemical fertilizers (Lucy et al. 2004). The development of valuable formulation is a challenging task for improving the efficacy of microbial inoculants. Actually, formulation is one of the crucial steps that determines the success or failure of a PGPR strain. However, this important step is generally neglected which results in less efficient outcome. The reason of this failure is the preparation of microbial formulation under lack of quality control and proper guidelines (Paau 1988; Berg 2009). The active ingredient in a microbial formulation is its viable culture. Regardless of the organism used, the success of bioagent depends upon the preparation of such inoculum having high level of viability and vigor (Jones and Burges 1998). In microbial formulation, the maintenance of bacterium in metabolically and physiologically active state is an important aspect for gaining maximum advantage (Paau 1998). In certain environmental conditions, where single-strain inoculum is unable to perform better, the development of multi-strain inoculum can be very effective (Domenech et al. 2006). Such multistrain inoculum would be more effective for enhancing plant growth and development due to the presence of more growth-promoting traits which might not be possible in single strain.

Another important aspect regarding formulation is carrier material, which plays active role in shelf life of formulation. It aids in the stabilization and protection of the microbial cells during storage and transport (Xavier et al. 2004). It also protects the active ingredient, i.e., microbe from environmental conditions, and enhances its activity in field (Deaker et al. 2004). Various organic and inorganic carrier materials are used for formulation development (Bashan and Levanony 1990; Bashan 1998). Organic carriers like peat have some advantages due to their higher nutrient content, and, however, complete sterilization by steam is difficult, and also during sterilization, toxic by-products are produced that may cause decrease in bacterial population (Weiss et al. 1987). Therefore, the use of inorganic carrier may be a good strategy for enhancing the effectiveness of the microbial formulation. However, the effectiveness of these inorganic carrier materials may also be different, as it is evident from the work of Saharan et al. (2010) who used talc and aluminum silicate powders to develop inorganic carrier-based formulation. They observed that the shelf life of talc powder-based formulation was higher compared to aluminum silicate-based formulation. It was also observed that both sterile and nonsterile carrier formulations significantly enhanced the growth of *Vigna mungo* and *Triticum aestivum*. The application of microbial inoculants in the form of granular or liquid form is also attaining much attention nowadays. For optimizing nodulation, granular inoculants particularly rhizobia can be placed below or at the side of seeds with appropriate equipment according to seeding depth and moisture availability (Stephens and Rask 2000). On the other hand, due to easy application of liquid inoculants, liquid formulation has also achieved much popularity (Xavier et al. 2004). However, both types of formulations have shown their effectiveness for enhancing the biomass yield of soybean (Atieno et al. 2012). They have also demonstrated that formulation of rhizobia and PGPR gave better response.

Bacterial Characters for Formulation Development

Although a good number of microbial strains are used for formulation development and also their performance is observed, however, there are various constraints for commercialization of microbial inocula. One of the challenges for developing PGPR inoculants on commercial basis is the selection of such strains which could have competitive advantage over indigenous population and also have the ability to maintain their growth under unfavorable environment. The most important aspect in this regard is the selection of such strains which have host plant specificity as well as adaptation to soil and climatic conditions (Bowen and Rovira 1999). An organism with properties like phosphate solubilization, phytohormone production, root colonization, siderophore, and indole acetic acid production is thought to be an ideal bioinoculant.

To develop a successful PGPR formulation, in addition to above-mentioned growth-promoting traits, bacteria should have the ability to tolerate harsh environmental conditions like drought, heat, salinity, and toxic metals. It should have high rhizosphere competence and compatibility with other rhizobacteria. Such bacteria should also have capability of multiplication and broad spectrum of action. In addition to possessing a number of other characteristics, a PGPR should also have great viability and good shelf life (Lianski 1985). Cost-effectiveness, shelf life, and delivery systems are very important aspects that should be kept in mind while preparing the microbial formulation.

Concluding Remarks and Future Prospects

The above discussion showed the effectiveness of PGPR for enhancing the growth and development of plants. These beneficial effects are obtained owing to a number of direct and indirect mechanisms including phosphate solubilization, production of plant growth regulators, iron sequestration by siderophores, production of antibiotics, synthesis of antifungal metabolites, production of fungal cell wall degrading enzymes, inducing systemic resistance, reducing deleterious effects of stress-induced ethylene by ACC-deaminase activity, and production of vitamins. These plant growth promoting abilities of microbes under normal as well as stress conditions have certified their role in sustainable agriculture. For better performance, the PGPR strain must be rhizosphere competent that should be able to survive and colonize (Cattelan et al. 1999). In addition to rhizosphere competency, the compatibility between the rhizodeposition of compounds by the plant host and the ability of the inoculated bacteria to utilize them are also very important (Strigul and Kravchenko 2006). However, there is still lack of evidence about the consistent performance of these microbes, particularly under field conditions. In certain cases, the results obtained in laboratory are not reproduced in the field (Zhender et al. 1999; Smyth et al. 2011). This may occur due to the low quality of the

inoculum and/or the inability of the bacteria to compete with the indigenous population (Brockwell and Bottomley 1995; Catroux et al. 2001). Therefore, the use of such technologies that enhance the agriculture production is indispensable to feed the burgeoning population. The application of multistrain bacterial consortium over single inoculation could be an effective approach for reducing the harmful impact of stress on plant growth. Strains that have the ability to protect the plant from diseases through biocontrol mechanisms may also be included in the formulation. The efficacy of such strains may be enhanced by ACC-deaminase gene (Hao et al. 2007). Therefore, the application of such strains which have multitraits for growth promotion should be preferred for inoculant formulation. It is also necessary to understand the interactions between microbial consortium and plant system. Understanding of such interactions could be very effective for improving plant growth (Raja et al. 2006).

It has been seen that certain growth-promoting traits may interact with each other and have influence on plant growth. For example, in one of our studies (submitted for publication), the strain having high ACC-deaminase activity and low IAA and/or high ACC-deaminase and high IAA performed better compared to a strain having high IAA and low ACC-deaminase. Therefore, such aspects need further research so that most effective strains or combinations of strains can be selected. Other beneficial aspects of bacterial inoculation also need special attention. For example, the addition of ice-nucleating bacteria to agriculture has potential benefits of protecting crops from frosts dropping below freezing, which might contribute to a solution of the worldwide problem of starvation and chronic hunger. Therefore, the application of these bacteria could be an effective technology for enhancing plant growth at low temperature. Similarly, cyanide-producing bacteria can be used effectively for disease suppression. Certain *Pseudomonas* strains produce allelochemicals that can be used as bioherbicides to minimize the use of chemicals and therefore eliminate environmental hazards.

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Chapter 3
Plant–Microbe Partnerships: Implications for Growth and Plant Health

N.S. Paulucci, G. González Anta, L.A. Gallarato, J.C. Vicario, A.B. Cesari, Y.B. Reguera, C. Kilmurray, M.A. Bueno, M.B. García, and M.S. Dardanelli

Contents

Abstract The rhizosphere can be defined as the zone of soil around plant roots whereby soil properties are influenced by the presence and activity of the root. Changes to the physical, chemical, and biological properties of rhizosphere soil have significant influence on the subsequent growth and health of plants. Interactions between plant roots and soil microorganisms are ubiquitous and are an essential component of ecosystem function. It has become increasingly evident that root interactions with soil microorganisms are intricate and involve highly complex communities that function in very heterogeneous environments.

N.S. Paulucci • L.A. Gallarato • J.C. Vicario • A.B. Cesari • Y.B. Reguera • C. Kilmurray
M.A. Bueno • M.B. García • M.S. Dardanelli (✉)
Departamento de Biología Molecular, Facultad de Ciencias Exactas,
Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto,
Ruta Nacional Nº 36, Km. 601, CP X5804BYA Río Cuarto, Córdoba, Argentina
e-mail: mdardanelli@exa.unrc.edu.ar

G. González Anta
Escuela de Ciencias Agrarias, Naturales y Ambientales, Universidad Nacional del Noroeste de la Provincia de Buenos Aires, Sede Pergamino Monteagudo 2772,
CP B2700 Pergamino, Buenos Aires, Argentina

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_3, © Springer India 2013

Although many plant-associated bacteria have beneficial effects on their host, their importance during plant growth and development is still underestimated. Plant-associated bacteria include endophytic, phyllospheric, and rhizospheric bacteria. Research into how plant growth can be promoted has mainly concentrated on rhizobacteria. More recently, however, attention has focused on the plant growth-promoting capacity of endophytes. Mechanisms of plant growth promotion by plant-associated bacteria vary greatly and can be broadly categorized into direct and indirect effects. The purpose of this chapter is to examine how microorganisms can help growth and plant health and its use in new area of research.

Introduction

The plant rhizosphere is an important soil ecological environment for plant–microorganism interactions, which involves colonization by a variety of microorganisms in and around the roots. The rhizosphere refers in general to the portion of soil adjacent to the roots of living plants. It supports a diverse and densely populated microbial community and is subjected to chemical transformations caused by the effect of root exudates and metabolites of microbial degradation. The bacterial communities associated with this microzone are thought to be determined by the quantity and composition of root exudates that serve as substrate for microbial growth. Plant growth promoting rhizobacteria (PGPR) are soil bacteria that have the ability to colonize roots and stimulate plant growth. PGPR activity has been reported for strains belonging to many different genera such as *Azoarcus*, *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Gluconoacetobacter*, *Pseudomonas*, and *Serratia* (Somers et al. 2004). *Rhizobium* can also be considered as a soil bacteria with PGPR activity. Plant growth promoting capacity has been related with different physiological activities: (1) synthesis of phytohormones, such as cytokinins, gibberellins, and auxins; (2) enhancement of factors affecting mineral nutrition, such as phosphorous solubilization; and (3) protection of plants against phytopathogens (Persello-Cartieaux et al. 2003; Somers et al. 2004).

This complex plant-associated microbial community, also referred to as the second genome of the plant, is crucial for plant health and growth, and it is essential to achieve higher crop yields while minimizing negative impacts on the environment (Dardanelli et al. 2010b; Berendsen et al. 2012). These microbial associations may result in endophytic, symbiotic, associative, or parasitic relationships within the plant, depending on the type of microorganisms, soil nutrient status, and soil environment (Parmar and Dadarwal 2001). Root exudates are believed to have a major influence on the diversity of microorganisms within the rhizosphere. Interestingly, specific compounds identified in root exudates have been shown to play roles in root–microbe interactions.

Root–Rhizobacteria Communication and Signal in the Rhizosphere

Plants produce a remarkably diverse array of about 100,000 low molecular mass natural products also known as secondary metabolites (Dixon 2001). These are organic compounds and inorganic ions that change the chemistry and biology of the rhizosphere and enhance adaptation to a particular environment (Crowley and Rengel 1999). Most root products are regulated plant compounds which become available as substrates of root-colonizing microorganisms (Badri et al. 2009). All chemical compounds secreted by plant are collectively named rhizodepositions, and they are released from living roots to the soil through several mechanisms that were defined by Somers et al. (2004) and Gregory (2006) such as:

1. Exudation of low molecular weight water soluble compounds, such as monosaccharides, which are lost passively without the involvement of plant metabolic activity
2. Secretions of simple compounds released by metabolic processes, such as enzymes, or complex organic compounds originating in root cells or from bacterial degradation or a gelatinous layer composed of mucilages and soil particles intermixed
3. Lysates released from sloughed off root cells and, with time, whole roots
4. Gases such as CO_2, ethylene, and hydrogen cyanide

Specific compounds identified in root exudates have been shown to play roles in root–microbe interactions. A chemotactic response toward root-secreted organic and amino acids is the first step in root colonization (Zheng and Sinclair 1996). Most root products are regular plant compounds which become available as substrates of colonizing microbes, including specific compounds typical of the secondary metabolism of each plant species (Badri et al. 2009). A good example is the molecular integration of legume flavonoid signals by compatible rhizobia during the initiation of nitrogen-fixing symbiosis. Several signaling molecules, including flavonoids, isoflavonoids, and phenolic compounds, secreted by the plant root are able to induce the expression of rhizobial nod genes. In response to these compounds, rhizobia produce a series of host-specific signal molecules, lipochitooligosaccharides (LCOs), also known as Nod factors (Schlaman et al. 1998). As a consequence, the formation of root or stem nodules occurs in response to the presence of rhizobia. The incorporation of atmospheric N_2 into organic material resulting from this rhizobia–legume symbiosis is estimated to account for one third of the total nitrogen needed for world agriculture. This unique intracellular association contributes significantly to agricultural yields (de Hoff and Hirsch 2003).

In addition to their *nod* gene-inducing or inhibiting properties, flavonoids interact with free-living rhizobia in several other ways. They can act as growth enhancers for some rhizobial strains and as antimicrobial agents for others, thereby influencing rhizosphere populations. A significant role of the phytoalexins and phytoanticipins in disease response, in particular in legumes, has been postulated because of their broad-spectrum in vitro antimicrobial activity (Dixon et al. 2002).

Simple isoflavone compounds, such as daidzein, glycitein, and formononetin glycosides, are accumulated constitutively by many legume species (Dakora and Phillips 1996), and the corresponding aglycones are inhibitory to the growth of microbial pathogens (VanEtten 1976; Kramer et al. 1984) and can thus be classified as phytoanticipins. Some flavonoids also elicit a chemotactic response in rhizobia. These bacteria can modify flavonoid structures, either by removal of glycosidic residues (Hartwig and Phillips 1991) or by degradation of flavonoid aglycones via C-ring fission mechanisms (Rao and Cooper 1994).

Information on exudate composition should be interpreted with care. Rhizodepositions collected from sterile plants growing under artificial conditions, such as on sterile filter paper or in sterile plant nutrient solution, are sufficiently concentrated to be analyzed successfully. Different factors can affect exudate composition, such as the physiological status of the plant, the presence of microbes, and the presence of products from rhizobacteria such as antibiotics (Lugtenberg and Kamilova 2009). Several microbial products, which are produced by common soil microorganisms such as *Pseudomona*s bacteria and *Fusarium* fungi, significantly enhanced the net efflux (i.e., exudation) of amino acids from roots. Data reported by Phillips et al. (2004) offer specific molecular examples of how microbial products can directly elicit changes in plant processes. In alfalfa, treating roots with 200 mM phenazine, 2,4-diacetylphloroglucinol, or zearalenone increased total net efflux of 16 amino acids 200–2,600 % in 3 h (Phillips et al. 2004). Major developments in our understanding of rhizosphere are expected. A combination of data analyses obtained from biochemistry, microbiology, and the new topics "omics" studies will further strengthen our capability to visualize a complete picture of plant–microbe interactions.

How Can Bacteria Help Plants?

Rhizobacteria are important for application in agriculture. Some rhizosphere microorganisms may be neutral or deleterious in regard to plant growth, whereas other microbes support their hosts (Raaijmakers et al. 2009; Dardanelli et al. 2008, 2010a). PGPR can stimulate plant growth, increase yield, reduce pathogen infection, as well as reduce biotic or abiotic plant stress without conferring pathogenicity (Welbaum et al. 2004; van Loon and Bakker 2005; Lugtenberg and Kamilova 2009). Through their ability to fix and solubilize mineral nutrients unavailable for plants, plant-associated bacteria can act as biofertilizers. Environmental conditions including biotic and abiotic stresses undoubtedly play a major role in limiting plant productivity. However, the information that we know is poor and a better understanding of how plants and microbes intimately interact with one another in an extremely complex environment is necessary and how this interaction leads to physiological changes in plants. Furthermore, knowledge is required of how plants prioritize their needs, such as investing resources into defense at the expense of growth and development, to develop sustainable strategies to improve plant health

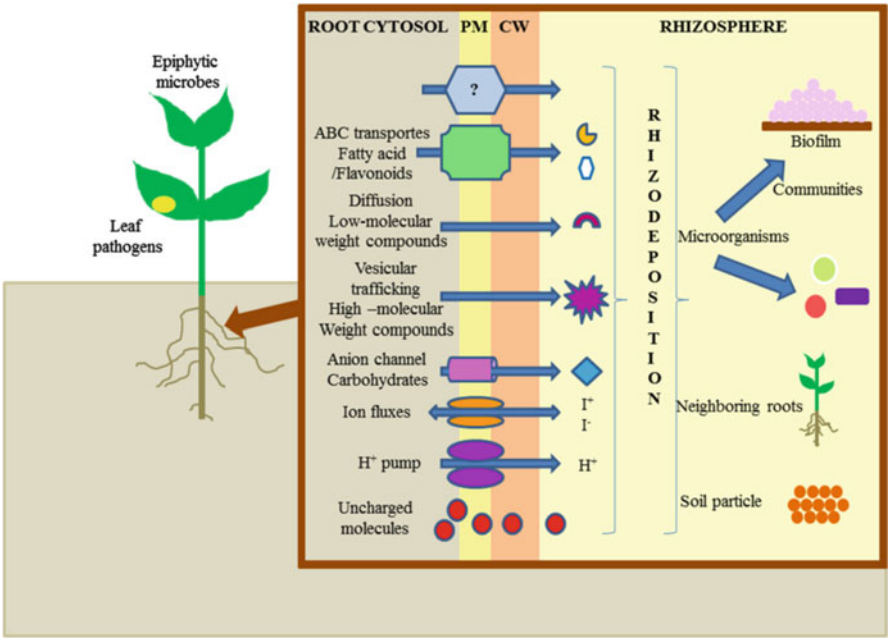

Fig. 3.1 Interactions between plants–microbes and root exudation. *PM* plasmatic membrane, *CW* cell wall (*Source*: Bais et al. 2004; Schenk et al. 2012)

in agriculture (Schenk et al. 2012). Figure 3.1 shows a general overview of interactions between microorganisms and plants.

Biofertilizers

PGPR can be used as biofertilizers because a number of mineral nutrients in the soil, including nitrogen, phosphorus, and iron, can be limited, thus restricting the growth of terrestrial plants. Strategies to minimize fertilizer inputs by promoting biological nitrogen fixation and acquisition of phosphorus and iron are important to achieve sustainable production.

Phosphorous (P) is considered as one of the insoluble elements in the nature with less than 5 % of the total soil P content available to the plants (Dobbelaere et al. 2003). Many soil microorganisms can solubilize mineral P through the production of organic acid (Zaidi et al. 2009), but the problem is the acidification of the surrounding soil.

Increased root growth and induction of metabolic processes can be mediated by rhizobacteria. However, the production of other metabolites beneficial to the plant by these microorganisms, such as phytohormones, antibiotics, or siderophores, has

created confusion about the specific role of P solubilization in plant growth and yield (Vargas et al. 2010). Rodriguez and Fraga (1999) reported that *Rhizobium* is one of the major P solubilizers, and bacteria of the genera *Mesorhizobium* and *Sinorhizobium* can solubilize P with different ability (Vargas et al. 2010). Antoun et al. (1998) and Alikhani et al. (2006) reported that only a few *Bradyrhizobium* were able to solubilize inorganic P, possibly because these generous alkali producers increase the pH of growth media, and this condition affects P solubility. Phosphorus is considered to be one of the most limiting nutrients for growth of leguminous crops in tropical and subtropical regions (Ae et al. 1990). At the current rate of usage of P fertilizer, readily available sources of phosphate rocks will be depleted over the next 60–90 years (Runge-Metzger 1995). At present, many tropical regions are faced with excessive mining of nutrients, including P, whereas some temperate regions with intensive, animal-based agricultural systems have, ironically, to deal with excessive soluble P in the soil that is threatening the ecosystem.

The other major group in P cycle and interaction with plants is the mycorrhizal fungi. Symbiosis with these fungi is very important to improve plant fitness and soil quality by increasing the plant uptake of P and nitrogen (N) by absorbing phosphate, ammonium, and nitrate from soil and also assists plant host in uptake of the relatively immobile trace elements such as zinc, copper, and iron (Zaidi et al. 2010). Moreover, mycorrhizal symbiosis improves plant health, increases protection against biotic and abiotic stresses, and improves soil structure through aggregate formation (Goicoechea et al. 1997; Barea et al. 2005; Zaidi et al. 2010). Other important organisms are rhizoplane or endophytic bacteria that colonize rhizoplane because they can release minerals, such as P, potassium, magnesium, or zinc, from the rocks and can live in extreme habitats (Puente et al. 2004). Under these conditions and the importance of plant to world agriculture, studies and applications of PGPR that have biofertilizing capacity are relevant.

Iron is essential for the growth of most microorganisms and plants. Despite being an abundant element in soil, its extreme insolubility at normal biological pH severely decreases its bioavailability. Harmsen et al. (2005) define that bioavailable iron is a portion of total iron that can be easily assimilated by one organism. To increase the iron in plants and to enrich the amount of bioavailable iron is a challenge of agriculture. The major challenge for microorganisms and plants is to acquire Fe (III) sufficient for growth. Plants and microorganisms have developed mechanisms of iron uptake and in many cases work cooperatively in the rhizosphere. Lemanceau et al. (2009) summarize processes as:

1. Acidification of soil solution mediated based on the excretion of protons or organic acids
2. Chelation of Fe (III) by ligands including siderophores with very high affinity for Fe^{3+}
3. Reduction of Fe^{3+} to Fe^{2+} by reductases and reducing compounds

The efficacy of these active iron uptake strategies differs among organisms, leading to complex competitive and synergistic interactions among microbes, plants, and between plants and microbes. The chemical properties of the soil in which they

occur have a strong effect on these interactions. In return the iron uptake strategies impact the soil properties and the iron status. Thus, multiple interactions between soils, plants, and microorganisms are driving a complex iron cycle in the rhizosphere (Lemanceau et al. 2009).

Interactions Benefiting to Sustainable Agroecosystem Development: PGPR–Rhizobium

Microorganisms able to establish nitrogen-fixing symbiosis with legume were discovered in the nineteenth century and were collectively named rhizobia and include more than 50 species distributed in genera *Rhizobium*, *Ensifer* (*Sinorhizobium*), *Mesorhizobium*, *Azorhizobium*, and *Bradyrhizobium* (Velázquez et al. 2010). Until 2011, rhizobia were reported as unique bacteria able to nodulate legumes, but Sy et al. (2001) showed the first non-rhizobial bacterium nodulating *Crotalaria* and was named *Methylobacterium nodulans* (Jourand et al. 2004). After that, other genera were reported: *Burkholderia* (Moulin et al. 2001), *Blastobacter* (van Berkum and Eardly 2002), *Devosia* (Rivas et al. 2003), *Phyllobacterium* (Valverde et al. 2005), *Ochrobactrum* (Trujillo et al. 2004), and *Shinella* (Lin et al. 2008). The rhizobia–legume interaction has been studied for over 100 years and has allowed better understanding of the mechanisms of interaction in the rhizosphere. The plant initiates the "molecular dialogue" by producing and secreting flavonoid compounds into the rhizosphere. Flavonoids are one of the largest groups of secondary metabolites and play an important role in plants as defense and signaling compounds in reproduction, pathogenesis, and symbiosis. Plant flavonoids are involved in response mechanisms against stress. Rhizobia respond to flavonoids by inducing the expression of *nod* genes and the production of Nod factors. Plant recognition of symbiotically relevant Nod factors triggers root hair deformation, cell division, and the production of an infection thread, which is necessary for the invasion of the host plant (Geurts et al. 2005). These events culminate in the development of root-borne nodules, which house nitrogen-fixing bacteria.

Different biotic and abiotic stresses can affect rhizobial symbiosis and PGPR could help. Salt stress is a major constraint in the production of legume crop species, particularly when the nitrogen needed for the growth of these plants is derived from symbiotic fixation. The impact of many chemical signals on the ecology of the rhizosphere is not as yet well understood. It is not clear, for example, how microorganisms modify the chemical signals and what the impact of changes are in the rhizosphere community or the abiotic stress on the flavonoid-mediated communication. A better understanding of the biology of root exudation should contribute to improvement of crop adaptation to stressful environments, such as saline lands, and to more sustainable and profitable farming (Dardanelli et al. 2012).

Inoculation with compatible rhizobia influences plant root exudation. Thus, when soybeans are inoculated with *Bradyrhizobium japonicum* USDA110, the root exudates contain higher concentrations of daidzein, genistein, and coumestrol

Table 3.1 Flavonoids detected in soybean root exudates in the presence or absence of *C. balustinum* Aur9 and salt stress

	Control	50 mM NaCl	Control + AUR9	50 mM NaCl + AUR9
Flavones				
7, 4′-Dihydroxyflavone	+[a]	+[a,b]	+[a]	+[a,b]
Apigenin	+[a,b]	+[a]	+[a]	+[a]
Flavonols				
Quercetin	+	+	–	–
Flavanones				
Naringenin	+	+	–	–
Isoflavones				
Daidzein	+[a]	–	+[a]	–
Genistein	+[a]	–	+[a]	–
Chalcone				
Isoliquiritigenin (4, 2′, 4′-trihydroxychalcone)	+[a]	+[a]	+[a]	+[a]
Coumarin				
Umbelliferone (7-hydroxy-2H-1-benzopyran-2-one)	+	+	+	+

Source: Dardanelli et al. (2010b)
The presence (+) or absence (–) of a flavonoid is indicated
[a]Glycosidated flavonoid
[b]Several peaks detected, possibly from different glycoside

in comparison with non-inoculated plants (Cho and Harper 1991). A change qualitatively in signal molecules has also been observed in soybean roots when were inoculated with PGPR in stress conditions. *Chryseobacterium balustinum* Aur9 changes qualitatively the pattern of flavonoids exuded when compared to control conditions. Thus, in the presence of *C. balustinum* Aur9, soybean roots did not exude quercetin and naringenin (Table 3.1) (Dardanelli et al. 2010a). Thus, microbial attenuation or alteration of flavonoid signals may be an important aspect of rhizosphere ecology and may play an essential role in the establishment of symbiosis (Shaw et al. 2006).

Dual inoculation with *Rhizobium* and *Azospirillum* and other plant growth promoting rhizobacteria was shown to significantly increase both upper and total nodule number of several legumes, acetylene reduction activities, faster 15N dilution, and the total N content of mineral macro- and micronutrients as compared to inoculation with *Rhizobium* alone (Sarig et al. 1986; Burdman et al. 1998; Rodelas et al. 1996, 1999). Inoculation of common bean or alfalfa (*Medicago sativa*) with *A. brasilense* in the absence of *Rhizobium* resulted in the production of plant root exudates in 6-day-old seedlings, with an increased capacity to induce *Rhizobium* nod-gene expression as compared to exudates of the non-inoculated controls (Burdman et al. 1998). This correlated with a change in the chemical composition of the root exudates and the quality of the flavonoids of inoculated plants (Burdman

et al. 1996; Volpin et al. 1996). Dardanelli et al. (2008) showed positive effect of *Azospirillum–Rhizobium* inoculation on *Phaseolus vulgaris* cv. Negro Jamapa at the level of root development, nitrogen fixation, production of more flavonoid signals, nod-gene transcription, Nod factor patterns, and relief of negative effects caused by NaCl on the above parameters. The results also suggest that *Azospirillum* allows a longer, more persistent exudation of flavonoids by bean roots. The positive trends obtained with *P. vulgaris* cv. Negro Jamapa are in agreement with the positive effects reported for many crops of agricultural interest (Dobbelaere and Okon 2007) after *Azospirillum* inoculation. Some authors (Jebara et al. 2001; Remans et al. 2008) have proposed that bacterial strain–plant genotype combination should be considered for selecting the most adapted microbe–plant combinations to environmental limitations like salinity. Estévez et al. (2009) indicated that co-inoculation with *C. balustinum* and rhizobia under mild saline conditions partially relieves the salt stress effects, although it does not always result advantageous for symbiotic N_2 fixation in legume plants. The co-inoculation pattern may also play a critical role in the outcome of results. Lucas García et al. (2004a, b) have demonstrated a competition effect among several PGPRs and rhizobia in *Ensifer fredii*–soybean and *Bradyrhizobium* sp.–lupine systems, when plants were at once co-inoculated compared with a delayed mode of inoculation, leading in the former case to no significant effects on plant growth. Therefore, the main conclusion that may be drawn for co-inoculation with PGPRs is that each symbiotic association requires a careful preliminary assessment in order to optimize the efficiency of the system under particular environmental conditions. Co-inoculation might contribute to enhance N_2 fixation in soybean and beans in the absence of salt but requires a careful selection of appropriated partners.

Conclusion

PGPRs are selected as a result of processes of coadaptation and coevolution between plants and microorganisms that develop under the influence of the roots. However, relatively few mechanisms have been unequivocally demonstrated to be an explanation for the increased resistance to environmental stresses of plants treated with PGPRs. Nevertheless, plant growth-promoting bacteria found in association with plants grown under chronically stressful conditions may have adapted to these conditions and could provide significant benefits to plants. Therefore, it is necessary to know the rhizosphere of each plant, molecular dialogue between microorganisms, and roots and plant growth promoting capacity of rhizobacteria. With all the information, we can hope to be able to develop crops under advantageous environmental conditions framed within ecological friendliness and sustainability.

Acknowledgements This research was partially supported by the Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto (SECyT-UNRC) and CONICET PIP 112-200801-00537 and PID Res. Ref MINCyT 113/201. NP, LG, JV, and AC are fellows of CONICET. MSD is member of the research career of CONICET, Argentina.

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Chapter 4
Plant–Microbe Symbiosis: Perspectives and Applications

Shivesh Sharma, K.P. Shukla, Vasudha Singh, Jyoti Singh, Shikha Devi, and Ashish Tewari

Contents

Abstract Plants and microbes, copious in the environment, can quietly coexist or fight for survival. Within their environment, plants interact with a wide range of microorganisms, some of which are pathogenic and cause disease and others are beneficial and stimulate plant growth or activate innate immune system. In this chapter, we consider the existing literature on interactions between plants, microorganisms and soils and include considerations of applications of these interactions. Some of

S. Sharma (✉) • K.P. Shukla • V. Singh • J. Singh • S. Devi • A. Tewari
Department of Biotechnology, Motilal Nehru National Institute of Technology, Allahabad, Uttar Pradesh, India
e-mail: shiveshs@mnnit.ac.in; ssnvsharma@gmail.com

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_4, © Springer India 2013

these interactions involve elaborate systems of communication, which in the case of symbiosis such as with arbuscular mycorrhiza are several hundreds of millions years old; others involve the release of exudates from plant roots, and other products of rhizodeposition that are used as substrates for soil microorganisms. Rhizosphere competence is an important requirement for the efficacy of the biocontrol strains. Therefore, over decades, multipurpose approaches have been combined to understand the molecular basis of bacterial traits involved in plant–microbe interaction. Here, we review recent advances and applications made in understanding the role of these interactions in modulating plant defence responses, plant growth promotion, sustainable agriculture, bioremediation and molecular aspect of these interactions.

Introduction

Though being nonmotile plants make up an excellent ecosystem for microorganisms. But they constantly encounter both abiotic stresses such as drought, salinity and metal toxicity and biotic stresses such as pathogenic bacteria, fungi, nematodes and oomycetes. The environmental conditions offered differ considerably between the highly variable aerial plant part and the more stable underground root system. Plants encounter a large and diverse community of microorganisms that compete and interact with each other and the host plant itself. Amongst the microbial population, a range of beneficial and pathogenic can be found, leading to the establishment of mutualistic and pathogenic interactions, respectively. Plant–microbe interactions can provide beneficial influence to plant growth through a variety of mechanisms, including fixation of atmospheric nitrogen by different classes of proteobacteria (Moulin et al. 2001), increased biotic and abiotic stress tolerance imparted by the presence of endophytic microbes (Schardl et al. 2004) and direct and indirect advantages imparted by plant growth-promoting rhizobacteria (PGPR) (Gray and Smith 2005). Bacteria can also interact with plants by producing protective biofilm or antibiotics operating as biocontrol agents against potential pathogenic fungi (Bais et al. 2004), by degrading plant- and microbe-produced compounds in the soil that would otherwise be allelopathic or even autotoxic or by even degrading the xenobiotic and recalcitrant inputs in the soil. However, rhizosphere bacteria can also have harmful effects on plant health and survival through pathogenic or parasitic infection.

Rhizosphere Interactions

The plant–microbe interactions engage highly coordinated cellular processes that determine the final outcome of the relationship and determine whether an interaction will be malevolent or benign. In the rhizosphere, plant–microbe interactions are responsible for a number of inherent processes such as carbon sequestration, ecosystem functioning and nutrient cycling (Singh et al. 2004). The rhizodeposition of root exudates, composed of small molecular weight metabolites, amino acids,

mucilage, secreted enzymes and cell lysates, can range from less than 10 % of the net carbon assimilation by a plant to as much as 44 % of a nutrient-stressed plant's total carbon (Patterson and Sims 2000). Soil microbes utilise this abundant carbon source, thereby implying that selective secretion of specific compounds may encourage beneficial symbiotic and protective relationships, whereas secretion of other compounds inhibits pathogenic associations (Bais et al. 2005). Symbiosis is close and long-term interaction between the two or more different biological species in which one organism lives on another or where one partner lives inside the other. Amongst the plant–microbe interactions, two symbiotic relationships have been very well studied. One is the root nodule symbiosis and another is arbuscular mycorrhizal symbiosis. Arbuscular mycorrhizal symbiosis is the most extensively studied interaction between the plants and microbes. Microorganisms associated with roots such as PGPR and arbuscular mycorrhizal fungi (AMF) can play important role in improving the plant growth and health.

Endophytes can also be beneficial to their host by promoting plant growth and also acting as biocontrol agents (Ryan et al. 2008). Despite their different ecological niches, free-living rhizobacteria and endophytic bacteria use the same mechanisms to promote plant growth and control phytopathogens (Compant et al. 2005). For example, they can affect plant growth by producing auxins such as indole-3-acetic acid (IAA) or cytokinins or by degrading the ethylene precursor ACC by ACC deaminase (Ryan et al. 2008). Application of bacterial inoculants as biofertilisers has resulted in improved growth and increased yield of cereal crops (Arcand and Schneider 2006).

Since nitrogen is a major factor limiting plant growth, the use of large amounts of chemically produced N_2-containing fertilisers has led to increased gains in crop productivity and was a major part of the original green revolution. However, such fertilisers cause deleterious environmental impacts, such as soil acidification and groundwater pollution, a situation which is exacerbated by the low efficiency of uptake by the crops to which they are applied (Reddy et al. 2002). In this context, biological nitrogen fixation (BNF) is increasingly being viewed as a viable alternative for supplying N_2 to plants.

The harmful interactions of microbes with plants lead to infectious diseases affecting only the plant kingdom. Losses in crop production due to plant disease average 13 % in the world and severely limit production of food (Agrios 1997). About 11,000 diseases that have been described in plants are caused by 120 genera of fungi, 30 types of viruses and eight genera of bacteria (including two genera of mollicutes) (Agrios 1997). Some soilborne microorganisms can promote plant growth as well as suppress diseases or induced systemic resistance (ISR).

Bioremediation

Sustained use of pesticides, chemical fertilisers and manures for increasing soil fertility and crop productivity frequently results in unexpected harmful environmental effects. Integrated nutrient management systems are essential to maintain agricultural productivity and protect the environment. Multipurpose bioinoculant

is a potential component of such management systems. In the last few decades, the rate of nitrogen, phosphorus and potassium (NPK) fertiliser application has increased tremendously. The International Fertiliser Industry Association reported that the three countries with the highest fertiliser use in 2006 were China, India and the USA, consuming 50.15, 21.65 and 20.83 million tonnes of NPK fertiliser, respectively, compared with consumption in 1961 of 1.01, 0.42 and 7.88 million tonnes, respectively (http://www.fertilizer.org/ifa). The challenge therefore is to continue agricultural productivity in a way that minimises harmful environmental effects of fertilisers.

Environments are contaminated with various levels of toxicants. Amongst these, pesticides, polycyclic aromatic hydrocarbons (PAHs) and total petroleum hydrocarbons (TPHs) from anthropogenic sources particularly from fuel combustion, pyrolytic processes, spillage of petroleum products, waste incinerators and domestic heaters pose an inevitable risk to human health (Meagher 2000). Traditional remediation of polluted sites requires soil excavation and transport, prior to off-site treatment by solvent extraction, thermal alkaline dechlorination, incinerations or land filling (Campanella et al. 2002). These techniques are costly, detrimental for the environment and, in many cases, practically infeasible due to the range of the contamination (Gerhardt et al. 2009). Therefore, nowadays there is a considerable interest in developing cost-effective alternatives based on microorganisms or plants. The importance of plant–microbe partnerships in the remediation of organic contaminants was confirmed in studies at the level of rhizosphere (Gerhardt et al. 2009), the phyllosphere and inside the plant (Gianfreda and Rao 2004). Rhizoremediation is considered as the most potential approach for PAHs' remediation in soil (Dean-Ross 1987). Soil microflora play vitally important role during rhizoremediation of xenobiotics (Glick 2003). The interaction amongst microbial degrader, plant and PAHs in soil might be regulated through rhizosphere processes (Grosser et al. 1991).

Rhizoremediation systems for PAHs rely on a synergistic relationship between suitable plants and their root-associated microbial communities (Glick 2003). Degradation is facilitated through a rhizosphere effect where plants exude organic compounds through their roots and thereby increase the density and activity of potential hydrocarbon-degrading microorganisms in the zone, surrounding the roots (Guerin and Jones 1988). Different approaches like rhizoremediation, combination of PGPR and specific contaminant-degrading bacteria, genetically engineered microbes, transgenic plants and enzyme technology can be used to improve the efficiency of bioremediation.

Gene Expression During Bacterial Plant Interactions

The availability of promoter fusion technology has allowed researchers to assess the contribution of bacterial traits to rhizosphere competence on the basis of gene expression. More precisely, promoter fusion technology has been used to monitor

the expression of genes involved in the biosynthesis of secondary metabolites and therefore associated with antibiosis. For example, the regulation of genes involved in the biosynthesis of 2,4-diacetylphloroglucinol (de Werra et al. 2008), phenazines (Chin-A-Woeng et al. 1998), oomycin A (Howie and Suslow 1991), pyoluteorin (Kraus and Loper 1995) and hydrogen cyanide (Jamali et al. 2009) has been assessed in the rhizosphere of different plant species. From these studies, there is a growing body of evidence suggesting that the expression of these secondary metabolites is influenced by a number of abiotic (e.g. ions availability, pH and temperature) and biotic factors (e.g. plant species, plant cultivar and plant age). The formation of specific rhizobacterial communities associated with distinct plant species is determined by 'complex epistatic interactions among many different genes product' (Rainey 1999). Development of genetic tools such as in vivo expression technology (IVET), along with 'omic' technologies (e.g. genomics, transcriptomics, proteomics, metabolomics), has provided opportunities to investigate global expression profiles of different bacterial strains in response to plant signals (Kiely et al. 2006).

Genetically Modified Plants (GMPs)

The engineering of genetically modified plants (GMPs) has great potential for future agriculture, but asks for a well-defined risk assessment as well. To date, environmental risk assessments regarding the cultivation of GMPs have mainly addressed above-ground effects. The underground component of GMP effects has been largely neglected, despite the recognised importance of soilborne organisms and processes and the dominant role of plants with respect to underground energy and carbon input. Microorganisms are the dominant soil organisms both in terms of biomass and activity (respiration), accounting for over 80 % of the total biomass (excluding roots), and largely determine the functioning of terrestrial ecosystems.

Techniques in Plant–Microbe Interactions

The interactions between plants and pathogens are complex (Dodds and Rathjen 2010). At the onset of plant–pathogen interaction, plants develop two strategies to detect and defend pathogen attack. One strategy involves the generation of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), whilst the other involves recognition by pathogen effectors, resulting in PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI), respectively (Dodds and Rathjen 2010). As a consequence, the plant switches on downstream signalling pathways and produces antimicrobial compounds to kill the pathogen and maintain homeostasis (De Wit 2007). This

very precisely controlled complex process involves a number of genes and a number of signalling pathways (Zipfel 2009). It is this complexity of plant–pathogen interactions, which makes it very difficult to discern, which anatomical features, metabolites and signalling pathways are activated: traditional biochemical and genetic experimental methods are inadequate tools for the task. Nowadays, the field of genomics provides powerful tools to investigate these critical factors. Transcript profiling techniques allow the simultaneous examination of thousands of genes and are used to study changes in gene expression that are transcriptionally regulated (Wang et al. 2009). DNA microarray is amongst the most common of profiling tools and is becoming more and more advanced with the availability of the genomic and EST (expressed sequence tag) sequences of plants simultaneous with the advancement in the computational biology tools. It helps in the study of defence mechanism of plants after pathogen attack, in the identification of pathogenesis-related genes and also to understand the interactions between different signalling pathways (Libault et al. 2010). Several genomes from causal agents of plant diseases, both viral and bacterial, have been completely sequenced and more are underway (wit.integratedgenomics.com/GOLD). Based on their analysis, new specific sequences could be used to design detection probes for different pathogens (van Sluys et al. 2002). The sequences of complete genomes in GenBank are available through NCBI (www.ncbi.nlm.nih.gov/Entrez/) and other databases.

Although beneficial for agriculture, exploitation of PGPRs as biocontrol or biofertiliser inoculants has been hampered by inconsistent results at the field scale (Mark et al. 2006). Therefore, over decades, efforts have been made to decipher the bacterial traits involved in rhizosphere competence (Lugtenberg and Kamilova 2009). The molecular basis of rhizosphere competence has been assessed by a combination of several approaches. For example, a number of bacterial functions such as motility (Capdevila et al. 2004), attachment (Rodriguez-Navarro et al. 2007), growth (Browne et al. 2010), stress resistance (Martinez et al. 2009) and production of secondary metabolites (Maunsell et al. 2006) have been linked to rhizosphere competence on the basis of gene inactivation in biochemical genetics evaluation studies. It follows that expression of rhizosphere competence genes is important for growth, survival and function of microbes in the rhizosphere.

This chapter focuses on the recent findings and applications in the biology of plant–microbe interactions. The chapter is organised into four sections. In the first section, positive and pathogenic processes, more importantly the constructive effects, are discussed. In the second section, application of PGPRs for sustainable agriculture is being focussed. The third section focuses on different approaches like rhizoremediation combination of PGPR and specific contaminant-degrading bacteria, genetically engineered microbes, transgenic plants and enzyme technology, strategies that are used to improve the efficiency of bioremediation. The fourth section includes the study of plant–microbe interaction vis-à-vis molecular aspects.

Positive and Pathogenic Processes and Responses

Nutrient Attainments

Mineral nutrients such as phosphorus and iron are very reactive and strongly bound to soil particles. Their availability is generally low, especially in calcareous soils. Plant species differ greatly in their capacity to take nutrients from soil. Some plants are capable of gaining phosphorus and iron or other ions from calcareous soils, whereas others cannot extract enough nutrients to persist on such soils (Lambers et al. 2008). Nutrient acquisition from calcareous soils involves rhizosphere processes, such as the exudation of phosphate mobilising carboxylates or the release of Fe-chelating phytosiderophores (Robin et al. 2008). Phytosiderophores also mobilise other micronutrients whose availability at high pH is low, e.g. Zn (Cakmak et al. 1996) and Cu (Michaud et al. 2008). Phosphate acquisition from soils with low P concentrations in solution as well as plant growth can be enhanced by mycorrhizal symbiosis (Richardson et al. 2009). However, even when P acquisition or plant growth is not enhanced in the presence of mycorrhizal fungi, the P taken up by the fungus may represent a major fraction of the total amount of P acquired by the mycorrhizal plant (Smith et al. 2003). Approximately 80 % of all higher plant species can form a mycorrhizal symbiosis; of these, the arbuscular mycorrhizal (AM) association is the most common (Brundrett 2009), especially on relatively young soils (Lambers et al. 2008). AM is also the most ancient amongst mycorrhizal symbiosis, the first evidence dating back to more than 400 million years ago (Brundrett 2002). Mycorrhizal association takes on a number of different morphologies, but they fall into two broad categories (Smith and Read 2008). In endomycorrhizal associations, such as arbuscular mycorrhizas (AM), the mycorrhizal fungus penetrates root cells in response to specific signals from the plant. In the cortical cells, the fungi differentiate into nutrient exchange structures, termed arbuscules. These are anatomically similar to the haustoria (feeding structures) formed by pathogenic fungi, although their function is very different. Gross changes in root morphology are not generally seen in this symbiosis, although subcellular modifications are extensive. By contrast, in ectomycorrhizal symbiosis fungi grow within the cortical cell walls and their hyphae form a sheath around the root. On somewhat older soils, AM are partly replaced by ectomycorrhiza and ericoid mycorrhiza, which are considered more advanced and diverse mycorrhizal symbiosis (Brundrett 2002); the latter symbiosis are capable of accessing forms of both P and N that are not available for AM fungi (Yao et al. 2001). Mycorrhizal associations are frequently beneficial for both symbiotic partners. Plants benefit from the fungi because these acquire nutrients, which are inaccessible for the plant because of distance from the roots, location in pores that are too small for roots to access, or, occasionally, occurrence as forms that are unavailable to plants. Conversely, fungi are ensured of C supply derived from photosynthesis by the plant (Smith and Read 2008).

In extremely poor soils, when virtually all P is strongly sorbed onto soil particles, the 'scavenging' strategy of mycorrhiza is not effective (Parfitt 1979). On such soils, which are common in old landscapes, species with root clusters that release a range of exudates that effectively 'mine' P are prominent (Lambers et al. 2008). Many species that produce root clusters are non-mycorrhizal, but some are capable of associating with mycorrhizal fungi as well as making clusters (Lambers et al. 2006). Indeed, many actinorhizal species and *Cyperaceae* with root clusters are common in acidic bogs or on calcareous dunes (Bakker et al. 2005). However, no systematic studies have focused on the role of root clusters in these environments, and further research is warranted. The non-mycorrhizal habit of many cluster-bearing plant species (Shane and Lambers 2005) presents an intriguing situation from an evolutionary perspective, because ancestors of these non-mycorrhizal species were most likely all arbuscular mycorrhizal (Brundrett 2002). We know that some of the non-mycorrhizal families with root clusters, e.g. *Proteaceae*, are as old as early to mid-Tertiary (Hopper and Gioia 2004), but there is no information about the time these lineages became non-mycorrhizal. Brundrett (2002) provided evidence for the view that the evolution of specialised strategies of nutrient acquisition, such as cluster roots and also new types of mycorrhizas, coincided with the origin of numerous plant families, which thereby became more competitive, especially so in certain nutrient-limited habitats. Such nutrient acquisition mechanisms may have provided a selective advantage to those plant lineages in which these new strategies evolved, resulting in increased nutrient acquisition, albeit presumably at increased C costs. Brundrett (2009) pointed out that cost/benefit analysis are rather complex to make, given that mycorrhizal plants remain dominant in most habitats, whilst a major group of non-mycorrhizal plant species is found in marginal environments, especially extremely infertile soils in the case of cluster-bearing species (Lambers et al. 2008). Non-mycorrhizal species also occur in waterlogged, saline, dry, metal-contaminated or cold habitats where plant productivity is low and inoculum of mycorrhizal fungi could be scarce (Brundrett 2002). Interestingly, at least one species in the *Proteaceae* is mycorrhizal as well as cluster bearing, i.e. *Hakea verrucosa*, which is endemic on ultramaphic soils, which have high nickel concentrations (Boulet and Lambers 2005).

Amongst the positive plant–microbe interactions that have been studied in the greatest detail are those in which bacteria or fungi enter into mutually beneficial symbiosis with higher plants (Smith and Read 2008). As is the case for the majority of plant–pathogen interactions, symbiosis are characterised both by their complexity and by their specificity; they are also of enormous importance for global agricultura1 productivity. Moreover, symbiosis provides model systems for studying fundamental plant or microbial processes, such as signal perception and transduction, control of the cell cycle and cellular differentiation. In most nitrogen-fixing symbiosis, soil bacteria of the unrelated genera *Rhizobium* and *Frankia* induce cell divisions in fully differentiated (and quiescent) cells in the root cortex or pericycle of plants in the families *Leguminosae* and *Rosaceae*. Bacteria enter the root and migrate, intercellularly or intracellularly, towards these foci of dividing plant cells. As cell division continues and the nascent structures mature into

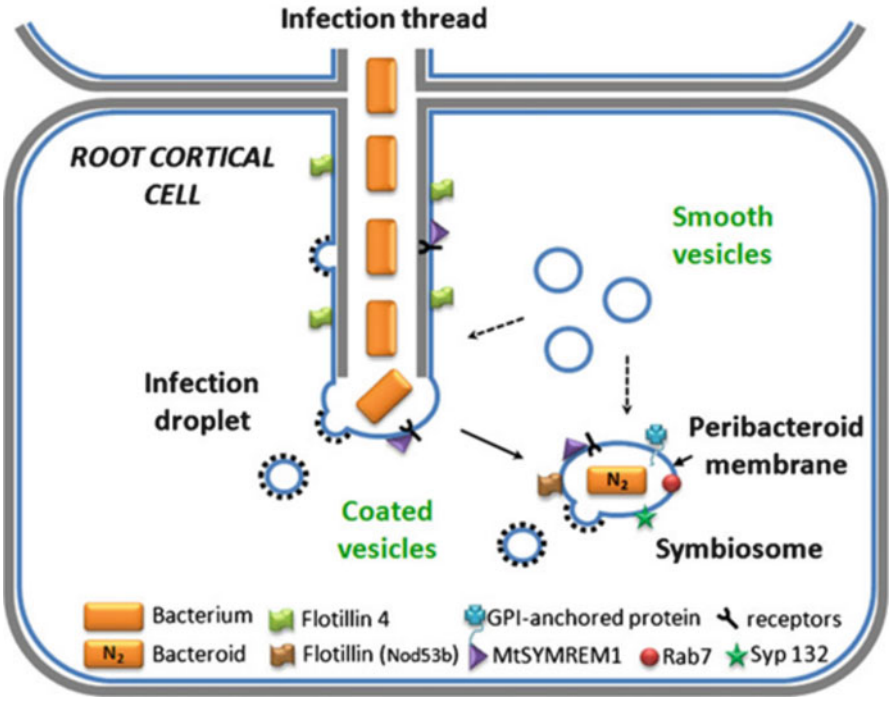

Fig. 4.1 Membrane dynamics in the rhizobial root nodule symbiosis. Rhizobia penetrate cortical cells via infection threads (IT). Rhizobia are released from unwalled IT droplets into the cell cytoplasm as host membrane-delimited compartments (symbiosomes) surrounded by the peribacteroid membrane and differentiate into N_2-fixing bacteria (bacteroids). Exocytosis (smooth vesicles) and endocytosis (coated vesicles) are thought to act in concert for IT growth, symbiosome formation and proliferation (not illustrated). Location of membrane markers associated with endocytosis and microdomains is indicated (*Source*: Castel et al. 2010)

nodules, the bacteria differentiate into forms that are capable of fixing nitrogen (i.e. reducing gaseous NP to compounds such as ammonia). The fixed nitrogen is transported throughout the plant and, in return, the bacteria are supplied with photosynthate and a protected environment in which to divide. Bacterial journey into the root cell layers starts with root hair curling, which entraps the rhizobia, followed by local degradation of the hair cell wall, invagination of the hair plasma membrane and secretion of plant cell wall components to form an apoplastic tube called infection thread (IT) that grows inwards towards dividing cells in the root cortex (Fig. 4.1 from Castel et al. 2010).

The most successful application of BNF in cropping systems is the inoculation of legumes with rhizobia, such as the inoculation of soybean (*Glycine max*) with *Bradyrhizobium*, which forms a symbiotic association capable of supplying 100 % of the N required for plant development and grain formation (Alves et al. 2003). The benefits of BNF have also been observed in nonlegumes (James 2000), with the most convincing evidence obtained from sugarcane and rice (Isawa et al. 2010).

In sugarcane, the diazotrophs that may contribute to BNF are *Gluconacetobacter diazotrophicus*, *Azospirillum amazonense* and *Herbaspirillum* sp. (Reis et al. 1994). Since 1990 there has been a growing interest in utilising endophytic diazotrophs within the genera *Gluconacetobacter*, *Azoarcus*, *Azospirillum*, *Klebsiella*, *Serratia*, *Rhizobium* and *Herbaspirillum* as PGPR. This is partly because of their occurrence on and within diverse plant tissues, where they form stable associations with plants of commercial importance, and also because of evidence for expression by them of N_2 fixation genes and proteins *in planta* (Iniguez et al. 2004). In addition, the aforementioned diazotrophic bacteria may also stimulate plant growth not only as a consequence of BNF (Iniguez et al. 2004) but also by the production of phytohormones, the control of phytopathogens and/or by enhancing the availability and uptake of minerals such as phosphate (Sessitsch et al. 2002).

There are many comparative features of the legume–*Rhizobium* symbiosis with those of the actinorhizal symbiosis that develop between plants and nitrogen-fixing Gram-positive bacteria of the genus *Frankia*. Some defence-related genes are induced in actinorhizal plants during *Frankia* infection, as they are in legumes. However, one significant difference between legume and actinorhizal nodules is the site at which root cells become reactivated to form nodule primordia. In rhizobial symbiosis, cortical cells are activated to re-enter the cell cycle, whereas in actinorhizal symbiosis, pericycle cells are activated. The early phases of actinorhizal nodule initiation are therefore quite similar to lateral root initiation. The usual condition of plants appears to be in a close interaction with endophytes. Endophytes show potential to increase crop yields, remove contaminants, inhibit pathogens and produce fixed nitrogen or novel substances. The selection of their effects and functions in plant has not been comprehensively defined. The challenge and goal is to be able to manage microbial communities to goodwill plant colonisation by beneficial bacteria. This would be acquiescent when a better knowledge on endophyte ecology and their molecular interactions is attained.

Effects of Endophytic Bacteria and Benefits to the Plant

The growth stimulation by the microorganisms can be a consequence of nitrogen fixation (Iniguez et al. 2004) or the production of phytohormones, biocontrol of phytopathogens in the root zone (through production of antifungal or antibacterial agents, siderophore production, nutrient competition and induction of systematic acquired host resistance, or immunity) or by enhancing availability of minerals (Sessitsch et al. 2002). The exposition of the mechanisms promoting plant growth will help to favour species and conditions that lead to greater plant benefits. Volatile substances such as 2,3 butanediol and acetoin produced by bacteria seem to be a newly discovered mechanism responsible for plant growth promotion (Ryu et al. 2003). It would be attractive to determine if volatiles could be produced inside plants. Endophytes produce adenine ribosides that stimulate growth and mitigate browning of pine tissues (Pirttilä et al. 2004). Endophytic bacteria of red clover seem to be responsible for the allelopathic effects observed with these plants over

maize, causing reduced plant emergence and plant height (Sturz and Christie 1996). Bacterial endophytes are capable of suppressing nematode proliferation, and this may benefit other crops in rotation with the host plants (Sturz and Kimpinski 2004). The frequent isolation of *Curtobacterium flaccumfaciens* as endophytes from asymptomatic citrus plants infected with the pathogen *Xylella fastidiosa* suggested that the endophytic bacteria may help citrus plants to better resist the pathogenic infection. Endophytes from potato plants showed antagonistic activity against fungi (Berg et al. 2005) and also inhibited bacterial pathogens belonging to the genera *Erwinia* and *Xanthomonas* (Sessitsch et al. 2004). Some of the endophytic isolates produce antibiotics and siderophores *in vitro* (Sessitsch et al. 2004). Inhibition of the oak wilt pathogen *Ceratocystis fagacearum* was obtained with 183 endophytic bacteria of 889 isolates tested (Brooks et al. 1994). Of 2,648 bacterial isolates analysed from the rhizosphere, phyllosphere, endosphere and endorhiza, only one, a root endophyte corresponding to *Serratia plymuthica*, was a highly effective fungal antagonist (Berg et al. 2005). Endophytic actinobacteria are effective antagonists of the pathogenic fungus *Gaeumannomyces graminis* in wheat (Coombs et al. 2004), and several endophytes showed antagonism against *Rhizoctonia solani* (Parmeela and Johri 2004). It is worth considering that most of the assays to test antagonism are *in vitro*, and it remains to be established if this correlates to effects in nature.

Prospect application may consider the use of genetically modified endophytes with biological control potential in agricultural crops. The endophytes *Herbaspirillum seropedicae* and *Clavibacter xyli* have been genetically engineered to produce and excrete the α-endotoxin of *Bacillus thuringiensis* to control insect pests (Downing et al. 2000). Bacteria degrading recalcitrant compounds are more abundant amongst endophytic populations than in the rhizosphere of plants in contaminated sites (Siciliano et al. 2001), which could mean that endophytes have a role in metabolising these substances. Engineered endophytic *Burkholderia cepacia* strains improved phytoremediation and promoted plant tolerance to toluene (Barac et al. 2004). There is an increasing interest on genetically modified endophytes (Andreote et al. 2004). The advantages and obstacles to use bioengineered endophytes have been clearly discussed (Newman and Reynolds 2005). Endophytic bacteria possess the capacity to solubilise phosphates, and it was suggested by the authors that the endophytic bacteria from soybean may also participate in phosphate assimilation (Kuklinsky-Sobral et al. 2004). A number of studies have been directed to find endophytes that could significantly increase the yields in different crops after their inoculation. To divulge the effects of endophytes, inoculation experiments have been performed, but it has been a problem to eliminate resident or indigenous endophytes from plants in order to have bacteria-free plants or seeds. Functional idleness of resident endophytes and added inocula may limit the effects observed from inoculation. Very complex microbial community plant interaction, poor rhizosphere competence with endogenous microorganisms (Sturz et al. 2000) and bacterial fluctuations with environmental conditions may also limit the applicability of endophyte inoculation in the field (Sturz and Nowak 2000). In addition, the large abundance and diversity of soil bacteria may be a rich source of endophytes, and, for this, inoculation effects may not be observed in the field. Since surface disinfection does not remove endophytes, procedures such as warming and drying seeds have been assayed to diminish bacterial populations inside

(Holland and Polacco 1994). Tissue culture has also been used to eliminate or reduce endophytes (Holland and Polacco 1994). Inoculants appear to be successful in micropropagated plants, as there are few or no other microorganisms with which to compete. In such cases, when the plantlets were inoculated, they were more vigorous and had increased drought resistance, an increased resistance to pathogens, less transplanting shock and lower mortality (Martínez et al. 2003).

Rhizodeposition

Rhizodeposition is the release of C compounds from living plant roots into the surrounding soil; it is a ubiquitous phenomenon (Jones et al. 2009). The thrashing of C from root epidermal and cortical cells leads to a proliferation of microorganisms inside (endophytes), on the surface and outside the root. Rhizodeposition consequences in different chemical, physical and biological characteristics in the rhizosphere compared with those of the bulk soil. The magnitude of these changes is determined by the amount and type of C released from the root as well as intrinsic soil characteristics. Rhizodeposition basically results from two different processes: (1) leakage of compounds over which the plant exerts little control and (2) exudation of specific compounds with a specific function and over which the plant exerts control (Jones 1998). Leakage of compounds as defined corresponds to a minor component of a plant's C budget, less than 5 % of all C daily fixed in photosynthesis (Lambers et al. 2008). Higher values cited in the literature probably include C released either by root respiration or from dying root cells (Lambers 1987). Plant roots may discharge huge amounts of organic compounds via rhizodeposition. At neutral pH and optimum P supply, rates tend to be low. Iron-efficient grasses release phytosiderophores when their growth is limited by the availability of Fe (Robin et al. 2008). The phytosiderophores are released from the root tips only (Marschner et al. 1987), predominantly during the early morning (Ma and Nomoto 1994). Robin et al. (2008) stressed that an efficient strategy to maximise the positive impact on Fe acquisition is by minimising the breakdown of phytosiderophores by rhizosphere microorganisms. Neighbouring Fe-inefficient plants may benefit from these released phytosiderophores (facilitation), and this knowledge can be applied in intercropping Fe-efficient crops with calcifuge ones, e.g. maize (*Zea mays*) with peanuts (*Arachis hypogaea*) (Zuo et al. 2000) or red fescue (*Festuca rubra*) with fruit trees (Ma et al. 2003).

Many plants enhance their rate of carboxylate exudation when their P supply is severely limiting (Vance et al. 2003). Massive exudation rates are exhibited by species that produce root clusters at very low P supply (Shane and Lambers 2005). Root cluster-bearing species, e.g. *Lupinus albus* (Watt and Evans 1999), *Hakea prostrata* (Shane et al. 2004) and *Schoenus unispiculatus* (Shane et al. 2006), release carboxylates in an exudative burst. According to model calculations of Darrah (1991), this ensures mobilisation of P before microorganisms have an opportunity to decompose the released exudates. Moreover, root clusters of *Lupinus albus* drastically reduce the cluster root rhizosphere pH, thus inhibiting bacterial

activity; they also release phenolics, which induce fungal sporulation, as well as chitinases and glucanases, which degrade fungal cell walls, prior to the exudative burst (Weisskopf et al. 2006). This complex strategy ensures minimal degradation and maximum efficiency of exuded carboxylates to mobilise scarcely available P and micronutrients.

Whilst rhizodeposition incurs a loss of C to the plant, there are obviously also major benefits. First, there is the signalling to microsymbionts, as discussed above; this only incurs a minor C cost. Second, exudation sensu stricto can have a major impact on nutrient acquisition. Phytosiderophores and other chelating agents play a pivotal role in acquiring Fe and other micronutrients, especially from calcareous soil (Robin et al. 2008). They invite a relatively small C cost, because release rates are relatively low and restricted in space (from root tips only) and time (in the early morning only) (Cakmak et al. 1996). Carbon costs associated with carboxylate release to chelate and detoxify Al or other metals are also relatively small, as only root tips appear to be involved in this process (Delhaize and Ryan 1995).

Response to Phytopathogens

In plant–microbe interactions, a compatible relation occurs when microbes are pathogenic, which ultimately results in disease, whilst a resistance to disease is referred to as an incompatible relation (non-host). Plants lack a circulatory system, but their defence relies on the innate immunity of each cell and on systemic signals deriving from infection sites. Local and systemic immune responses are known as the hypersensitive response (HR), which is characterised by rapid cell death at the site of infection (Mur et al. 2008) and the systemic acquired resistance (SAR), which confers long-lasting protection to the whole plant (Durrant and Dong 2004), respectively. The plasma membrane (PM) appears as a critical barrier that senses microorganisms and eventually allows their entry or the uptake of microbial molecules. Disease suppression can occur through microbial antagonism or induction of resistance in the plant. The spectrum of diseases against which PGPR elicit ISR conferring enhanced resistance overlaps partly with that of pathogen-induced SAR (van Loon 2007). Both ISR and SAR represent a state of enhanced basal resistance of the plant that depends on the signalling compounds jasmonic acid and salicylic acid, respectively, and pathogens are differentially sensitive to the resistances activated by each of these signalling pathways (van Loon 2007).

Plant Growth Promotion by Bioaugmentation

Plant roots offer a niche for the proliferation of soil bacteria that thrive on root exudates and lysates. Population densities of bacteria in the rhizosphere may be up to hundred fold higher than in bulk soil and up to 15 % of the root surface may be

covered by micro-colonies of a variety of bacterial strains. Whilst these bacteria utilise the nutrients that are released from the host for their growth, they also secrete metabolites into the rhizosphere. Several of these metabolites can act as signalling compounds that are perceived by neighbouring cells within the same micro-colony, by cells of other bacterial colonies/strains that are present in the rhizosphere or by root cells of the host plant (Kiely et al. 2006). Growth promotion by soil microorganisms is far from uncommon (Ryu et al. 2005) and can be considered part of a continuum in which interactions between plants and microorganisms range from deleterious (pathogens) to beneficial (PGPR).

Many bacteria in soil have similar properties (Compant et al. 2005), but in a number of cases, rhizobacteria can enhance plant growth in gnotobiotic systems (Van Loon and Bakker 2003). The ability to fix atmospheric nitrogen is present in various bacterial species that are either free-living in the soil or associated with plant roots by growing endophytically (Dobbelaere et al. 2003). Poorly soluble inorganic nutrients that are rate limiting for growth can be made available through the solubilising action of bacterial siderophores or the secretion of organic acids (Vessey 2003). The high population densities of bacteria in the rhizosphere stimulate nutrient delivery and uptake by plant roots. Other mechanisms of growth promotion involve modulation of plant regulatory mechanisms through the production of hormones or other compounds that influence plant development (Frankenberger and Arshad 1995).

Enhanced lateral root formation increases the capacity to take up nutrients. For *Azospirillum brasilense* it has been shown that auxin is responsible for its growth-promoting action in wheat and pearl millet, as bacterial mutants that had lost 70 % of their capacity to produce IAA had lost their plant growth-promoting activity (Barbieri and Galli 1993).

Fertiliser use in agriculture and the technological advances in agriculture are helping meet the food needs of an ever-increasing world population. Although the population is growing, land available for agriculture is shrinking. Intensive agriculture that involves heavy and continuous use of fertilisers has ensured high crop productivity. As an example, increased use of fertilisers played an important role in the immense success in food productivity during the period of the green revolution (Tilman 1998). However, reports have shown that continuous use of fertilisers is generating environmental problems. Low efficiency in the uptake of fertiliser is a major factor that aggravates the negative environmental effects (Barlog and Grzebisz 2004). Akanbi et al. (2007) showed that foliar spray of compost extracts from cassava (*Manihot esculenta*) peel and Mexican sunflower (*Tithonia rotundifolia*) helps produce fluted pumpkin (*Telfairia occidentalis*) plants with comparable growth to those that received NPK fertiliser. In a different study with strawberry, Hargreaves et al. (2009) reported that compost tea enhanced the uptake of most macronutrients and micronutrients in strawberry plants in amounts that compared with municipal solid waste compost, ruminant compost and inorganic mineral fertilisers. However, it is important to emphasise

that agro-environmental problems are not limited to the use of chemical fertilisers but also occur with manures and compost (Mitchell and Tu 2006). Both animal waste and chemical fertilisers have the potential of environmental pollution (Jarecki et al. 2008). Organic manures (fertilisers) contain N-rich materials and high extractable nutrients (P, K, calcium (Ca), magnesium (Mg), copper (Cu) and zinc (Zn)) and can significantly raise soil fertility in the medium to long term (Hinsinger et al. 2001; Mitchell and Tu 2006). Mitchell and Tu (2006) noted that continued application of poultry waste will increase levels of soil nutrients but could cause a build-up of some nutrients and loss of nutrients to the environment apart from the soil contamination issues. A sustainable alternative to these practices is the use to these methods is the use of microbial inoculants that is a process of artificial augmentation of useful microbes in the soil or more specifically in the rhizosphere. Microbial inoculants include three major groups: (1) arbuscular mycorrhiza fungi (AMF), (2) PGPR and (3) nitrogen-fixing rhizobia. There is some discussion in the scientific literature on the role of specific strains of PGPR and AMF in plant growth promotion, N_2 fixation, biofertiliser activities or biological control of plant diseases (Morrissey et al. 2004), but there is a need for more attention now especially in regard to nutrient interactions. Based on the beneficial effects of PGPR and AMF, studies using inoculant mixtures are very promising (Berg 2009). Benefits to plants from plant–PGPR interactions have been shown to include increases in seed germination rate, root growth, yield, leaf area, chlorophyll content, nutrient uptake, protein content, hydraulic activity, tolerance to abiotic stress, shoot and root weights, biocontrol and delayed senescence (Yang et al. 2009). The mechanisms behind plant–PGPR interactions are complex phenomena involving a combination of direct and indirect mechanisms, the details of which can be seen in the work by Glick et al. (2007). One specific proposed mechanism by which PGPR affect nutrient uptake is by enhancing growth and development of plant roots, leading to root systems with larger surface area and increased number of root hairs, which are then able to access more nutrients (Adesemoye et al. 2008). The capacity of AMF to influence plant growth, water and nutrient content has been widely reported over the years (Giovannetti et al. 2006).

In exploring the interactions between PGPR and AMF, for better plant use efficiency of inorganic fertilisers or manures, synergism is most likely, but one must be cognizant that antagonism between PGPR and AMF is also a possibility. Many PGPR and AMF have been used separately and as combinations to investigate the impacts on the uptake of individual or multiple elements. Even though the applications of the tools to sustainable agriculture are yet to be well understood, advances in genomic technology have provided substantial information in plant–PGPR and/or plant–AMF interactions.

Further studies with focus on similar issues with other elements and the molecular mechanisms of the impacts of microbes on plant nutrition and fertility management will help improve our understanding of how to use microbial inoculants to decrease harmful effects of fertilisers.

Bioremediation Strategies Using PGPRs

Remediation of Organic Contaminants by PGPR–Rhizoremediation

Phytoremediation is an emerging technology that uses plants and associated bacteria for the treatment of soil and groundwater contaminated by toxic pollutants (Salt et al. 1998). Phytoremediation has several advantages: (1) preserves the natural properties of soil, (2) acquires energy mainly from sunlight, (3) high levels of microbial biomass in the rhizosphere can be achieved, (4) it is low in cost and (5) has the potential to be rapid (Huang et al. 2004). Even though phytoremediation has been shown to efficiently reduce chemical hazards associated with various classes of organic and inorganic pollutants, it also suffers serious limitations that prevent large-scale field applications (Iimura et al. 2002). One of the main challenges that have so far prevented full-scale application of phytoremediation technologies is that contaminant-induced stress frequently leads to low rates of seed germination, slow rates of plant development and decreases in plant biomass. In many cases, this problem can be solved by using PGPRs (Glick 2003). The term rhizoremediation has been used to describe the combination of phytoremediation and bioaugmentation with contaminant-degrading PGPR. Rhizoremediation is a specific form of phytoremediation involving plants and their associated rhizospheric microorganisms (bacteria and fungi). Rhizoremediation either can occur naturally or can be facilitated by inoculating soil with microorganisms capable of degrading environmental contaminants. The presence of PGPR in rhizosphere can enable plants to achieve high levels of biomass in contaminated soils despite in extreme conditions (Siciliano and Germida 1998). Generally, PGPR function in three different ways: (1) by synthesising particular compounds for the plants (Glick 1995), (2) by facilitating the uptake of certain nutrients from the environment (Siddiqui and Mahmood 2001) and (3) by preventing the plants from diseases (Raj et al. 2003).

Although PGPR were first used for prompting the plant growth and for the biocontrol of plant diseases, much attention has recently been paid on bioremediation (Huang et al. 2005; Bisht et al. 2010; Shukla et al. 2012). Facing a variety of environmental contaminants such as total petroleum hydrocarbons (TPHs), remediation technology even with both PGPR and plants may still be low in efficiency. Different approaches like rhizoremediation, combination of PGPR and specific contaminant-degrading bacteria, genetically engineered microbes, transgenic plants and enzyme technology can be used to improve the efficiency of bioremediation.

Genetically Engineered Microbes (GEMs) for Enhanced Bioremediation

The bacteria capable of degrading certain kind of organic pollutant, such as polychlorinated biphenyls (PCBs), have been isolated from a range of sites, and the pathways and encoding genes have also been well studied (Brazil et al. 1995). But

most of these bacteria cannot survive in the near-starvation conditions found in the rhizosphere (Normander et al. 1999). Several effective methods have been developed to improve the degradation efficiency and the tolerance of bacteria to contaminants in soils. Using biotechnology, bacterial strains can be engineered to produce specific enzymes capable of degrading toxic organic substances. For some pollutants such as trichloroethylene (TCE) and polychlorinated biphenyls (PCBs), the molecular mechanisms of degradation have been clearly studied (Brazil et al. 1995). Mostly pesticide degradation genes have been shown to reside on plasmids (Holben et al. 1992). The first described organophosphorus-degrading (opd) gene was found in *Pseudomonas diminuta* and was shown to be present on a plasmid (Serdar et al. 1982). An organophosphate-degrading gene (opd) was isolated from various organisms and diverse geographic locations (Serdar et al. 1982). In most of the studies, opd genes were found to be plasmid based and had similar DNA sequences. However, an opd A gene from *Agrobacterium radiobacter* was located on the chromosome but had a similar sequence to the opd gene from other bacteria. The organophosphorus hydrolase enzyme (OPH), which is encoded by the opd gene, exhibits broad substrate specificities and high hydrolytic activities against organophosphates.

GEMs can be equipped with new metabolic routes by overexpression of certain genes or operons (Timmis and Pieper 1999). Many new techniques are being developed and improved and may be (more) suitable for routine testing for GEMs. GEMs developed from PGPRs can be used in future along with their host plant for bioremediation of contaminated sites.

Transgenic Plants

An electrifying option to the use of plant-associated bacteria to degrade toxic organic compounds in soil is the use of recombinant DNA technology to generate transgenic plants expressing bacterial enzymes resulting in improved plant tolerance and metabolism of toxic organic compounds in soil. Transgenic plants have been produced for phytoremediation of both heavy metals and organic pollutants (Eapen et al. 2007). Transgenic poplar plantlets expressing bacterial mercuric reductase were shown to germinate and grow in the presence of levels of ionic mercury that are normally toxic (Rugh et al. 1998) and to release elemental mercury, thereby transporting soil-bound mercury efficiently out of the soil. *Arabidopsis thaliana* was engineered to express a modified organomercurial lyase (Bizily et al. 1999), and these transgenic plants grew vigorously on a wide range of concentrations of highly toxic organomercurials, probably by forming ionic mercury which should accumulate in the disposable plant tissues. The first report of genetically modified plant for the transformation of xenobiotic contaminants to nontoxic material was reported by French et al. (1999). They reported that *Enterobacter cloacae* PB2 is capable of growth with trinitrotoluene (TNT) as a nitrogen source. The pentaerythritol tetranitrate reductase, an enzyme involved in the degradation of nitrate esters, is capable of reducing the aromatic ring of TNT and causing liberation of nitrite (French et al. 1999). Tobacco plants (*Nicotiana tobacum*) were modified by

the insertion of the gene responsible for 2,3-dihydroxybiphenyl ring cleavage, *bph*C, from the PCB-degrader Gram-negative bacterium *Comamonas testosteroni* (Francova et al. 2003). A transgenic *Arabidopsis* plant was developed which expressed a secretory laccase, LAC1, from cotton (*Gossypium arboreum*) (Wang et al. 2004). The LAC1 plants showed enhanced resistance to a variety of phenolics as compared to wild types in growth media. In a similar study, the laccase of fungus *Coriolus versicolor* was expressed in tobacco resulting in the secretion of laccase into the rhizosphere and the enhanced degradation of bisphenol A and pentachlorophenol (PCP) in hydroponics (Sonoki et al. 2005). Genetically engineered organisms with novel pathways will be used to generate new or improved activities hold a great potential for enhanced bioremediation. Using genes encoding the biosynthetic pathway of bio-surfactants can enhance biodegradation rates by improving the bioavailability of the substrates, and genes encoding resistance to critical stress factors may enhance both the survival and the performance of designed catalysts. Thus, genetic engineering of indigenous microflora, well adapted to local environmental conditions, may offer more efficient bioremediation of contaminated sites and making the bioremediation more viable and ecofriendly technology.

Molecular Aspects

The formation of specific rhizobacterial communities associated with distinct plant species is determined by 'complex epistatic interactions among many different genes product' (Rainey 1999). Development of genetic tools such as *in vivo* expression technology (IVET), along with 'omic' technologies (e.g. genomics, transcriptomics, proteomics, metabolomics), has provided opportunities to investigate global expression profiles of different bacterial strains in response to plant signals (Kiely et al. 2006). An alternative approach is to use metagenomics to identify processes that are important for the function of rhizospheric bacteria by determining whether particular classes of genes are enriched in populations of rhizosphere bacteria. Plant-derived extracellular metabolites and signals can influence the behaviour of bacteria in the rhizosphere (Berg and Smalla 2009). Due to the difficulty of obtaining sufficient material under *in situ* conditions, the effects of chemical plant signals on global bacterial gene expression have been primarily assessed on synthetic media containing seed exudates (Pothier et al. 2007) and root exudates (Mark et al. 2005) or specific plant signals such as phytohormones (Yuan et al. 2008). More recently, protein expression profiles of bacterial strains exposed to root exudates were also investigated using a proteomic approach (Cheng et al. 2009). In addition, the influence of live roots on bacterial gene expression has also been monitored directly (Liu et al. 2011) or in the presence of soil (Silby et al. 2009). The vast majority of these studies have investigated changes of gene expression of *Pseudomonas* species within the 'rhizosphere' of different plant species such as maize (Matilla et al. 2007), rice (Rediers et al. 2003), poplar (Attila et al. 2008), canola (Cheng et al. 2009) and sugar beet (Silby

et al. 2009). The availability of multiple data sets provides an opportunity to determine which functions of the core *Pseudomonas* genome are consistently expressed and therefore likely to be important in plant–microbe (rhizosphere microbe) interactions. Although masking the specific functionality of different species, this approach highlights common pathways employed by *Pseudomonas* sp. to colonise the rhizosphere. Ability of PGPR to use a large panel of nutrients combined with efficient nutrient scavenging systems is likely to be a decisive factor for a successful colonisation of the rhizosphere. PGPR also have to overcome the chemical stress to efficiently colonise plant roots. Therefore, numerous genes coding for stress response and detoxification proteins have been reported to be induced in the soil (Nishiyama et al. 2010), in response to exudates (Cheng et al. 2009) or in the rhizosphere. One of the first steps employed by bacteria to cope with a toxic compound is to extrude it out of the cell. Efflux of toxic molecules such as antibiotics, heavy metals and solvents, as well as endogenous toxic compounds, is generally performed by multidrug resistance (MDR) pumps (Martinez et al. 2009). Many bioinformatics software that assist in microarray analysis (a platform to measure the expression levels of thousands of genes in a sample in a single experiment, thereby creating an expression profile or 'transcriptome' for the sample understudy to create a global picture of cellular function) are available that can be used to perform the analytical techniques (Lodha and Basak 2012). Some of the important software are listed in Table 4.1.

A symbiotic relationship between microarray technology and high-throughput sequencing in the future will enable new questions to be addressed in the area of plant–microbe interactions.

Conclusion

In the current state PGPRs, biofertilisers, rhizoremediation and molecular aspects of plant–microbe interaction are being worked upon across the world. However, to attain maximum exposure, the regulations controlling bioproducts have to be framed exclusively. Bioremediation provides an attractive alternative to the conventional methods of degradation. Keeping in mind the increasing demand for un-degraded polluted products, such methods can be carried out at a much larger scale with the proper understanding of the plant–microbe interactions. Further, study of gene level will open up new avenues for gaining insight on the regulatory mechanism of various applications and provide scope for their manipulation using molecular tools like recombinant DNA technology to suit various applications. With the collaborative efforts of scientists from all over world, effective solutions to the biotechnological development of plant–microbe interaction for human health and environmental protection are expected to be available in the future.

Acknowledgement Authors are thankful to the Director, MNNIT Allahabad, and Department of Biotechnology (DBT), New Delhi, for providing support and necessary financial support.

Table 4.1 List of important software available for microarray data analysis (Lodha and Basak 2012)

Software name	Functions performed	Source
TM4 (MeV)	MultiExperiment Viewer (MeV) is a Java application designed to allow the analysis of microarray data to identify patterns of gene expression and differentially expressed genes	http://www.tm4.org/
EDGE	EDGE (Extraction of Differential Gene Expression) is an open-source, point-and-click software program for the significant analysis of DNA microarray experiments. EDGE can perform both standard and time course differential expression analysis	http://faculty.washington.edu/jstorey/edge
R	R is a language and environment for statistical computing and graphics	http://cran.at.r-project.org/
CYBER-T	Web interface port test, regularised *t*-test, etc.	http://visitor.ics.uci.edu/genex/cybert/
FiRe	FiRe (Find Regulons) is an Excel macro that quickly surveys microarray data by establishing lists of 'interesting' candidate genes that follow a given pattern of mRNA accumulation. Genes are selected depending on their fold-change ratios over different experimental conditions	http://www.unifr.ch/plantbio/FiRe/FiRe_2.2.xls
Cluster, TreeView	Standard for hierarchical clustering and viewing dendrograms and also creates self-organising maps and performs principal components analysis	http://rana.lbl.gov/EisenSoftware
GeneCluster 2.0	This software is used for constructing self-organising maps. The latest version now also finds nearest neighbours and performs other supervised methods. Written in Java, this program can essentially run under any computer operating system	http://www.genome.wi.mit.edu/cancer/software/genecluster2
MultiExpression Viewer	Creates self-organising maps and performs hierarchical clustering as well as finding principal components. This package also includes a component for support vector machines but at present offers little for documentation. The software is written in Java, and a licence for the source code of the software is also available	http://www.tigr.org/software
MAExplorer	Performs many aspects of microarray processing, including the raw image analysis. It contains few analytical techniques, including hierarchical clustering. The software is written in Java, and the source code is freely available for modification	http://maexplorer.sourceforge.net/
RELNET	Creates relevance networks. The software is written in Java, and a licence for the source code is also available	http://www.chip.org/relnet

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Chapter 5
Soil Rhizobacteria Regulating the Uptake of Nutrients and Undesirable Elements by Plants

Stefan Shilev

Contents

Abstract Numerous rhizosphere bacteria are known to be beneficial for plant growth. Such bacterial species are generally recognized as plant growth-promoting rhizobacteria. In this chapter, different mechanisms are discussed by which, depending on the specific conditions, plants benefit from growth and development of rhizobacterial population. Such mechanisms directly or indirectly influence plant growth and development. Direct mechanisms are related to phosphorus solubilization, nitrogen fixation, iron chelation, production of phytohormones, and degradation of ethylene

S. Shilev (✉)
Department of Microbiology and Environmental Biotechnologies, Agricultural University Plovdiv, 12 Mendeleev Str., 4000 Plovdiv, Bulgaria
e-mail: stefan.shilev@au-plovdiv.bg

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_5, © Springer India 2013

production, while the indirect are fitted to suppression of plant phytopathogens and induced systematic resistance in plants. The combination of mechanisms is possible to exist in a habitat where a microbial community composed of plant-growth-promoting rhizobacteria finds suitable niches for development. This chapter also reviews different combinations of mechanisms presented in soils.

Introduction

Plants present different symptoms of lack of nutrient elements during their growth. As a result, plant production suffers decrease in quantity and quality that has significant economical impact. Plant nutrition depends mostly on physicochemical characteristics of soil, presence of water and nutrient elements, and existence of pathogens but also on beneficial soil microorganisms and especially on the soil rhizobacteria. So, the rhizosphere can be defined as a zone where the soil properties are actively influenced by presence of the root nearby. Since germination of the seed, all properties of this zone are influenced basically by the stage of development of plant and the interactions with the physicochemical and biological properties of soil (Darrah 1993). In addition, populations of microorganisms in soil play a crucial role in modification of soil properties, thus changing the plant nutrition (Pate et al. 2001; Mukerji et al. 2006). Furthermore, soil nutrients are transferred into plant root from rhizosphere not without the active role of soil rhizobacteria. Rhizobacteria take important and beneficial part in plant growth and development through various ways (Glick 1995): fixing atmospheric nitrogen and transferring it to the plant; producing siderophores which bound soil iron and provide it to the plant that is able to take up the complex of bacterial siderophores and iron; synthesizing phytohormones such as cytokinins, gibberellins, and auxins, which can regulate the plant development; solubilization of phosphorus between other elements, thus making it more available to plant; and synthesizing the enzyme 1-aminocyclopropane-1-carboxilate (ACC) deaminase, which can lower plant ethylene level (Glick 1995; Glick et al. 2007a; Kidd et al. 2009; Richardson et al. 2009).

All the above-mentioned mechanisms are the main part of the so-called rhizosphere effect described first in 1904 (Hiltner 1904). The reason for that effect is exudation of nutrient molecules from plant roots to the surrounding soil – rhizoplane and rhizosphere. Many of these microbial populations not only benefit from plant exudates but have positive impact on the plant growth and development. These effects are cumulative result of the interaction between plant and plant-growth-promoting rhizobacteria (PGPR), antagonists, and pathogens (Schippers et al. 1990). Now many PGPR are used as bacterial inoculants for biofertilization, biocontrol agents, etc. (Shilev et al. 2012).

The focuses of this chapter are the abilities of PGPR and the mechanisms on which soil beneficial rhizobacteria improve plant nutrition.

Characteristics of Plant Growth Promoters

PGPR are widespread in almost all environmental conditions and include many genera like Cyanobacteria, Proteobacteria, *Bacteroides*, and *Pseudomonas* among many others (Tilak et al. 2005). In many cases, initial investigation in cultivated soil included study of the existence and activity of PGPR in order to estimate the capacity and necessity of the site. Thus, principal efforts were directed to change the chemical tools, as pesticides and fertilizers, with biological ones or environmental friendly via biotechnological approaches. This way could improve in times the safety of food, decreasing traces of undesirable compounds into the food chain.

Generally, the interactions between plants and bacteria can be divided into three parts: positive, negative, and neutral (Whipps 2001). Most autochthonous plant-associated rhizobacteria benefit from the interaction, while it is neutral for the plant. Many rhizobacteria in some conditions could negatively influence the growth and development of the plants because of pathogenic or parasitic activity and secretion of phytotoxic substances (Beattie 2006). In opposite, PGPR through direct and indirect mechanisms improve plant health. Glick et al. (2007a) generalize that the direct mechanisms are those affecting the balance of growth regulation of the plant, improving plant nutrition and stimulating plant resistance. On the other hand, the indirect mechanisms are related to biocontrol, including antibiotic production, chelation of available Fe in the rhizosphere, and extracellular enzyme synthesis in the rhizosphere (Zahir et al. 2004).

The PGPR possess different mechanisms that depending on the behavior could be described as biofertilizer, phytostimulator, or biocontrol agent. Biofertilizer is defined as substance containing microbial population that could colonize seeds, root surface, and other plant parts or soil and promotes plant growth through improved nutrient supply. In this case, the possible ways or mechanisms are related to the nitrogen fixation or utilization of insoluble phosphorus (Fuentes-Ramírez and Caballero-Mellado 2006; Vessey 2003). Another important term is based on the phytohormone production (cytokinins, gibberellins, and auxins) together with possession of ACC deaminase, thus decreasing interior plant concentration of ethylene. These are the phytostimulators. They have the ability to modify the concentration of plant growth regulators such as indole acetic acid (IAA) and ethylene (Somers et al. 2004). Finally, the biocontrol agents suppress the development of plant pathogens, thus indirectly stimulating plant growth (Vessey 2003; Somers et al. 2004). These abilities are possible due to antibiotic production, antifungal enzymes, systematic resistance, etc. Presently, the above-mentioned terms are widely applied in scientific papers, although sometimes it is difficult to be exact in determination of the effect of some PGPR due to combined impact on plant health.

According to Kloepper and Schroth (1978), bacterial populations that present one or more of these abilities are denominated as PGPR. Bashan and Holguin (1998) suggested the existence of two types of PGPR: plant-growth-promoting bacteria (PGPB) and biocontrol PGPB. This may include beneficial rhizosphere or non-rhizosphere bacteria. Also, Vessey (2003) consider that numerous species of soil

bacteria which live in plant rhizosphere may grow in, on, or around plant tissues stimulating plant growth by an abundance of mechanisms and are nominated as PGPR. In addition to these functional grouping, PGPR can be classified according to the plant compartment that they occupy as intracellular (iPGPR, symbiotics) or extracellular (ePGPR, free living), depending on the level of association with the root cells. The iPGPR live inside the root cells, generally in specialized structures, such as nodules, while the ePGPR are present on the root surface (rhizoplane) or between cells of root cortex (Gray and Smith 2005).

Impact of Rhizobacteria on Plant Nutrition

Nowadays, the use of rhizobacteria and microorganisms as a whole in agriculture to improve nutrient supply for plants is a very important practice (Freitas et al. 2007). Rhizobacteria-named biofertilizer could influence plant growth by direct or indirect mechanisms (Glick 1995). Direct stimulation may include benefits to the plants as fixed nitrogen, phytohormones, sequestered iron by bacterial siderophores, solubilized phosphate, and low ethylene level, while indirect plant stimulation is attributed to the biocontrol (antagonistic interrelations with soilborne phytopathogens) (Glick and Bashan 1997).

Direct Impact

Nitrogen Fixation

The nitrogen as a very important element for living beings, particularly for plants, part of the amino and nucleic acids, is a limited nutrient for plant growth and generally for agricultural production. Although the N presents 78 % of the atmosphere, it remains unavailable to the plants. The molecular N should be converted into ammonia – the available form for plants. There are three processes by which the atmospheric N is converted to plant useful compound: (1) oxidation of molecular N to oxides in atmosphere, (2) catalytic conversion of N to ammonia using very high temperatures, and (3) biological fixation of atmospheric N to ammonia by microorganisms through enzyme complex nitrogenase (Kim and Rees 1994). Soil bacteria that have the ability to "absorb" atmospheric N and convert it in form (ammonia) suitable for plants play a crucial role. The process name "nitrogen fixation" could be of two kinds: nonsymbiotic and symbiotic. The first one is realized by free-living diazotrophs stimulating growth of non-legume plants (Antoun et al. 1998). A lot of free-living soil bacteria and endophytic microorganisms that can use the atmospheric nitrogen, converting it into nitrogen-containing compounds needed for their growth are known (Cocking 2003). Generally this is the ability of genera of common rhizosphere-occupying bacteria as *Azotobacter*, *Acetobacter*, *Azospirillum*,

Burkholderia, *Enterobacter*, and *Pseudomonas* (Baldani et al. 1997; Vessey 2003; Mirza et al. 2006). Some of them are determined as endophytes. Endophytic diazotrophs may have advantage over rhizoplane-associated microorganisms, as they can colonize the root interior of plants and dispose their own niches that are more suitable to effective N_2 fixation and consequent transfer of the fixed compound to host plants (Baldani et al. 1997).

Because of high energy requirements for N fixation and the low metabolic activity of the free-living diazotrophs, together with the huge competition for exudated root compounds, the capacity and respectively the importance of nonsymbiotic bacteria to fix N are limited. Although in in vitro studies they show good capacity to fix N, in greenhouse or field experiments, the capacity is lower. According to the investigations of Dobbelaere et al. (2003), rhizobacteria are able to provide to plants significant quantities of N. In earlier studies, Okon and Labandera-Gonzalez (1994) calculated a contribution of 5 kg N ha^{-1} $year^{-1}$, as a result of inoculation of *Azospirillum* in rhizosphere of sorghum, maize, and wheat plants. Comparing such quantity to the habitual application of N fertilizers of 150–200 kg N ha^{-1} $year^{-1}$, the contribution of rhizobacteria seems insignificant. Different authors suggested range values describing the contribution of rhizobacteria to the soil nutrient supply. Their studies suggested that yearly amount per hectare due to the free-living diazotrophs is between 1 and 15 kg (Unkovich and Baldock 2008; Peoples et al. 2002). These results suggested that the free-living fixation is not an important ability for PGPR.

On the other hand, the role of symbiotic rhizobacteria is significant for their host, the legume plants. According to Höflich et al. (1994) and Franche et al. (2009), 90 % of legume plants' requirements are covered by symbiotic rhizobia that provide fixed atmospheric N_2 in the form of ammonia. The symbiotic fixation by bacteria is the most important mechanism but unfortunately exists only with host like legumes, some trees (*Frankia*), and shrubs. The genera widely presented as symbionts are *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Mesorhizobium* (Zahran 2001). They are members of family Rhizobiaceae, Gram-negative bacteria, which are able to infect the host, provoking nodule formation with active fixation of atmospheric N inside of the nodules. The fixation of N_2 is carried out by nitrogenase enzyme complex encoded by *nif* genes (Kim and Rees 1994). The essence of nitrogenase enzyme was elucidated by Dean and Jacobson (1992). The enzyme consists of two components: (1) dinitrogenase reductase, representing an iron protein, and (2) dinitrogenase, which has a metal cofactor. On the basis of the cofactor were identified three different N-fixing systems: Mo-dinitrogenase, V-nitrogenase, and Fe-nitrogenase. The existence of one or another fixing system depends on corresponding genera (Bishop and Joerger 1990).

Phosphorus Solubilization

Phosphorus (P) is an essential plant nutrient which has low availability in many agricultural soils. It is required for different metabolic processes such as photosynthesis, respiration, energy transfer, signal transduction, and macromolecular

biosynthesis (Khan et al. 2009). Also, it is one of the most important elements which limits plant growth (Fernandez et al. 2007). On the other hand, due to high application of fertilizers in the past years, soils have a high total P content. According to Rodríguez et al. (2006) and Richardson et al. (2009), much of this soil P is not available to plants due to its rapid rate of fixation/complexation with other soil elements. The P ion concentrations range between 0.1 and 10 μM, while the required are in the range of 1–5 μM for grasses and 5–60 μM for crops like pea (*Pisum sativum*) and tomato (*Lycopersicon esculentum*) (Raghothama 1999). It is present in soil in organic and inorganic form. The organic form is in humus, decayed animal, plant, and microbial tissues and represents between 20 and 80 % of total soil P (Richardson 1994). Other authors (Borie et al. 1989; Turner et al. 2002) suggested that the portion of organic P is between 30 and 50 % of the total one. The major part of inorganic forms of P is present as calcium phosphates in alkaline soils (Goldstein and Krishnaraj 2007) and aluminum and iron phosphates in acid soils (Mullen 2005).

Normally in agriculture, the solution of this problem is the application of P fertilizers, although it is expensive, less effective, and environmentally unsafe method. An alternative for improving crop production are phosphate-solubilizing bacteria (PSB) which may provide available P forms to plants. Such bacteria are considered as viable and promising biofertilizers because they can supply plants with otherwise unavailable forms (Khan et al. 2006). According to the same authors, the mechanisms of solubilization of phosphorus compounds are related to formation of organic chemicals such as organic acids (chelate mineral ions in soils), exopolysaccharides (hold the free P from the insoluble one in soils), enzymes (phytases and acid phosphatases mineralize organic P), assimilation of P (indirect dissolution of organic Ca–P compounds), and excretion of H^+ (from organic and inorganic acid leading the acidification of the solution).

Generally, the ability to solubilize insoluble forms of P has been attributed to their capacity to reduce pH by secreting organic acids (gluconic, citric, lactic, or succinic) or protons from NH_4^+ (Gyaneshwar et al. 1999; Mullen 2005). PSB are characterized by their capacity to solubilize precipitated forms in laboratory conditions and mainly are presented by members of genera *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, and *Pseudomonas* (Chung et al. 2005; Hariprasad and Niranjana 2009; Oliveira et al. 2009). Phosphorus in labile organic compounds normally is mineralized as available inorganic P or can be immobilized in the organic matter (McKenzie and Roberts 1990). On the other hand, the effectiveness and performance of PSB are affected by the environmental factors (Ahemad and Khan 2010). In spite of this, authors reported beneficial effect of inoculation of PSB alone or together with other rhizosphere microorganisms (Chen et al. 2008; Zaidi and Khan 2006).

It is evident that the solubilization of phosphates is not the unique tool for plant growth promotion of PSB. Many of them are characterized as PGPR and enhance the plant nutritional status through other mechanisms as synthesizing important growth substances (Mittal et al. 2008; Vassilev et al. 2006), antibiotics (Fernando et al. 2006), or biocontrol tools against soilborne pathogens.

Sequestering Iron by Bacterial Siderophores

PGPR secrete compounds named siderophores to sequester iron in the environment. Iron is essential for cellular growth and metabolism, so the Fe acquisition through siderophores plays an essential role in for the bacteria to colonize plant roots and to compete with other microorganisms in the rhizosphere (Crowley 2006). The siderophores secreted by the PGPR are low molecular weight iron chelators which are released under iron-limited conditions in the surroundings, possess high binding affinity and specificity for iron (III), and facilitate their transport into the bacterial cell (Schalk et al. 2001). They are small molecules (most of them are less than 1 kDa). Siderophores consist of lateral chains and functional groups that possess ligands with strong affinity to bind to the ferric ion (Neilands 1995). They are classified as catecholates, hydroxamates, and α-carboxylates depending on the nature and binding sites with the iron (Winkelmann 2002). In spite of this, siderophores produced by *Pseudomonas* species (typically PGPR) are classified as "mixed," e.g., pyoverdines contain hydroxamate and catecholate functional groups (Meyer and Stintzi 1998). The siderophores are produced as free ligands that become complexed with iron as released into extracellular environment. A ferric complex is then transported into the cell via specific transport receptor proteins. Inside the cell, the siderophore is freed from the transport receptor and again released outside as free ligand and can repeat the cycle (Kuhad et al. 2004). The secretion of siderophores may be assayed easily by a sample and universal method that is a modification of the method of Schwyn and Neilands (1987) made by Pérez-Miranda and coworkers (2007).

PGPR that produce siderophores combat the pathogenic microorganisms sequestering Fe^{3+} near the roots (Siddiqui 2006). The bacterial siderophores are used often by plants as iron source in spite of the total concentration is low for an important contribution for plant nutrition. On the other hand, plants have their own mechanisms to mobilize iron: converting Fe^{3+} into Fe^{2+} or production of phytosiderophores (Crowley 2006). In a number of studies, siderophore-producing bacteria have been isolated (Carrillo-Castañeda et al. 2002; Shilev et al. 2010). Fluorescent pseudomonads, among many others, are known to produce siderophores, the pyoverdines which are available in both homologous and heterologous uptake systems (Sharma and Johri 2003). Therefore, microbial activity plays an important role in iron acquisition in the rhizosphere. It is reported that under non-sterile soil system, plants show no iron-deficiency symptoms and have fairly high iron level in roots in contrast to plants grown in sterile system (Masalha et al. 2000). All these bacterial characteristics support the symbiotic interactions in the rhizosphere zone for mutual benefits of plants and microorganisms.

Phytohormone Production

Another direct mechanism by which PGPR improve plant growth is the production of phytohormones that are considered to enhance root surface and shoot biomass (Glick 1995; Vessey 2003). Most common phytohormones that have been well

characterized are auxins, cytokinins, and gibberellins (Patten and Glick 1996; Arshad and Frankenberger 1998). The indole-3-acetic acid (IAA, auxin) is a powerful phytohormone produced by PGPR. It controls a wide range of processes related to the plant development and growth and also has a key role in promoting root growth especially in lateral and polar hairs together with vesicular tissue differentiation and meristem maintenance (Aloni et al. 2006; Fukaki et al. 2007). According to Patten and Glick (1996), the biosynthesis of IAA by microorganisms involves (1) formation via indole-3-pyruvic acid and indole-3-acetic aldehyde, which is the most common mechanism in bacteria like *Pseudomonas*, *Rhizobium*, *Bradyrhizobium*, *Agrobacterium*, *Enterobacter*, and *Klebsiella*; (2) as an alternative way the transformation of tryptophan to indole-3-acetic aldehyde producing tryptamine (this pathway is characteristic for *Pseudomonas* and *Azospirillum*); (3) the synthesis of IAA producing indole-3-acetamide by some pseudomonads and pathogenic bacteria as *Agrobacterium tumefaciens*, *Pseudomonas syringae*, and *Erwinia herbicola* and some symbiotic bacteria as *Rhizobium*, *Bradyrhizobium*, and *Azospirillum*; and (4) transformation of tryptophan to indole-3-acetonitrile. Many genera are known to synthesize IAA in promoting plant growth. From this point of view, the rhizosphere bacteria are very important in converting tryptophan into auxin. Only few specific genes and proteins involved in IAA biosynthesis have been characterized till now that too in a small number of PGPR.

Shilev and coauthors (2010) reported growth promotion of sunflower plants in salt stress condition when population of IAA producing PGPR *Pseudomonas fluorescens* biotype F was applied into sand-peat growth substrate. The positive effect resulted in increase in fresh weight by more than 10 %, together with less Na^+ and more K^+ accumulation. So, there was positive effect on K^+/Na^+ ratio combined with improved root growth. On the other hand, PGPR was used in improving root growth rate and root biomass. A *Bacillus subtilis* strain which produces IAA was applied as a suspension on the surface of an edible plants of *Dioscorea rotundata* L. (Swain et al. 2007). As a result, an increase in roots and stems and of root-to-shoot ratio was observed. In a number of PGPR, genes involved in IAA production are regulated by several stress factors presented in the soil and in the rhizosphere (e.g., acidic pH, toxic ions, and osmotic stress). They have been shown to be activated by extracts of plant (amino acids such as tryptophan, tyrosine and phenylalanine, and auxins) (Ona et al. 2005; Prinsen et al. 1991; Van de Broek et al. 1999).

Cytokinins stimulate plant cell division, regulate root meristem differentiation, and inhibit primary root elongation and lateral root formation (Riefler et al. 2006; Silverman et al. 1998). The production of cytokinin has been reported in various PGPR such as *Arthrobacter*, *Azospirillum*, and *Pseudomonas fluorescens* among others (Cacciari et al. 1989; de Salamone et al. 2001; Perrig et al. 2007). However, because the involvement of genes in biosynthesis of bacterial cytokinins is not well studied in PGPR, their role in plant growth promotion is still consequence of conjectures.

Gibberellins enhance the development of stem tissue and promote root elongation and lateral root extension (Barlow et al. 1991; Yaxley et al. 2001). Production of gibberellins has been found in various PGPR such as *Azospirillum*, *Gluconobacter diazotrophicus*, *Azotobacter*, *Bacillus pumilus*, *Bacillus licheniformis*, *Herbaspirillum*

seropedicae, and rhizobia (Bottini et al. 2004; Gutiérrez-Mañero et al. 2001). The genes involved in production of gibberellins in bacteria are not yet identified.

Ethylene is a key phytohormone that can inhibit root elongation, nodulation, and auxin transport and promote seed germination, senescence, and abscission of various organs and fruit ripening (Bleecker and Kende 2000; Glick et al. 2007b). Ethylene is required for the induction of systemic resistance in plants during associative and symbiotic plant-bacteria interactions and, if high concentrations are present, is involved in plant defense pathways against pathogens (Broekaert et al. 2006; Glick et al. 2007b). A better knowledge is needed in order to determine growth-promoting effect of PGPR producing ethylene.

Lowering Ethylene Concentration

Some PGPR can lower plant ethylene level, thus stimulating plant root growth. Such mechanism is well known and consists in the action of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase on ACC (deamination on the plant ethylene precursor) forming NH_3 and α-ketobutyrate. Glick and collaborators (2007a) suggested that ACC is a source of N for the PGPR and some of them could utilize it as sole carbon source, thus lowering the ACC concentration – the immediate precursor of ACC. Thus, the ACC concentration in root surroundings is decreased, and the plant tries to maintain the equilibrium by exuding more ACC in the rhizosphere, lowering the internal levels. The ACC exudation is stimulated by the ACC deaminase containing bacteria, which is capable to utilize the compound as a unique source of carbon and nitrogen. The continuous exudation conducts to acceleration of growth of the population of bacteria containing ACC deaminase in the immediate vicinity to the roots. A main result is that the internal ethylene biosynthesis level is reduced as a consequence of lower concentrations of ACC (Glick et al. 1998).

This model has been validated in the case of *Azospirillum*, where the genome of the bacteria was complemented with an *acdS* gene from *Pseudomonas putida*, thus enhancing the beneficial effects of PGPR on both tomato and canola (Holguin and Glick 2001, 2003). A number of studies reported that the growth promotion effect of ACC deaminase in rhizobacteria is most effective in stress environments such as in flood, heavy-metal contamination, or salinity (Cheng et al. 2007; Farwell et al. 2007) and in response to phytopathogens (Wang et al. 2000).

It is clear that the PGPR effect occurs as a result of a combination of various mechanisms. A model has been proposed by Glick et al. (2007a) to describe effects of auxin and ethylene in both PGPR and plants. From the IAA effect, it is clear that in response to root exudates containing tryptophan, PGPR produce IAA that can be taken up by plant cells. Besides the direct effect of IAA on plant cell proliferation and elongation, it also induces the synthesis of ACC in plants and thus the production of ethylene (Abel et al. 1995). The inhibition of ethylene by the transcription of auxin response factors would lead to a decrease of ACC synthase activity and of ACC and ethylene biosynthesis (Glick et al. 2007a).

Indirect Impact

Although plant growth in agricultural soils is influenced by both abiotic and biotic factors, physical and chemical approaches are predominantly used to manage the soil environment and increase crop yields. The application of microbial products for this purpose is less common despite the enormous attention attracted to their role in reducing plant diseases. Significant control of plant pathogens and enhancement of plant development have been demonstrated by PGPR in the laboratory and in the greenhouse conditions. PGPR can influence plant growth by indirect mechanisms such as an antagonistic activity against harmful insects (Antoun and Prevost 2005), plant pathogenic bacteria, fungi, and nematodes (Oostendorp and Sikora 1989, 1990; Hasky-Günter et al. 1998; Frankenberger and Arshad 1995; Kim et al. 1998; Kumar et al. 2009). PGPR that indirectly enhance plant growth through suppression of phytopathogens use different mechanisms as well. The effect of these rhizobacteria has also been attributed to their ability to produce various compounds including iron-chelating siderophores (Neilands 1986; Carson et al. 1994) that make it unavailable to pathogens and hydrogen cyanide, which suppress the growth of fungal pathogens (Hassanein et al. 2009). They are able to synthesize antifungal antibiotics and fungal cell wall lysing enzymes or to compete with other soil microorganisms during root colonization for an ecological niche or a substrate. Rhizobacteria are capable to induce systemic resistance to pathogens (Compant et al. 2005; Haas et al. 2000) and abiotic stresses in host plants (Mayak et al. 2004; Nowak and Shulaev 2003). Despite their different ecological niches, free-living rhizobacteria and endophytic bacteria use some of these mechanisms to promote plant growth and control phytopathogens (Bloemberg and Lugtenberg 2001; Hallman et al. 1997; Lodewyckx et al. 2002; Maheshwari 2011). Direct mechanisms of plant growth promotion can be demonstrated in the absence of rhizosphere microorganisms including plant pathogens. Indirect mechanisms involve the ability of rhizospheric microorganisms to reduce the deleterious effects of plant pathogens on crop yield. Even in simplified model laboratory systems, the study of biocontrol involves interactions among a minimum of three organisms. Therefore, despite its potential in agricultural applications, biocontrol is one of the most poorly understood areas of plant–microbe interactions, and using bacterial species as biocontrol agents has not been extensively explored.

The production of antibiotics is considered to be one of the most powerful and studied biocontrol mechanisms against phytopathogens and the main characteristics of PGPR. In many cases, this is one of the reasons for screening rhizobacteria. There are numerous reports of the production and importance of antimicrobial metabolites. For instance, it was found that oomycin A is responsible for 70 % of the ability of *Pseudomonas* to reduce *Pythium* root infection of cotton and 50% of its ability to increase cotton seed emergence (Howie and Suslow 1991). The antibiotics produced by PGPR include butyrolactones, zwittermycin A, kanosamine, oomycin A, oligomycin A, phenazine-1-carboxylic acid, pyoluteorin, pyrrolnitrin, viscosinamide, xanthobaccin, and 2,4-diacetylphloroglucinol (2,4-DAPG) (Whipps 2001).

To demonstrate a role of antibiosis in biological control, mutants lacking production of antibiotics have been used. Mutant strain of *Erwinia herbicola* Eh1087 (Ant2) can grow at the same rate as wild-type strain Eh1087 but did not suppress development of the disease caused by *Erwinia amylovora* (Whipps 2001). Many other microbial metabolites have been studied for their antimicrobial activity, range, and mode of action. Many of them have a broad-spectrum activity. For example, the broad-spectrum activity of pyrrolnitrin, produced by *Pseudomonas* and *Burkholderia* species, has shown activity against a wide range of *Basidiomycetes*, *Deuteromycetes*, and *Ascomycetes*, including several economically important pathogens, and against several Gram-positive bacteria and in particular *Streptomyces* species (Raaijmakers et al. 2002). However, the classic and commercially successful biocontrol, based on the antibiotic-producing strains, is the application of nonpathogenic *Agrobacterium* against *Agrobacterium tumefaciens* (Whipps 2001).

Another widely studied microbial metabolites with low molecular weight (<1 kDa) are the siderophores. Although some siderophores are known to chelate other ions, their specificity to iron is the most consistent feature (Chincholkar et al. 2007). Several evidences indicate that siderophore production, when iron is limited, is responsible for the antagonism by some strains of *P. aeruginosa* against *Pythium* spp. (Antoun et al. 2005). Also, hydrogen cyanide (HCN) expression and production by *Pseudomonas* is dependent on iron availability (Keel et al. 1989) and may act synergistically with siderophores. Siderophores produced by rhizosphere microorganisms have been considered to not only improve rhizosphere colonization of producer strain but also play an important role in iron nutrition of plant (Vansuyt et al. 2007).

PGPR compete with communities of other microorganisms associated with the host plants, growing in the rhizosphere or on and in the host tissues (Compant et al. 2005). This competition in the rhizosphere plays main role when microorganisms compete for scarce nutrient resources. Even, if nutrients are limiting, the region around the root is relatively rich in nutrients due to the loss of as much as 40 % of plant photosynthates from the roots. The establishment of beneficial organisms on the roots limits the chance that a pathogenic organism that arrives later will find space to become established. It is competitiveness-related plant defense. Thus, high populations of PGPR may affect colonization not only of plant pathogens, but the greatest benefit of seed treatment may be inhibition of slightly parasitic or nonparasitic but toxigenic microorganisms, which is a significant advantage of the bioaugmentation.

Case Studies for PGPR-Based Immobilization of Heavy Metals

The following case studies are related to the immobilization of undesirable (toxic) metals in soil with the purpose to improve safety of food crops grown in such fields. The soil was industrially polluted in the past from a nonferrous

Table 5.1 Studied parameters in soil and compost on the basis absolute dry weight

Parameter	Method	Unit	Contaminated soil	Compost
Nitrogen – available	BDS ISO 14255	mg/kg	16.5 ± 0.8	609 ± 15
Phosphorus – available	Egner-Riem	mg/kg	33.2 ± 1.5	2,770 ± 75
Total nitrogen	VLM A29/A03	g/kg	1.35 ± 0.09	24.52 ± 0.77
Total phosphorus	VLM A29/VVLM 005	g/kg	0.31 + 0.02	9.01 ± 0.20
Organic carbon	BDS ISO 14235	g/kg	10.65 ± 0.57	342.7 ± 12.5
Organic matter (humus)	BDS ISO 14235	g/kg	18.36	590.8
Cadmium	ISO 14870	mg/kg	17.1 ± 1.2	0
Lead	ISO 14870	mg/kg	606 ± 16	0.9 ± 0.07
Zinc	ISO 14870	mg/kg	840 ± 31.7	9.3 ± 0.42

metalworks with Cd, Pb, and Zn. Although the soil is calcareous, in some sites, the availability of these metals is significant. According to the Bulgarian state standards (BDS), maximum permissible limits of heavy metals at pH 7.5 are as follows: Pb, 80 mg/kg; Cd, 2.5 mg/kg; and Zn, 340 mg/kg. In Table 5.1 are presented some of the most important parameters measured in the soil and compost.

The compost was result of composting of organic waste and mycelium from enzymatic and pharmaceutical production.

Effect of Compost Incorporation on Microbial Activity and Metal Bioavailability in Soil

In this section are presented results of investigation on immobilization of heavy metals in soil and the role of autochthonous microbial population. The experiment was carried out in boxes of 1 liter under controlled conditions with three treatments: contaminated soil, contaminated soil with 1 % of compost, and contaminated soil with 10 % of compost, and three repetitions for each treatment. During the experiment, the parameters observed were soil respiration, electroconductivity (EC), pH, dehydrogenase, and arylsulfatase soil activity (Alef and Nannipieri 1995), as well as available Cd, Pb, and Zn (ISO 14870).

From first day of the experiment, the microbial activity increased. This was evident through soil microbial respiration (Fig. 5.1), and it was highly pronounced in the treatment with 10 % compost. The enzyme β-glucosidase (β-d-glucoside glucosidase, EC 3.2.1.21) is limiting regarding microbial degradation of cellulose to glucose. The enzyme catalyzes the hydrolysis of glycosides in presence of water. Since the 15th day of the beginning of experiment, the formation of *p*-nitrophenol was increased in the treatments with addition of compost (Fig. 5.2). The activity of this enzyme was higher in treatment with 10 % compost comparing with the rest. When no compost was added, β-glucosidase activity maintained almost constant, without fluctuations during the study.

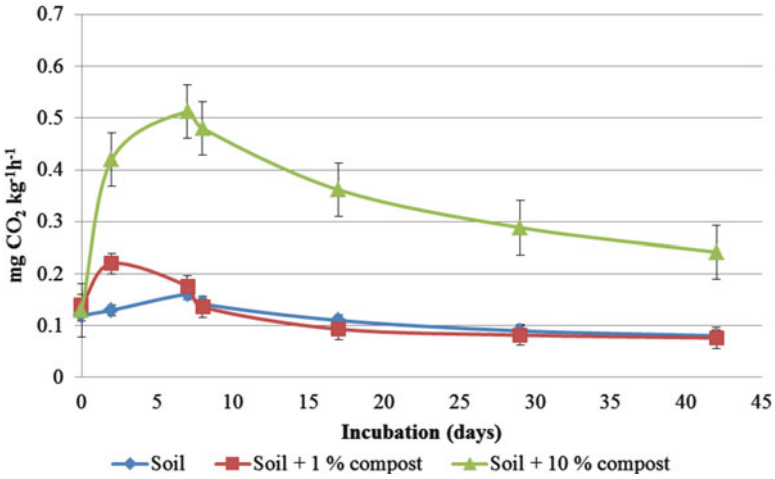

Fig. 5.1 Dynamics of intensity of soil respiration expressed per milligrams of CO_2 per kilogram of soil per hour. Results represent the mean value of three repetitions and the standard error

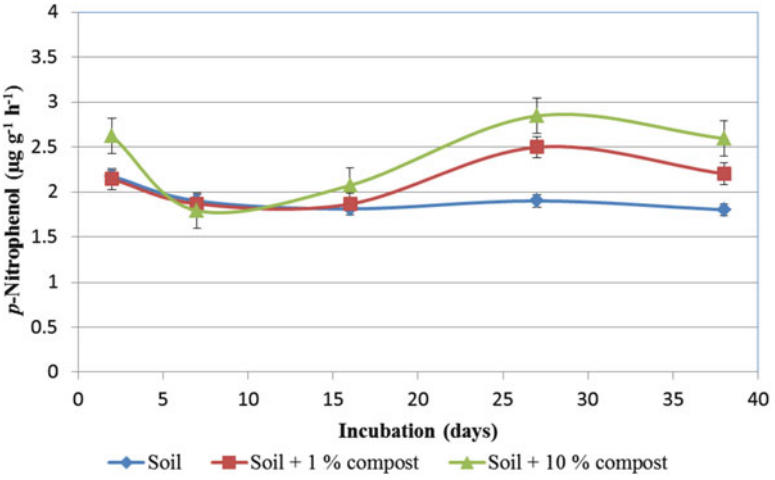

Fig. 5.2 Dynamics of β-glucosidase activity in soil expressed in milligrams of *p*-nitrophenol secreted per gram of soil per hour for each treatment. Results represent mean of three repetitions and the standard error

Generally the results regarding heavy-metal bioavailability suggested decreasing of availability when compost is presented. Moreover, higher concentration of compost decreased even more the available soil concentration of metals. It was strongly pronounced in case of Cd.

Fig. 5.3 Aspects of plants at the end of experiment

Role of Compost and PGPR on Growth and Metal Accumulation in Radish Plants

We carried out a pot experiment on immobilization of Cd and Pb in soil inoculating rhizobacteria *Pseudomonas fluorescens* biotype F for improving safety of radish (*Raphanus sativus* var. *radicula*) plants. The experimental design included four treatments: contaminated soil, contaminated soil supplemented with 10 % compost, contaminated soil supplemented with 10 % compost and rhizobacteria *P. fluorescens* biotype F, and contaminated soil supplemented with rhizobacteria *P. fluorescens* biotype F. In this experiment, same soil and compost was used as described in Table 5.1. The inoculation of rhizobacteria was made twice during the experiment, as liquid suspension in exponential phase on basis of concentration 10^6 c.f.u./cm^3 of soil. Plants were watered on the basis of 70 % water holding capacity (WHC). After 45 days, the plants were removed, and their fresh and dry weight was measured, while digested tissue samples were analyzed for the accumulation of Cd and Pb.

In Fig. 5.3 is presented the aspect of the plants at the end of experiment. The difference between the treatments (with or without compost) is very clear. The plants grown on contaminated soil without any supplementation were very weak and chlorotic, while those in treatments 2 and 3 were quite good in comparison to the first treatment (Table 5.2).

Generally, the accumulation of Pb and Cd was much higher in plants grown in contaminated soil without any supplementations. This resulted in tremendous reduction of plant fresh weight in this treatment. Although the fresh weight in treatment

Table 5.2 Accumulated concentration of Pb and Cd and fresh weight of radish plants at the end of experiment

	Cd		Pb		Fresh weight	
Treatments	Tubers	Shoots	Tubers	Shoots	Tubers	Shoots
Contaminated soil	18.4 ± 2.7	148 ± 51	36.2 ± 4.8	117 ± 33	2.1 ± 0.2	20 ± 2
Contaminated soil + compost	5.4 ± 0.6	62.9 ± 5.5	30.3 ± 7.1	46 ± 2	7.4 ± 0.5	74 ± 8
Contaminated soil + compost + PGPR	5.1 ± 0.1	49 ± 1.3	16.8 ± 1.1	48.6 ± 9	11 ± 0.4	102.9 ± 10
Contaminated soil + PGPR	10.2 ± 1	78 ± 17	25 ± 3.6	48 ± 4.7	3.2 ± 0.4	41.4 ± 3.2

The results represent the mean ± standard error of three replicates

with PGPR *P. fluorescens* was higher than those in plants grown in contaminated soil alone, it was much lower than in treatments supplemented with compost. The best results (for various plant parameters) were observed by treatment with compost and PGPR. Finally, it is possible to summarize from both the experiments that the optimal way of growing plants (radish in this case) with purpose to obtain maximum immobilization grade is a combination of matured compost with PGPR.

Conclusion

The use of PGPR is a very promising, proven, and environmentally friendly way to increase agricultural production. Because of the great variation in soil ecology from one region to other, each and every PGPR cannot be used separately as inoculant. The capabilities of PGPR to support plant growth have to be considered in their totality together with the plant-based mechanisms as solubilization and protection against pathogens. Although the combined effect of PGPR as well as the interactions of PGPR and plants are not very well understood, our opinion is that more important is the result of these interactions and it should be promoted.

Acknowledgements We acknowledge the financial support of Fund "Science investigation" of the Bulgarian Ministry of Education, Youth and Science for Bulgarian part of project COST Action FA0905 "Mineral improved crop production for health food and feed."

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Chapter 6
The Complex Molecular Signaling Network in Microbe–Plant Interaction

María A. Morel and Susana Castro-Sowinski

Contents

M.A. Morel
Laboratory of Molecular Microbiology, Clemente Estable Institute of Biological Research, Av. Italia 3318, CP 11600 Montevideo, Uruguay

S. Castro-Sowinski (✉)
Laboratory of Molecular Microbiology, Clemente Estable Institute of Biological Research, Av. Italia 3318, CP 11600 Montevideo, Uruguay

Department of Biochemistry and Molecular Biology, Faculty of Science, University of the Republic, Igua 4225, CP 11400 Montevideo, Uruguay
e-mail: s.castro.sow@gmail.com; scs@fcien.edu.uy

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_6, © Springer India 2013

Abstract Soil bacteria living around plants exert neutral, beneficial, or detrimental effects on plant growth and development. These effects are the result of signal exchange in which there is a mutual recognition of diffusible molecules produced by the plant and microbe partners. Understanding the molecular signaling network involved in microbe–plant interaction is a promising opportunity to improve crop productivity and agriculture sustainability. Many approaches have been used to decipher these molecular signals, and the results show that plants and microorganisms respond by inducing the expression of, and releasing, a mixture of molecules that includes flavonoids, phytohormones, pattern recognition receptors, nodulins, lectins, enzymes, lipo-chitooligosaccharides, exopolysaccharides, amino acids, fatty acids, vitamins, and volatiles.

This chapter reviews current knowledge of the diverse signaling pathways that are turned on when plants interact with beneficial microbes, with emphasis on bacteria belonging to the genera *Rhizobium*, *Azospirillum*, and *Pseudomonas*.

Beneficial Rhizospheric Microbes

Mutualistic association between microbes and plants brings benefits to the interacting partners. Some mutualistic microbes (plant–arbuscular mycorrhizal fungi interactions have been excluded from this chapter) are rhizospheric bacteria known as plant growth promoting rhizobacteria (PGPR) (Glick 1995) because they exert a positive influence on plant growth. Over the last decade, several PGPR have been isolated and used as bio-fertilizers, giving insight into good agronomical practices (Morel et al. 2012). Their contribution to plant growth promotion (PGP) can be exerted through direct and/or indirect mechanisms. Bacteria that use direct PGP mechanisms secrete metabolites such as hormones and polysaccharides, among other molecules, that influence root and shoot development. Indirect PGP effects include the secretion of bacterial metabolites with deleterious properties against the growth of phytopathogens (Lopez-Bucio et al. 2007). These bacteria are collectively called biocontrol agents.

The best-known microbe–plant mutualistic interaction is the diazotrophic microbial association with plants. Diazotrophs are free-living or symbiotic microbes that fix and reduce atmospheric nitrogen to ammonia. This process, called biological nitrogen fixation (BNF), is catalyzed by the bacterial enzyme nitrogenase (Masson-Boivin et al. 2009; Bhattacharjee et al. 2008). Examples of bacterial diazotrophs are *Azotobacter* (free-living diazotroph), *Azospirillum* (associative symbiont), *Azoarcus* and *Gluconacetobacter diazotrophicus* (endophytic non-nodular symbionts), and rhizobia (endophytic nodular symbionts). PGPR also produce phytohormones (Cassán et al. 2009), iron-sequestering siderophores (Yadegari et al. 2010), phosphate-solubilizing molecules (Wani et al. 2007), and/or 1-aminocyclopropane-1-carboxylate deaminase (Remans et al. 2007), among others. Examples of non-diazotrophic PGPR are *Pseudomonas* and *Bacillus* (Parmar and Dufresne 2011).

There is an exchange of signaling molecules between both interacting partner cells in mutualistic PGPR–plant interactions, leading to changes in gene expression.

This chapter reviews the progress in molecular signaling research involving beneficial microbe–plant interactions reported in recent years.

Rhizobia–Legume Symbiotic Association

The rhizobia–legume association is the best-known endosymbiotic microbe–plant interaction and, together with plant–mycorrhizal fungi interactions, is recognized for its importance in sustaining agricultural ecosystems and productivity. Rhizobia consist of several genera of the subclass Alpha- and Betaproteobacteria that are well known for their ability to form mutualistic associations, especially (but not exclusively) with leguminous plants (*Fabaceae*). Rhizobia induce the formation of root nodules where BNF occurs (Bapaume and Reinhardt 2012). The rhizobia–legume association is specific (each rhizobium establishes a symbiosis with only a limited set of host plants and vice versa). Plants mutually compatible with the same species of *Rhizobium* are called "cross-inoculation groups" (Morel et al. 2012).

Root colonization by rhizobia is accompanied with important changes in root architecture and gene expression in root and shoot, which lead to the nitrogen-fixing phenotype. During the process of BNF, rhizobia provide reduced nitrogen to the plant in exchange for carbohydrates and a micro-aerobic environment for the effective functioning of the oxygen-sensitive nitrogenase. Establishment of the symbiosis requires the reciprocal recognition of partners and the production of various signaling molecules that are required to regulate nodule initiation and differentiation and nitrogen fixation. Briefly, nitrogen fixation is preceded by root morphological changes that include highly coordinated events. Most legumes constitutively release root-diffusible attractant signal molecules (flavonoids), which trigger rhizobial production of specific lipo-chitooligosaccharides known as nodulation factors (Nod Factors or NFs) (Hassan and Mathesius 2012) (see section "Extracellular Polysaccharides"). NFs are among the most important molecules in the microbe–plant dialog, mediating rhizobia recognition by the plant root and nodule organogenesis (Masson-Boivin et al. 2009). NF recognition is accompanied by curling of root hairs, where bacteria are entrapped, and formation of plant-derived infection threads (IT) that carry the rhizobia into the dividing cells of the inner cortex, the nodule primordium (Fournier et al. 2008). Then, rhizobia are released into the nodule primordium where they differentiate into bacteroids, the symbiotic rhizobial form that expresses the nitrogen-fixing enzyme, nitrogenase (Oldroyd et al. 2011).

Rhizobia–legume symbiosis is regulated by transcriptional reprogramming of host cells that ensures the functioning of the nodule. Many reprogrammed genes are membrane proteins with important roles in signaling, intracellular accommodation, and nutrient transport (Bapaume and Reinhardt 2012; see section "PGPR and Plant Root Attachment"). In addition to BNF, most rhizobia have been found to produce auxins. The roles of auxins in rhizobia–legume interactions are related to plant growth and nodule organogenesis (Lambrecht et al. 2000; see section "Phytohormones Production").

Azospirillum–*Plant Association*

Bacteria belonging to the genus *Azospirillum* are free-living, nitrogen-fixing, surface-colonizing, and, sometimes, endophytic diazotroph Alphaproteobacteria (family Rhodospirillaceae). *Azospirillum* spp. establish associations that are beneficial to plants, but with no apparent preference for specific plants, and can be successfully applied to plants that have never been colonized before by azospirilla (Bianco and Defez 2011; Guerrero-Molina et al. 2011; Reis et al. 2011). Currently, there is a limited market for commercial bio-fertilizers for non-legume crops based on *Azospirillum* spp., but they have been shown to be efficient PGPR (Figueiredo et al. 2010).

Azospirillum is a nitrogen-fixing microbe, but given that azospirilla promote plant growth even in nitrogen-rich conditions, PGP by *Azospirillum* might be attributed to other mechanisms rather than BNF (Okon and Kapulnik 1986), such as deamination of the ethylene precursor 1-aminocyclopropane-1-carboxylate and siderophore (Tortora et al. 2011), auxin, or nitric oxide production (Baudoin et al. 2010; Spaepen et al. 2007). Among these PGP properties, auxin production is thought to be the main mode of action of *Azospirillum brasilense*. This assumption was corroborated in experiments using genetically modified azospirilla that showed enhanced auxin production (Baudoin et al. 2010; Spaepen et al. 2007, 2008). Many other workers have also reported that plant hormone production by *Azospirillum* spp. is the main mechanism that explains the PGP effect (Reis et al. 2011; Bashan et al. 2004; Lambrecht et al. 2000; Okon and Labandera-Gonzalez 1994). Auxin production by azospirilla promotes root development and proliferation, leading to enhanced nutrient uptake (Lambrecht et al. 2000) and increased root exudation of molecules to the rhizosphere. Molecules exuded by the root act as chemoeffectors that attract azospirilla to the rhizosphere (chemotaxis), thereby increasing the chance of root–bacterial interactions. This was, and still is, the mechanism that in fact explains how azospirilla promote plant growth (Hayat et al. 2010).

Azospirilla are considered "helper" bacteria that promote rhizobia–plant interactions (Morel et al. 2012). Co-inoculation with azospirilla stimulates nodulation (early nodulation and more nodules), nodule function, and plant growth and development when compared with inoculation with rhizobia alone (Bianco and Defez 2011; Remans et al. 2008). The evidence supports a mix of molecules secreted to the rhizosphere being involved in improving rhizobia–legume association. Auxin production by azospirilla, during co-inoculation, stimulates morphological and physiological changes in the root system, increasing the number of potential sites for rhizobial infection, thus leading to a much higher number of nodules (Bianco and Defez 2011). Some direct evidence also suggests that during co-inoculation, *Azospirillum* spp. induce the synthesis of chemoattractant flavonoids by roots of chickpea, common bean, and alfalfa (Star et al. 2012; Dardanelli et al. 2008; Burdman et al. 1996; Volpin et al. 1996).

Other PGPR–Plant Interactions

There is a long list of microbes that establish beneficial interactions with plants, but some endophytes and *Pseudomonas* head the list.

Endophytes

Endophytes are bacteria that infect and colonize the plant apoplast, evading or suppressing the host plant defense system. Many facultative endophytic bacteria can also survive in the rhizosphere, where they can enter their host plant via the roots (Badri et al. 2009). PGPR are bacteria that live in soil near the root, colonize the root surface, reside in root tissue, or live inside plant cells in specialized structures, promoting plant growth; thus, most endophytes might be considered PGPR. Given the semantic overlap and the difference between PGPR and endophytes, many researchers have adopted two simple terms: intracellular PGPR (iPGPR), for bacteria residing inside plant cells, and extracellular PGPR (ePGPR) for those bacteria living outside plant cells, root surface, or rhizosphere (Gray and Smith 2005). However, the definition of endophytes is still controversial. Many authors claim that ePGPR are simply epiphytes and iPGPR are just endophytes (Ikeda et al. 2010).

In endophyte–plant interactions, bacteria are not restricted to a specific compartment within the plant but can be found in roots, stems, and leaves. Like rhizobia, most endophytes commonly used as inoculants are diazotrophs that improve plant growth. Examples of endophyte–plant interactions are *Burkholderia* and sugarcane, *Herbaspirillum* and a broad range of host plants, and *Azospirillum* and rice (Govindarajan et al. 2008). It has been shown that crop yield increase after endophyte inoculation is mainly due to BNF. Details about endophytes for non-legumes can be read in Bhattacharjee et al. (2008).

Pseudomonas

The genus *Pseudomonas* includes the most diverse and ecologically significant group of bacteria, belonging to the class Gammaproteobacteria. They are ubiquitously distributed in terrestrial and marine environments and have been found associated with animals and plants (Kiil et al. 2008). Their genetic diversity is a reflection of their ecological diversity (Silby et al. 2009). Many *Pseudomonas* spp. have been extensively studied as PGPR. There is evidence that some *Pseudomonas* spp. produce siderophores (Rosas et al. 2006), phenolic compounds (Combes-Meynet et al. 2011), lytic enzymes (Egamberdieva et al. 2010), and phytohormones (Pallai et al. 2012; Khalid et al. 2011; Khakipour et al. 2008); solubilize phosphate (Azziz et al. 2012); act as biocontrol agents of phytopathogenic microbes (Quagliotto et al. 2009); and induce systemic resistance (Bakker et al. 2007), thus

promoting plant growth. Moreover, some rhizospheric *Pseudomonas* spp. interact synergistically with other PGPR, assisting PGPR–plant colonization and suppressing plant pathogens (Parmar and Dufresne 2011). Many studies support the action of *Pseudomonas* spp. as "helper" bacteria during the establishment of the rhizobia–legume interaction, evidenced by the promotion of plant growth during co-inoculation (Morel et al. 2012; Malik and Sindhu 2011). This helper effect might be explained by the production of phytohormones (Malik and Sindhu 2011; Egamberdieva et al. 2010), a qualitative change in plant-secreted flavonoids (Parmar and Dadarwal 1999), or the solubilization of non-available nutrients (mainly refixation of exogenously applied phosphorus), among other actions (Medeot et al. 2010).

Delftia

Recently, a new genus has emerged as a PGPR. Bacteria belonging to the genus *Delftia* have been described as novel PGP microbes (diazotrophic and biocontrol agents against various plant pathogens). They fix atmospheric nitrogen, produce the auxin indole-3-acetic acid and siderophores, promote alfalfa and clover growth under nitrogen-rich conditions, and assist as a "helper" bacterium during rhizobia–legume interaction, probably due to auxin production (Ubalde et al. 2012; Morel et al. 2011; Han et al. 2005).

Early Signaling Events: The Role of Root Exudates

The root system of plants imports water and nutrients from the soil solution but also releases low- and high-molecular-weight compounds to the rhizosphere. Root exudates are composed of a broad range of root-secreted molecules that act as a complex chemical cocktail that mediates interactions occurring in the rhizosphere and shapes soil microbial communities (Okumoto and Pilot 2011). Their chemical composition is influenced by environmental conditions, plant genotype, and the multipartite interactions occurring in the rhizosphere, among other factors.

Carbon-based compounds are the main constituent of this complex cocktail, but ions, oxygen, and inorganic acids are also important components with relevant roles during rhizospheric interactions (Badri and Vivanco 2009). Exuded molecules include low-molecular-weight compounds, such as sugars and phenolics, and high-molecular-weight compounds such as polysaccharides and proteins, which often compose a larger proportion of the total mass of the exudate (Cai et al. 2012). Even though these chemicals are root-secreted, many rhizobacteria also secrete metabolites that contribute to the pool of molecules that mediate rhizospheric interactions (Badri et al. 2009). Table 6.1 summarizes examples of these bacterial-secreted compounds and their general role in plants. The sections below describe current knowledge of different plant and bacterial metabolites involved in microbe–plant interactions.

Table 6.1 Some bacterial-secreted compounds and their role in plant physiology and architecture

Chemical group	Bacterial metabolite	Plant response	Reference
Phytohormones	Salicylic acid, jasmonic acid, and ethylene	Immune plant defense activation through SAR[a] (mainly) and ISR[b]	Bent (2010), Bakker et al. (2007), Ping and Boland (2004)
		Inhibition of legume response to NF and rhizobia	Oldroyd and Downie (2008), Ramos Solano et al. (2009), Ding et al. (2008), Sun et al. (2006)
	Cytokinins, auxins, and gibberellins	Phyto-stimulation. Morphogenesis	Morel et al. (2011), Cassán et al. (2009), Ferguson and Beveridge (2009), Boiero et al. (2007), Remans et al. (2007), (2008), Lopez-Bucio et al. (2007), Spaepen et al. (2007)
	Auxins	Pathogenesis (i.e., gall induction, necrotic lesions)	Ding et al. (2008), Chalupowicz et al. (2006), Robert-Seilaniantz et al. (2007), Lambrecht et al. (2000)
N-acyl-L-homoserine lactones (AHLs) and QS[c]-related signals	AHLs	Modulation of root system architecture	Ortiz-Castro et al. (2008a), von Rad et al. (2008)
		Induction of ISR	Schuhegger et al. (2006)
	AHL-degrading lactonases	Interference with QS signals required for virulence in phytopathogens	Friesen et al. (2011)
Volatile organic compounds	Acetoin, butanediol, 1-octen-3-ol, and butyrolactone	Modulation of root system architecture	Gutierrez-Luna et al. (2010), Lopez-Bucio et al. (2007)
		ISR	Ryu et al. (2005), Ping and Boland (2004)
Phenolic compounds	Flavonoids, phenolic acids	*nod*-gene inducers	Mandal et al. (2010), Parmar and Dadarwal (1999)
		Antimicrobial agents, ISR	Combes-Meynet et al. (2011), Parmar and Dufresne (2011)
Lipopolysaccharides (LPS) and extracellular-related factors	Siderophores, LPS	ISR	Ping and Boland (2004)

[a] *SAR* systemic acquired resistance
[b] *ISR* induced systemic resistance
[c] *QS* quorum sensing

Phytohormones Production

Phytohormones are chemical messengers produced by plants and microorganisms, which coordinate plant cellular activities at low concentrations (Ferguson and Beveridge 2009). Common phytohormones belong to five major classes: auxins, cytokinins, gibberellins, abscisic acid, and ethylene. Other known phytohormones are brassinosteroids, salicylic acid, jasmonates, polyamines, nitric oxide, strigolactones, etc. (Pieterse et al. 2009). The following microbes are known phytohormone producers: *Pseudomonas* (Khakipour et al. 2008), *Azospirillum* (Khalid et al. 2011), rhizobia (Etesami et al. 2009), *Bacillus* (Lim and Kim 2009), and *Delftia* (Morel et al. 2011). Microbial secreted hormones, mainly cytokinins (CKs) and auxins, act as signaling molecules that coordinate changes in plant cell division and differentiation, affecting root and shoot architecture and functioning (Boiero et al. 2007; Lopez-Bucio et al. 2007; Ryu et al. 2005). In this section, we review information concerning phytohormones (auxins and CKs) that positively correlate with PGP during microbe–plant interaction (Tables 6.2 and 6.3).

The information supports the view that a mix of phytohormones, rather than a single effector, acts to control plant cellular processes at multiple levels (Yoshimitsu et al. 2011), including major effects on plant growth and the induction of plant immune defenses. During the microbe–plant interaction, bacterial-produced phyto-hormones, mainly auxins and CKs, also have phyto-stimulation effects (Robert-Seilaniantz et al. 2007). Most of the information that supports this affirmation was gathered working in the areas of rhizobia–legume and azospirilla–wheat interactions.

CKs are purine derivatives produced in root tips and developing seeds and are transported via the xylem from roots to shoots (Ortiz-Castro et al. 2009). Some effects of CKs in plants are the induction of root and shoot cell division, cell growth and dedifferentiation, apical dominance, lateral bud growth, leaf expansion, and delayed senescence. Zeatin is the most common CK, but other cytokinin-like substances are known: isopentenyladenine, isopentenyladenosine, zeatin riboside, and dihydrozeatin riboside (Davies 2010). CKs are probably the most studied phytohormones involved in nodule organogenesis (Ariel et al. 2012; Op den Camp et al. 2011; Oldroyd and Downie 2008; Murray et al. 2007; Tirichine et al. 2007). They have been proposed as secondary signal molecules that perceive NF at the root epidermis. In response to NF application at roots, a local increase in CK levels is detected, which induces nodule primordial development in the cortex cells, thus influencing bacterial infection (Heckmann et al. 2011; Ding et al. 2008; Murray et al. 2007; Oldroyd 2007; Tirichine et al. 2007). For instance, Murray et al. (2007) and Tirichine et al. (2007) showed that plant CK signaling pathway activation by rhizobial cells is necessary (and sufficient) to activate nodule formation in *L. japonicum*. CK production by plant-associated bacteria, other than rhizobia, has also been well documented. Some examples of CK-producing bacteria are *Bacillus megaterium* and *Azospirillum* (Ortiz-Castro et al. 2008b).

Many plant pathogenic bacteria also secrete CK analogs or activate plant CK production to form gall structures, leading to delayed senescence activity and suppression of plant basal defense mechanisms (Chalupowicz et al. 2006).

Table 6.2 Effects of bacterial auxins on microbe–plant interaction: root architecture and/or physiology

PGPR–plant system	Strategy used during the study	Effect	Possible mechanism of action	Reference
Sinorhizobium meliloti–*Medicago truncatula*	Proteomics of roots. Effect of inoculation and exogenous application of auxin (without inoculation)	Similar accumulation level in inoculated and auxin-treated plants	Auxin is a positive regulator of nodulation initiation	Van Noorden et al. (2007)
Azospirillum brasilense–*Triticum* sp. (wheat)	Plant growth. Inoculation with overproducing IAA[a]—*A. brasilense*	Increased shoot biomass, thinner roots, and no significant effect on root biomass (a month after inoculation)	Transient positive effect of bacterial IAA on root development	Baudoin et al. (2010)
Bacillus subtilis and *B. licheniformis*–*Capsicum* sp. (red pepper) and *Solanum lycopersicum* (tomato)	Plant growth and seed germination. *Bacillus* co-inoculation and *Bacillus* purified auxins exogenous application	Increased root, stem, and leaf growth and seed germination	Bacterial auxins are major factors responsible for plant growth promotion	Lim and Kim (2009)
Pseudomonas aeruginosa and *A. brasilense*–wheat and rice	Plant growth and yield. Inoculation under field conditions	Increased number of tiller, straw and grain yield	Auxin-producing PGPR positively affect plant growth	Khalid et al. (2011)
B. japonicum and *A. brasilense*–*Glycine max* (soybean)	Plant growth and seed germination. Inoculation and co-inoculation	Promotion of seed germination and PGP of soybean seedlings in co-inoculated plants	PGPR excretion of IAA (auxin) promotes young seedlings	Cassán et al. (2009)
Rhizobium galegae and *Pseudomonas* spp.–*Galega orientalis* (Galega)	Plant growth and nodulation. Inoculation and co-inoculation experiments	Increased shoot and root dry matter, number of nodules, and nitrogen content of co-inoculated plants	*Pseudomonas* spp. produce auxin and cellulase as mechanism to enhance symbiotic performance of rhizobia	Egamberdieva et al. (2010)
Mesorhizobium sp. and *Pseudomonas* spp.–*Cicer arietinum* (chickpea)	Plant growth and nodulation parameters. Inoculation and co-inoculation experiments	Increased shoot and root dry matter, number, and biomass of nodules in co-inoculated plants	Enhanced nodulation in chickpea by auxin secretion	Malik and Sindhu (2011)

[a]*IAA* indol-3-acetic acid

Table 6.3 Effects of cytokinins (CKs) on microbe–plant interaction: root architecture and/or physiology

PGPR–plant system	Strategy	Effect	Possible mechanism	Reference
Mesorhizobium loti–Lotus japonicum	Nodule organogenesis. Inoculation and CK application (without inoculation) of plant *hit1*[a] mutants	Hit1 roots are insensitive to exogenously applied CK and rhizobia inoculation	A CK receptor (LHK1) is required for the activation of *Nin*[b] and nodule organogenesis	Murray et al. (2007)
M. loti–L. japonicum	Nodule organogenesis in a *L. japonicum snf2* mutant[c]	Spontaneous development of root nodules in absence of rhizobia or rhizobial signal molecules	CK signaling is required for nodule initiation and is a key element in dedifferentiation of cortical cells	Tirichine et al. (2007)
M. loti–L. japonicum	Plant growth and nodule organogenesis. Inoculation and exogenous CK application to plant mutants for CK response	Formation of discrete and easily visible nodule primordial. Expression of nodulin genes	Root cortical cell activation by CK depends on LHK1	Heckmann et al. (2011)
B. subtilis (CK-producing strain)–*Lactuca sativa* (lettuce)	Plant growth and hormone determination of shoot and root. Inoculation under drought stress	Increase of CKs in shoot. Weight increase in shoots and roots	Inoculation with CK-producing bacteria may have a beneficial result under moderate drought	Arkhipova et al. (2007)
B. megaterium–Arabidopsis thaliana	Plant growth. Inoculation of plants with mutations in three CK receptors (single, double, and triple mutants)	Reduced PGP in single and double mutant. Non-PGP in triple mutant	PGP can be mediated by different CK receptor homologs	Ortiz-Castro et al. (2008b)
B. japonicum and *A. brasilense–G. max*	Plant growth and seed germination. Inoculation and co-inoculation	Promotion of seed germination, PGP of soybean seedlings	PGPR excrete zeatin (CK) at sufficient concentration to produce PGP in young seed tissues	Cassán et al. (2009)

[a] *Hit1* genetic suppressor of the hyperinfected phenotype; abundant infection threads formation but failed cortical cell division
[b] *Nin* nodule inception regulator
[c] *snf2* an allele of a lotus histidine kinase LHK1 gene; spontaneous nodule formation

The production of CKs enhances pathogenicity and modulates the physiology of host plants (Choi et al. 2011). In contrast, plant-derived CKs may be involved in plant resistance to pathogen infection (Choi et al. 2011). However, the molecular mechanisms of CK action in disease resistance against a wide spectrum of pathogens and the reason for the opposite effects of CKs on plant responses against pathogens are still unclear.

In addition to CKs, auxins also influence plant growth. Auxins are compounds with aromatic ring and carboxylic acid groups. The increasing amount of data about bacterial strains with the ability to increase the pool of auxins available to plants leads to the assumption that their production is one of the major direct factors that promote root and plant growth (Khalid et al. 2011; Ali et al. 2009). Auxins act on root architecture increasing the number of lateral roots and root hair elongation. They are also responsible for apical dominance acting as a signaling molecule in root and shoot growth (Ferguson and Beveridge 2009). As a result of increased root bulk, the plant may scavenge a larger area for nutrient and water uptake, and the root has a larger number of potential niches for beneficial or pathogenic microbial infection.

Tryptophan is an amino acid commonly found in root exudates, and it is the main precursor of auxin biosynthesis (Etesami et al. 2009). Indole-3-acetic acid (IAA) is the main auxin in plants, controlling cell enlargement and division, tissue differentiation, and responses to light and gravity. Many PGPR, such as *Azospirillum*, *Pseudomonas*, *Delftia*, and *Rhizobium* species, induce root proliferation through IAA production (Morel et al. 2011; Spaepen et al. 2007, 2008; Kapulnik et al. 1985). However, various phytopathogens also have the ability to produce IAA and/or alter its levels in plants, facilitating host infection and virulence and causing uncontrolled growth in plant tissues (mainly tumor and gall induction) (Chalupowicz et al. 2006; Robert-Seilaniantz et al. 2007). *Agrobacterium*, *Pseudomonas*, and *Erwinia* produce IAA as part of their pathogenic behavior (Lambrecht et al. 2000). Other indolic compounds with auxin activity are indole-3-butyric acid, indole-3-pyruvic acid, indoleacetamide and indole-2-carboxylic acid (Lim and Kim 2009).

Gibberellins and brassinosteroids also play an important role during nodule formation (Oldroyd and Downie 2008). It has been shown that brassinosteroids act together with auxins on many developmental plant processes (Yoshimitsu et al. 2011). Strigolactones are plant hormones that contribute to apical dominance (Ferguson and Beveridge 2009). They are exuded by roots in extremely low concentrations (Steinkellner et al. 2007). They act as chemical signals for root colonization by symbiotic arbuscular mycorrhizal fungi and inhibit shoot branching. Even though there are no reports of microbial production of strigolactones, it has been suggested that this class of phytohormones has biological signaling functions in the rhizosphere (Tsuchiya and McCourt 2009; Steinkellner et al. 2007).

Other Secondary Metabolites

Plants produce an extremely diverse array of low molecular mass compounds, often called secondary metabolites, which include, among others, alkaloids, essential oils

or essences, steroids, terpenoids, and phenolic compounds. Some secondary metabolites are commonly found in plants, but others are specific to only a few related plant species and/or are produced in particular conditions (Pichersky and Gershenzon 2002). Most of them are signaling molecules, and even if their roles in signaling are unknown, some are strictly necessary, like flavonoids. Here, we summarize some of the highlights of plant secondary metabolites involved in plant–microorganism interaction, other than phytohormones, which have been covered in section "Phytohormones Production."

Volatile Organic Compounds (VOCs)

VOCs are molecules that have high vapor pressure and vaporize to the atmosphere under normal conditions (Ortiz-Castro et al. 2009). The first report of a plant VOC was the plant hormone ethylene in the year 1910 (recognized as cell-to-cell signal transmission in 1934 by Gane) (Bleecker and Kende 2000). Since then, it has been accepted that plants produce and release a variety of diffusible compounds, including low molecular weight compounds, such as terpenoids, modified fatty acids, benzenoids, and other scented substances (Ortiz-Castro et al. 2009; Ping and Boland 2004). Improved techniques for the collection and analysis of volatiles, such as gas chromatography-electroantennographic detection, have allowed the detection of new plant VOCs (Pichersky and Gershenzon 2002). VOCs act as plant growth regulating substances that affect other organisms, acting, for example, as attractants and/or repellents. Recently, some authors demonstrated that some PGPR can produce VOCs as signals that stimulate the growth of plants (Gutierrez-Luna et al. 2010). Examples of PGPR-producing VOCs are *B. megaterium* (acetoin and butanediol producer) (Lopez-Bucio et al. 2007) and *Bacillus* spp. (1-octen-3-ol and butyrolactone producer) (Gutierrez-Luna et al. 2010). Many VOCs have been detected in rhizospheric soil, but their role in microbe–plant interactions is still uncertain. It has been suggested that VOCs may have antibiotic functions acting in the control of plant pathogens, induce different phytohormonal signaling networks (Ortiz-Castro et al. 2009), or activate induced systemic resistance (ISR) via a salicylic acid- and jasmonic acid-independent pathway (Ping and Boland 2004). For example, the VOCs 2,3-butanediol and acetoin, released by *Bacillus* spp., trigger growth promotion of *Arabidopsis* seedlings and induce systemic resistance against *Erwinia carotovora* (Ryu et al. 2005). It was concluded that VOCs activate a CK-dependent pathway for PGP and an ethylene-dependent signaling pathway for ISR (Ping and Boland 2004).

Phenolic Compounds

Phenolic compounds are produced by plants and microbes as well, but they differ in chemical structure (Combes-Meynet et al. 2011; Mandal et al. 2010; Parmar and Dadarwal 1999). Increasing evidence suggests that root-secreted polyphenols initiate and modulate the dialog between roots and soil microbes (Badri and Vivanco 2009).

Table 6.4 Plant-secreted flavonoids induce *nod* genes

PGPR	Plant	Flavonoid(s)	Effect	Reference
Sinorhizobium meliloti	Alfalfa	Luteolin(3′,4′, 5,7-tetrahydroxyflavone)	*nod*-gene inducer	Peters et al. (1986)
S. meliloti	Alfalfa	4,4′-dihydroxy-2′-methoxychalcone, 4′,7-dihydroxyflavone, 4′-7-dihydroxyflavanone	Flavonoids, other than luteolin, are *nod*-gene inducers	Maxwell et al. (1989)
S. meliloti	Alfalfa	Chrysoeriol and luteolin	*nod*-gene induction	Hartwing et al. (1990)
Azospirillum brasilense (co-inoculation with *Rhizobium tropici* and *Rhizobium etli*)	Common bean	Daidzein, naringenin, genistein, and coumestrol	Increased root hair formation, nodule number, *nod*-gene induction	Burdman et al. (1996)
Rhizobium leguminosarum	Pea and lentil	Hesperetin and naringenin	*nod*-gene induction	Begum et al. (2001)
Bradyrhizobium japonicum	Soybean	Coumestrol	Increased number of nodules, high degree of biofilm formation. Weak *nod*-gene induction	Lee et al. (2012)

Flavonoids are plant phenolic compounds recognized as important signaling molecules in microbe–plant interaction events. The main subclasses of flavonoids include chalcone, flavone, isoflavone, flavonol, flavanone, and isoflavonoid compounds (Cesco et al. 2012). The effects of flavonoids in the rhizosphere depend on their chemical composition and concentration. In the rhizosphere, they have a critical role in early stages of the rhizobia–legume symbiotic interaction and in plant defense. The best-known roles attributed to plant flavonoids are in chemoattraction of rhizobia to the legume root and as primary molecular signals for rhizobial *nod-gene* induction and NF production (Mandal et al. 2010; Badri et al. 2009; Oldroyd and Downie 2008; Steinkellner et al. 2007). A wide variety of plant flavonoids have been shown to induce NF production in different rhizobia–legume interactions (Table 6.4). In the presence of compatible rhizobial strains, the legume host increases the exudation of a particular set of flavonoids, e.g., in the presence of *Sinorhizobium* strains, alfalfa produces increased amounts of the flavonoid luteolin. Flavonoids protect dividing cells from oxidative damage due to their antioxidant properties and ability to modulate several enzymes (Ariel et al. 2012; Cesco et al. 2012).

The genome-wide transcriptional response of *Bradyrhizobium japonicum* to genistein showed that 100 genes were induced, including all *nod* box-associated

genes and flagellar and transport process genes, suggesting that genistein has a much broader function than *nod*-gene induction (Lang et al. 2008). Flavonoids (naringenin and hesperetin) are also factors that influence rhizobial competitiveness in soils, as showed in several biovars of *Rhizobium leguminosarum* (Maj et al. 2010). Flavonoids also participate in plant host specificity for few rhizobial species. Plants secrete a characteristic group of inducing and non-inducing flavonoids that are recognized by rhizobial outer membrane protein, NodD (the LysR-type transcriptional regulator that mediates the expression of *nod* genes and a key determinant of host specificity). Both inducing and non-inducing flavonoids bind NodD and mediate conformational changes at *nod*-gene promoters, but only a few set of flavonoids are capable of promoting *nod* genes. The production of non-inducing flavonoids may be a mechanism by which legumes prevent overnodulation (Peck et al. 2006). The rhizospheric microbial community may also alter the amount and composition of *nod*-inducing signals secreted by the plant. Many reports showed that the inoculation of leguminous plants with *Azospirillum* induces the secretion of a particular set of *nod*-inducing flavonoids that facilitate the establishment of the rhizobia–plant interaction, even under stress conditions (Burdman et al. 1996; Volpin et al. 1996; Dardanelli et al. 2008).

Flavonoids shape rhizosphere microbial community structure because they may be used as potential carbon sources or may act as toxic substances for microbes that do not possess flavonoid biodegradation pathways (Shaw et al. 2006). They may also accelerate the biodegradation of xenobiotics, since the chemical structures of many flavonoids and xenobiotics are similar (Cesco et al. 2012; Shaw and Burns 2003), and flavonoids may have allelopathic effect on other plants (Cesco et al. 2012).

The role of phenolic compounds as signaling compounds in pathogenic microbe–plant interactions is undeniable. Usually, phenolic compounds released from seeds and roots act against soilborne pathogens and have high antifungal, antibacterial, and antiviral activities (Mandal et al. 2010). For example, *Pseudomonas* produces 2,4-diacetylphloroglucinol (DAPG), a phenolic compound with antibiotic properties, and a signal molecule that induces systemic resistance in plants and stimulates root exudation and branching (Combes-Meynet et al. 2011). The secretion of catechin by *Combretum albiflorum* interferes with the production of virulence factors by *P. aeruginosa* (Vandeputte et al. 2010).

Quorum Sensing Responses

Quorum sensing (QS) is a phenomenon where microbes communicate and coordinate activities by the accumulation of signal molecules at sufficient concentration (Adonizio et al. 2008). Both pathogenic and symbiotic bacteria require QS to interact successfully with their hosts (Badri et al. 2009). In Gram-negative bacteria, QS is typically mediated by *N*-acyl-L-homoserine lactones (AHLs). AHLs are freely diffused through the bacterial membrane and distributed within the rhizosphere where they regulate the behavior of rhizospheric bacteria. Increasing evidence

indicates that higher plants may produce metabolites that mimic AHLs, interfering with rhizospheric QS behavior (Gao et al. 2003). For example, *Medicago sativa* produces multiple signal molecules, including L-canavanine, capable of interfering with QS gene expression in *S. meliloti* (Keshavan et al. 2005). Canavanine is an arginine analog commonly found in seed and root exudates of a variety of legumes. Cai et al. (2009) found that canavanine is toxic to many soil bacteria but not to some rhizobia and suggest that host legumes may exude canavanine to optimize the bacterial population and select beneficial rhizobia in their rhizospheres. The role of these plant AHL-like compounds is still unclear (Ortiz-Castro et al. 2009), but some authors report direct effects on plant development in a similar way to auxins, by modulating root system architecture (more lateral roots and root hairs) (Ortiz-Castro et al. 2008a; von Rad et al. 2008). Plant AHL-like compounds are also involved in protection against pathogens. Vandeputte et al. (2010) reported the secretion by *Combretum albiflorum* of the flavonoid catechin that interferes in the QS signaling of *Pseudomonas aeruginosa* PAO1, as the first line of defense against this pathogen. Some PGPR can also protect plants by disrupting the QS signals required for pathogen–pathogen communication, interfering with the expression of virulence genes. For example, *Bacillus*, *Arthrobacter*, and *Klebsiella* produce AHL-degrading lactonases which inactivate AHLs (Friesen et al. 2011). Moreover, QS in the rhizosphere can also be disrupted by abiotic factors such as alkaline pH (Reis et al. 2011). Other PGPR secrete AHLs that induce plant systemic resistance to pathogens. For example, AHL molecules produced by *Serratia liquefaciens* MG1 and *P. putida* IsoF induce ISR in tomato plants against *Alternaria alternata* via a salicylic acid and ethylene-dependent pathway (Schuhegger et al. 2006). It is important to highlight the relevance of disrupting bacterial QS signaling as a strategy to fight against phytopathogens. This field is still unexplored.

Extracellular Polysaccharides

Bacterial extracellular polysaccharides (exopolysaccharides, EPSs; lipopolysaccharides, LPSs; capsular polysaccharides, CPSs; and cyclic β-glucans) are usually accumulated on cell surfaces and/or secreted into the cell surroundings (Gray and Smith 2005). They have multiple roles, such as protection against stress (Qurashi and Sabri 2012; Upadhyay et al. 2011), attachment to surfaces (Tsuneda et al. 2003), plant invasion (Fraysse et al. 2003; Troch and Vanderleyden 1996), and inhibition of the plant defense response in plant–microbe interactions (Kyungseok et al. 2008). PGPR also produce EPS and other surface polysaccharides as essential components that promote interaction with plants (Upadhyay et al. 2011).

Rhizobial surface polysaccharides are highly important during the early steps of microbe–legume interaction. They are essential for the formation of infection thread (IT), for nodule development, and for adaptation and survival of rhizobia under different environmental conditions (Fischer et al. 2003). In rhizobia, surface

polysaccharides form a hydrated matrix that contributes to protection against abiotic factors and plant products secreted as a defense response during the infection process. Moreover, CPSs may have an active signaling role during beneficial infections (Parada et al. 2006; Becker et al. 2005).

LPSs are anchored to the surface membrane by a lipidic moiety and inserted into the bacterial phospholipid monolayer, and the saccharidic part is oriented outside. Although LPS is a constitutive component of the bacterial membrane in Gram-negative bacteria, it is commonly found in very low concentrations in growth media, being released from cells in vesicles (Becker et al. 2005), and consequently it seems likely that LPSs may act as long-distance signaling molecules to target cells (Fraysse et al. 2003). They play various roles at different stages of the symbiotic process, act as inhibitors of plant defense responses, and/or help bacteria to adapt to the endosymbiotic environment. Experimental evidence demonstrates that root exudates, mainly plant-exuded flavonoids, induce changes in the PGPR-extracellular polysaccharide (EPS, LPS-O antigen, and CPS) composition, affecting the PGPR–plant interaction (Fischer et al. 2003; Fraysse et al. 2002, 2003; Reuhs et al. 1994; Dunn et al. 1992).

The importance of bacterial surface polysaccharides during the symbiotic process has been extensively demonstrated. *Azorhizobium caulinodans* mutants with LPS deficiency (Mathis et al. 2005) and LPS with reduced rhamnose content (Gao et al. 2001) established defective interactions with *Sesbania rostrata*, suggesting that both correct LPS amount and composition are required to sustain an effective rhizobia–legume interaction. In addition, LPS affects competitiveness and colonization as demonstrated by working with *Mesorhizobium loti* mutants defective in LPS and cyclic β-glucans (D'Antuono et al. 2005) and LPS mutants of *A. brasilense* in maize (Jofre et al. 2004), respectively.

EPSs are mostly species-specific heteropolysaccharides with an important role for an efficient symbiotic process. Bacterial mutants which fail to produce EPS induce nodules on the roots of the host plant but fail to invade these root nodules. Rhizobial EPSs are involved in the invasion process, IT formation, bacteroid and nodule development, and plant defense response and also confer protection to rhizobia when exposed to environmental stress (Bomfeti et al. 2011). EPSs are also involved in plant colonization and cell aggregation, as widely shown in *Azospirillum* species (Bahat-Samet et al. 2004; Jofre et al. 2004; Fischer et al. 2003; Burdman et al. 2000). The data showed that aggregation and root colonization properties of *Azospirillum* depend on the concentration and composition of EPS. The influence of EPS during aggregation on rhizospheric soil results in increased water and fertilizer availability to inoculated plants (Qurashi and Sabri 2012). Some PGPR-EPS can also bind cations, including Na^+, suggesting a role in mitigation of salinity stress by reducing the content of Na^+ available for plant uptake (Upadhyay et al. 2011). EPS produced by specific rhizobacteria can also elicit plant-induced resistance against biotic stress. For example, inoculation with the EPS-producing *Paenibacillus polymyxa* on peanut seeds significantly suppressed crown rot disease caused by *Aspergillus niger* (Haggag 2007), and the purified EPS from the PGPR *Burkholderia gladioli* induced resistance against *Colletotrichum orbiculare* on cucumber (Kyungseok et al. 2008).

Among extracellular polysaccharides, the rhizobial lipo-chitooligosaccharide known as nodulation factor (Nod factor or NF) is the most studied and probably the "movie star" of rhizobia–legume interaction. NFs have an oligomeric backbone of β-1,4-linked *N*-acetyl-D-glucosamine, N-acylated at the nonreducing terminal residue, and trigger the nodule developmental process. Depending on the rhizobial species, NFs have different chemical structures (variation in acyl chain, substitutions at the reducing and nonreducing terminal sugar, and additional decorations) (D'Haeze and Holsters 2002; Geurts and Bisseling 2002). Rhizobia perceive plant-secreted flavonoids by binding to NodD, a member of the LysR family of transcriptional regulators that triggers NF synthesis. NodD binds to conserved DNA sequences, known as *nod* boxes, found in the promoter regions of inducible *nod* genes. NF synthesis is commanded by the common *nodABC* genes which encode enzymes involved in the core structure, and many other *nod* genes are involved in decorations. Properties and functions of NFs are described throughout the body of the text of this chapter.

PGPR and Plant Root Attachment

Successful colonization and persistence in the rhizosphere are required for PGPR to exert their beneficial effect on plants. Many studies have shown that rhizobacteria are attracted to seed and root (chemotaxis) by plant-exuded molecules, the "rhizosphere effect" (Bais et al. 2006). Plant roots provide a carbon-rich environment and produce signals that are recognized by microbes which in turn produce others signals that initiate colonization. What are the most important traits in root–microbe interaction events? Motility, chemotaxis, and electrotaxis (the ability to use electric potentials produced at the root surface which act as attractants) enhance competitiveness during root colonization. Many microbe–plant interactions are mediated by the flagella which modulate attachment of the microbial cell to the root system. This process is well known in root colonization by azospirilla. Azospirilla undergo a biphasic attachment process, with an initial flagella-dependent adsorption phase, followed by an irreversible anchoring of the bacterium to the surface, and then the formation of bacterial aggregates embedded within the fibrillar material (Reis et al. 2011; Troch and Vanderleyden 1996).

A model described by Genre and Bonfante (2007) suggests alternative routes to biotrophy in interactions between plants and PGPR, endophytes, and pathogens, where precontact signaling contributes to the recognition of rhizobacteria as beneficial or pathogenic. A weak, nonspecific, and reversible first contact occurs mediated by lectins, bacterial surface proteins, CPS, and/or flagella (Rodriguez-Navarro et al. 2007). Then, a direct contact occurs characterized by a rapid translocation of the cytosolic and subcellular elements to the contact site (localized secretion). In beneficial interactions, this secretion leads directly to (1) epiphyte–bacterial aggregates on the plant surface or (2) a preemptive assembly of an intracellular apoplastic compartment to host the endophyte (Genre and Bonfante 2007). In this step,

extracellular polysaccharides are the main determinants, required for tight and irreversible binding of bacteria (Rodriguez-Navarro et al. 2007).

In the rhizobia–legume interaction, the endophytes access the root by the ITs, tubular structures derived from plant plasma membranes that act as "tourist guides" to the root cortex. The process of rhizobia accommodation into the nodule primordium may be explained by a sequence of events described by Held et al. (2010). The extracellular colonization of roots by rhizobia leads to the uptake of cells through an intracellular (through root hairs) or intercellular ("crack-entry") infection (Held et al. 2010). The latter is thought to be the ancestral mechanism of root infection and involves the formation of transcellular ITs within the root cortex (Downie 2010). The next section gives a brief but more detailed description of rhizobia–legume interaction events.

Proteins Involved in Rhizobia–Plant Interaction

Proteomics, the identification of a set of proteins under specific conditions, is a valuable tool to decipher part of the complex network involved in plant and microbe communication. Most works dealing with plant–microbe exchange of information through a proteomic approach have been performed on plant tissues after bacterial inoculation, bacteroids, or nodules. Additional information has also been achieved by transcriptomic and metabolomic analysis (Stacey et al. 2006).

It has been shown that rhizobia inoculation induces or increases the level of several proteins in soybean root hairs (calcium/calmodulin kinase, lipoxygenases, phospholipase D, ascorbate peroxidase, phosphoglucomutase, lectin), roots (enzymes involved in energy, carbohydrate, amino acid, and flavonoid metabolism), and bacteroids (proteins involved in carbon and nitrogen metabolism, stress response and detoxification, ABC transporters and receptors) (Mathesius 2009). In addition, large amount of information has been generated about the regulation of signal transduction involved in bacterial infection and nodule organogenesis and long-distance signaling to control nodule number (Oka-Kira and Kawaguchi 2006; Popp and Ott 2011). However, few experiments have analyzed proteins secreted in the rhizosphere or those that are associated with the bacterial outer membrane. These experiments involve plant growth in liquid media, protein concentration by lyophilization or precipitation, desalting, two-dimensional gel electrophoresis, and protein identification by mass spectrometry (Jayaraman et al. 2012). In addition, proteins secreted by bacteria or associated with their outer membrane have been found using a classical approach, by the analysis of culture medium after adding plant-secreted molecules, or a genomic approach through the study of mutants. Using different approaches, many proteins secreted to the rhizosphere and involved in plant–microbe communication have been identified.

Rhizobial proteins are secreted by general secretion (Sec) and two-arginine (Tat) systems of general use (NodO, adhesins, PlyA and PlyB polysaccharide lyases, ExoK and ExsH succinoglycan depolymerases, calsymin, cellulose, etc.) and by specialized secretion systems (Nops or nodulation outer proteins secreted by the

type III secretion system, Msi059 and Msi061 by the type IV secretion system, ribose-binding protein-like by the type V and VI secretion systems) (Downie 2010; Deakin and Broughton 2009; Tseng et al. 2009; Fauvart and Michiels 2008). Plant roots secrete compounds mainly by passive process mediated diffusion, ion channels, and vesicle transport. But excretion of high-molecular weight compounds by roots, including proteins, generally involves vesicular transport. Rhizobial cells secrete adhesins such as rhicadhesin that plays an important role in attachment to root hairs (Smit et al. 1992), hydrolytic proteins such as cellulase that erodes the noncrystalline cellulose in the root hair cell wall allowing rhizobial penetration (Robledo et al. 2008), and glycanases that cut emerging EPS produced by rhizobia and are required for biofilm formation (Russo et al. 2006). Many extracellular glycanases, involved in nodulation and EPS modification, have been identified and characterized in rhizobia: PlyA and PlyB of *R. leguminosarum* bv. *viciae* and ExoK and ExsH of *S. meliloti*. The secreted nodulation-signaling protein NodO was purified from the supernatant of cultures of *R. leguminosarum* bv. *viciae* supplemented with flavonoids (Sutton et al. 1994). NodO is a calcium-binding protein that forms cation-selective channels in membranes and may complement NF function by promoting the movement of cations across the root hair membrane (Downie 2010). *M. sativa* inoculation with *S. meliloti* caused an increase in the secretion of plant hydrolases (chitinases that use NFs as substrates, glycosidases, and peptidases), peroxidase precursors, pathogenesis-related proteins (thaumatin-like protein), lectins, bacterial superoxide dismutase, glycine betaine-binding ABC transporter, and a putative outer membrane lipoprotein transmembrane (De la Peña et al. 2008).

Rhizobia–Legume Interaction Events

Rhizobia–legume signaling strategies are mainly based on sugars such as the NFs, EPSs, lipopolysaccharides and capsular polysaccharides, as well as cyclic β-glucans. However, roots and microorganisms also produce diverse proteins that play a dynamic role in the process of signaling and recognition that occurs during their interaction. A picture of events implicated in legume–rhizobia interaction involving carbohydrates, flavonoids, phytohormones, and proteins may be summarized as follows (Fig. 6.1).

Plant roots release species-specific mixtures of molecules, such as phytohormones and flavonoids (that act as bacterial attractants), that initiate the symbiotic chemical dialog. Rhizobial cells recognize flavonoids by their binding to NodD, an extracellular membrane protein that works as an environmental sensor and master transcriptional activator of genes downstream of promoters known as *nod* boxes. In response to *nod*-gene activation, rhizobia produce and release the signaling molecule NF that is identified by plant root receptor-like kinases (NFR-LKs). Many NFR-LKs have been identified, e.g., LysM-type RLKs NFR5/NFR1 of *L. japonicus*, NFP/LYK3/LYK4 of *M. truncatula*, SYM10/SYM2 of *Pisum sativum*, and NFR5αβ/NFR1β of *G. max*. After the NFR-LK-ligand recognition,

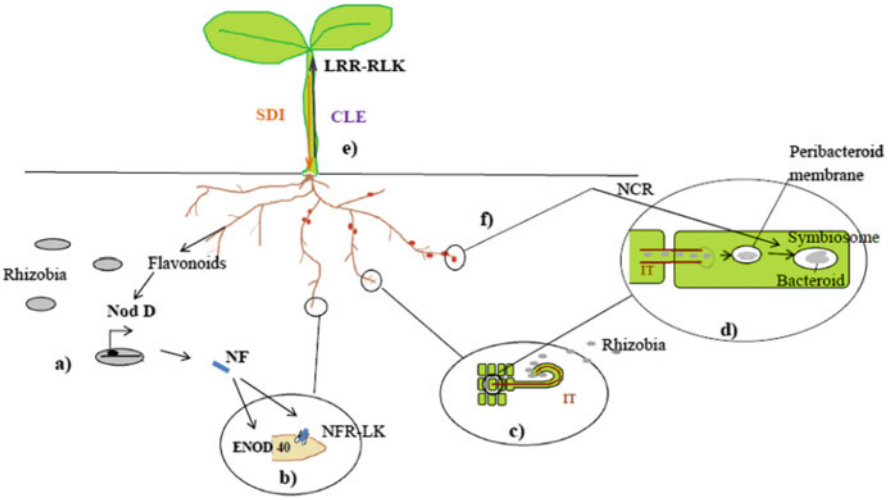

Fig. 6.1 Overview of rhizobia–legume interaction events. (**a**) Induction of *nod* genes by root-exuded flavonoids and NF production; (**b**) NF perception by NFR-LK elicits calcium signaling that leads to localized CK biosynthesis. CK induces the ENOD40 production and downstream signaling for activation of symbiotic response and nodule organogenesis; (**c**) deformation of root hair and formation of IT. Bacteria move through the IT; (**d**) rhizobia penetrate cortical cells via IT. They are released from unwalled IT into the host cell cytoplasm as membrane-delimited symbiosome into bacteroids; (**e**) CLE peptide synthesis in the nodule and recognition by shoot-specific receptor kinase (LRR-RLK). Production of shoot-derived inhibitor (*SDI*) that regulates nodule number (*AON*); (**f**) indeterminated nodule produces NCRs that induce bacteroid differentiation

many physiological events are turned on, such as root hair deformation and IT initiation, depolarization of the plasma membrane, rhizosphere alkalinization, Ca spiking by a calcium-dependent calmodulin kinase (CCaMK), cytoskeletal rearrangement, early nodulin gene expression, and finally nodule formation.

In addition to NF, some rhizobia secrete proteins involved in host specificity and symbiotic efficiency by a type III secretion system or T3SS. T3SS delivers virulence proteins called effectors directly into the host cells. Rhizobial effector proteins are known as Nops (nodulation outer proteins). Rhizobial NopL and NopP interfere with plant signaling pathways acting as positive effectors that enhance nodule formation. These and other Nops effectors might contribute to suppression of plant innate immune response or modulate cytoskeletal rearrangements in root cells during nodule formation. Thus, rhizobial effectors could facilitate bacterial release from IT, initiate symbiosis, and/or promote or maintain persistence of bacteroids (Saeki 2011; Deakin and Broughton 2009).

The invading bacteria move through the IT and are taken into the plant cell by a type of endocytosis in which they are surrounded by a plant-derived peribacteroid membrane. Nodule organogenesis, cell proliferation and dedifferentiation, and bacteroid differentiation are driven by plant hormones and systemic signaling peptides (ENOD40, CLE, NCR) (Ding et al. 2008; Batut et al. 2011). Ethylene, jasmonic

acid, and abscisic acid (ABA) regulate NF signaling and affect the nature of NF-induced calcium spiking, with ABA being capable of coordinating regulation of diverse development pathways associated with nodule formation (Ding et al. 2008).

CLE (CLAVATA3/endosperm surrounding region) are peptides that have been identified in a wide variety of plants. They are key molecules in the regulation of nodulation acting as a root-derived ascending signal to the shoot. This peptide is probably recognized as a ligand for a leucine-rich repeat (LRR) autoregulation receptor kinase that controls multiple aspects of shoot development, jasmonate signaling, and the production of a shoot-derived inhibitor (produced in leaves) that regulates root nodule number. These LRR receptor kinases (GmNARK, *Glycine max* nodule autoregulation receptor kinase of soybean; HAR1, hypernodulation and aberrant root of *Lotus japonicus*; SYM29, symbiosis of pea; and SUNN, super numeric nodules of alfalfa) are key regulators of the autoregulation of nodulation (AON) signaling pathway that controls a hypernodulated unproductive phenotype (Staehelin et al. 2011; Popp and Ott 2011; Miyazawa et al. 2010; Kinkema and Gresshoff 2008; Oka-Kira and Kawaguchi 2006). AON is the major pathway that controls nodulation events acting through the inhibition of nodule development in a long-distance signaling fashion between root and shoot. NF is also involved in the expression of several early nodulin (ENOD) genes (ENOD12 y ENOD40).

It has been suggested that CK is an epidermal cell synthesized secondary signal, which after translocation to cortex cells triggers the initiation of nodule primordial ahead of the upcoming IT (see section "Phytohormones Production"). CK induces the expression of the *enod40* gene serving as an amplification mechanism, thus triggering a localized hormone imbalance, a state that initiates cell divisions in the root cortex (Fang and Hirsch 1998). The *enod40* gene codes for two short conserved peptides, A and B, which strongly bind the cytosolic sucrose synthase (SuSy) enzyme-stimulating sucrose breakdown activity. The data support the view that Enod40 peptide may participate in phloem uploading, increasing the carbon sink strength in pre-dividing root cortical cells and in mature nodule tissues (Batut et al. 2011). CK induces the expression of the *Nin* transcriptional regulator within the root cortex through the activation of the LHK1 cytokinin receptor, subjected to HAR1-mediated autoregulation (Heckmann et al. 2011).

Some legumes such as *Medicago*, *Pisum*, *Vicia*, and *Trifolium* maintain active apical meristems that produce indeterminate nodules. This type of nodule undergoes an irreversible differentiation mediated by nodule-specific cysteine-rich (NCR) peptides. NCRs are produced by the host cells and targeted to bacteroids where they interfere with the rhizobial cell cycle affecting terminal bacterial differentiation. In addition, NCRs resemble antimicrobial peptides (Batut et al. 2011; Van de Velde et al. 2010). Findings suggest that after the root epidermal cell recognition of NF, several kinase receptors are activated, working as a signal transduction cascade responsible for the control and progression of IT, nodule organogenesis, and nitrogen fixation (activation of downstream common *nod* and *sym* genes). These kinase receptors are regulated by E3-ubiquitin ligases that act as dynamic modulators of cellular reprogramming during rhizobial infections (Popp and Ott 2011; Mathesius 2009). Hundreds of proteins from nodule, xylem, root, and shoot have been

implicated in rhizobia–legume interaction (Mathesius 2009), but insufficient work has been done on proteins secreted in the soil by roots and bacteria during microbe–plant interaction.

A large variety of regulatory molecules, including kinases, transcriptional factors, and other regulatory molecules, are involved in symbiotic nodule organogenesis, and recent reports showed that sRNAs, especially microRNAs (miRNAs), are also key regulatory factors of this process. Thus, miRNAs are emerging as riboregulators that control gene networks in plant cells through interactions with specific target mRNAs. Only a few nodulation-responsive miRNAs have been linked to nodule formation: among other miRNAs, miR169 and miR166 overexpression in *M. truncatula* led to lower densities of lateral roots and nodules, and they might be responsible for nodule meristematic zone regulation during nodule differentiation into nitrogen-fixing cells; soybean miR482 targets the resistance gene receptor kinase involved in the defense response, playing a role during nodule initiation; miR1511 and miR1512 target transcripts encoding signaling proteins, including a calmodulin-binding protein (Bazin et al. 2012; Khan et al. 2011; Voinnet 2008). In addition, there is strong evidence that there is a connection between miRNA regulation and hormone response. Some miRNAs facilitate hormone-induced responses, e.g., the miRNAs miR160, 167, and 393 that are implicated in the regulation of auxin signaling target transcripts to reduce lateral root production and are potentially involved in nodulation (Simon et al. 2009; Bazin et al. 2012).

Concluding Remarks

Compounds exuded by plants and microbes provide a cocktail of molecules (carbohydrates, phytohormones, flavonoids, amino acids, and proteins) that constitute the words of a chemical dialog between plants and microbes in the rhizosphere (Fig. 6.2). The massive variety of metabolites released by plants suggests that they provide a specific language for communication. Researchers are deciphering the content and significance of the cells' signaling and responses. Recent advances in analytical skills and biochemical and molecular approaches have provided new tools for evaluating the natural roles of these substances and for investigating the mechanisms underlying their regulation.

In brief, the picture of microbe–plant interaction events involves a huge number of molecules that span our imagination. Every year a new signaling molecule is found, and the overall scene is getting much bigger and more complex. The new information on proteins involved in two-component signal transduction systems that allow sensing and responding to different stimuli, transcriptional regulators, and plant-derived peptides is far from completing the picture of the microbe–plant interaction. In this chapter, only some recent and relevant earlier information related to molecules involved in microbe–plant interaction have been used to present a partial panorama.

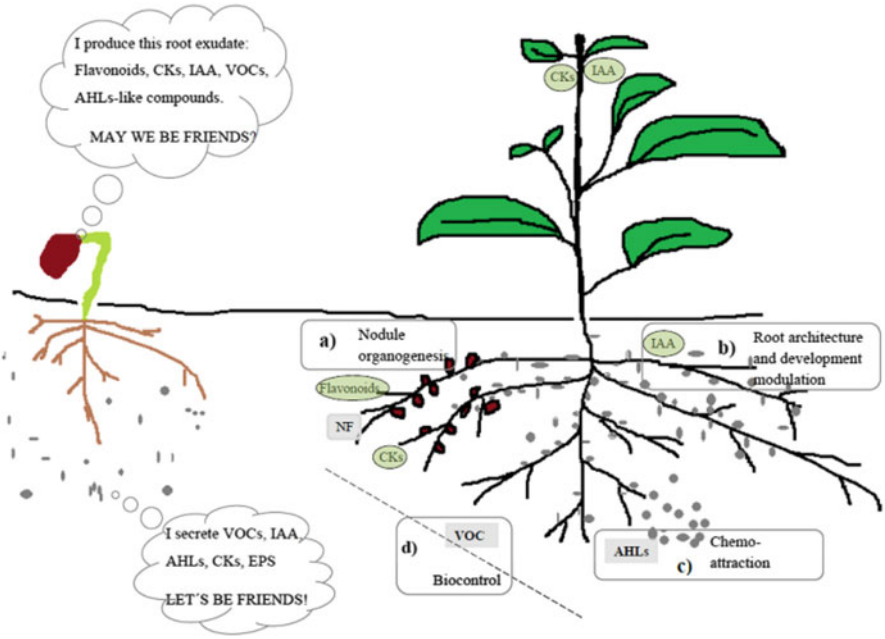

Fig. 6.2 Summary of early signaling events in microbe–plant interaction. *Left*: Some bacterial and root-exuded compounds involved in microbe–plant interaction. *Right*: Four mechanisms of PGP: (**a**) In legume–rhizobia symbiosis, root-secreted flavonoids trigger rhizobial production of NF and nodule formation (see Fig. 6.1); (**b**) bacteria-produced IAA modulate root architecture and development. In legume–rhizobia interaction, they act on nodule functioning; (**c**) bacterial AHLs and plant AHL-like compounds regulate the behavior of rhizobacteria, selecting beneficial ones and interfering with QS of non-beneficial ones. AHLs may also modulate root architecture in an auxin-like fashion; (**d**) VOCs may function as biocontrol compounds against phytopathogens

Acknowledgements We thank Programa de Desarrollo de las Ciencias Básicas (PEDECIBA). The work of M. Morel was supported by Agencia Nacional de Investigación e Innovación (ANII). Dr. Valerie Dee revised linguistic aspects of this manuscript.

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Chapter 7
The Contribution of New Technologies Toward Understanding Plant–Fungus Symbioses

Raffaella Balestrini, Stefano Ghignone, and Fabiano Sillo

Contents

Abstract Symbiotic associations between beneficial soil fungi and the roots of about 90 % of land plants, commonly known as mycorrhizae, exist in a wide range of terrestrial ecosystems. During the interaction, both the plant and the fungus benefit from the relationship. Complete genome sequences give useful information to deeper understanding of the molecular mechanisms underlying the symbiotic lifestyle and several genome sequencing projects on mycorrhizal fungi have been launched. Genomic projects are currently coupled to transcriptome analysis, which represents the starting point for the post-genomic activities, in which research is focused to ascribe function to genes. The introduction of new sequencing techniques (next-generation sequencing, NGS), which produce short-read sequences in large quantity, has been accompanied by advances in bioinformatics. In this chapter we will review recent advances in plant/fungus symbiotic interactions, focusing on the recent fungal genome projects and on the NGS application in this field.

R. Balestrini (✉) • S. Ghignone • F. Sillo
Istituto per la Protezione delle Piante del CNR, UOS Torino and Dipartimento di Scienze della Vita e Biologia dei Sistemi, UniTO, Viale Mattioli 25, 10125 Torino, Italy
e-mail: r.balestrini@ipp.cnr.it

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_7, © Springer India 2013

Introduction

In their natural habitats, plants interact with a large number of microbes, and among these many are fungi. While some microbes colonize the plant for their own benefit, others can directly cooperate with plants in a mutually beneficial manner. In addition, microbes can indirectly affect plants by altering their environments (Schenk et al. 2012). Plant-fungi associations play an important role in terrestrial ecosystem and vary from pathogenic interactions to mutualistic associations (Cox et al. 2010). Symbiotic associations between beneficial soil fungi and the roots of about 90 % of land plants, commonly known as mycorrhizae, exist in a wide range of terrestrial ecosystems. During the interaction, both the plant and the fungus benefit from the relationship: the fungus supplies the plant with nutrients, such as phosphate and nitrogen, while the plant supplies the fungus with carbohydrates. Mycorrhizal fungi play a central role in the capturing of nutrients in natural as well as in agricultural systems, in which they can contribute to plant health and productivity, increasing the tolerance to biotic and abiotic stress (Smith and Read 2008; Balestrini et al. 2012a). According to their ability to penetrate the roots cells, mycorrhizae can be classified as two main types: endomycorrhizae and ectomycorrhizae (ECM) (Balestrini et al. 2012a). Recent research has focused on soil organisms involved in symbioses, which play a key role in plant/microbial communities and in ecosystem processes (Finlay 2008). While the researches, originated from the several plant genome projects, have pointed out the plant genes involved in symbiosis (Güimil et al. 2005; Güther et al. 2009; Dermatsev et al. 2010; Hogekamp et al. 2011), less information is available on the fungal side (Bonfante and Genre 2010). Understanding how fungi with different nutritional strategies achieve their lifestyle is crucial to understand their ecological functions, their interactions with other organisms, and their impact on plant communities and productivity (Martin et al. 2011).

Complete genome sequences are seen as valuable tools to help obtain a deeper understanding of the molecular mechanisms that underlie the symbiotic lifestyle. Generally, genomic projects are associated with transcriptome analysis, which represent a good starting point for post-genomic activities, in which research is focused on the assignment of a function to the gene dataset discovered in an organism. Bioinformatics can provide powerful tools to identify and evaluate candidate genes through database searches and through the analyses of expression profiling. The knowledge of expressed genes is essential to interpret the functional elements of the genome and to reveal the molecular determinants of physiological processes, i.e., during the development and life cycle of an organism, as well as the processes that occur during interaction with other organisms. The transcriptomics approaches that are commonly applied are large-scale approaches, i.e., microarrays (Breakspear and Momany 2007) and next-generation sequencing (NGS) (Wang et al. 2009; Nagalakshmi et al. 2010). Recent papers have shown the great potential of transcriptome analysis in fungi (Tisserant et al. 2011, 2012; Zuccaro et al. 2011), as it unravels the biological

processes that occur in fungi life. In the last few years, with the advancements in NGS technologies (Zhang et al. 2011), the sequencing of fungal genomes/transcriptomes has become simpler and less expensive, becoming a relatively routine approach to data collection for all areas of mycology. A deeper understanding of the evolutionary history of the Fungi Kingdom is necessary to complement and expand our knowledge of the natural evolution of ecosystems and to enhance the development of tools that will possibly allow the recognition of undescribed species (Hibbett et al. 2007).

JGI Fungal Genomics Program

About 80,000 described species belonging to the Fungi Kingdom, but the diversity in the group has been estimated to involve about 1.5 million species, and it thus represents one of the largest branches of the Tree of Life. In the early 2000s, the Assembling the Fungal Tree of Life project (AFTOL; http://aftol.org/) was launched to increase the understanding of the evolution of the Fungi Kingdom (Hibbett et al. 2007). Recently, thanks to the advances in massive-scale DNA sequencing and analysis capabilities, the Joint Genome Institute (JGI) of the US Department of Energy has launched the Fungal Genomics Program (FGP; http://genome.jgi.doe.gov/programs/fungi/index.jsf), with the aim of exploring fungal diversity for energy and environmental sciences and applications (Grigoriev et al. 2011). They started with the sequencing of a few single fungal genomes (Martinez et al. 2004, 2008, 2009; Jeffries et al. 2007; Martin et al. 2008a) and then moved onto higher-scale system-level genomics.

In the frame of FGP, two main research lines are currently under way:

1. The Genomic Encyclopedia of Fungi (http://genome.jgi.doe.gov/programs/fungi/GE_Fungi.jsf)
2. The 1000 Fungal Genomes Project (F1000) (http://genome.jgi.doe.gov/programs/fungi/1000fungalgenomes.jsf).

The former focuses on three areas of research related to bioenergy: (1) Plant Feedstock Health, which encompasses symbiosis, plant pathogenicity, and biocontrol; (2) Biorefinery, which involves the analysis of lignocellulose degradation, sugar fermentation, and industrial organisms; and (3) Fungal Diversity.

The latter, in collaboration with an international research team, has the goal of filling the gaps in the Fungal Tree of Life by sequencing at least two reference genomes from more than 500 recognized families of fungi (Spatafora 2007).

In the DOE JGI framework, fungi (as symbionts, pathogens, and biocontrol agents) are considered key organisms that can exert an impact on the maintenance of plant health and on the sustainable growth of biofuel feedstock. In this perspective, the optimization of bioenergy feedstock plant growth and productivity depends on the understanding of the molecular mechanisms that are involved in the interactions between plants and mycorrhizal fungi.

In this contest, JGI has started the Mycorrhizal Genomics Initiative, which targets several fungal species from different orders (Table 7.1) (Grigoriev et al. 2011; Plett and Martin 2011; http://mycor.nancy.inra.fr/IMGC/MycoGenomes/). At the moment, there are 72 ongoing or completed genome/transcriptome projects in the frame of the "Exploring the Genome Diversity of Mycorrhizal Fungi to Understand the Evolution and Functioning of Symbiosis in Woody Shrubs and Trees" proposal, which is coordinated by Francis Martin (INRA). On the basis of previous genome projects conducted on *Laccaria bicolor* (Martin et al. 2008a) and *Tuber melanosporum* (Martin et al. 2010), the main goal of this project is to explore the genomics sequence of a phylogenetically and ecologically diverse suite of mycorrhizal fungi (Basidiomycota and Ascomycota), which includes the major clades of symbiotic species associating with trees and shrubs, including endo- and ectomycorrhiza.

The JGI Genome Portal (http://genome.jgi.doe.gov/) offers an access point to all the sequencing genome project managed by the DOE JGI. A specialized tool for the analysis and exploration of fungal genomes, named MycoCosm (http://genome.jgi.doe.gov/fungi), was released in March 2010, in response to a request from the fungal community to integrate all fungal genomes and interactive analytic tools in one place (Grigoriev et al. 2012). Newly sequenced and annotated fungal genomes from JGI and elsewhere (e.g., *T. melanosporum* sequenced and annotated by Genoscope) are available to the public, and new annotated genomes are being added to this resource upon completion of their annotation. MycoCosm offers useful web-based genome analysis tools that can be used to search through sequenced genomes and explore them in different contexts. Genome-centric tools offer the Genome Browser, BLAST, and the possibility of searching within the data for a single genome. Predicted gene models and annotations are displayed within the Genome Browser along with different lines of evidence in support of these predictions (e.g., gene and protein expression profiles). The Genome Browser also displays other types of data mapped to a genome assembly, G+C profiles, and annotation features, including regions of homology, domains, repeats, and noncoding genes. The functional profiles of genomes are based on summaries of predicted gene annotations, according to the GO (The Gene Ontology Consortium 2000), KEGG (Kanehisa et al. 2008), and KOG (Koonin et al. 2004) classifications. Genome conservation and synteny can be explored using the *VISTA* tool, which has been designed for the visualization and analysis of pairwise and multiple DNA alignments and which makes the analysis of whole-genome alignments, functional profiles, and gene clusters possible. The cluster analysis enables the exploration of gene families within a given group of organisms. Clusters are built using the Markov clustering algorithm MCL (Enright et al. 2002) and all-against-all BLAST alignments of the proteins from the entire dataset. Registered users participating in a particular genome project can validate and improve predicted gene models and annotations.

The JGI initiative has the main goal of providing new useful information that, through a comparative analysis, can be used to improve the understanding of fungal lifestyles, their interaction with plants, and their evolution. An example of this is the work by Eastwood and colleagues (2011). They have conducted, through the sequencing of the brown rot wood decay fungus *Serpula lacrymans*, a genome comparison with sequenced fungal species that represent several functional niches and

Table 7.1 List of fungal species sequenced in the frame of JGI proposal "Exploring the Genome Diversity of Mycorrhizal Fungi to Understand the Evolution and Functioning of Symbiosis in Woody Shrubs and Trees" and related genome projects of interest in mycorrhizal research (updated May 2013). Modified after Martin and Bonito (2012)

Fungal sequencing project	Genome release
Tier 1 – 2011 [14 species]	
Basidiomycotina:	
Laccaria amethystina (Agaricales, Hydnangiaceae)	v.1.0
Hebeloma cylindrosporum (Agaricales, Cortinariaceae)	v.2.0
Paxillus involutus (Boletales, Paxilineae)	v.1.0
Paxillus rubicundulus (Boletales, Paxilineae)	v.1.0
Pisolithus microcarpus (Boletales, Sclerodermatineae, Pisolithaceae)	v.1.0
Pisolithus tinctorius (Boletales, Sclerodermatineae, Pisolithaceae)	v.1.0
Piloderma croceum (Atheliales)	v.1.0
Scleroderma citrinum (Boletales, Sclerodermataceae)	v.1.0
Sebacina vermifera (Sebacinales, forms endomycorrhiza [orchid])	v.1.0
Tricholoma matsutake (Agaricales, Tricholomataceae)	v.3.0
Tulasnella calospora (Cantharellales, Tulasnellaceae)	v.1.0
Ascomycotina:	
Cenococcum geophilum (Dothideomycetes)	v.2.0
Oidiodendron maius (Leotiomycetes)	v.1.0
Terfezia boudieri (Pezizales, Pezizaceae)	v.1.0
Tier 2 – 2012 [13 species]	
Basidiomycotina:	
Amanita muscaria (Agaricales, Amanitaceae)	v.1.0
Boletus edulis (Boletales, Boletineae)	v.1.0
Cantharellus cibarius (Cantharellales)	In progress
Cortinarius glaucopus (Agaricales, Cortinariaceae)	In progress
Gymnomyces xanthosporus (Russulales)	
Lactarius quietus (Russulales)	In progress
Gyrodon lividus (Boletales)	In progress
Suillus luteus (Boletales)	v.1.0
Thelephora terrestris (Thelephorales)	In progress
Tomentella sublilacina (Thelephorales)	In progress
Ascomycotina:	
Meliniomyces bicolor (Helotiales)	v.2.0
Meliniomyces variabilis (Helotiales)	v.1.0
Rhizoscyphus ericae (Helotiales)	In progress
Others	
Laccaria bicolor (Agaricales, Hydnangiaceae)[a]	v.2.0
Tuber melanosporum (Pezizales, Tuberaceae; Genoscope)[b]	v.1.0
Tuber magnatum (Pezizales, Tuberaceae)	In progress
Rhizophagus irregularis (Glomeromycota)	v.1.0
Piriformospora indica (Sebacinales)[c]	v.1.0
Suillus brevipes (Boletales)	v.1.0

[a]Martin et al. (2008a)
[b]Martin et al. (2010)
[c]Zuccaro et al. (2011)

have demonstrated that the evolution of ectomycorrhizal biotrophy and brown root saprotrophy is accompanied by a reduction and losses in specific gene families, which would suggest an adaptation to intercellular colonization of the plant tissue.

Early Findings in Mycorrhizal Genomics

It is still unknown whether several mycorrhizal fungi have a common core set of genes that are necessary for the symbiosis development or whether the mechanisms required for the symbiotic interaction changed during the evolution (Plett and Martin 2011). The first sequenced mycorrhizal fungi were the basidiomycete *Laccaria bicolor* (Martin et al. 2008a) and the ascomycete *Tuber melanosporum* (Martin et al. 2010), and other ECM genome sequencing projects are currently under way (Table 7.1). *L. bicolor* has a genome of 64.9 Mb in size with ~19,000 estimated protein-coding genes, while *T. melanosporum* has a 125 Mb genome and only ~7,500 predicted protein-coding genes, showing a relatively small complement of predicted proteins in comparison with other sequenced filamentous fungal genomes (Martin et al. 2010). This expansion in truffle genome size results from a proliferation of repeated transposable elements, which account for ~58 % of the genome. Although both fungi are ectomycorrhizal species and form similar symbiotic structures, they encode different proteomes: large with many expanded multigene families in *Laccaria versus* compact with very few multigene families in *Tuber*. Differences can be seen in symbiosis-regulated genes. Both genomes reveal a reduced set of plant cell wall (PCW) degrading enzymes, but there are significant differences in the enzyme repertoire in the two fungi and in the expression during the symbiosis (Martin et al. 2010). Moreover, the effector-like proteins expressed in *Laccaria* (i.e., MiSSPs) are not expressed in *T. melanosporum* ectomycorrhizae. Looking at the different symbiosis-related toolboxes in the two genomes, the evolution of the ectomycorrhizal lifestyle seems to be quite divergent (Plett and Martin 2011). The results of genome sequencing of more ECM fungi that are currently being sequenced will allow the symbiotic fungal strategies developed by different ECM fungal lineages to be compared. As far as endomycorrhizal fungi are concerned, *Oidiodendron maius* (ericoid symbiont) and *Tulasnella calospora* (orchid symbiont) genome sequencing has recently been released, thus providing the possibility of comparing different mycorrhizal strategies.

A sequencing project of an arbuscular mycorrhizal (AM) fungus genome (*Glomus intraradices* DAOM197198, now named *Rhizophagus irregularis*) was started in 2004 and is currently under way (Martin et al. 2008b; Lanfranco and Young 2012). The first sequencing data provided an estimate of the genome size of about 150 Mb (Martin et al. 2008b), and this value has recently been confirmed experimentally (Sedzielewska et al. 2011). On the other hand, the *G. intraradices* mitochondrial genome and the mitochondrial genome of two *Gigaspora* isolates have already been completed (Lee and Young 2009; Formey et al. 2012; Pelin et al. 2012; Nadimi et al. 2012). In the absence of a complete genome sequence, the

knowledge of the *G. intraradices* DAOM197198 genome has recently been expanded through the publication of genome-wide transcriptomic data (Tisserant et al. 2012). The expression of genes encoding membrane transporters and small secreted proteins has been found in the intraradical mycelium, along with a lack of expression of hydrolytic enzymes acting on PCW polysaccharides, which would suggest that *G. intraradices* shares some features with obligate biotrophic pathogens (Spanu 2012; Kemen et al. 2011) and ECM symbionts (Plett and Martin 2011). However, the obligate biotrophy of *G. intraradices* does not seem to be associated with a large reduction in metabolic complexity, as observed in many obligate biotrophic pathogens; in this way, the ability to interact with the soil environment, regarding the nutrient uptake, is maintained in the symbiotic fungus. The work of Tisserant et al. (2012) on *G. intraradices* is the first comprehensive gene inventory of a Glomeromycotan fungus and can be considered a keystone for accessing symbiosis-related functional features in other members of this unique phylum.

The Microarray Era

The transcriptome, i.e., the mRNA pool of a cell at any one moment, has long been analyzed using methods such as expressed sequence tag (EST) sequencing (through cDNA libraries construction) and cDNA (macro) microarrays, in which gene-specific oligonucleotides are spotted on a solid surface (Wilkes et al. 2007). These techniques have led to the rapid identification of expressed genes in several organisms, thus providing data for the large-scale analysis of thousands of genes. In the first work using cDNA arrays to study mycorrhizal symbiosis, gene expression was analyzed during the ECM symbiosis between *Eucalyptus globulus* and *Pisolithus tinctorius*. A comparison of signals from the free-living partners and symbiotic tissues has led to the identification of many plant/fungus symbiosis-regulated genes, thereby demonstrating the utility of this technique in the study of gene expression changes during symbiosis development (Voiblet et al. 2001). Liu et al. (2003) then used cDNA arrays to examine transcript profiles in *M. truncatula* roots during interaction with the AM fungus *Glomus versiforme* and during growth under different phosphorous concentrations. Interestingly, most genes showing increased transcript levels in AM roots did not change in response to high phosphorus level, suggesting that the changes in transcript levels during symbiosis were a consequence of the AM fungus rather than a secondary effect due to the improved phosphorus nutrition (Liu et al. 2003). To date, thanks to the increase in plant genome sequencing, genome-wide cDNA arrays are available for several mycorrhizal plants such as rice, tomato, grapevine, *Populus trichocarpa*, *Lotus japonicus*, and *Medicago truncatul*a (Rensink and Buell 2005). Over the last 10 years, major changes in gene expression that accompany the establishment of symbiosis and a wide spectrum of genes involved have been revealed, providing insight into the molecular mechanism that underlie symbiosis both for AM (Hohnjec et al. 2005; Küster et al. 2007; Güther et al. 2009; Dermatsev et al. 2010) and ECM symbiosis (Le Quéré et al. 2005; Duplessis et al.

2005; Heller et al. 2008). Microarrays have also been applied, in the frame of genome projects, to symbiotic fungi such as *L. bicolor* and *T. melanosporum* (Martin et al. 2008a, 2010). This approach has been used to verify changes in gene expression during the three stages of the complex life cycle of a symbiotic ectomycorrhizal fungus: free-living mycelium, ectomycorrhiza, and fruiting bodies. A global characterization of the endophytic fungus *Piriformospora indica* transcriptional responses has recently been performed (Zuccaro et al. 2011) during the colonization of living and dead barley roots using microarrays (60-mer probes) containing also 265 barley genes (including genes related to defense and transport). Microarrays, constructed using the nonredundant virtual transcripts obtained with the Sanger and 454 sequencing technologies from germinated spores, extraradical mycelium, and symbiotic roots, have been developed to study gene expression in several life cycle stages of the AM fungus *Glomus intraradices*, including RNA extracted from arbuscule-containing cells collected using laser microdissection (LMD). Over the last few years, LMD has been used to study cell specificity in AM symbiosis, and particular attention has been paid to the cortical cells containing the main feature of the symbiosis: the arbuscules. Several works on AM symbiosis have been focused on verifying the expression of specific plant-fungal genes, which appeared previously regulated in microarrays, in different cell-type populations (Güther et al. 2009; Gomez et al. 2009; Hogekamp et al. 2011). In addition, in order to obtain insight into cell-specific reprogramming in AM symbiosis, transcriptome analyses of several cell types have been performed using an LMD approach combined with microarray hybridization (Gaude et al. 2011).

Despite the wide use of this approach to detect differential gene expression in symbiotic interactions, the microarray construction (probe design and synthesis) remains limited to organisms with a sufficient level of information on gene sequences.

The Advantages of High-Throughput Sequencing

With the improvements in the fields of microfluidics, nanotechnology, and informatics, alternative technologies have recently emerged that can increase the large-scale DNA/RNA sequencing (Margulies et al. 2005; Branton et al. 2008; Wang et al. 2009). The term NGS is commonly used to describe technologies other than Sanger sequencing that have the ability to produce an enormous volume of data cheaply.

There are several commercially available NGS platforms, and, of these, the Roche/454 (http://www.454.com/), Solexa/Illumina (http://www.illumina.com/), and Life Technology/SOLiD (http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/solid-next-generation-sequencing.html) are currently dominating the market.

Microarrays, although still an accurate and useful tool in gene expression studies, are now being replaced by seq-based methods, which can identify and quantify

rare transcripts without prior knowledge of the gene sequences and can provide information on alternative splicing and sequence variation in identified genes (Malone and Oliver 2011). Whole-transcriptome sequencing using NGS technologies, also known as RNA sequencing (RNA-seq), has started to reveal the complex landscape and dynamics of a transcriptome. However, the manipulation and the interpretation of the millions of short-read sequences produced by a typical NGS experiment still present significant computational challenges (Zhang et al. 2011; Martin and Wang 2011).

The obtained short reads can be (1) aligned to a reference genome, to obtain a quantitative measure of the transcript expression level, which is measured as read coverage (Mortazavi et al. 2008; Wilhelm and Landry 2009); and (2) de novo assembled, without an existing genome reference (Martin and Wang 2011).

To date, high-throughput transcriptome sequencing has been performed on only a few symbiotic (*L. bicolor*, *T. melanosporum*, *G. intraradices*) and two endophytic fungi (*Epichloe festucae*, *P. indica*) with the aim of improving the genome annotation as well as of identifying specific genes expressed during symbiosis (Larsen et al. 2010; Martin et al. 2010; Tisserant et al. 2012; Eaton et al. 2010; Zuccaro et al. 2011).

Larsen and colleagues (2010), using the RNA-seq approach in *L. bicolor*, have corrected most of the gene models that in the previous oligoarray analysis resulted to be differentially expressed during symbiosis, including genes related to carbon metabolism, membrane permeability and transport, and intracellular signaling (Martin et al. 2008a). Moreover, RNA-seq data obtained from fully developed *L. bicolor*/*Populus tremuloides* ECMs have been used to predict metabolomic models of mycorrhizal systems (Larsen et al. 2011). The deep RNA sequencing short reads have been used to identify significantly expressed gene models belonging to specific metabolic pathways. This approach allows the transcript profiles of the plant and its symbiotic fungus to be simultaneously determined, providing information on how the two partners cooperate to form this important symbiotic association. The mycorrhizal metabolome model suggests that *L. bicolor* can synthesize nitrogen compounds (i.e., glycine, glutamate, allantoin) via pathways not expressed in *P. tremuloides* roots, and these compounds might be exchanged with the photosynthetically derived sugars of the plant (Larsen et al. 2011).

In 2011, Tisserant and colleague deep sequenced the *T. melanosporum* transcriptome at three different developmental stages (free-living mycelium, fruiting body, ectomycorrhiza). These data have improved the *T. melanosporum* genomic structural annotation and led to the identification of 91 previously unidentified transcripts, exons, untranslated regions (UTRs) that extended in silico gene models, and alternative splicing events. In addition, RNA-seq transcript profiling, which provides a global view on the transcriptome complexity, has been used for detailed analyses of specific groups of genes (Balestrini et al. 2012b; Ceccaroli et al. 2011; Montanini et al. 2011; Rubini et al. 2011). To date, among the 72 JGI proposals on mycorrhizal fungi, ten projects have been aimed at fungal transcriptome and annotation.

Perspectives

Democratization of genome sequencing and the low cost and high quantities of the data being produced by new sequencing technologies will surely result in an avalanche of new sequenced genomes. This will enable the ECM/AM research community to go beyond the sequencing of new single genomes and allow already sequenced organisms to be "resequenced" (e.g., resequencing of a dozen strains of the ectomycorrhizal model species *L. bicolor*; Plett and Martin 2011), with the aim of verifying the intraspecific variability. In addition to elucidating the role of the mycorrhizal symbioses in nutrient cycling and plant health, genomic and transcriptomic sequencing projects have the goal of identifying the common core of symbiosis-related genes, as determinants of the symbiotic lifestyle. However, genome sequencing is only the first step toward knowledge of an organism and its capabilities to interact with the environment and with other organisms. The integration of functional, structural, molecular, cellular, and bioinformatics approaches is still required to obtain a deep understanding of the function of genes/proteins and the multiplicity of processes that occur inside an organism. Laser microdissection, for instance, is a powerful tool that can be used to isolate selected tissues/cell types from sectioned specimens, which allows DNA, RNA, proteins, and even metabolites to be extracted. It represents a useful and innovative technique that can be used to study plant-fungus interactions and in the analysis of gene expression in specific target cells/fungal compartments (Fig. 7.1) (Balestrini et al. 2009 and references therein; Hogekamp et al. 2011; Hacquard et al. 2013).

The metagenomics and metatranscriptomics studies that are currently under way, coupled with microarray construction, can provide a powerful approach to the analysis of environmental microbial transcriptomes, in order to uncover the functions encoded in the genomes of thousands of soil fungal species that cannot be cultured and sequenced directly (Martin and Martin 2010).

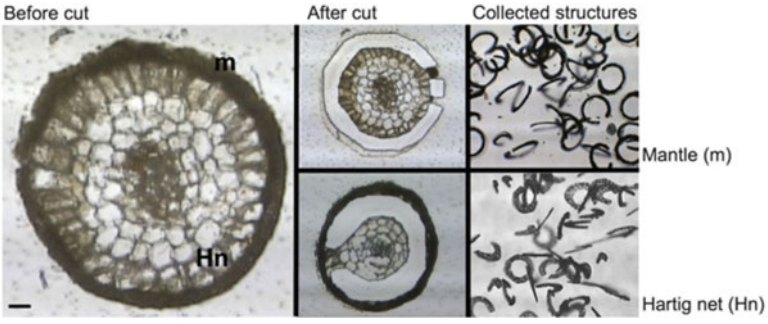

Fig. 7.1 Laser microdissection of the two compartments presents in an ectomycorrhiza from paraffin sections (mantle and Hartig net). *Bar* corresponds to 25 μm

Acknowledgements The authors would like to thank Francis Martin's lab, JGI and the Mycorrhizal Genomics Initiative consortium to access genome information before publication. RB was funded by Premio DAA2009 (CNR). FS's PhD fellowship was funded by Università di Torino.

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Chapter 8
Legume Root Nodule Associated Bacteria

G. Selvakumar, P. Panneerselvam, and A.N. Ganeshamurthy

Contents

Abstract Root nodules have intrigued mankind ever since their role in the maintenance of soil fertility has been known. The earlier school of thought amongst microbiologists and agronomists was that root nodules are highly specialised structures rich in leghaemoglobin, which house the diazotrophic bacterium *Rhizobium*, whose primary role was to fix atmospheric nitrogen in association with the host plant. But several path-breaking discoveries over the past few decades have thrown light on the plethora of bacterial occupants of the root nodules and their possible role in nodulation and N fixation besides several other beneficial roles. Recent technological advances in bacterial taxonomy and microbial ecology have unearthed a wide range of microbial nodule occupants, some of which have been encompassed under the classical umbrella of rhizobia, purely based on their ability to nodulate the host and fix atmospheric nitrogen, while other closely or even distantly related bacterial genera devoid of the ability to

G. Selvakumar (✉) • P. Panneerselvam • A.N. Ganeshamurthy
Division of Soil Science and Agricultural Chemistry, Indian Institute
of Horticultural Research, Bangalore 560089, India
e-mail: gselva74@rediffmail.com

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_8, © Springer India 2013

nodulate and fix nitrogen in nodules are often referred to as endophytes or simply nodule inhabitants. This chapter attempts to capture the existing knowledge on the root nodule associated bacteria both rhizobial and non-rhizobial and their possible roles in sustaining plant growth.

Introduction

The world over, legumes hold an important place in sustaining soil fertility and ensuring the nutritional security of both the human and animal populations. The uniqueness of this group of plants arises from the fact that they bear nodules, which serve as sites of nitrogen fixation, thereby enabling access of plants to ammoniacal nitrogen, a reduction product of atmospheric nitrogen. This process is mediated by the prokaryote – exclusive enzyme nitrogenase. Legumes are estimated to have evolved nearly 59 million years ago, with all three subfamilies recognisable soon after. Amongst the three subfamilies of legumes, nodulation is widespread in Papilionoideae, frequent in Mimosoideae and rare in Caesalpinioideae. This observation assumes significance since the subfamily Papilionoideae is thought to have been preceded by Mimosoideae and Caesalpinioideae (Allen and Allen 1981). Hence nodulating legumes are postulated to have evolved at a later time period in comparison to their non-nodulating relatives. An interesting question that arises at this point of time is that why nodulation evolved in some groups of legumes alone. Several lines of evidence suggest that, about 55 million years ago, when nodulated legumes evolved, there was a major peak in atmospheric carbon dioxide, temperature and humidity (Bowen et al. 2004), thereby creating an atmosphere of excess carbon dioxide. Since it is a well-established fact that the process of nitrogen fixation uses a significant amount of the carbon fixed by the host plant, a possible driving force behind the evolution of nodules could have been an excess of carbon dioxide coupled with deficiency of nitrogen. The first organisms that nodulated and colonised legume nodules presumably gained entry by the direct epidermal or crack infection. Subsequently, this led to two distinct modes of nodule development: one involving transcellular infection tubes, while the second mode was devoid of these specialised structures (Sprent 2007).

The recent past has noticed a surge of information on bacteria belonging to the α- and ß-proteobacterial groups, which are known to infect and nodulate legumes and have broadly accommodated under the umbrella term rhizobia. Apart from these, several bacterial genera and species exist in root nodules in the cryptic mode and are not known to harbour nodulation traits. Some of the early non-rhizobial bacteria isolated from legume nodules included *Agrobacterium* (De Lajudie et al. 1999) and *Bacillus* spp. (Bai et al. 2002). But later findings indicate that the nodule occupants can be as diverse as members of the genera *Inquilinus*, *Bosea*, *Rhodopseudomonas*, *Paracraurococcus*, *Phyllobacterium*, *Starkeya*, *Sphingomonas*, *Pseudomonas*, *Agromyces*, *Microbacterium*, *Ornithinicoccus* and *Paenibacillus*. Interestingly,

most of these non-rhizobial bacterial genera are not known to play a role in nodule formation (Zakhia et al. 2006). The mode of entry of such bacteria into root nodules and their possible roles in plant metabolism still remain to be clearly deciphered. This chapter attempts to capture the existing knowledge on various legume root nodule associated bacteria and their possible roles in sustaining plant growth and soil fertility.

The Legume Root Nodule as an Ecological Niche for Bacteria: An Evolutionary Perspective

A unique feature of rhizobia that sets them apart from plant-associated bacteria is their ability to ultimately become intracellular symbionts within nodule cells. The two major papilionoid nodule groups, namely, the dalbergioid and genistoid legumes, appeared early, about 55 million year ago. The dalbergioid legumes are characterised by the presence of aeschynomenoid nodules that are devoid of uninfected cells in the infected region, and their infection processes do not involve transcellular infection tubes (Lavin et al. 2001). The genistoid legumes also share similar characteristics but have an indeterminate growth pattern unlike the dalbergioid nodules that have a determinate growth pattern. It has been postulated that the default position for infection in these legumes lies directly between the epidermal or cortical cells (Sprent and James 2007). It has been observed that as rhizobia pass between cells, they may be surrounded by some of the extra cellular components normally found in transcellular infection tubes. This mode of infection accounts for approximately 25 % of all legume genera (Brewin 2004).

The second mode of nodule development takes place in legumes, which are thought to have evolved later, probably in between 55 and 50 million years ago. This involves the transcellular infection tubes, although in some cases the tubes might not be necessarily involved in the infection process. The entry of transcellular infection tubes into newly formed meristematic cells is accompanied by cessation of later phases of mitotic division. This leads to polyploid cell development and the cells become enlarged, thereby enabling them to house vast numbers of nitrogen-fixing bacteria. Individual cells are infected by branches of the transcellular infection tubes, and the active nitrogen-fixing tissue contains a mixture of both infected and uninfected cells. This pattern of nodule development appears common in some members of Mimosoideae and all members of Caesalpinioideae (Sprent and James 2007). While the mode of entry, colonisation behaviour and the nodulation process of the rhizobial group of bacteria have been well established, the mode of entry of the non-rhizobial species and their colonisation behaviour still continue to intrigue microbiologists. It has been widely speculated that the non-rhizobial bacterial species either enter nodules through the cracks that appear at the time of lateral root emergence or some species may even hitch a ride along with rhizobia, while a

later school of thought is that the non-rhizobial groups that exist in nodules are simply endophytes that exist within a nodule tissue without causing any external symptoms.

A Historical Perspective of Legume Root Nodule Associated Bacteria

In the beginning of the twentieth century, only one nodulating bacterium had been described, *Bacillus radicicola* (subsequently renamed as *Rhizobium*). This development was followed by the discovery of fast- and slow-growing rhizobia, which were subsequently given different generic names (*Rhizobium* and *Bradyrhizobium*). Subsequently, several genera of rhizobia infecting a wide variety of legumes and plant parts were recognised. The rhizobial genera initially associated with legume nodules were *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Sinorhizobium* (*Ensifer*), *Rhizobium* and *Mesorhizobium* (Zakhia and de Lajudie 2001). Currently, the International Committee on Systematics of Prokaryotes (ICSP), Subcommittee on Taxonomy of Rhizobium and Agrobacterium-Diversity, Phylogenetics and Taxonomy recognises 17 bacterial genera capable of nodulating and fixing atmospheric nitrogen in symbiosis with leguminous plants (http://edzna.ccg.unam.mx/rhizobial-taxonomy/node/4). These include 14 α-proteobacterial genera and three genera of β-proteobacteria. The latest genus to be included in this list is *Microvirga*, which is found to encompass three nodulating species in taxonomically separate legume hosts (Ardley et al. 2012).

A landmark discovery in rhizobial ecology was the discovery of the ability of *Burkholderia* and *Cupriavidus*, both belonging to the ß-class of proteobacteria to nodulate legumes (Chen et al. 2001; Moulin et al. 2001). This gains significance since it was believed that the nodulation trait was exclusively distributed amongst the α proteobacteria to which the classical *Rhizobium* and its related genera belong. A later development in β-proteobacteria was the inclusion of the genus *Herbaspirillum* as a nodulating bacterial species (Valverde et al. 2003). To encompass this massive development, the terms rhizobia/root-nodulating bacteria (RNB)/legume-nodulating bacteria (LNB) have been coined and have been used by various workers. But the underlying feature of all these terms is the ability of the bacterial species to nodulate and fix atmospheric nitrogen in association with various legume species. Some of the significant milestones in the discoveries of association between legumes and their root-nodulating bacteria are listed in Fig. 8.1.

Bacteria Associated with Legume Root Nodules

For the sake of brevity and better understanding, we have classified the root nodule-associated bacteria into two sections, namely, rhizobial and non-rhizobial, with emphasis on the non-rhizobial bacteria that are associated with nodules and their functional role in plant growth and development.

- 1679-Malphigi describes "lumps" on legume roots
- 1829-Meyen makes observations on legume root associated bacteria
- 1866-Woronin confirms Meyen's Hypotheses
- 1888-Hermann Hellriegel and Hermann Wilfarth discover the nitrogen contribution potential of nodules
- 1888-Martinus Beijerinck isolated the nodule associated bacteria by enrichment technique and names them *Bacillus radicicola*
- 1889-Renamed as *Rhizobium leguminosarum* by Frank
- 1932-Fred proposes the cross inoculation grouping of Rhizobia
- 1942-Cohn discovers *Agrobacterium*
- 1964-Distinguishing of fast and slow growing rhizobia by Graham
- 1982-Discovery of *Bradyrhizobium*
- 1988-Discovery of *Azonhizobium* and *Sinorhizobium*
- 1997-Discovery of *Mesorhizobium* and presence of several non rhizobial bacteria in root nodules
- 1998-Discovery of *Allorhizobium*
- 2001-Discovery of root nodulation by β-proteobacteria
- 2004-Claim of nodulation by γ proteobacteria
- 2012-*Microvirga* included as a root nodulating bacteria

Fig. 8.1 Significant milestones in legume root associated bacterial discovery

Rhizobial Occupants of Legume Root Nodules

The evolution of rhizobial taxonomy from a single species to the present-day umbrella of rhizobia has been a long winding path, where several candidate genus and species were included/excluded over extended periods of time, while several original genera/species and some others have not been proved conclusively. Currently, the ICSP Subcommittee on Taxonomy of Rhizobium and Agrobacterium-Diversity, Phylogenetics and Taxonomy recognises 17 bacterial genera, namely, *Allorhizobium*, *Aminobacter*, *Azorhizobium*, *Bradyrhizobium*, *Devosia*, *Ensifer*, *Mesorhizobium*, *Methylobacterium*, *Microvirga*, *Ochrobactrum*, *Phyllobacterium*, *Rhizobium*, *Shinella*, *Sinorhizobium* (*Ensifer*), *Burkholderia*, *Cupriavidus* and *Herbaspirillum*, which are capable of nodulating and fixing atmospheric nitrogen in symbiosis with leguminous plants (http://edzna.ccg.unam.mx/rhizobial-taxonomy/node/4). The last three genera that are listed above constitute the beta-proteobacterial group within the rhizobial framework (Fig. 8.2).

The genus *Burkholderia* comprises of the following nodulating species, namely, *Burkholderia tuberum* (Vandamme et al. 2002), *B. phymatum* (Vandamme et al. 2002), *B. mimosarum* (Chen et al. 2006), *B. nodosa* (Chen et al. 2007), *B. sabiae* (Chen et al. 2008), *B. caribensis* (Chen et al. 2003), *B. contaminans* (Vanlaere et al. 2009), *B. fungorum* (Coenye et al. 2001), *B. lata* (Vanlaere et al. 2009) and *B. symbiotica* (Sheu et al. 2012). The symbiosis-related genes of *Burkholderia* are thought to have diverged over a long period within *Burkholderia* without substantial horizontal gene transfer between species complexes (Bontemps et al. 2010). An interesting feature of rhizobial taxonomy is that often the same genus or even species contains both rhizobial and non-rhizobial strains; for example, the genus *Methylobacterium* contains one rhizobial species *M. nodulans* (Jourand et al. 2004), in addition to several saprophytic species. Similarly, *Cupriavidus* (formerly *Ralstonia taiwanensis*) species is known to have been isolated from nodules as well as clinical samples (Chen et al. 2001). Therefore, it would be ideal to assess the nodulation potential of a bacterial strain and detect the presence of *nod* and *nif* genes, before assigning it to the

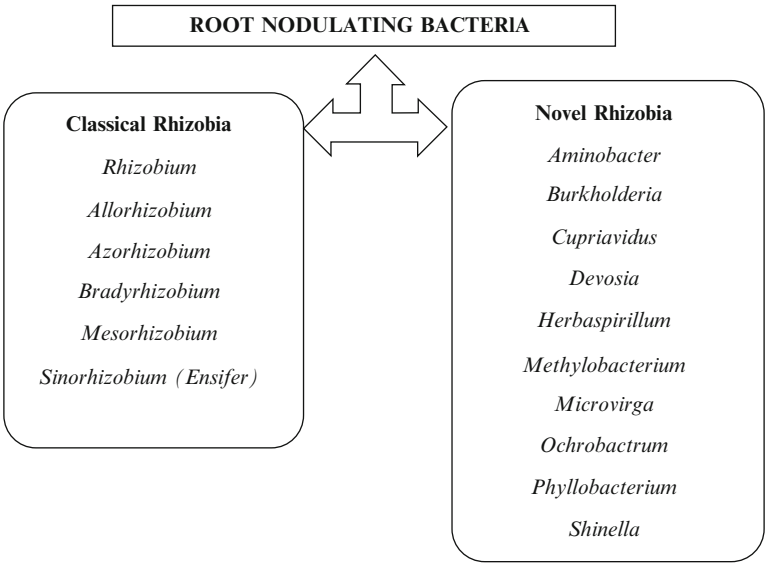

Fig. 8.2 Classical and novel rhizobial genera that are known to nodulate legumes

broad umbrella of rhizobia. Table 8.1 presents a list of the nonclassical rhizobia that have been known to be associated with various leguminous plants.

Though each rhizobial species has a specific host spectrum, there is no strict correlation between legume and bacterium taxonomy, and very often the same bacterium has been recovered from more than one host. However, some associations are known to be favoured in nature (e.g. *Azorhizobium–Sesbania* and *Burkholderia–Mimosa*).

Novel Rhizobia and Diazotrophy

Till date nodulation and diazotrophy remain the buzz words in rhizobial explorations, and hence, the world has witnessed an explosion in the number of nodulating and N-fixing bacterial species. With the explosion in knowledge on novel rhizobial, one would naturally expect the utilisation of these bacterial strains in inoculum development for crop production. But, unfortunately, the converse is true and till date the most favoured rhizobial strains used for crop production are limited species of the classical *Rhizobium* and *Bradyrhizobium* and to a limited extent *Sinorhizobium* (*Ensifer*). If one were to explore the reasons for this, it is evident that most novel nodulating bacteria have been discovered from wild legumes and their nitrogen-fixing abilities have been attributed solely to the presence of the *nod* and *nif H* gene. With the exception of a handful of experiments, very few studies

Table 8.1 Published nonclassical rhizobia associated with various legumes and their features

Bacterial species	Host	Feature	Reference
Blastobacter denitrificans	*Aeschynomene indica*	Presence of *nif* HDK gene	Van Berkum and Eardly (2002)
Devosia neptuniae	*Neptunia natans*	*nod* D gene of *Devosia* is closely related to *R. leguminosarum*	Rivas et al. (2002)
Devosia yakushimensis	*Pueraria lobata*	Isolated from the nodules, renodulation not reported	Bautista et al. (2010)
Ensifer adhaerens	*Sesbania grandiflora*, *Medicago sativa*, *etc.*	Isolated from multiple genera	Merabet et al. (2010), Willems et al. (2003)
Ensifer arboris	*Acacia senegal*, *Prosopis chilensis*	Isolated from multiple genera	Nick et al. (1999)
Ensifer fredii	*Glycine* spp., *Vigna unguiculata* and *Cajanus cajan*	Isolated from multiple genera	Chen et al. (1988)
Ensifer kostiense	*Acacia senegal*, *Prosopis chilensis*	Isolated from multiple genera	Nick et al. (1999)
Ensifer kummerowiae	*Kummerowiae stipulacea*	Single host	Wei et al. (2002)
Ensifer medicae	*Medicago* spp.	Isolated from multiple hosts	Rome et al. (1996)
Ensifer meliloti	*Medicago* spp., *Melilotus* spp.	Isolated from multiple genera	Rome et al. (1996)
Ensifer mexicanus	*Acacia* spp., *Phaseolus vulgaris*	Isolated from multiple genera	Lloret et al. (2007)
Ensifer morelense	*Leucaena leucocephala*	Single host	Wang et al. (2002)
Ensifer numidicus	*Argyrolobium uniflorum*	Single host	Merabet et al. (2010)
Ensifer saheli	*Sesbania* spp.	Single host	De Lajudie et al. (1994)
Ensifer teranga	*Sesbania* spp.	Isolated from multiple hosts	Lortet et al. (1996)
Ensifer xinjiangense	*Glycine max*	Single host	Chen et al. (1988)
Methylobacterium nodulans	*Crotalaria* spp.	*M. nodulans* contains *nod* ABC gene and genes encoding structural nitrogenase enzyme	Sy et al. (2001)
Ochrobactrum lupini	*Lupinus albus*	The nodulating and nitrogen-fixing genes (*nod* and *nif* genes) were detected in all the *sym* plasmids using *nif H* and *nod D* probes	Trujillo et al. (2005)
Ochrobactrum cytisi	*Cytisus scoparius*	Single host	Zurdo-Pineiro et al. (2007)

(continued)

Table 8.1 (continued)

Bacterial species	Host	Feature	Reference
Ochrobactrum cytisi	*Lupinus albus*, *Lupinus honoratus*	Single host	Trujillo et al. (2005)
Phyllobacterium trifolii	*Trifolium pratense*, *Trifolium repens* and *Lupinus albus*	It harbours symbiotic plasmids which have nodulating and nitrogen-fixing genes	Valverde et al. (2005)
Phyllobacterium ifriqiyense	*Trifolium pratense*, *T. repens* and *Lupinus albus*	Isolated from multiple genera	Mantelin et al. (2006)
Phyllobacterium leguminum	*Astragalus algerianus* and *Argyrolobium uniflorum*	Isolated from multiple genera	Mantelin et al. (2006)
Shinella kummerowiae	*Kummerowia stipulacea*	Unable to renodulate the original host plant	Lin et al. (2008)
Burkholderia caribensis	*Mimosa pudica*, *M. diplotricha*	Single host	Chen et al. (2003), Vandamme et al. (2002)
Burkholderia dolosa	*Alysicarpus glumaceus*	Only one strain isolated from the host plant	Vandamme et al. (2002)
Burkholderia mimosarum	*Mimosa pigra*, *M. scabrella*	Single host	Chen et al. (2006)
Burkholderia nodosa	*Mimosa bimucronata*, *M. scabrella*	Single host	Chen et al. (2007)
Burkholderia phymatum	*Machaerium lunatum*	Presence of *nod* ABC shows the capability to produce nod factors to initiate nodulation	Vandamme et al. (2002)
Burkholderia tuberum	*Aspalathus carnosa*	Presence of *nod* ABC shows the capability to produce nod factors to initiate nodulation	Vandamme et al. (2002)
Burkholderia sabiae	*Mimosa caesalpineafolia*	Single host	Chen et al. (2008)
Cupriavidus taiwanensis	*Mimosa* spp.	Single host	Chen et al. (2003)
Cupriavidus necator	*Mimosa caesalpineafolia* , *L. leucocephala*, *Macroptilium atropurpureum*, *P. vulgaris* and *Vigna unguiculata*	Isolated from multiple genera	da Silva et al. (2012)
Herbaspirillum lusitanum	*Phaseolus vulgaris*	Single host	Valverde et al. (2003)

Note: The nomenclature of some published species has not been validated by the ICSP Subcommittee on the Taxonomy of *Rhizobium* and *Agrobacterium*

have been carried out with the novel rhizobial in order to establish their diazotrophic potential. In one of the available studies, Garu et al. (2009) studied the symbiotic capabilities of the beta-proteobacteria *Burkholderia phymatum* STM815^T and *Cupriavidus taiwanensis* LMG 19424^T, when inoculated onto the papilionoid legumes *Rhynchosia ferulifolia*, *R. caribaea*, *Rhynchosia minima* and *Macroptilium atropurpureum* (Siratro). The root nodule bacteria isolated from *R. minima* and *R. totta* were also included in the study. The level of N fixation by this symbiosis was reported to be almost as efficient as that of the *Medicago* symbiosis. While the molecular evidence and taxonomic validation of such novel strains are definitely of interest, the utility of such rhizobia in terms of N contribution to the host and the soil on which it grows has been poorly established. This requires the determination of the ability of the novel rhizobia to nodulate a wide spectrum of cultivated legumes, besides studies such as the classical acetylene reduction assay (ARA) and ^{15}N studies. But the unfortunate part is that these crucial studies have not received the attention of microbiologists and agronomists the word over, and hence to this day the realm of rhizobial inoculant usage has not moved beyond the boundaries of a handful of species. The ^{15}N isotope dilution technique (Talbott et al. 1982) continues to be a preferred method for determination of the N-fixing potential of any legume–rhizobia symbiosis. Hence, much more information needs to be generated by this technique using the novel rhizobial and a wide range of host legumes.

Non-rhizobial Occupants of Legume Root Nodules

The later part of the last century was dotted with findings that lead to a surge in the explorations of various legume root nodules and exposed a plethora of bacteria that were hitherto known to exist in association with legume nodules. The observations that legume root nodules play hosts to diverse microbes like *Bacillus*, *Streptomyces*, *Herbaspirillum*, Arbuscular Mycorrhizal Fungi and *Agrobacterium* (Sturz et al. 1997; De Lajudie et al. 1999; Tokala et al. 2002; Valverde et al. 2003; Scheublin et al. 2004) gave rise to a school of thought that they were probably endophytes. But the term ‘endophyte’ has been much debated, and for a bacterial species to be denoted as a ‘true endophyte’ more stringent evaluation than mere isolation from surface-sterilised plant tissue is suggested (Schulz and Boyle 2006). Hence, most non-rhizobial bacteria found in root nodules are commonly referred to as nodule inhabitants. It would not be far-fetched to say that many of these initial observations probably led to our present-day understanding of the novel nodulating bacterial genera that fall outside the classical *Rhizobium*.

Sturz et al. (1997) made a novel observation that the legume root nodule is known to accommodate several eubacterial genera apart from rhizobia and their population densities are reported to be in the range of 10^4 viable bacteria per gram of fresh nodule tissue. A pioneering observation made by them was that clover root nodules were host to 12 bacteria species other than rhizobia, including eight tissue-specific ones. Interestingly, it was reported that *R. leguminosarum*

bv. *trifolii* constituted only 8.8 % of all the root nodule bacteria recovered. In another early report, Bai et al. (2002) reported the isolation of putative endophytic *Bacillus* including a growth-promoting *Bacillus thuringiensis* strain from the nodules of soybean plants. This was followed by a dramatic claim that nodules of the legume *Hedysarum* were nodulated by bacteria belonging to the class Gammaproteobacteria (Benhizia et al. 2004). This claim was based on the lack of any rhizobial-like sequence on amplification of the bulk of microbial cells obtained from the squashed nodules. The authors therefore speculated that the exclusive occupants of the nodules formed by the three plants belonged to the orders Enterobacteriales or Pseudomonadales. The bacterial species implicated in the nodulation process include *Pantoea agglomerans*, *Enterobacter kobei*, *Enterobacter cloacae*, *Leclercia adecarboxylata*, *Escherichia vulneris* and *Pseudomonas* sp. But till date, this finding is yet to gain credibility amongst rhizobial workers, and the status of the bacterial species remains more of endophytes rather than true nodulants.

Later Wang et al. (2006a) detected the presence of *Pantoea*, *Erwinia*, *Salmonella*, *Enterobacter*, *Citrobacter* and *Klebsiella* in nodules of the tree species *Conzattia multiflora* grown in Mexico. The presence of *Agrobacterium* strains in nodules, but incapable of nodulating their hosts, has been frequently reported from the nodules of different legumes, and various possible mechanisms have been proposed to explain the existence of these bacteria within nodule tissue (De Lajudie et al. 1999; Han et al. 2005). Wang et al. (2006b) proved that the *Agrobacterium* strain CCBAU 81181, which was originally isolated from the root nodules of *Onobrychis viciifolia*, and a symbiotic strain of *Sinorhizobium meliloti* CCBAU 10062 could actually co-inhabit the root nodules of *Melilotus dentatus*. Kan et al. (2007) concluded from a study of 61 root nodule isolates from diverse legumes, namely, *Vicia*, *Oxytropis*, *Medicago*, *Melilotus* and *Onobrychis* species grown in Qinghai–Tibet plateau, that in addition to nodulating genera like *Rhizobium leguminosarum*, *S. meliloti*, *Sinorhizobium fredii*, *Mesorhizobium* sp. and *Phyllobacterium* sp., two non-symbiotic groups related to *Agrobacterium* and Enterobacteriaceae were present in their nodules. Selvakumar et al. (2008) reported the presence of diverse plant growth promoting strains of bacteria such as *Bacillus thuringiensis*, *Enterobacter asburiae* and *Serratia marcescens* from the nodules of the legume *Kudzu* (*Pueraria thunbergiana*) grown in the Indian Himalayan Region. Dashti et al. (2009) made an unusual finding that the surfaces of root nodules of *Vicia faba* and *Lupinus albus* were colonised by bacterial consortia that utilised oil and fixed nitrogen. This finding has immense value in the realm of nitrogen-poor desert soils where anthropogenic oil spills are quite common. The nodules of peanut grown in Argentina were found to harbour Gammaproteobacteria predominantly belonging to the genera *Pseudomonas* spp., *Enterobacter* spp. and *Klebsiella* spp. These strains enhanced plant yield and colonised preformed nodules when co-inoculated with an effective bradyrhizobial strain (Ibáñez et al. 2009). The presence of endophytic bacteria belonging to Alphaproteobacteria, Betaproteobacteria, Actinobacteria and Firmicutes phyla encompassing nine different

Table 8.2 Some non-rhizobial bacteria associated with legume root nodules and their features

Bacterial species	Host plant	Features	Reference(s)
Agrobacterium-like strains	*Phaseolus vulgaris*, *Acacia*, *Prosophis*, *Chamaecrista*	Nitrogen-fixing genes were detected	Mhamdi et al. (2002), De Lajudie et al. (1999)
Labrys neptuniae	*Neptunia oleracea*	Novel species	Chou et al. (2007)
Microbacterium sp. and *Starkeya* sp.	Spontaneous legumes	Presence of *nif* H-like gene detected	Zakhia et al. (2006)
Bacillus megaterium, *Brevibacillus choshinensis* and *Microbacterium trichothecenolyticum*	*Medicago sativa*	Plant growth promotion traits	Stajković et al. (2009)
Bacterial isolates with maximum similarity to *Bacillus subtilis*, *Bacillus simplex* and *Agrobacterium tumefaciens*	*Vigna radiata*	IAA production, P solubilisation	Tariq et al. (2012)

genera, namely, *Arthrobacter*, *Bacillus*, *Bradyrhizobium*, *Burkholderia*, *Dyella*, *Methylobacterium*, *Microbacterium*, *Rhizobium* and *Staphylococcus*, from the nodules of the legume *Lespedeza* sp. grown in two different locations in South Korea was reported by Palaniappan et al. (2010). Most of the isolates they studied showed multiple plant growth promotion activity, i.e. indole acetic acid production, ACC deaminase activity, siderophore production and phosphate solubilisation.

The knowledge about the plethora of bacterial nodule inhabitants has expanded and some interesting reports have started to emerge. The existence of plant-borne lineages of *Salmonella* was an interesting observation, with public health implications (Wang et al. 2006a). Muresu et al. (2010) observed that nodules of three wild legumes of the genus *Hedysarum* grown in Algeria harboured potential human pathogenic bacterial strains such as *Enterobacter cloacae*, *Enterobacter kobei*, *Escherichia vulneris*, *Pantoea agglomerans* and *Leclercia adecarboxylata*. These strains exhibited pathogenic traits such as cytotoxicity, vital strain exclusion and adhesion to epithelia. In a recent report, the presence of coccobacilli was reported from the root nodules of fenugreek (*Trigonella foenum-graecum*). An interesting observation here is that 64.7 % of the bacterial occupants were Gram-negative coccobacilli and 29.41 % were Gram-positive bacilli. Two isolates possessing maximum positive PGP features belonged to the genus *Exiguobacterium* (Rajendran et al. 2012), which is probably the first for this genus. The existence of *Micromonospora* in nodules of *Lupinus angustifolius* collected from Spain has been recently reported by Trujillo et al. (2010). Table 8.2 lists some of the non-rhizobial bacteria that are known to occur in legume root nodules.

Functional Role of Legume Root Nodule Associated Bacteria

While the primary role of rhizobia is the conversion of atmospheric nitrogen to ammonia, through an energy-intensive reduction process, it has been variably argued that the micro-symbiont drains the host of its energy resources (Vance and Heichel 1991). But this school of thought has been debated since the overall benefit the plant and the surrounding ecosystem derive as a result of the nitrogen fixation process far outweighs the drain on the carbon resources of the plant. In contrast to the rhizobia whose role is largely confined to diazotrophy, non-rhizobial nodule occupants seem to have a diverse influence on the plant survival, nodulation and growth promotion and yield (Remans et al. 2008; Selvakumar et al. 2008). It has been hypothesised that IAA of bacterial origin from the nodules is transported to other plant parts (Basu and Ghosh 1998) and dually occupied nodules serve as hot spots for lateral gene transfer of symbiotic genes from rhizobia (Valverde et al. 2005). In general, the non-rhizobial bacteria are thought to synergistically act with rhizobia and increase nodulation and yield possibly by production of growth hormones like IAA production, solubilisation of nutrients, N fixation and siderophore production. In view of this, researchers tend to focus their attention towards the isolation and characterisation of non-rhizobial bacteria from legume nodule and utilising these strains to improve nodulation and crop growth yields.

The symbiotic effectiveness of rhizobia can be improved by co-inoculation with suitable non-rhizobial beneficial bacteria in most legume crops (Lazdunski et al. 2004). In some studies, *Azotobacter*, *Azospirillum*, *Burkholderia*, *Enterobacter* and *Kurthia* have also been evaluated with rhizobia and were found to improve plant growth (Pandey and Maheshwari 2007). Hung et al. (2007) isolated endophytic bacteria from surface-sterilised stems, roots and nodules of wild and cultivated soybean varieties. They analysed various phenotypic traits that are expected to be involved in the persistence and functions of these bacteria. Most of the isolates from soybean were motile, indole acetic acid producers capable of cellulase and pectinase activities. A strain of *Bacillus thuringiensis* originally isolated from nodules of the wild legume *Kudzu* was able to promote plant growth and nodulation of soybean when inoculated with *Bradyrhizobium japonicum* (Mishra et al. 2008). The same Bt strain when co-inoculated with *R. leguminosarum* improved plant growth and nodulation of pea and lentil (Mishra et al. 2009).

The role of nodule bacteria in the selection of the rhizobial stains was revealed by Mrabet et al. (2006) who observed in soils of Tunisia, that nodulation of common beans showed a biased genetic structure, with high levels of inhibition of *Rhizobium gallicum*, while nodulation by *Sinorhizobium medicae* was favoured. The co-inoculation of non-sterile soils with *R. gallicum* and *Agrobacterium* confirmed these findings. In vitro antibiosis assays indicated that agrobacteria possessed a significant antagonism against *R. gallicum*. The positive effect of co-inoculation of non-rhizobial endophytes isolated from sterilised root nodules of alfalfa (*Medicago sativa* L.) and *Sinorhizobium meliloti*, on nodule numbers in alfalfa, as compared to

S. meliloti inoculation alone has been reported by Stajković et al. (2009). The existence of 99 % similarities in the *nif* H genes of *Bradyrhizobium japonicum* and the endophytic *Bacillus* strains strongly indicates the possibility of horizontal transfer of symbiotic genes between the symbiotic bacteria and the endophytes (Li et al. 2008). The nodulation behaviour of soybean seems to have an effect on the endophytic occupants of soybean stems. A greater abundance of Firmicutes was observed in Nod^{-} (non-nodulating) and Nod^{++} (hyper-nodulating mutants) of soybeans, compared to the wild type (Okubo et al. 2009). A few interpretations that can be drawn from the available information are that non-rhizobia occur in significant numbers and influence the rhizobial microbial composition of the nodule. Most of these isolates posses plant growth promotion traits. But their definite role within the legume root nodule needs to be established.

Conclusion

Interest in legume nodule microbiology has grown by leaps and bounds over the years, and with the continuing addition to the existing knowledge on nodule-occupying bacteria and their functional role, it is being increasingly recognised that the nodule harbours not only symbiotic 'rhizobia' but also a wide plethora of non-rhizobial organisms that play both well-established and cryptic roles in plant metabolism. The frontiers of science are being pushed beyond their boundaries, which is much evident from the numbers of nodulating and nitrogen-fixing genera, which have been broadly accommodated under the umbrella term 'rhizobia'. But most of these developments remain as artefacts of academic interest, and their utility in terms of inoculant production and utilisation has remained largely unexplored. Similarly, the ever-expanding knowledge on non-rhizobial nodule-associated bacterial species has also remained confined to the pages of academic journals, while their practical utility has not seen the dawn of the day. Some future lines of research could be:

1. Determination of the cross inoculation potential of the novel rhizobia in association with cultivated legumes
2. Establishment of the N-fixing potential of the novel rhizobia, by classical methods such as the ARA assay and ^{15}N dilution studies
3. Quantification of the diazotrophic benefits of the novel rhizobial–legume association by isotopic and non-isotopic methods
4. Exploration and utilisation of the non-rhizobia as inoculants in association with rhizobia, preferably in the consortia mode in order to promote effective nodulation.

Therefore, it needs to be emphasised that the utility of both rhizobial and non-rhizobial–legume nodule associated bacteria will be realised fully only when they move beyond the confinement of the publication space and tend to be utilised as inoculants in order to harness their potential for the nutritional security of mankind.

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Chapter 9
Legume–Rhizobia Symbiosis and Interactions in Agroecosystems

Asmita Rajwar, Manvika Sahgal, and Bhavdish N. Johri

Contents

A. Rajwar • M. Sahgal (✉)
Department of Microbiology, G. B. Pant University of Agriculture and Technology, Pantnagar 263 145, Uttarakhand, India
e-mail: sahgal.manvika@gmail.com

B.N. Johri
Department of Biotechnology, Barkatullah University, Bhopal 462 026, Madhya Pradesh, India

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_9,

Abstract In the present scenario, when the population of the world is expected to become 8–9 billion by 2040, the major concern is to maintain sustained food supply. Production of high-quality protein-rich food is extremely dependent on the availability of sufficient nitrogen. Nitrogen though abundant on Earth is unavailable to plants. Indiscriminate use of nitrogenous chemical fertilisers has significantly increased food production and quality but at the same time affected ecosystem sustainability. Hence, the process of biological nitrogen fixation (BNF) has gained considerable significance. BNF is both free-living as well as symbiotic. Symbiotic N_2 fixation accounts for about 65 % of the total biologically fixed nitrogen. *Frankia* and rhizobia are two groups that fix atmospheric nitrogen symbiotically. Out of these, rhizobia–legume symbiosis accounts for about 45 % of nitrogen being used in agriculture. Rhizobia and legumes both are diverse. Currently 98 species of legume-nodulating bacteria have been identified within 13 bacterial genera, 11 in α-proteobacteria, whereas 2 in β-proteobacteria. Similarly, 13,000 species have been identified in 700 legume genera. Specificity of nodulation is an important attribute of legume–rhizobia symbiosis and is governed by both legume and rhizobial signals. For any successful legume–rhizobia symbiosis, interaction with other belowground microbes like AM fungi is also important. Here we give an account of rhizobial diversity and systematics, signals governing legume–rhizobia symbiosis, genes regulating nodulation and nitrogen fixation and legume–rhizobia–AM interactions.

Rhizobia?

Rhizobia are classically defined as soil bacteria capable of eliciting and invading root and stem tissue forming nodules on leguminous plants. Inside the nodules, the rhizobia convert dinitrogen into ammonia and ammonium compounds, and this process is known as nitrogen fixation. Legume–rhizobium association is both ‘symbiotic’ as well as ‘mutualistic’. It is symbiotic because bacteria live in intimate association with the plant and mutualistic because both partners gain.

‘Rhizobium’ *Versus ‘Rhizobium’*

The word ‘rhizobium’ is actually derived from two Greek words ‘rhizo’ meaning root and ‘bium’ meaning home, together conveying the meaning ‘root dweller’; ‘rhizobium’ is single bacterium and ‘rhizobia’ several bacteria. ‘*Rhizobium*’ is the

formal taxonomic name of a bacterial genus, and this certainly cannot be written as Rhizobia. Since the late nineteenth century (Frank 1889), all legume root-nodule bacteria were placed in the genus '*Rhizobium*'. Gradually it was realised that they were rather diverse. A few slow-growing rhizobia were split off into a new genus '*Bradyrhizobium*'. In the 1984 edition of Bergey's *Manual of Systematic Bacteriology* (Krieg and Holt 1984), all rhizobia were placed in the family *Rhizobiaceae* which included *Bradyrhizobium* and *Rhizobium*. Since then, the number of bacterial genera representing rhizobia has increased rapidly (Sy et al. 2001); presently, rhizobia are placed in genera that have been created to describe other non-nodulating bacteria as well (Willems 2006). Thus, the genus name is no longer a good criterion to describe whether a bacterium will be a rhizobium.

Importance of Legume–*Rhizobium* Symbiosis

In the present scenario, the population of the world stands at 6 billion and is projected to increase and stabilise at 8–9 billion by 2040; the major concern is to maintain sustained food supply to feed an ever-increasing global population. The adequate food production is possible using intensive agricultural practices, that is, increased use of chemical fertilisers and irrigation. As currently practised, agriculture will require an additional 40 and 20×10^6 million tonnes of N and P, respectively, to meet food production needs in the year 2040. The use of chemical fertilisers has increased agricultural production, but it is accompanied by deteriorating soil health and environmental quality (Tilman et al. 2001; Trewavas 2001).

Although nitrogen is amongst the most abundant element on Earth, it is the critical limiting element for growth of plants due to its unavailability (Graham and Vance 2000). Production of high-quality protein-rich food is extremely dependent upon availability of sufficient nitrogen. Plants acquire nitrogen from two principal sources: (a) the soil, through commercial fertilisers and manure/mineralisation of organic matter, and (b) biological fixation of atmospheric nitrogen (BNF). The first option that is the intense use of chemical fertilisers has been practised since 1960s and accounts for about 25 % of Earth's fixed nitrogen. About 50 % of the nitrogenous chemical fertilisers that are applied to agricultural fields are leached, and this has led to contamination of soil, increased concentration of toxic nitrates in drinking water and eutrophication of lakes and rivers. This has adversely affected biodiversity and ecosystem sustainability. Thus, in the present scenario, BNF has gained importance.

BNF is estimated to add nearly 90 % of 180×10^6 metric tonnes of the total nitrogen fixed annually in the terrestrial environment (Sahgal and Johri 2003; Gage 2004) which is equivalent to generation of resources equivalent to US $160–180 billion. This process is catabolised by prokaryotes only. Prokaryotes fixing atmospheric nitrogen are diverse. These include 2 genera of archaea, 38 genera of bacteria and 20 genera of cyanobacteria. The process of biological nitrogen fixation is both free-living as well as symbiotic. Symbiotic nitrogen fixation is restricted to a limited number of bacterial

groups, i.e. *Frankia* and rhizobia. *Frankia* is a filamentous Gram-positive actinomycete that induces nodules on a variety of woody plants in the families *Betulaceae*, *Casuarinaceae*, *Coriariaceae*, *Datiscaceae*, *Elaeagnaceae*, *Myricaceae*, *Rhamnaceae* and *Rosaceae* (Benson and Clawson 2000). Rhizobia are Gram-negative bacteria that induce nodules on stem and roots of plants belonging to family *Leguminosae*. They represent 13 genera spread over α- and β-proteobacteria.

There are approximately 700 genera and about 13,000 species of legumes, only 20 % of which have been examined for nodulation and shown to have the ability to fix nitrogen. Symbioses of rhizobia with 100 agriculturally important legumes contribute about 70 million tonnes of nitrogen year^{-1}. Legume–rhizobia symbiosis, apart from reducing the use of chemical nitrogen fertilisers, also contributes to carbon sequestration. The biological nitrogen fixation of 45×10^6 metric tonnes of nitrogen per year by legume–rhizobia symbiosis is equivalent to sequestering an additional 770 to 990×10^6 metric tonnes of carbon year^{-1} (Vance 2001). Thus, in conclusion it can be said that BNF is of substantial economic importance in low-input sustainable agriculture, agroforestry and land reclamation.

Diversity of Rhizobia Versus Taxonomy

Rhizobial Classification Based on Specificity of Symbiotic Plant Range

Rhizobia have legume host preferences for nodulation and nitrogen fixation. Nobbe and co-workers (1891, 1895) observed that bacteria isolated from legume *Pisum sativum* were very specific and were unable to nodulate plants belonging to the legume tribes Genisteae and Hedysareae. Thus, earliest classification of rhizobia was based on the hosts it nodulated and fixed nitrogen (Hiltner and Störmer 1903). Fred et al. (1932) recognised six species in the genus *Rhizobium*, namely, *R. japonicum* (*Lathyrus*, *Lens*, *Pisum* and *Vicia*), *R. lupini* (*Lupinus*), *R. meliloti* (*Melilotus*, *Medicago*, *Trigonella*), *R. phaseoli* (*Phaseolus*) and *R. trifolii* (*Trifolium*) based on their host range for nodulation. A few years later, Wilson (1939), while testing the host ranges of rhizobia isolated from 31 different genera of legumes on 160 different legume species, observed that on an average a particular rhizobial isolate nodulated 33 % of the total legume species. He also reported that *Rhizobium* sp. strain NGR234 nodulated 112 out of 160 legume genera tested, and *R. fredii* USDA257 nodulated 77 genera, whereas *Vigna* was a promiscuous host that was nodulated by several rhizobial species. Now it is well established that a single rhizobial species is able to nodulate different legume genera, and that many legumes can be nodulated by several rhizobial species (for review see Sahgal and Johri 2003; Perret et al. 2000). It is only six decades later in the early 1960s that rhizobia were separated into different groups based on extensive microbiological criteria (Graham 1964; Moffett and Colwell 1968). At the same time Norris (1965) observed differences in growth rate of rhizobia and proposed that it was associated with their symbiotic affinity. Slow

growers were largely associated with tropical legumes and fast growers with temperate legumes (Allen and Allen 1981; de Lajudie et al. 1994). But several workers (Dreyfus and Dommergues 1981; Scholla and Elkan 1984; Jenkins et al. 1987; Fulchieri et al. 1999) reported the presence of both fast- and slow-growing rhizobia in tropical legumes. The roots of tropical legume *Phaseolus vulgaris* were nodulated by ten different *Rhizobium* species. These species include *Bradyrhizobium japonicum*, *Mesorhizobium loti*, *Rhizobium etli*, *R. tropici*, *R. leguminosarum* bvs. *trifolii* and *viciae*, *Rhizobium* spp. NGR234 and GRH_2, *Sinorhizobium fredii* and *S. meliloti* (Michiels et al. 1998). Similarly, there are *Rhizobium* strains which are relatively non-specific for their legume partner, e.g. *Rhizobium* sp. strain NGR234 that has broad host range and is able to elicit nodules on 50 % of the known legumes (Pueppke and Broughton 1999). Hence, classification of rhizobia on the basis of host range and biological and physiological properties has serious shortcomings.

Polyphasic Approach for Taxonomy

In the 1990s emerged the concept of polyphasic taxonomy. Polyphasic taxonomic approach includes characterisation based on biochemical, physiological and genetic fingerprinting methods along with host range for nodulation in case of rhizobia. This has led to the description of the new genera and reorganisation of the existing genera. PCR-based genetic fingerprinting methods and base sequence comparisons of 16S rRNA genes as well as other housekeeping genes have been used extensively for characterising and classifying rhizobia (Willems and Collins 1993; Chen et al. 1995; Wang et al. 1999a; Willems et al. 2001; Zeigler 2003). Several bacterial isolates located outside traditional rhizobial genera in class α-proteobacteria have been reported from legume nodules that are capable of nitrogen fixation. In the year 2001 β-proteobacteria were reported in legume nodules for the first time when *Burkholderia* spp. were described from the nodules of the South African legume *Aspalathus carnosa* (Moulin et al. 2001) and *Ralstonia taiwanensis* in *Mimosa* nodules from Taiwan (Chen et al. 2001). Tripathi (2002) has reported *Ralstonia* from *Mimosa* nodules from India and how a good science was left behind in the publication race. Other new lines that contain N_2-fixing legume symbionts include *Methylobacterium* (Jourand et al. 2004), *Devosia* (Rivas et al. 2002), *Ochrobacterium* (Trujillo et al. 2005) and *Phyllobacterium* (Valverde et al. 2005), all α-proteobacteria. Till 2003, 36 rhizobial species distributed amongst seven genera were recognised (Sahgal and Johri 2003). In the subsequent 3 years, eight new rhizobial species were described. By 2006, 44 species of nodule bacteria on legumes were recognised within 11 genera (Sahgal and Johri 2006). With the use of genetic characteristics (DNA–DNA, DNA–rRNA hybridisations, rRNA catalogues, rDNA sequencing) and sequence analysis-based systematics, more diversity has been discovered amongst rhizobia, their relationships recognised and relationships with other groups of bacteria became apparent. In α-proteobacteria, a single species *Allorhizobium undicola* (de Lajudie et al. 1998) was reported within genus *Allorhizobium*. *Sinorhizobium* is now *Ensifer* with two species (Young 2003). Amongst β-proteobacteria single species, *Ralstonia*

taiwanensis within genus *Cupriavidus* (Chen et al. 2001; Vandamme and Coenye 2004) has been identified. Other species described were *Devosia neptuniae* for strains from *Neptunia natans* from India (Rivas et al. 2002) and *Methylobacterium nodulans* for strains from *Crotalaria* (Jourand et al. 2004; Sy et al. 2001). *Ochrobacterium lupines* was described from *Lupinus* species (Trujillo et al. 2005), *Phyllobacterium lupinii* for isolates nodulating *Trifolium* and *Lupinus* (Valverde et al. 2005) and *Shinella kummerovia* from *Kummerowia stipulacea* (Lin et al. 2008). All these new nodulating bacteria have 16S rDNA distinct from traditional rhizobial genera but carry *nod* genes similar to those of rhizobia. Thus, currently 98 species of legume-nodulating bacteria have been identified within 13 bacterial genera, 11 in α-proteobacteria and 2 in β-proteobacteria (Weir 2012). The above-mentioned number is severalfold less than the expected number considering the great number and vast distribution of leguminous hosts. Approximately 19,700 legume species are present globally, and rhizobia characterised and described are mainly from a small portion of legumes, mainly crops. A few bacterial isolates have been characterised and described from wild annuals and woody tree legumes. Out of 43 rhizobial species known till 2005, only ten were from tree legumes.

The present-day classification of rhizobial species is based on 16S rDNA sequence comparisons and physiological and biochemical properties. It does not reflect symbiotic features of rhizobia particularly host plant range. Although it is widely agreed that phylogenies based on stable chromosomal genes are necessary to establish biologically meaningful rhizobial taxonomy, a proper definition of broad host range should consider the diversity of symbiotic (sym) genes rather than the diversity of species that carry them. Thus, characterisation and the phylogenetic classification of sym genes must be included in the minimal standards for the description of new rhizobia (Laguerre et al. 2001).

Legume–Rhizobia Interactions

Legume–*Rhizobium* symbiosis is a marriage between two vastly different genomes. Rhizobial genome totals about 6–9 Mbp (Perret et al. 2000). In contrast, genome of legumes is larger with total DNA contents in the range of 450–4,500 Mbp per haploid genomes (Arumuganathan and Earle 1991). Legume genomes are thus at least 50 times larger than those of their microsymbionts. Nevertheless their respective contributions are almost similar. Rhizobia provide fixed nitrogen to the plants and bacteria are supplied with nutrients (Lodwig and Poole 2003) as well as protected inside nodule structure (van Rhijin and Vanderleyden 1995).

Nodule Development

Nodule development is a multistep process. It consists of recognition of host plants by bacteria, attachment of bacteria to root hair, root hair curling and formation of

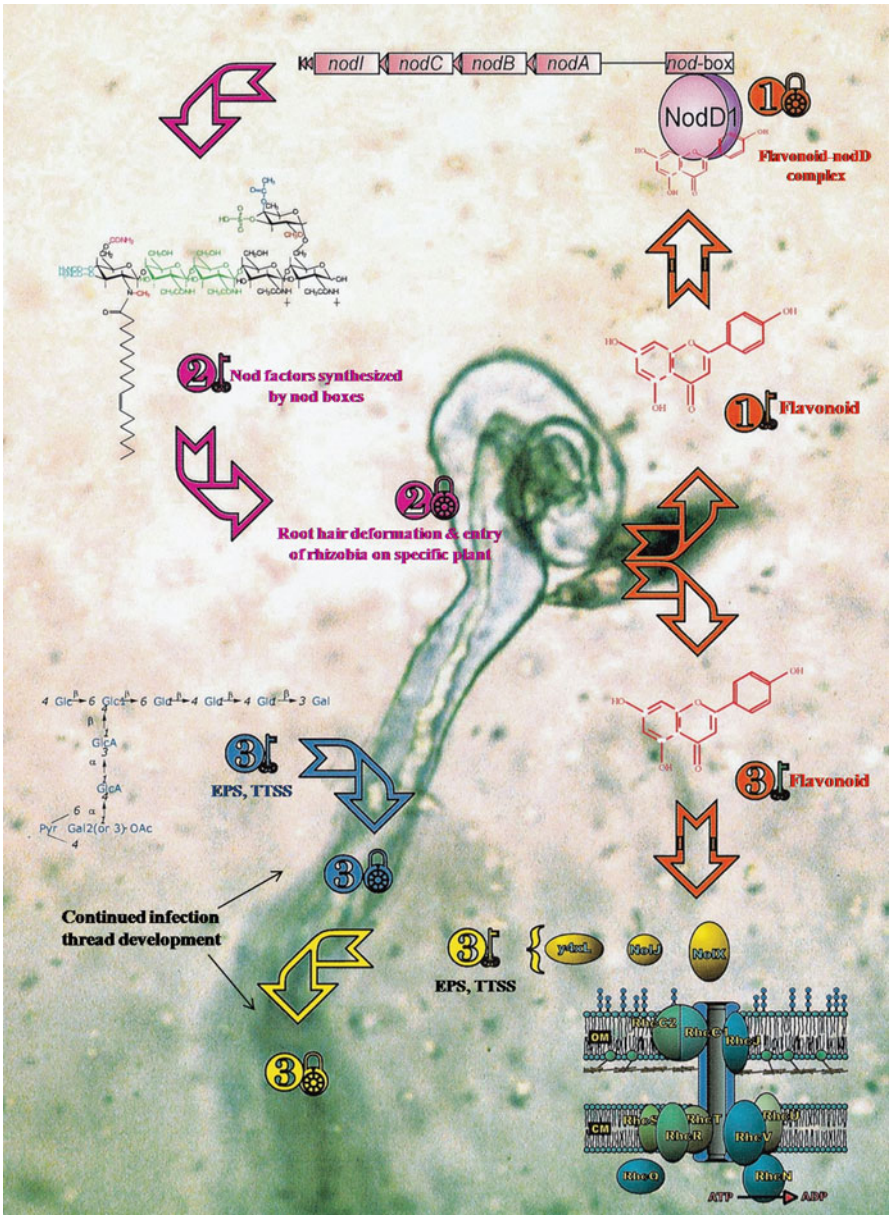

Fig. 9.1 Signals involved in legume–*Rhizobium* symbiosis (*Source*: Broughton et al. 2000)

infection thread and nodule development. Inside the nodules, bacteria differentiate into bacteroids, the site of biological nitrogen fixation.

At least three different set of symbiotic signals are exchanged between legumes and rhizobia during nodule development (Fig. 9.1). Legume roots secrete flavonoids and betaines that accumulate in the host plant rhizosphere. Since legumes are

nonmotile, so bacterial partner (rhizobia) senses the flavonoids and betaines secreted by legume host and advances into its rhizosphere and courtship begins. These flavonoids activate rhizobial Nod D proteins and form a flavonoid–Nod D complex. This complex acts as transcriptional regulator of nodulation genes (Broughton and Perret 1999). As a result nodulation genes (*nod*, *nol* and *noe* genes) secrete Nod factors that are chemically lipochitooligosaccharides (LCOs) (Spaink 2000). Nod factors are a second set of signals that trigger root hair curling and allow rhizobia to enter through infection thread. Infection thread reaches nodule primordium and releases the bacteria into cytoplasm. The meristematic activity of root cortex and active multiplication of rhizobia lead to nodule formation. The third set of signals necessary for the completion of nodule development are extracellular polysaccharide (EPS), lipopolysaccharides, K antigens, cyclic glycans, lectins and proteins exported by type three secretion system (TTSS).

Host Specificity

The interaction between rhizobia and legume is host specific. It means that rhizobial species are specific for nodulating a legume or that not all rhizobia nodulate all legumes. Based on host specificity, rhizobia are classified as broad and narrow host range rhizobia. For example, *Rhizobium leguminosarum* bvs. *viciae* and *trifolii*, though closely related, are specific for their legume partner. *R. leguminosarum* bv. *viciae* nodulates *Lathyrus*, *Lens*, *Pisum* and *Vicia*, whereas *R. leguminosarum* bv. *trifolii* nodulates *Trifolium* spp. NGR234 is a broad host range rhizobia and nodulates at least 35 different legumes. Hence, the degree of host specificity varies tremendously amongst rhizobia. The amount and the structural variation of Nod factors are important in determining the host specificity in rhizobia–legume symbiosis (Bladergroen and Spaink 1998).

All the Nod factors consist of a backbone of two to six β-1,4-linked *N*-acetyl-d-glucosamine residues. The nonreducing terminal end of *N*-acetyl-d-glucosamine is substituted on the C-2 position with a fatty acid whose structure is variable, and the reducing end may be substituted with a sulphate group or with a d-arabinose, l-fucose or 2-*O*-methyl fucose. The Nod factor synthesis is induced by flavonoids secreted by legumes. Thus, composition and concentration of flavonoid mixture liberated into the rhizosphere by the legume host are important in determining the nodulation preferences of rhizobia. The flavonoids produced via phenylpropanoid biosynthetic pathways are strongest inducers of nod gene expression (Stafford 1997; Werner 1998), whereas those related glycosides or related conjugates are less active in inducing nod genes (Hartwig and Phillips 1991). Compositions of flavonoids in seeds, roots and root exudates of *Glycine max*, *P. vulgaris*, *Medicago sativa*, *Trifolium repens* and *Vicia sativa* are significantly different from each other, and this determines their preferences for nodulation with rhizobial species (Perret et al. 2000). It was observed that *G. max*, *P. vulgaris*, *Robinia pseudoacacia* and *Sesbania rostrata* were nodulated by a broad host range *Rhizobium* sp. strain NGR234, in addition to their homologous *Rhizobium*, and thus were highly

non-selective for rhizobia, whereas *M. sativa* and *Vicia* sp. have restricted host ranges and are thus highly selective for rhizobial partners.

Symbiotic Genes in Rhizobia

Several rhizobial and symbiotic genes are required for legume–rhizobia symbiosis. Rhizobial genes include those involved in Nod factor synthesis (van Rhijin and Vanderleyden 1995), nodule development, synthesis of nitrogen-fixing apparatus and bacteroid metabolism. Amongst these are nodulation (*nod*, *nol*, *noe*) and nitrogen fixation (*nif*, *fix*) genes, whereas plant genes expressed in root tissues as a consequence of the interaction with rhizobia are nodulin genes (Verma et al. 1992). Symbiotic genes are located on either plasmid or chromosome. In the genus *Rhizobium*, *nod* genes are located on a large plasmid known Sym plasmid, pSymA, pSymB in *Rhizobium meliloti* (now *Sinorhizobium meliloti*) (Galibert et al. 2001), whereas in *Azorhizobium* spp., *Bradyrhizobium* and *Mesorhizobium loti* on the chromosomes (Kaneko et al. 2000). The organisation of these genes in operons is very similar in *Rhizobium* and *Bradyrhizobium* (Fig. 9.2).

The Nodulation Genes

In all there are 13 *nod* genes. The nodulation genes have been classified into two groups: common and host specific (hsn). The common *nod* genes are *nod*A,-B,-C,-I,-J. The common nodulation genes *nod* ABC are found in all rhizobial isolates studied so far (Martínez et al. 1990; Goethals et al. 1992) and are structurally conserved and functionally interchangeable between the rhizobial species without altering host range; another essential gene is nod D, which is present in one or more alleles depending on the rhizobial species. The nod D gene behaves as a common *nod* gene for nodulation on some host plants, while in other cases it represents an important determinant of host specificity (Gyorgypal et al. 1991; Schlaman 1992). Nod D gene is present in a single allele in *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii*; in four alleles *nod* D, *nod* D_2, *nod* D_3 and syr M in *R. meliloti*; and two alleles *nod* D_1 and *nod* D_2 in *B. japonicum*. The *nod* hsn genes are specific and determine its host for nodulation and are not conserved amongst rhizobia. The host-specific *nod* genes include *nod* FE, *nod* L and *nod* M common to all *Rhizobium* sp., *nif* W (formerly *nod* O) in *R. leguminosarum* bv. *viciae*, *nod* H and *nod* PQ in *S. meliloti* (formerly *R. meliloti*) and nod Z in *B. japonicum*, respectively.

The Nitrogen Fixation Genes

These are *nif* and *fix* genes. Rhizobial *nif* genes are structurally homologous to 20 *Klebsiella pneumoniae nif* genes (Arnold et al. 1998), but their organisation in rhizobia is different than those in *K. pneumoniae*, in which 20 adjacent *nif* genes

Table 9.1 The list of *nif* and *fix* genes of *S. meliloti*, *B. japonicum* and *A. caulinodans* and their functions

S. no.	Gene	Product and/or (proposed) function
a.	***nif* genes**	
i.	*nif* H	Fe protein of nitrogenase
ii.	*nif* D	α-subunit of MoFe protein of nitrogenase
iii.	*nif* K	f3 subunit of MoFe protein of nitrogenase
iv.	*nif* N	Involved in FeMo cofactor biosynthesis
v.	*nif* B	Involved in FeMo cofactor biosynthesis
vi.	*nif* S	Cysteine desulphurase activation of sulphur for metallocluster synthesis?
vii.	*nif* W	Unknown function; required for full activity of FeMo protein
viii.	*nif* X	Unknown function
ix.	*nif A*	Positive regulator of *nif*, *fix* and additional genes
b.	***fix* genes**	
i.	*fix* ABCX	Unknown function; required for nitrogenase activity; Fix X shows similarity to ferredoxins
ii.	*fix* NOQP	Microaerobically induced, membrane-bound cytochrome oxidase
iii.	*fix* GHIS	Redox process-coupled cation pump?
iv.	*fix* LJ	Oxygen-responsive two-component regulatory system involved in positive control of *fix* K (*Sm*, *Bj*, *Ac*) and *nif* A (*Sm*)
v.	*fix* K/*fix* K_2	Positive regulator of *fix* NOQP (*Sm*, *Bj*, *Ac*), *nif* A (*Ac*), *rpo*Nj, and 'nitrate respiration' (*Bj*); negative regulator of *nif* A and *fix* K (*Sm*)
vi.	R_m*fix*K′	Reiterated, functional copy of *fix* K
vii.	*Bj fix* K_1	*Fix* K homolog of unknown function; not essential for nitrogen fixation
viii.	*fix* R	Unknown function; not essential for nitrogen fixation
ix.	*Nfr* A	Regulation of *nif* A

Source: Fischer (1994)
Sm: *S. meliloti*, *Bj*: *B. japonicum*, *Ac*: *A. caulinodans*

are organised in eight operons within 24 kb of DNA. At least nine different *nif* genes have been identified so far in *S. meliloti* (formerly *R. meliloti*), *B. japonicum* and *A. caulinodans* (Table 9.1). These are *nif* HDK and *nif* E, N, B, S, W, X and A. These *nif* genes play a similar role in rhizobia as in *K. pneumoniae*. The *nif* HDK is a good marker for nitrogen fixation as it is not constitutively expressed and is regulated in response to factors that control nitrogen fixation. Amongst *nif* HDK, *nif* H gene is widely used as a nitrogen fixation marker (Haukka et al. 1998) because large sequence data is available for this gene. The 'fix' genes play an important role in nitrogen fixation but do not have a homologous counterpart in *K. pneumonia*. The 'fix' genes represent genes originally involved in the development and metabolism of bacteroids but at the same time may also play an important role in other processes not related to nitrogen fixation or may even be present in non-diazotrophs.

S. meliloti carries two megaplasmids pSymA of 1,400 kb and pSymB of 1,700 kb (Young 2000). In *S. meliloti*, *nif* and *fix* genes are organised into two clusters (Fig. 9.2a). Cluster I includes *nif* HDKE, *nif* N, *fix* ABCX, *nif* A, *nif* B and *frd* X and cluster II includes *fix* LJ, *fix* K, *fix* NOQP *and fix* GHIS, and both

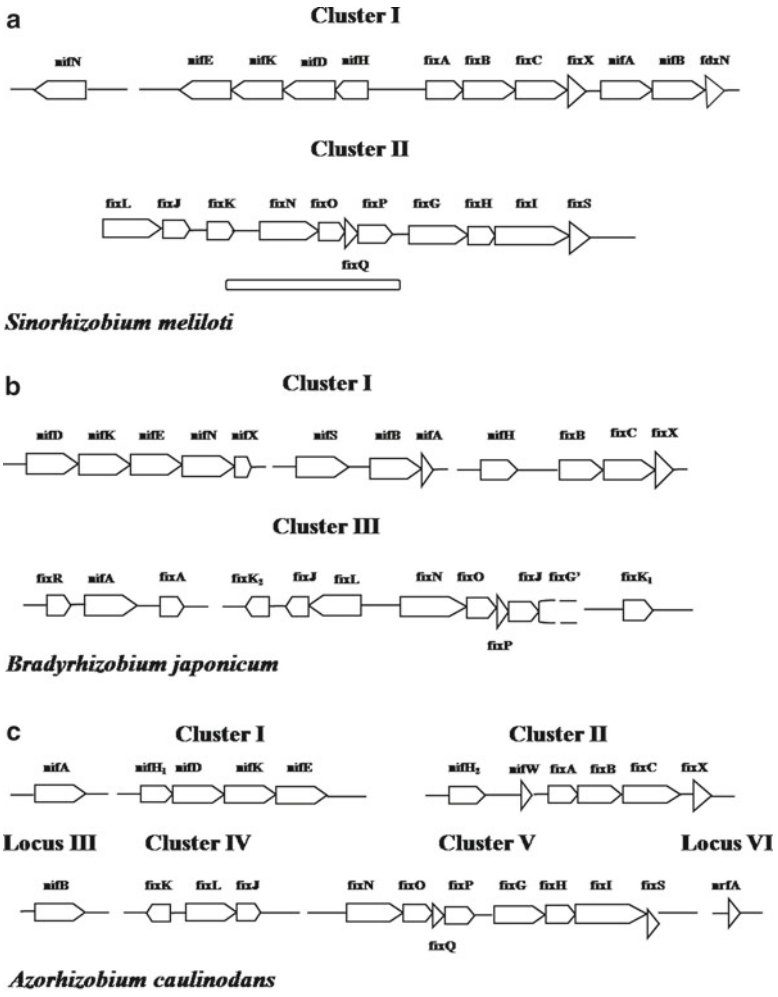

Fig. 9.2 Organisation of *nif* and *fix* gene clusters in (**a**) *S. meliloti*, (**b**) *B. japonicum* and (**c**) *A. caulinodans* (*Source*: Fischer 1994)

the clusters are present on megaplasmid pSymA. The cluster II genes are located 220 kb downstream of the *nif* HDKE operon and are transcribed in opposite orientation to it. Additional genes that are required for effective symbiosis are located on megaplasmid pSymB. Rhizobial species *B. japonicum* and *A. caulinodans* do not have plasmids. Hence, *nif* and *fix* genes are located on chromosomes and organised as shown in Fig. 9.2b and c, respectively. In *B. japonicum*, *nif* and *fix* genes are organised into four clusters and along with common nod genes are located within 100 kb on chromosome. Thus, it can be presumed that symbiotic gene region of *B. japonicum* was located originally on a plasmid and became part of chromosome by integration. Alternatively, the symbiotic

plasmids of *S. meliloti* (or other rhizobia) might have evolved by excision of a chromosomal region. In *A. caulinodans* four clusters of *nif* or *fix* genes and two additional loci carrying nitrogen-fixing genes *nif* B and *nif* A are present. Moreover, additional gene regions that are located in nodulation (nod UV) (Göttfert et al. 1990) or expressed under symbiotic conditions (*rpo*N) (Kullik et al. 1991), *gro* ESL_3 (Fischer et al. 1993) and *ndp* (Weidenhaupt et al. 1993), are present close to the segment harbouring essential *nif* and *fix* genes (Kündig et al. 1993).

nif Genes

The nitrogenase enzyme complex is composed of two multisubunit metalloproteins component I and II. Component I is composed of two heterodimers encoded by *nif* K and *nif* D genes and has active sites for nitrogen reduction. The *nif* D and *nif* K genes specify α- and β-subunits, respectively, of $\alpha_2\beta_2$FeMo protein (component I or dinitrogenase, Mr ≈ 220,000). Component II is composed of two identical subunits encoded by nif H and transfer electrons and protons to component I. *nif* H encodes homodimeric Fe protein (component II or dinitrogenase reductase, Mr ≈ 60,000). In *S. meliloti*, the *nif* HDK genes are organised in an operon along with *nif* E, whereas in *A. caulinodans*, *nif* HDK and *nif* E form two separate transcriptional units. The *nif* H gene is present in two alleles, *nif* H and *nif* H_2, differing in two nucleotides. In *A. caulinodans*, *nif* H_2 is found in cluster II along with *fix* ABCX genes, and in *R. leguminosarum* bv. *phaseoli*, three identical and functional copies of *nif* H genes are present. The products of *nif* genes *nif* E, *nif* N and *nif* B are required for the synthesis of FeMo cofactor of component I.

fix ABCX Genes

These are present in *S. meliloti*, *B. japonicum*, *A. caulinodans*, *R. leguminosarum* bv. *trifolii* and *R. leguminosarum* bv. *phaseoli*. They are organised in a single operon in all species except *B. japonicum*, in which *fix* A and *fix* BCX form distinct transcriptional units present in clusters II and I, respectively. The products of *fix* ABCX genes are involved in electron transport to nitrogenase. Mutation in any one of the *fix* ABCX genes of *S. meliloti*, *B. japonicum* and *A. caulinodans* completely stops nitrogen fixation. These include genes encoding MoFe protein and Fe protein as well as accessory genes for electron transfer proteins, metal cluster synthesis and regulation (Dean and Jacobsen 1992).

fix NOQP Genes

The products of *fix* NOQP genes constitute a membrane-bound cytochrome oxidase (Kahn et al. 1993; Mandon et al. 1994; Preisig et al. 1993). This oxidase complex

supports bacteroid respiration under low oxygen conditions present in root nodules (Hennecke 1993; Preisig et al. 1993). These were first described in *S. meliloti* and are expressed under symbiotic conditions. These were limited to regulatory genes *fix* LJ and *fix* K. Subsequently, they have been identified in *B. japonicum* (Preisig et al. 1993), *A. caulinodans* (Mandon et al. 1994) and *R. leguminosarum* bv. *viciae*. The *B. japonicum* and *S. meliloti* fix NOQP mutants are defective in symbiotic nitrogen fixation, whereas a corresponding mutant of *A. caulinodans* showed 50 % wild-type nitrogenase activity.

fix GHIS Genes

These are present downstream of the *fix* NOQP operon in cluster II of *S. meliloti*. All four *fix* GHIS gene products are transmembrane proteins. *fix* G is likely to be involved in redox process, and *fix* I is homologous to the catalytic subunit of bacterial and eukaryotic ATPases involved in cation pumping.

fix R

This is present in *B. japonicum* and is located downstream of the regulatory *nif* A gene. The product of *fix* R is involved in redox-dependent activation and inactivation of the Nif A protein.

Regulation of nif *and* fix *Genes*

S. meliloti, *B. japonicum* and *A. caulinodans* all use largely identical regulatory elements (FixL, FixJ, FixK, NifA and RpoN); however, these are integrated into different species-specific networks.

Intracellular Oxygen Tensions

Oxygen concentration controls the expression of *nif* and *fix* genes (Soupene et al. 1995). Enzyme nitrogenase is extremely sensitive so inside nodule oxygen concentration has to be very low. However, the colonising rhizobia require oxygen to generate ATP, which is required in large amounts for the energy-intensive process of nitrogen fixation. Tightly packed plant cortical cells adjacent to the surface of the nodule form an oxygen diffusion barrier and leghaemoglobin present in the nodule cytoplasm tightly binds to oxygen. Hence, diffusion of oxygen to actively respiring bacteroids is prevented. Rhizobia sense oxygen concentration through two proteins, Fix L and Nif A. At low oxygen concentrations, these proteins are active and are responsible for the induction of genes involved in fixation of atmospheric nitrogen.

FixL–FixJ

In *S. meliloti*, the FixL–FixJ two-component system is the master regulator of all nif and fix genes (Agron and Helinski 1995). The FixL is a membrane-bound histidine kinase which at low levels of oxygen autophosphorylates and then transfers the phosphoryl group to FixJ (Gilles-Gonzalez and Gonzalez 1993; Lois et al. 1993). Phosphorylated FixJ activates transcription of regulatory *fix* K and *nif* A genes. The products of *fix* K and *nif A* genes regulate transcription of the rest of the nitrogen fixation genes. The FixL–FixJ system is one of the few two-component systems whose signal-responsive autophosphorylation and phosphotransfer have been reconstituted in vitro. Anoxic conditions enhance FixL autophosphorylation, whereas phosphorylation of FixJ is independent of oxygen status.

FixK

It is a regulatory protein whose expression is activated by FixJ in response to low concentrations of oxygen (Kaminski et al. 1998). It is homologous to the regulator Fnr except that cysteine residues are not present at N-terminal domain. Fix K can act either as an activator or as a repressor depending on the position of its binding site within the target promoter. In *S. meliloti*, Fix K activates the transcription of *fix* NOQP and *fix* GHIS operons and negatively regulates its own expression as well as the expression of *nif* A (Waelkens et al. 1992; Foussard et al. 1997).

Nif A

It is a transcriptional regulator whose expression and activity are inhibited by high oxygen concentrations. It does not belong to a family of two-component systems because it does not contain a receiver domain. Nif A protein is a homolog of Ntr C. It acts in conjugation with sigma 54 and requires hydrolysis of an ATP molecule to activate transcription. In the absence of oxygen, Nif A activates the expression of its own gene as well as that of nif HDKE and fix ABCX operons (Fischer 1994, 1996). It also induces transcription of genes involved in the synthesis of rhizopines.

Rhizopines in Sinorhizobium*–Plant Interaction*

Rhizopines are nutritive compounds produced by bacteroids of certain strains of rhizobia, i.e. *S. meliloti* and *R. leguminosarum* bv. *viciae*. They are synthesised by 11 % of *S. meliloti* and 12 % of *R. leguminosarum* bv. *viciae* strains. Structurally, rhizopines are 3-*O*-methyl-scyllo-inosamine (3-O-MSI) and scylloinosamine (SI) (Dessaux et al. 1998). In *S. meliloti*, genes involved in rhizopine synthesis (*mos* genes)

and rhizopine catabolism (*moc* genes) are located on the symbiotic megaplasmid pSymA, along with nitrogen fixation genes. The *mos* locus is regulated by symbiotic nitrogen fixation regulator NifA; hence, it is co-ordinately regulated with nitrogen fixation and controlled by low oxygen levels. Rhizopine catabolic gene (*moc*) is not expressed in bacteroid (Saint et al. 1993), but catabolic products of rhizopines affect intraspecies competition for nodulation (Murphy et al. 1995). Although very few rhizobia synthesise rhizopines, it is possible that new classes of rhizopines might be discovered and this phenomenon may be more universal amongst rhizobia (Brencic and Winans 2005).

Regulation of Bradyrhizobium*–Soya Bean Symbiosis*

Symbiotic interaction of *Bradyrhizobium* with soya bean (*G. max*) is influenced by both the bacterial and host genotypes. Soya bean genotypes, including cultivars and plant introductions (PI), have been shown to be differentially nodulated by specific stains or genotypes of *B. japonicum* (Cregan and Keyer 1986; Sadowsky et al. 1987). The nodulation of *Glycine max* by *B. japonicum* USDA 110 and USDA 123 is controlled by legume host genotype and bacterial population density (Jitacksorn and Sadowsky 2008). Nodulation was enhanced when soya bean plants received low cell diversity inocula (10^5 cell ml^{-1}), whereas it was suppressed when plants received high diversity inocula (10^9 cell ml^{-1}). The regulation of nod gene expression in the *Bradyrhizobium* occurs via three regulatory pathways involving *nod* D, *nod* VW and *nol* A (Loh and Stacey 2001). *B. japonicum* produces two Nod D proteins (Nod D_1 and Nod D_2). Nod D1, a LysR-type regulator, is a positive transcriptional activator and responds to plant-secreted isoflavones (Göttfert et al. 1992), whereas $NodD_2$ represses nod D1 expression (Loh and Stacey 2003). Although initial studies by Göttfert and colleagues (1992) showed that there was no role of $nodD_2$ gene in inoculation of soya bean plants, subsequent studies by the same group have shown that nodulation of soya bean plants was delayed in $nodD_2$ deletion mutant of *B. japonicum* as compared to wild-type stain. Nod VW is essential for the nodulation of cowpeas, siratro and mung bean but not for soya bean and provides an alternative pathway for nod gene activation in NodD mutants that are able to nodulate soya bean. The third pathway is regulated by NolA, a MerR family of regulatory proteins, and was identified as the product of genotype-specific nodulation gene. NolA activates the expression of $NodD_2$ which in turn represses nod gene expression in *Bradyrhizobium*. *B. japonicum* strain USDA 110 grown to high cell density secretes an extracellular quorum-responsive signal molecule, bradyoxetin. Bradyoxetin induces NolA which in turn leads to nod gene repression. The production of bradyoxetin is regulated in a population-density-dependent manner; the greatest production occurs in high population density and iron-depleted conditions. Thus, expression of nod genes in the *Bradyrhizobium* is modulated by quorum-responsive signal molecules. The functional copy of the nodD1 gene is required for diversity-dependent

enhanced nodulation of soya bean, and that *B. japonicum* strain with mutation in *nol* A and *nod* D_2 can be used to enhance the nodulation of soya bean at high inoculum densities. In nitrogen-fixing bacteroids, carboxylic acids are a major source of carbon and energy, necessary for the generation of ATP and reducing power needed for nitrogenase activity (Kaminski et al. 1998). However, dicarboxylic acids also inhibit the expression of nod genes, e.g. *B. japonicum* (Yuen and Stacey 1996).

Rhizobia Associated with Annual Legumes

Agricultural soils often contain diverse indigenous rhizobial populations. Rhizobia have great potential for improving growth of host plants (Becki et al. 2004; Bogino et al. 2008). Their performance in field is affected by host plant specificity, environmental factors as well as soil conditions (Diouf et al. 2007). Correlations between the rhizobial genomic groups and their geographic origins have been detected amongst symbionts of faba bean (*Vicia faba*) (Tan et al. 2007) and epidemic legumes growing on the Qinghai–Tibet plateau (Hou et al. 2009). Several other studies have shown that both abiotic (pH, rainfall, soil, temperature) and biotic (genotypes of host plants and their distribution) conditions might affect the diversity of the rhizobial species in soil (Hagen and Hamrick 1996; Handley et al. 1998; Bromfield et al. 2001).

Host plant plays a central role in site-specific selection of rhizobia. Wang et al. (1999a) observed that *R. etli* from root nodules of *Mimosa affinis* growing in Mexico was different in *nif* H gene and host specificity as compared with *R. etli* strains nodulating *P. vulgaris* L. They proposed new biovariety for *R. etli* strains nodulating *M. affinis*. Thus, repeated cultivation of legumes like *M. affinis* is likely to reduce rhizobial diversity to a marked strain than repeated cultivation of a promiscuous legume like *P. vulgaris* which is nodulated by genetically diverse rhizobia, namely, *Bradyrhizobium* spp., *R. leguminosarum* bv. *phaseoli* (Andrade et al. 2002), *R. tropici* (Martínez-Romero et al. 1991), *R. etli* (Graham et al. 1982), *R. giardinii* and *R. gallicum* (Amarger et al. 1997). Nodulation of rhizobia on heterologous host (cross-nodulation pattern) is an important trait in defining their diversity. But association between rhizobia and their host under laboratory conditions is less important than in natural environment because such species of rhizobia can form nodules with legumes under laboratory conditions from which they have never been isolated in the field, e.g. nodulation of *R. huautlense* on *Leucaena leucocephala* in in vitro studies (Wang et al.1998).

Geographical locales can also influence genetic diversity amongst rhizobial populations. Han et al. (2008) characterised genetic and symbiotic rhizobial diversity from three introduced (*Lathyrus odoratus*, *Robinia pseudoacacia* and *V. faba*) and nine wild legumes, *Astragalus* spp., *Alhagi sparsifolia*, *Caragana jubata*, *Halimodendron halodendron*, *Lotus* sp., *Oxytropis glabra*, *Sophora alopecuriodes*, *Vicia hirsuta* and *Orobus* (Lathyrus) *luteus*, growing in the Xinjiang region of

China. They identified nine genomic species amongst 111 rhizobial strains associated with 25 legume species within 12 legume genera. Regardless of the composition of sampled legumes, *Rhizobium* was the most predominant bacteria (genomic sp. I and II), *Mesorhizobium* (genomic sp. V and VI) second largest and *Bradyrhizobium* populations were least dominant. This implied that highly alkaline and saline soils in Xinjiang were dominant in acid-producing strains of *Rhizobium*, *Mesorhizobium* and *Ensifer* than alkaline-producing *Bradyrhizobium* strains. The characterisation of nodule bacteria from unexplored legumes will reveal additional diversity and novel species are likely to be described (Wolde-Meskel et al. 2005). Moreover, an introduced legume in an area might trap rhizobial populations that exist locally as a minority in the soil. Consequently, both sampled legumes and local environment may affect the composition of rhizobial community. Chen and co-workers (1988, 1995) have reported that soya bean plants in Xinjiang region have been nodulated by *Mesorhizobium tianshanense* and *Sinorhizobium fredii*, whereas in other regions with bradyrhizobia. Similarly, Velázquez et al. (2001) observed that bean isolates recovered from León (France) belonged to *R. leguminosarum* bvs. *viciae* and *trifolii*, whereas those from Andalucia were more diverse and belonged to *R. etli*, *R. gallicum*, *R. giardinii*, *R. leguminosarum* bv. *viciae* and bv. *trifolii* and *S. fredii*. Similarly, Bernal and Graham (2001), while studying bean rhizobia in Ecuador and Northern Peru, observed that *R. etli* strains from the Mesoamerican region were phenotypically and phylogenetically separated from those associated with beans in the Andean region. Physical properties of soil also affect the genetic diversity amongst rhizobial populations. Andrade et al. (2002) reported higher rhizobial diversity in limed soils in the *P. vulgaris*-growing region of Brazil. Shifts observed in genetic diversity amongst the population of *S. meliloti* (formerly *R. meliloti*) and *R. leguminosarum* nodulating *M. sativa* growing in Italy have been attributed to chemical and physical differences between soil (Paffeti et al. 1996), history of N fertilisation (Caballero-Mellado and Martínez-Romero 1999) and land management practices (Palmer and Young 2000). The observation that there is a correlation between geographical regions and rhizobial diversity has been strengthened by studies of rhizobia from legume-growing regions in China. Lu et al. (2009) studied the rhizobial diversity associated with endemic *Caragana* species, *C. bicolor*, *C. erinacea*, *C. franchetiana*, *C. intermedia* and *C. jubata*, growing in three ecoregions of China, ecoregion A (Eastern Inner Mongolia having prairie with sandy soils), ecoregion B (Northern Shanxi hills with saline/alkaline soil) and ecoregion C (hillside/forest land with fertile soil in north-western Yunnan). Ecoregions A and B represented temperate condition, whereas ecoregion C, a tropial soil and climatic conditions. Rhizobial communities associated with *Caragana* species were different in the three ecoregions of China. *Caragana* species in region A were nodulated by *Mesorhizobium* genospecies I, II, IV, VI and VII, and in region B by genospecies *M. temperatum*, *M. tianshanense*, *M. septentrionale*, *M.* genospecies III, *R. yanglingense* and *Rhizobium* sp. IV, whereas with *M. plurifarium*, *M.* genospecies V and VII and *Rhizobium* sp. IV in region C. In conclusion, the above study demonstrated that *Caragana* species could be nodulated with distinctive populations mainly with *Mesorhizobium* spp. (82.8 %) and occasionally with

Rhizobium and *Bradyrhizobium*. The same group of workers observed identical results while studying *Caragana* isolates in another ecoregion in Northeastern China (Yan et al. 2007) but different from those in which *Rhizobium/Agrobacterium-related* strains were predominant in *C. intermedia*-associating rhizobia (Gao et al. 2002).

Most of the *Mimosa* species are native to Central and South America (Barneby 1991) with Cerrado region of Central Brazil being the major centre of diversification (Barneby 1991; Simon and Proenca 2000). It has long been known that *Mimosa* plants are nodulated by diverse rhizobial species. Prior to year 2000, all had been ascribed to known α-rhizobial genera (Barret and Parker 2005; Wang et al. 1999a; Moreira et al. 1993; Oyaizu et al. 1993). Since the first report of β-rhizobia from legume nodules (Moulin et al. 2001), β-rhizobia belonging to genera *Ralstonia* (now *Cupriavidus*) and *Burkholderia* have been reported from legumes, and a majority of them have been reported from *Mimosa* spp. (Chen et al. 2001, 2003; Verma et al. 2004). Chen et al. (2005), while investigating the diversity of nodule isolates from *Mimosa* spp. in South America, observed that most of the nodule isolates belonged to *Burkholderia* and none belonged to *Cupriavidus*, which appears strange considering that *Cupriavidus taiwanensis* is dominant in Taiwan (Chen et al. 2003) and possibly India (Verma et al. 2004). The possible explanation for this could be that *C. taiwanensis* is an Asian bacterium that has acquired its symbiosis genes from *Burkholderia* strains resident within *Mimosa* nodules that were introduced in Asia from tropical America and Caribbean by European colonists. The study of genetic diversity of rhizobia in medicinal legumes, namely, *Abrus precatorius*, *Mucuna pruriens*, *Melilotus officinalis*, *Trigonella foenum-graecum* and *Vicia angustifolia*, growing in the sub-Himalayan tract of Uttarakhand defined six rDNA genotypes within these rhizobia, and their phylogenetic relationships were intertwined within *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium* (Pandey et al. 2004).

Traditionally chickpea-nodulating rhizobia were rather host specific with two described species, *Mesorhizobium ciceri* (Nour et al. 1994) and *M. mediterranean* (Nour et al. 1995). However, Romdhane et al. (2009), while studying nodulation of chickpea in Tunisia under water-deficient conditions, reported that its nodulation by *M. mediterranean* was reduced, while with *Ensifer meliloti* was favoured. *E. meliloti* has also been reported from chickpea growing in the Terai and Almora regions of Uttarakhand Himalayas. When characterised, rhizobial isolates recovered from the nodules of various annual legumes, *Lens culinaris*, *Cicer arietinum*, *T. foenum-graecum*, *P. sativum* and *Trifolium* species, were genetically diverse, and symbiosis of *E. meliloti* with chickpea was effective. An interesting finding from the above study is the presence of *Rhizobium*, *Sinorhizobium* and *Burkholderia* from *Lens culinaris* nodules. This is the first report of *Burkholderia* from *Lens culinaris* nodules in India (Fig. 9.3).

The extensive survey of rhizobial diversity from various legumes, *Amorpha fruticosa*, *Astragalus*, *Glycyrrhiza* spp., *Gueldenstaedtia* spp. and *Lespedeza* spp., in the Northwestern region of China has led to the recovery of novel forms within the *Bradyrhizobium* (Yao et al. 2002), *Mesorhizobium* (Wang et al. 1999b), *Rhizobium* (Tan et al. 2001; Wei et al. 2002, 2003) and *Sinorhizobium* (Wei et al. 2002). From

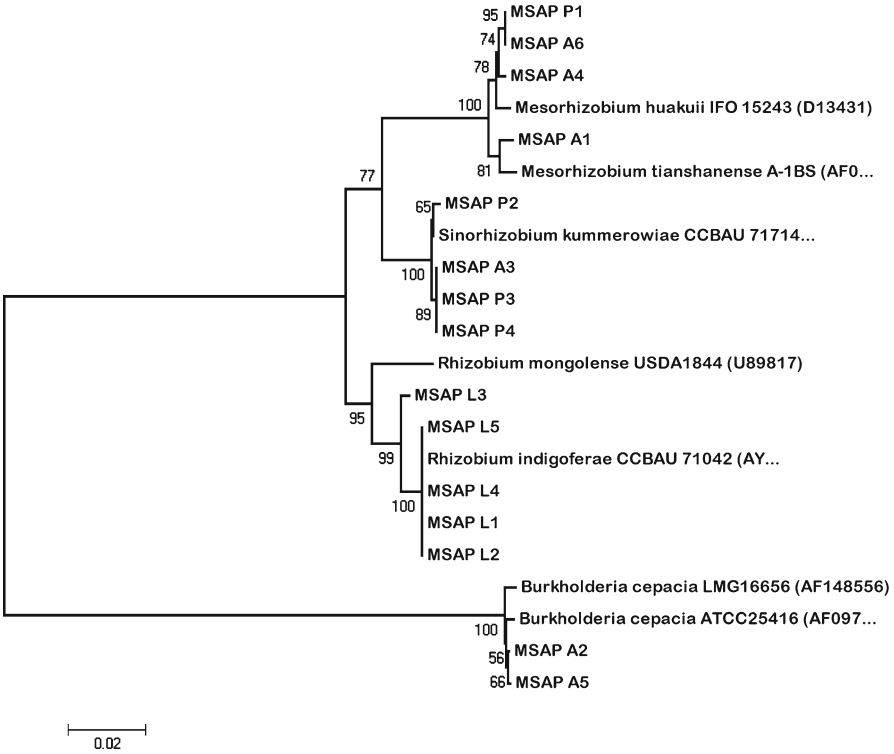

Fig. 9.3 Phylogenetic relationships based on full 16S rDNA sequences amongst rhizobial isolates from annual legumes of Uttarakhand (*Source*: unpublished)

these studies it has emerged that rhizobia in temperate regions are as diverse as those in tropical regions. Moreover, genetically diverse rhizobia are present at any single site and closely related strains could be found in varied geographic locations (Zhang et al. 1999). *Bradyrhizobium* strains nodulating genistoid legumes (brooms) in Canary Islands, Morocco, Spain and the Americas were highly diverse. Phylogenetic analysis of *Bradyrhizobium* strains using ITS, *atp*D, *gln* II and *rec*A sequences revealed that these belonged to four distinct evolutionary lineages, one representing *B. japonicum*, another representing *B. canariense* and the other two representing unnamed genospecies. Strains of *B. canariense* did not nodulate *Glycine max* but nodulated diverse legumes in tribes Genisteae and Loteae (Vineusa et al. 2005). Bacterial strains from nodules of *Genista tinctoria* were similar to slow-growing bradyrhizobia and genetically heterogenous. They did not nodulate *G. max*, *Lupinus corniculatus*, *M. sativa*, *P. vulgaris*, *T. repens* and *Vigna sativa* (Kalita and Malik 2004). Rodríguez-Navarro et al. (2004) reported that *Bradyrhizobium* strains nodulating legume *Pachyrhizus* were highly diverse and related to *B. elkanii*, *B. japonicum*, *B. liaoningense*, *B. yuanmingense* and *B. betae*. Nodule isolates from *Macrotyloma uniflorum* growing in the Almora region of Uttarakhand formed two genetic lineages: lineage I, representing fast-growing strains, and lineage II, very

slow-growing strains. The bacterial isolates from lineage I did not form nodules on homologous host but nodulated *G. max*, whereas slow-growing isolates nodulated *M. uniflorum* but not *G. max* (Agarwal 2009).

Rhizobia Associated with Tree Legumes

Ecological interaction between tree legumes and rhizobacteria is beneficial from three angles: increased biomass and amelioration of degraded sites on the account of improved water and nutrient uptake, prevention of soil erosion and increased soil fertility through N_2 fixation and greater organic matter production and recycling of nutrients. The leguminous trees are well nodulated under drought stress conditions. Species of *Acacia* are prevalent in Africa, Asia, Australia and Central America, and with the exception of *A. brevispica* from Africa, all nodulate effectively (Odee and Sprent 1992; Masutha et al. 1997; Tissue et al. 1997) with both fast- and slow-growing rhizobia (Barnet and Catt 1991). Other leguminous trees forming effective symbiosis with rhizobia are *Albizia* and *Leucaena*. A few leguminous trees can fix about 43–581 kg of N ha^{-1}, as compared with 15–210 kg of N ha^{-1} (Dakora and Keya 1997). Rhizobia of *Acacia senegal* and *Prosopis chilensis* are phenotypically and genotypically diverse (Zhang et al. 1991; Haukka and Lindström 1994; Haukka et al. 1996; Nick 1998; Dhabhai and Batra 2012). Zhang et al. (1991) placed *Acacia* rhizobial strains from Sudan in nine different clusters based on numerical analysis. Genetic characterisation based on 16S rRNA gene analysis (Haukka et al. 1996) showed that most Sudanese and Kenyan strains belonged to the genus *Sinorhizobium* and a few to *Mesorhizobium*. Nick and co-workers (1999) subsequently utilised DNA–DNA hybridisation on Sudanese and Kenyan isolates and grouped them into two clusters which showed low similarity with already described species of other tree legumes. Lafay and Burdon (2001) grouped nodule isolates from Australian acacias into nine genomospecies represented in genera *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium*, eight representing novel forms. He also proposed that majority of strains represented *Bradyrhizobium* spp. Hoque and co-workers (2011) genetically characterised the nodule symbionts of *A. salicina* and *A. stenophylla* growing across South-eastern Australia and reported the presence of *Burkholderia*, *Devosia*, *Ensifer*, *Mesorhizobium*, *Phyllobacterium* and *Rhizobium*. Dhabhai and Batra (2012) identified two genospecies inside the nodules of *Acacia nilotica* L., one showing homology to *Mesorhizobium loti* and second intermediate between *R. leguminosarum* and *Rhizobium hainanense*.

Rhizobia nodulating a diverse pool of forest legume species in Brazil were investigated by Moreira et al. (1998) who found six novel sequences amongst 44 strains from 29 leguminous tree species belonging to 13 tribes of *Leguminosae*. Studies undertaken with *Dalbergia sissoo*, *L. leucocephala*, *Mimosa* and *Prosopis* reveal that rhizobial isolates recovered from them are also diverse (Dupuy et al. 1994; de Lajudie et al. 1998; Nick et al. 1999). The long-term association between the symbionts allows gradual differentiation and diversity in compatible rhizobial

Table 9.2 Rhizobia described from tree legumes

Microsymbiont	Tree species	Reference
Mesorhizobium chacoense	*Prosopis alba*	Velázquez et al. (2001)
M. plurifarium	*Acacia*, *Leucaena*	de Lajudie et al. (1998)
R. tropici	*Leucaena* sp.	Martínez-Romero et al. (1991)
R. huautlense	*Sesbania herbacea*	Wang et al.(1998)
Ralstonia taiwanensis	*Mimosa* sp.	Chen et al. (2001)
Sinorhizobium arboris	*Acacia senegal*, *Prosopis chilensis*	Nick et al. (1999)
S. kostiense	*Acacia senegal*, *P. chilensis*	–do–
S. saheli	*Sesbania* sp.	de Lajudie et al. (1994)
S. terangae	*Acacia* sp.	de Lajudie et al. (1994)
S. morelense	*Leucaena leucocephala*	Wang et al. (2002)

populations resident in native soils. Rhizobial strains isolated from root nodules of native and exotic woody legumes (*Albizia gummifera*, *Erythrina brucei* and *Millettia ferruginea*) growing in Ethiopia showed very little metabolic and genomic relatedness to reference strains, hence representing probably novel forms. Phenotypic characterisation of the above gene pool showed a large diversity including very-fast- and extraslow-growing forms (Wolde-Meskel et al. 2004). Molecular systematics of *Sesbania* microsymbionts from Venezuelan wetlands using *rrs*, *atp*D, *rec*A and *nif* H sequence analysis identified them as *Mesorhizobium plurifarium* and *Rhizobium huautlense* (Vineusa et al. 2005). Amongst 98 rhizobial species known so far from legumes, 10 are from tree legumes (Table 9.2).

We observed considerable variability in rhizobia isolated from *Dalbergia sissoo* growing in various ecozones of Northern India (Sahgal 2002; Sahgal and Johri 2003). Out of 35 isolates, all were able to nodulate the homologous host, *D. sissoo*, while only 22 nodulated heterologous host *Sesbania aculeata*; only three nodulated *L. leucocephala* and *Vigna mungo* (Sahgal et al. 2004). Based on amplified rDNA restriction analysis of 16S and IGS, these isolates were grouped into seven rDNA types wherein none was identical to reference strains representing *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium*. Further extension of this work by Samant (2003) showed that six isolates from *D. sissoo* clones CPT5 and CPT6 were genetically different from those of the previous study (Sahgal 2002) and did not match any of the reference strains. The geographical origin appears to have considerable influence on the heterogeneity of rhizobia that nodulate wild tree legumes and those microsymbionts with restricted host ranges are limited to specific niches and represent specialisation of widespread and more ancestral promiscuous symbiosis.

Legume–Rhizobia–Mycorrhiza: A Tripartite Relationship

Legumes form tripartite symbiotic associations with nodule-inducing soil bacteria of the genera *Rhizobium*, *Bradyrhizobium* or *Azorhizobium* (Caetano-Anollés and Gresshoff 1991; Hirsch 1992) and with arbuscular mycorrhizal (AM) fungi

(Koide and Schreiner 1992). AM fungi and rhizobia are two of the most important plant symbionts to assess the capacity of plants to acquire nutrients. Mycorrhiza benefits the host through mobilisation of phosphorus from non-labile sources, whereas rhizobia fix N_2 (Scheublin and Vander Heijden 2006). Both the rhizobial and fungal microsymbionts improve the mineral nutrition of the host plant in exchange for assimilates provided by the latter. The nitrogenase enzyme of rhizobia fixes atmospheric nitrogen in the nodules (Thorneley 1992), and fungal hyphae facilitate the uptake of ions, mainly phosphate, in mycorrhizal roots (Smith and Gianinazzi-Pearson 1988). There are many similarities between rhizobial and AM symbioses, which suggest common properties in interactions with plants. Both are surrounded by plant-derived membranes in the established stage of the symbiosis: the peribacteroid membranes in the infected nodule cells and the perihaustorial membranes around arbuscules in the mycorrhizal roots, respectively. These interfaces are characterised by symbiosis-specific proteins (Perotto et al. 1994).

When soya bean (*G. max* [L.] Merr.) roots were co-inoculated with *B. japonicum* 61-A-1O1, considerable enhancement of colonisation by the mycorrhizal fungus *Glomus mosseae* was observed. In association with AM fungi, the rhizobia–bean symbiosis is benefitted by a better supply of phosphorus (Sanginga et al. 2000). Plants do not acquire phosphorus in organic form but AM is also able to help in this process (Bucher et al. 2001). Bargaz and colleagues (2011) reported that nitrogen fixation was significantly limited by P deficiency, and plants deficient in P show decreased nodule number and biomass. When compared with the control treatments, it was found out that dual inoculation with AM and rhizobia decreased the harmful influence of sulphate salinity on plant growth and nutrient accumulation (P and N) in *Lathyrus sativus* (Jin et al. 2010). Xie et al. (1995) described that highly purified Nod factors also increased the degree of mycorrhizal colonisation. Nod factors differed in their potential to promote fungal colonisation on the basis of their acetylation and sulphation. The acetylated factor NodNCR-V (MeFuc, Ac), added at concentrations as low as 10^{-9} M, promoted AM colonisation of plant roots, whereas the sulphated factor NodNCR-V (MeFuc, S) did not. The plant flavonoids mediate the Nod factor-induced stimulation of mycorrhizal colonisation in soya bean roots similar to determining host specificity in rhizobia–legume symbiosis. Thus, both symbioses share parts of signalling pathways, indicating intimate interactions between all three partners during co-evolution (Demir and Akkopru 2007; Xiao et al. 2010).

Mycorrhiza and Rhizobia: Common Signalling Factors

For the establishment of *Rhizobium* symbiosis, elucidation of the Nod factor structure was a major step to unravel the signalling pathway in legumes. Rhizobial Nod factors are lipochitooligosaccharides (LCOs) consisting of three to five

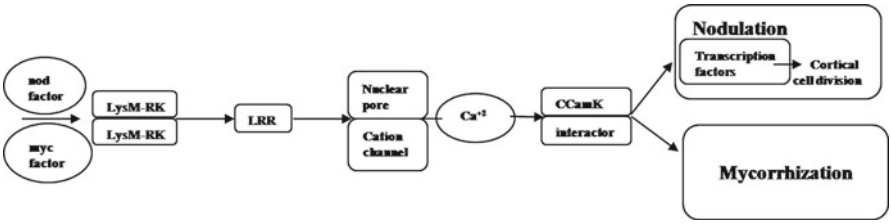

Fig. 9.4 Schematic representation of rhizobia–mycorrhiza symbiosis common signalling pathway (*Source*: Streng et al. 2011)

N-acetyl-glucosamines; the amino group of the nonreducing glucosamine is acylated with a fatty acid of 16–20 C-atoms in length (C16 to C20). Furthermore, terminal glucosamines contain species-specific substitutions, thereby determining that specific Nod factor structure is determined by the specific legume host plant. The modifications may be glycosylation, sulphation, acetylation and methylation, for which a particular *Rhizobium* species harbours specific nodulation (*nod*) genes (Gardes and Bruns 1993; Parniske 2008). Hence, it is assumed that the perception of Nod factors by legume host plants has co-evolved with their corresponding rhizobial symbionts. The calmodulin-binding domain and calcium-binding motifs of CCaMK (calcium–calmodulin-dependent kinase) allow the protein to sense calcium, which makes it a prime candidate for the response to calcium signatures induced by AM fungi (Kosuta et al. 2008) or the Nod factor that induces calcium spiking. The legume–rhizobia symbiosis and legume–mycorrhizal symbiosis pathways have interrelated factors. A deregulated version of CCaMK can trigger spontaneous nodule formation in the absence of rhizobia, which indicates that deregulation of CCaMK alone is sufficient to trigger the nodule formation. Also, in *Medicago truncatula*, three genes, called *DMI* (for *Does Not Make Infection*)-1, *DMI*-2 and *DMI*-3, are needed for infection by AM. These encode a protein that is a receptor-like kinase present on the cell membrane. Their one region extending to the outside of cell can bind to signal molecules such as growth factors, whereas an interior segment regulates other proteins by adding phosphate groups to them. This can conclude that DMI protein might be part of recognition machinery for Nod factors.

It is well known that in legumes, mycorrhizal and rhizobial symbioses share some common symbiotic genes. This has been first of all unravelled in pea (*P. sativum*) and the model species of legume, *M. truncatula* (medicago) and *Lotus japonicus* (lotus), respectively (Kouchi et al. 2010). In both the model species, the common symbiotic signalling pathway comprises a conserved set of six genes, encoding a plasma membrane receptor kinase (MtDMI2 and LjSYMRK), several components in the nuclear envelope including a cation channel (MtDMI1, LjCASTOR and LjPOLLUX), a nuclear localised calcium–calmodulin-dependent kinase (CCaMK; MtDMI3 and LjCCaMK) and a CCaMK interacting protein (MtIPD3 and LjCYCLOPS) (Fig. 9.4). Nod factor perception and signal transduction in the plant involve calcium spiking and

lead to induction of nodulation gene expression; mycorrhizal symbiosis bifurcation also takes place from this step. Mycorrhizae and rhizobia induced signalling bifurcates downstream of CCaMK, possibly due to a different nature of the calcium signal (Kosuta et al. 2008). Rapid calcium influxes are induced by both Nod factors (Oldroyd and Downie 2004) and AM fungal exudates (Kosuta et al. 2003). SYMRK perceives both mycorrhizal and rhizobial signals, probably at the junction of the common pathway (Parniske 2008; Oldroyd and Downie 2004). It encodes a leucine-rich repeat (LRR) receptor-like kinase.

Conclusion and Future Scenario

Rhizobial or fungal (AM) invasions of plant roots are decidedly beneficial for both their host plants and the world's agricultural systems. Plant–AM symbiosis helps plant to acquire phosphate from the soils, whereas legume–rhizobia symbiosis converts atmospheric nitrogen into the form required for plant growth. Unravelling the molecular underpinnings of these symbiosis shows that these associations share some common signalling factors concluding that both the associations are interrelated. For legume–rhizobia interactions, nodule development is an important event. Legume–rhizobia symbiotic control is exercised at three points: flavonoids in Nod D proteins, Nod factors in Hac or bacterial entry, as well as EPS and/or TTSS proteins in infection thread. Legume roots secrete flavonoids and betaine which are sensed by rhizobial partner that aids in the activation of Nod proteins and in turn secretion of Nod factors which assist in nodule development. The nodulation genes (*nod*, *nol*, *noe*) and nitrogen fixation genes (*nif*, *fix*) are key symbiotic genes in rhizobia, whereas nodulin genes that are expressed in root tissues as a consequence of interaction with rhizobia are symbiotic genes in plants. Host preferences of rhizobial partner are due to Nod D protein that can be activated by a large variety of flavonoids, production of more than 80 different types of Nod factors and the fact that its Nod D protein can be activated by both EPS and TTSS proteins. Diverse AM fungi produce small, diffusible factors that trigger the activity of one of the same genes activated by Nod factors. Hence, fungal and rhizobial Nod factors may play an analogous role. In conclusion, legume–rhizobia interactions are incomplete without mycorrhizae. The chemical nature of various Nod factors is known. It is expected that in the near future, the chemical nature of fungal factor and flavonoid–Nod factor association is elucidated. We must investigate rhizobial partners of yet unexplored legumes, their natural variations and responsiveness with biodiversity collections of important crop plants. The long-term aim is to identify or design crop–rhizobia–fungus combinations with optimised performance so that fertiliser and energy input can be reduced.

Acknowledgements Our work on tree and legume rhizobia has been supported through the Centre for Research on Bacteria and Archaea, under All India Coordinated Project on Taxonomy (AICOPTAX) of the Ministry of Environment and Forests, Govt. of India. Authors thank Mr. Mukesh Samant and Ms. Komal Agarwal for use of unpublished data.

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Chapter 10
Biological Nitrogen Fixation: Importance, Associated Diversity, and Estimates

Márcia do Vale Barreto Figueiredo, Adália Cavalcanti do Espírito Santo Mergulhão, Júlia Kuklinsky Sobral, Mario de Andrade Lira Junior, and Ademir Sergio Ferreira de Araújo

Contents

M. do Vale Barreto Figueiredo (✉)
Vegetal Production Department, Agronomical Institute of Pernambuco IPA/SEAGRI, 1371, Gen. San Martin Avenue, 50761-000 Recife, PE, Brazil

National Research and Technological Development, Brazil
e-mail: mbarreto@elogica.com.br

A.C. do Espírito Santo Mergulhão
Vegetal Production Department, Agronomical Institute of Pernambuco IPA, 1371, Gen. San Martin Avenue, 50761-000 Recife, PE, Brazil

J.K. Sobral
Agronomy Department, Federal Rural University of Pernambuco, Garanhuns, PE, Brazil

M. de Andrade Lira Junior
National Research and Technological Development, Brazil

Agronomy Department, Federal Rural University of Pernambuco, UFRPE/DEPA, s/n D. Manoel de Medeiros St, 52171-900 Recife, PE, Brazil

A.S.F. de Araújo
National Research and Technological Development, Brazil

Agronomy Department, Federal University of Piaui, UFPI, Campus of the Socopo, 64049-550 Teresina, PI, Brazil

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_10,

Abstract Several processes mediated by soil microorganisms play an important role in nutrient cycling. One such process is biological nitrogen fixation (BNF) by representatives of various bacterial phylogenetic groups, which are called diazotrophs. These bacteria can be free-living, associate with plant species, or even establish symbiosis with legumes. Studies with diazotrophic organisms are of great importance due to their contribution to the nitrogen supply in different ecosystems, including natural and managed systems. It is estimated that global BNF adds 122 Tg of N yearly with cultivated agricultural systems fixing from 33 to 43 Tg, which occurs mostly by legume-rhizobia symbiosis. There is a large potential of BNF contribution by associative systems with tropical grasses, but there is uncertainty in these estimates due to several assumptions in the estimation process and fewer studies with this system when compared to the legume-rhizobia symbiosis. Recent progress in the understanding of diversity, colonization ability, action mechanisms, formulation, and application of these biological systems should facilitate their development as reliable components in the management of sustainable agricultural systems. Several efforts have been made to develop commercial inoculants using these organisms. The current progress in using microorganisms that fix nitrogen in a variety of applications is summarized and discussed herein.

Introduction

Microorganisms that carry out biological nitrogen fixation (BNF) have great importance because this element is an essential component of proteins, nucleic acids, and other nitrogen compounds. Therefore, nitrogen is an essential component of life for all living beings (Döbereiner 1997). The process of BNF performed by symbiotic nitrogen-fixing bacteria with legume species, which are commonly known as alpha and beta rhizobia, provides high sustainability for ecosystems (Bomfeti et al. 2011). These microorganisms can help promote plant growth not only by supplying nitrogen but also by other mechanisms, such as production of siderophores, exopolysaccharides (EPS), and phytohormones; phosphate solubilization; and protection against phytopathogenic fungus (Dakora 2003; Figueiredo et al. 2008; Moreira et al. 2010).

Diazotrophs are found in a wide variety of habitats: free-living in soil and water, associative symbioses with grasses, symbiotic association in termite guts, actinorhizal association with woody plants, cyanobacterial symbioses with various plants, and root-nodule symbioses with legumes (Dixon and Kahn 2004). The two most important types of symbioses are N_2 fixation and acquisition of P and other nutrients by mycorrhizae (Rengel 2002; Bonfante and Anca 2009). For cultivation of legumes, relationship of rhizobia and mycorrhiza is of great importance because these bacteria influence the infection rate and mineral nutrition as well as the physical and chemical conditions of the soil by adding organic waste and increasing the growth of these plants (Andrade et al. 2000; Parniske 2008).

Symbiotic or even mutualistic relationships involving rhizobia depend on chemical signals between the two organisms. These signals define the rhizobia host specificity in the relationship. Selecting for the optimal combination of the rhizobia and the host generally results in more effective symbiosis and better growth of the host plant (Rengel 2002; Araújo et al. 2012).

Biofertilizers that can cater to the different needs of growing plants act as a consortium in addition to other microorganisms in the rhizosphere. Understanding the interaction between the consortium of microbial inoculants and plant systems will lay the foundation for harnessing more benefits from microbial inoculants for improving plant growth and yield (Raja et al. 2006). Single inoculants and combinations of plant growth-promoting bacteria (PGPB)/plant growth-promoting rhizobacteria (PGPR) are common inoculants, and their use is increasing in agricultural practices (Díaz-Zorita and Fernández-Canigia 2009). PGPB affect plants through a multitude of mechanisms. Several comprehensive and critical reviews describing the operational mechanisms of PGPB/PGPR have been published in recent years (Bashan et al. 2011; de-Bashan et al. 2012).

The formulation step is a crucial aspect of producing microbial inoculants, and it determines the success of a biological agent (Brahmaprakash and Sahu 2012). In recent years, the strong potential of biopolymers to be used as inoculants has been studied (Borschiver et al. 2008; Silva et al. 2009). Biopolymers have demonstrated potential as bacterial carriers for microbial inoculants. Another recent possibility for development of new inoculants or biofertilizers is the use of biofilm (Seneviratne et al. 2009). Furthermore, the role of these compounds in stress adaptation may be an important criterion for the selection of inoculant strains to raise plant productivity by BNF under different soil and climatic conditions (Bomfeti et al. 2011).

BNF as the Key for Ecological Success

Nitrogen is the most abundant element in the atmosphere, and it is mainly present in the diatomic form (N_2). Nitrogen is an essential macronutrient for plant species. Some bacteria have enzymes with the ability to reduce N_2 and turn it into ammonia. Subsequently, ammonia is used in the synthesis of essential elements, which is a process known as BNF (Hungria et al. 2007; Di Ciocco et al. 2008). BNF can be symbiotic when there are mutualistic associations between plant species and fixing microorganisms (mainly rhizobia) or can be asymbiotic when it is carried out by free-living fixing microorganisms, such as species of the genera *Azotobacter* and *Beijerinckia* (Freitas 2007).

Rhizobia are distributed in different taxonomic groups according to their morphological, physiological, genetic, and phylogenetic characteristics (Lindström et al. 2006). Currently, they are classified into α- and β-rhizobia (Bomfeti et al. 2011). The genera *Agrobacterium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Devosia*, *Mesorhizobium*, *Methylobacterium*, *Ochrobactrum*, *Phyllobacterium*, *Rhizobium*, and *Sinorhizobium* belong to the group of α-proteobacteria, and bacteria of the genera

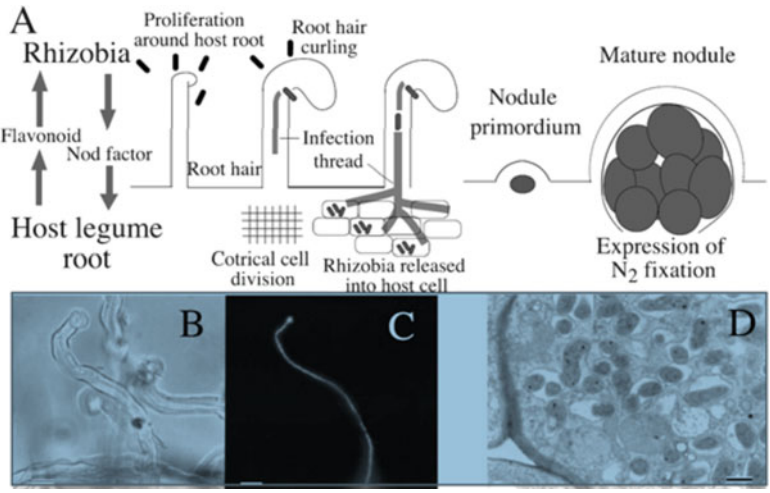

Fig. 10.1 Nodulation by rhizobia. (**a**) Scheme of chemical signal exchanges and infection processes involving rhizobia. (**b**) Root-hair curing in *Lotus japonicus*. (**c**) *Mesorhizobium loti* cells tagged with constitutive *gfp* gene in an infection thread in the root hair shown in *panel* (**b**). (**d**) *M. loti* bacteroids in infected cells of a mature *L. japonicus* nodule. *Bar* indicates 10 **μ**m (**b**, **c**) and 1 **μ**m (**d**) (Adapted from Okasaki et al. 2004)

Burkholderia, *Cupriavidus*, and *Herbaspirillum* belong to the β-proteobacteria group (Weir 2011).

The effectiveness of the legume-rhizobia symbiotic system and the development of nodules result from the exchange of molecular chemical signals between plant and its symbiont (Okasaki et al. 2004; Zilli et al. 2009, 2011) (Fig. 10.1). Although legumes form root nodules mainly in response to Nod factors, the plant perception of endogenous signals, particularly plant hormones, is also thought to be important for the establishment of proper symbiotic interactions between rhizobia and legumes (Caetano-Anolles and Gresshoff 1991). The native species of nitrogen-fixing bacteria perform BNF at a low degree of efficiency. Therefore, it is necessary to obtain elite strains of rhizobia for efficient BNF (Figueiredo et al. 2008; Zilli et al. 2011).

Microorganisms that fix nitrogen require 16 mol of adenosine triphosphate (ATP) to reduce each mole of nitrogen (Hubbell and Kidder 2009). These organisms obtain this energy by oxidizing organic molecules. Non-photosynthetic free-living microorganisms must obtain these molecules from other organisms, and photosynthetic microorganisms, such as cyanobacteria, use sugars produced by photosynthesis to obtain these molecules. Associative and symbiotic nitrogen-fixing microorganisms obtain these compounds from the rhizosphere of their host plants (National Research Council 1994; Hubbell and Kidder 2009). Different N_2-fixing organisms and symbioses found in agricultural and terrestrial natural ecosystems are shown in Fig. 10.2.

Advances in agricultural sustainability will require an increase in the use of BNF as a major source of nitrogen for plants. Long-term sustainability of agricultural systems must rely on the use and effective management of internal resources. The process of BNF offers an economically attractive and ecologically sound means of

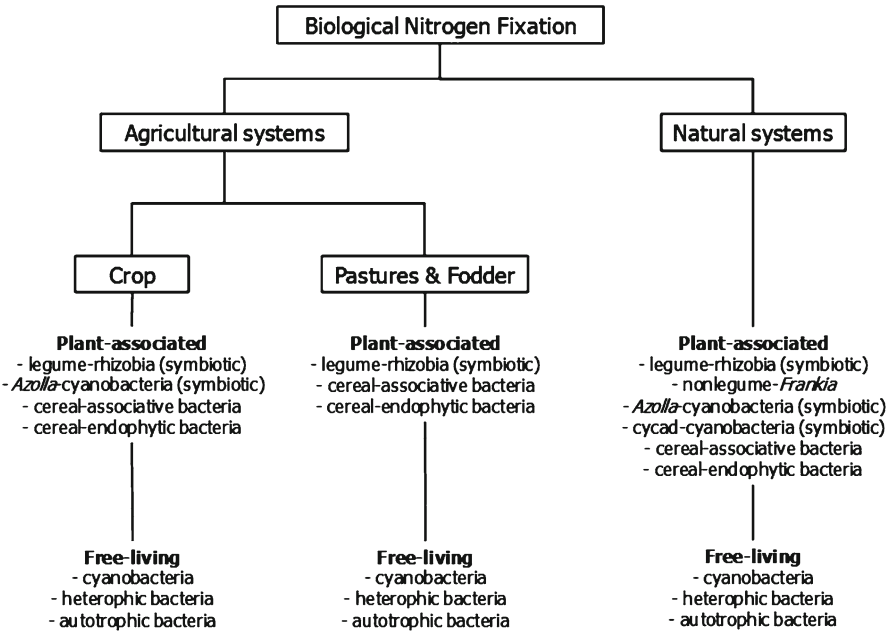

Fig. 10.2 Biological N_2-fixing agents in agricultural and terrestrial natural systems (Adapted from Herridge et al. 2008)

reducing external nitrogen input and improving the quality and quantity of internal resources. Clearly, it is unreasonable to consider sustainable agriculture on a broad scale without BNF. Further research is needed to optimize the contribution of BNF to sustainable agriculture (Saikia and Jain 2007). The study of efficient use of N yields multiple advantages, such as the reduction of fertilizer doses to maintain productive levels and even genetic improvements to adapt plants to nitrogen-poor soils. The study of N acquisition and use should be linked to the understanding of the absorption, assimilation, and redistribution of this nutrient in cells as well as its balance between storage and use (Majerowicz et al. 2002).

Currently, new methods designed to increase nitrogen use efficiency are being intensely studied, especially through the recognition of biochemical and molecular pathways of absorption and assimilation in plants. Agroecological methods, such as BNF, are proposed to allow the sustainable use of this nutrient without production loss (Herridge et al. 2008).

Diversity of BNF Systems

The high genetic variability of diazotrophs enables the occurrence of BNF in different systems (Franche et al. 2009), and this variability is important for the study of phylogenetic relationships. It is important for the study of phylogenetic

relationships, and diversity of bacterial genes is based not only on the taxonomic position but also on the need to fully exploit the potential of biotechnology (Woese 1994; Meitanis et al. 2008; Vale et al. 2008). According to Van Elsas and Boersma (2011), the study of microbial populations that inhabit the natural environment is essential in understanding the functioning of ecosystems. Microbial diversity is generally considered to be the number of individuals of different taxa and their distribution among taxa, and genetic diversity is the variation of genes and genotypes within groups (Lynch et al. 2004).

In the past, the study of microbial diversity was based on techniques that were dependent on cultivation, providing limited information due to lack of culture media which accurately reproduce the different ecological niches in a laboratory environment. Therefore, only a small fraction of microbial diversity existing in environmental systems can be cultivated in vitro, which causes an underestimation of the natural diversity (Tyson and Banfield 2005). Currently, microbial diversity can be assessed more broadly through the use of molecular biology techniques (both dependent on and independent of microorganism culture) enabling the detection of nucleic acids (Andreote et al. 2009; Roesch et al. 2010).

The diversity of the composition of ribosomal genes has been greatly discussed, especially for phylogenetic studies. In prokaryotes, such as diazotrophs, the 16S rRNA gene is the most widely used and is highly conserved. This gene is considered to be the most suitable for studies of microbial ecology and phylogeny and allows identification at the level of genus and species. 16S rRNA even allows for the analysis and correlation between genotype and the studied niche (Chéneby et al. 2000; Gribaldo and Brochier 2009). Diversity studies have identified different groups of diazotrophic bacteria: rhizobia (α-proteobacteria); Frankia (in Actinobacteria); cyanobacteria; bacteria belonging to various bacterial genera, such as *Bacillus*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Methylobacterium*, *Paenibacillus*, *Pantoea*, *Pseudomonas*, and *Stenotrophomonas*, which colonize the surface of plant tissues without formation of differentiated structures; and endophytes (Kuklinsky-Sobral et al. 2004; Franche et al. 2009; Ribeiro et al. 2009; Lindstrom et al. 2010; Tripp et al. 2010; Monteiro et al. 2012). BOX A1R-based repetitive extragenic palindromic (BOX) polymerase chain reaction (PCR) is among several existing techniques in molecular biology that depend on the isolation and cultivation of bacterial communities in the laboratory. BOX PCR uses the same technique of repetitive extragenic palindromic sequence (REP) PCR, which is based on finding and amplifying repetitive regions of the bacterial genome. In this technique, repetitive and highly conserved regions of the bacterial genome are amplified, including Box elements, which are divided into three groups: BoxA, BoxB, and BoxC with BoxA being the most common. The BOX A1R primer allows a more detailed characterization of isolates, and it produces robust fragments of fingerprints with a complex pattern. Therefore, the BOX A1R primer is generally used to differentiate bacterial strains (Marques et al. 2008; Lee and Wong 2009). Torres et al. (2008) found a large diversity among endophytic bacteria isolated from root nodules formed by the symbiosis between rhizobia and bean (*Phaseolus vulgaris* L.).

Table 10.1 Examples of molecular techniques for the study of the *nif* H gene

Application	Molecular technique	Reference
Diversity analysis	Terminal restriction fragment length polymorphism analysis (T-RFLP)	Bannert et al. (2011), Beneduzi et al. (2008)
	Denaturing gradient gel electrophoresis (DGGE)	Li et al. (2012), Dias et al. (2012), Coelho et al. (2009), Martensson et al. (2009)
Gene quantification	Real-time PCR (qPCR)	Dias et al. (2012), Bannert et al. (2011), Coelho et al. (2009), Martensson et al. (2009)
Gene expression	Oligonucleotide microarray	Duc et al. (2009)
	Cloning and RT-PCR (reverse transcription) analysis	Honga et al. (2012), Thaweenut et al. (2011)

Diazotrophs have the nitrogenase enzyme complex that is responsible for BNF. This enzyme system is composed of three subunits, and it is regulated by a complex system with multiple genes. Nitrogenase 1 (classic) is dependent on iron and molybdenum, and it is encoded by the *nif* gene. Nitrogenase 2 is dependent on vanadium, and it is encoded by the *vnf* gene. Nitrogenase 3 is dependent on iron, and it is encoded by the *anf* gene (Franche et al. 2009; Canfield et al. 2010). In free-living and associative microorganisms, the *nif* genes are responsible for encoding highly conserved subunits (Zehr et al. 2003; Falkowski et al. 2008; Franche et al. 2009). Because of the high conservation of these genes, phylogenetic studies based on these genes have shown similar results to those obtained using 16S rRNA. Thus, the *nif* gene has been used to characterize the genetic diversity of diazotrophs (Zehr et al. 2003).

The *nif* genes include *nif* D, *nif* H, and *nif* K, which all encode proteins of the nitrogenase enzyme complex. The *nif* H functional gene, which encodes the Fe-protein of nitrogenase, is well preserved and well studied as compared to other *nif* genes, which have been used for phylogenetic analysis of the diazotrophic bacterial community (Zehr et al. 2003; Franche et al. 2009). However, Gaby and Buckley (2011) assessed the global diversity of nitrogen-fixing microorganisms through the construction and analysis of an aligned database of 16,989 *nif* H sequences. They concluded that the diversity of diazotrophs is still poorly described and that many organisms remain to be discovered.

The techniques that evaluate bacterial diversity using the isolation and cultivation of bacteria in a laboratory followed by DNA extraction do not allow the study of uncultured microorganisms present in the sample environment, thereby restricting the diversity found. However, the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) technique allows access to the diversity of bacterial communities directly from their habitat without cultivation (Andreote et al. 2009). Therefore, various molecular techniques are being used to study the diversity, quantification, and analysis of *nif* H gene expression (Table 10.1).

BNF Inputs to Agricultural Systems

While the Haber-Bosch nitrogen fertilizer production system is considered to have saved untold millions of people throughout the world, it is not without the following major concerns: the severalfold increase in reactive nitrogen cycling throughout the ecosphere; the relative lack of efficiency of reactive nitrogen under agricultural use, which may lead to major ecological issues of water contamination and eutrophication; and the demand for fossil fuel, which is generally demanded as natural gas (Rockström et al. 2009; Good and Beatty 2011; Kim et al. 2011; Sutton et al. 2011).

In contrast, even with all of these caveats, the growing population and its desirable increase in income will only demand higher levels of food production, particularly of meat and dairy products (Godfray et al. 2010). Meeting these demands will be impossible without a reliable nitrogen source. While chemically fixed fertilizer will necessarily be a part of the various options deployed by agricultural and soil scientists throughout the world, an increased reliance on BNF is one of the major alternatives for both maintaining and/or increasing agricultural yield and reducing both environmental and economical concerns linked to nitrogen fertilizer use (Doane et al. 2009; Hvistendahl 2010). One point to keep in mind is that these alternatives are not either/or solution sets and should not be thought as such. Both alternatives are simply tools to increase agricultural yield to allow human resources needs to be fulfilled in such a way that future generations will have access to at least the same pool of natural resources as previous ones did.

Unfortunately, even though obtaining global nitrogen fertilizer estimates is relatively easy, obtaining estimates for biologically fixed nitrogen is not easy (Herridge et al. 2008; Peoples et al. 2009); this factor may be a major constraint on an increased dependency on this source. One of the first reasons for this difficulty is the sheer number of possible biological systems, which all have different BNF capabilities, natural ranges, cultivated ranges, areas, and potential yields (Fig. 10.3). Burris (2008) has been quoted by Herridge et al. (2008) as having said that "potential authors could use a variety of methods to fill in the values in the N cycle, from gazing at crystal balls, consulting sages to cranking out computer-generated random numbers." However, the most common method is a literature review, and choosing the numbers thought to be a more logical approach. This difficulty in estimating global BNF can be roughly divided into several different reasons:

1. Methodological problems in field-scale BNF estimation
2. Highly variable BNF rates, which are strongly affected by environmental and agricultural concerns
3. Difficulty in estimating individual cropping systems, worldwide distribution, and cultivated areas

The first and second reasons intermingle with the high variation in BNF rates and lead to highly variable estimates, for example, for soybeans (*Glycine max*), which range from 0 to 450 kg shoot $N.ha^{-1}$ according to different sources cited by Peoples

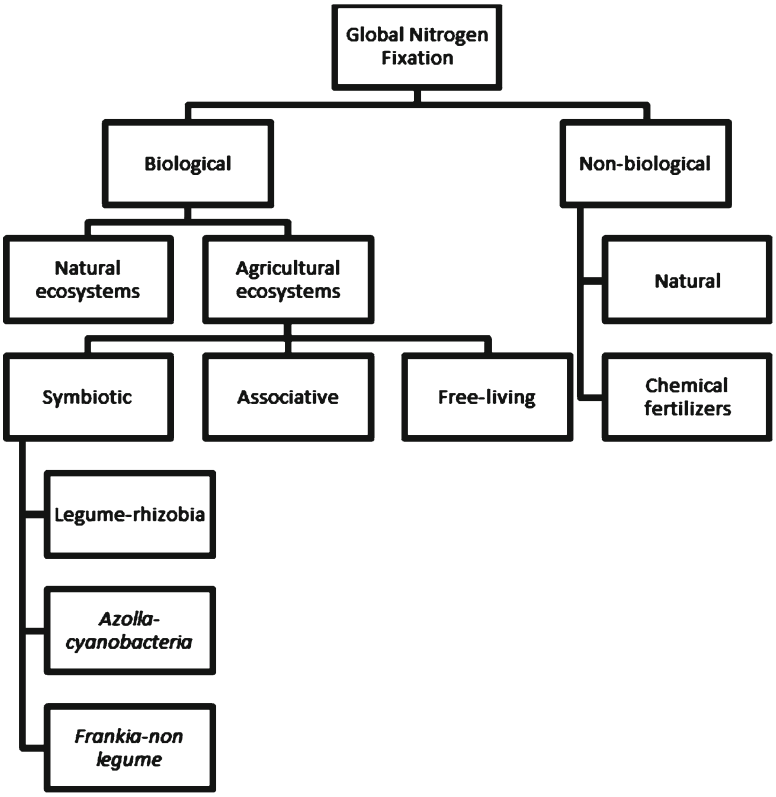

Fig. 10.3 Main sources of biologically available nitrogen, not including mining the soil organic matter reserve

et al. (2009). In addition, another major problem is that the root system is routinely not included in the BNF estimates, which may lead to soybean going from a net exporter (Di Ciocco et al. 2011) to a net fixer of soil N (Singh et al. 2004). When considering the importance and number of agricultural systems in which soybean participates, this change may have significant effects on overall N balance.

Even with all of these caveats, it is still highly important to achieve an overall estimate, which has been well executed in several recent reports on a global or national scale (Herridge et al. 2008; Peoples et al. 2009; Yang et al. 2010). One common approach has been to obtain an estimate for the average BNF rate per hectare and then to multiply by another estimate of total cultivated area of the specific system. This approach may also be performed with some type of subdivision as exemplified by the recognition of different BNF rates for Argentinean-, Brazilian-, Chinese-, and North American-grown soybeans (Gan et al. 2002; Nicol et al. 2002; Okogun et al. 2005; Hungria et al. 2006; Oberson et al. 2007; Schipanski et al. 2010; Di Ciocco et al. 2011).

As indicated by the previous examples, a further point to consider is that BNF estimation is much more common for the legume-rhizobia symbiosis than

for other systems, such as grass-endophyte associations or *Azolla*-cyanobacteria symbiosis, which results from a better knowledge of the system, a much more precise estimate of occurrence, a vastly higher number of punctual experiments from which to derive raw data, and the sheer importance of the legume-rhizobia symbiosis in world agriculture.

Most estimates for global BNF are 120 Tg of N per year (estimations summarized by Herridge et al. (2008)) with the crop legume-rhizobia symbiosis accounting for approximately a sixth of that estimation (according to several authors summarized by Peoples et al. (2009)). These values are less than those estimated for the nitrogen fertilizer industry of *ca*. 140 Tg of N per year (Canfield et al. 2010). In contrast, while most estimates indicate that approximately 1–2 % of global energy consumption is directly linked to N fertilizer production and that *ca*. 300 Tg of fossil C is derived just from its production (not including the large fossil C cost of its distribution), there is close to zero fossil fuel use linked to BNF, which does not mean that there is not any C emission linked to BNF. There should also be a lower NO_x emission derived from BNF, and thus lower glasshouse effects, because all of the N is in an organic form by definition and, thus, should not be available for denitrification most of the time (Jensen et al. 2012). This consideration is also important when considering that there is an international tendency to demand a more sustainable agriculture with less resource consumption for a given yield level, which could be maximized through further use of BNF as a rule in agricultural systems (Wilkins 2008), and if non-BNF advantages of legume inclusion are considered, such as the reduction in disease incidence or nutrient mining of deeper layers allowed by their greater root system (Sileshi et al. 2008; Köpke and Nemecek 2010; Fornara 2011).

All of these advantages require a greater need for management knowledge, which is one of the primary reasons for the greater reliance on N fertilizer as an alternative. The greater need for management knowledge is due to the highly localized effects of environment and cropping systems, which demand a high level of knowledge of the farmer to maximize their efficiency as exemplified by the variable effects of species and cultivars of legumes seeded with barley, the effect of a fertility gradient on BNF from legume-grass mixtures (Schipanski and Drinkwater 2012), or the impact of cutting management and *Desmodium* species in a mixture with corn (Kifuko-Koech et al. 2012).

Mycorrhizal Infection of Legume Roots to Stimulate Nodulation

A mycorrhiza (Greek; mycos = fungus and rhiza = root) is a symbiotic association between certain soil fungi and plant roots (Bonfante and Anca 2009). Based on morphoanatomy of colonized roots, mycorrhizae are grouped into ectomycorrhiza, ectendomycorrhiza, and endomycorrhiza (Azcón 2000). Ectomycorrhizae are characterized by the formation of a mycelial mantle on the root with only intercellular penetration of the cortex by fungal mycelium and the formation of a "Hartig

net" (Bonfante 2001). Ectendomycorrhizae are generally ectomycorrhiza with intracellular penetration but with anatomical differences according to the host plant. Endomycorrhizae are the most common type of mycorrhiza formed by arbuscular mycorrhizal fungi (AMF) that penetrate intercellularly and intracellularly in the host root cortex, currently belonging to the phylum Glomeromycota with 3 classes (*Archaeosporomycetes*, *Glomeromycetes*, and *Paraglomeromycetes*) composed of 5 orders (*Archaeosporales*, *Diversisporales*, *Gigasporales*, *Glomerales*, and *Paraglomerales*), 14 families, 29 genera, and approximately 230 described species (Oehl et al. 2011) (Fig. 10.4). These fungi are distributed in most ecosystems representing a broader association between plants and fungi, and they are present on more than 80 % of plant species (Smith and Read 2008), including almost all species of agronomic interest and pastoral and tropical forest (Moreira and Siqueira 2002; Bonfante and Genre 2008).

Leguminosae is the third largest family of angiosperms and is the second most economically important in the world only behind *Poaceae*. With three subfamilies (*Mimosoideae*, *Caesalpinioideae*, and *Papilionoideae*), 727 genera, and 19,325 species, this family has a cosmopolitan distribution (Lewis et al. 2005). In Brazil, there are approximately 210 genera and 2,694 species of *Leguminosae* (Lima et al. 2010). Legumes play a key role in the balance of nitrogen in ecosystems due to their ability to fix nitrogen and improve soil quality in agroforestry, silvopastoral, and forestry (Foelkel 2012). Legumes are also ecologically important because they are well adapted to the first colonization and exploitation of different environments due, in part, to their association with nitrogen-fixing bacteria or mycorrhizal fungi (Silva and Tozzi 2011).

The rhizobia-legume symbiosis is responsible for producing annual levels of at least 35 million tons of nitrogen (Freire 1992). Nitrogen-fixing bacteria, including rhizobia and mycorrhizal fungi, form mutualistic symbiotic associations with legumes. In this association, which is known as tripartite (Bonfante and Anca 2009; Vega et al. 2010), the mycorrhizal mycelia through the network may increase the absorption and solubilization of phosphorus by translocating phosphorus in the soil to rhizobia located on plant nodules. Rhizobia fix nitrogen and provide it in the form of ammonia to the plant, which, in turn, provides carbohydrate to microsymbionts (Silveira et al. 2001; Gross et al. 2004). For cultivation of legumes, this relationship between rhizobia and mycorrhiza is of great importance because it influences the infection rate and mineral nutrition as well as the physical and chemical conditions of the soil by adding organic waste and increasing the growth of these plants (Andrade et al. 2000).

Under conditions of phosphorus deficiency, legumes have low nodulation and nitrogen fixation unless their roots are colonized by mycorrhizas or if the soil is fertilized with high phosphorus levels. Moreover, the mycorrhizal condition influences the efficient competition among strains of rhizobia to occupy the nodules in the roots of the host (Miranda and Miranda 2002; Garg and Manchanda 2008). Kaschuk et al. (2010) studied the AMF-rhizobia symbiosis in 12 legume species, and they reported an increase in the photosynthetic rate and grain yield of legumes. This result is important because this rate compensates for the transfer of plant

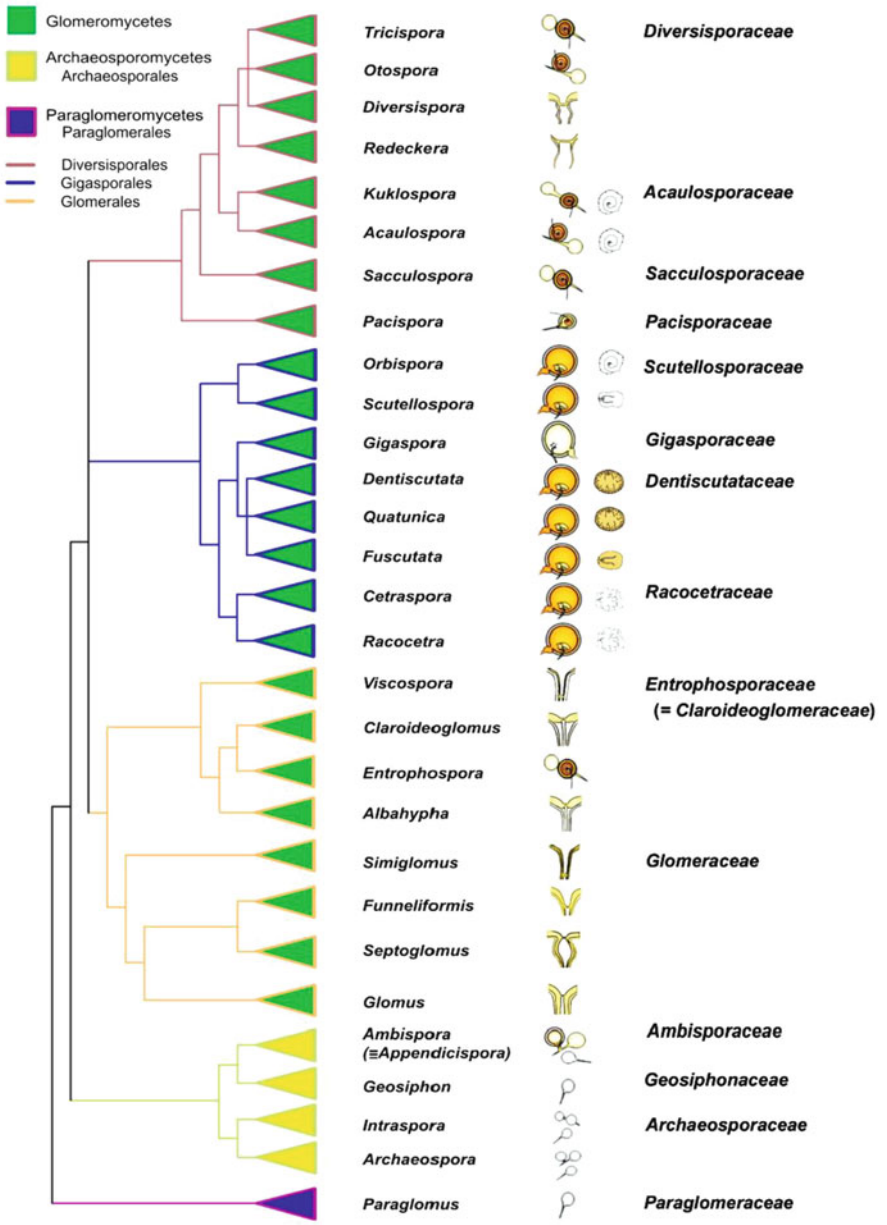

Fig. 10.4 Representative tree of the phylum Glomeromycota based on molecular and morphological analyses (*Source*: Oehl et al. 2011)

photosynthates for microorganisms. Burity et al. (2000) studied thrush (*Mimosa caesalpiniaefolia*) inoculated with *Glomus etunicatum*, *Acaulospora morrowae*, *A. longula*, and *Rhizobium* sp., and they observed a larger increase in nodulation and root colonization by mycorrhiza. Jesus et al. (2005) studied two species of legumes

(*Piptadenia gonoacantha* (Mart.) Macbr. and *Piptadenia paniculata* Bentham) and found that these two species depend on mycorrhiza for satisfactory growth and nodulation with rhizobia. The synergy between the mycorrhizal fungi and rhizobia microsymbionts on legumes has been well documented in several studies (Mergulhão et al. 2001; Diniz et al. 2002; Jesus et al. 2005; Kaschuk et al. 2010; Lima et al. 2011; Mendes Filho et al. 2011). However, according to Scotti (1997), the benefit of these microorganisms to the host plant depends on the compatibility between the strain of rhizobia and mycorrhizal fungi inoculated.

Some strains of bacteria can positively influence and establish symbiosis with mycorrhizal fungi (Garbaye 1994; Frey-Klett et al. 2007), and these synergistically effective are called "mycorrhiza helper bacteria" (MHB) (Duponnois and Garbaye 1991; Garbaye 1994). Importantly, MHB have specificity for fungi but do not have specificity for plants (Garbaye 1994; Duponnois and Plenchette 2003). Duponnois and Garbaye (1991) were the first to observe the effect of bacteria *Pseudomonas fluorescens* to significantly stimulate the formation of ectomycorrhizal fungus *Laccaria laccata*. After the review on the effects observed in MHB work by Garbaye (1994), Frey-Klett et al. (2007) provided further information on MHB functionality during symbiosis. Frey-Klett et al. (2007) reported that MHB improve the conductivity of soil and responsiveness to root fungus by plant-fungus recognition and establishment of symbiosis, and they also reported that MHB promote survival, germination of propagules, and mycelial growth of fungi. Moreover, they reported that both the fungus and root select the bacterial population in the rhizosphere soil, promote the growth of fungus, and determine the receptivity of the root to the fungus. The strains of MHB identified to date belong to gram-negative Proteobacteria (*Agrobacterium*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Rhizobium*, and *Klebsiella*), gram-positive Firmicutes (*Bacillus*, *Brevibacillus*, and *Paenibacillus*), and gram-positive Actinomycetes (*Rhodococcus*, *Streptomyces*, and *Arthrobacter*) (Frey-Klett et al. 2007). This demonstrates the diversity of bacteria having potential use in biotechnological processes. Rigamonte et al. (2010) suggested that the first effect of MHB on ectomycorrhiza is related in stimulating the growth of these fungi. Another feature of MHB in mycorrhizal plants is acting as the stimulus for the formation of lateral roots in addition to growth of the fungus, which may lead to an increased number of sites of interaction between plant and fungus (Schrey et al. 2005), thereby increasing mycorrhizal colonization on the host plant (Rigamonte et al. 2010). Moreover, MHB may improve the nutritional status of mycorrhiza by providing nitrogen and contributing to solubilization of soil minerals. Some strains of MHB are able to compete with bacteria that inhibit mycorrhiza (Garbaye 1994); thus, these strains reduce the concentration of antifungal metabolites in the plant rhizosphere, which favors the release of fungi exudates that serve as nutrients for the bacteria (Rigamonte et al. 2010). According to Frey-Klett et al. (2007), MHB enhance spore germination and mycelial growth by producing growth factors, inhibiting competitors, inhibiting antagonists, and promoting detoxification of antagonistic substances. According to Bonfante and Anca (2009), the release of bioactive molecules and physical contact between bacteria and mycorrhizal fungi are important in the establishment of their interactions, which are the main

signaling mechanisms observed in the tripartite rhizobia/mycorrhiza/legume association (Oldroyd and Downie 2008). Legumes have an exceptional ability to form a symbiotic association with rhizobia and mycorrhizal fungi, and the positive effects of the symbiosis depend on the combination of interactions among the host plant, symbionts, and environmental factors. Thus, additional research is needed to better understand the functions and mechanisms of the interaction between fungi and bacteria in and on the roots of these host plants (Kannan et al. 2011).

Bacterial Biofertilizers

Plant yield is dependent on nutrients, such as nitrogen (N), and farmers usually need to apply at least 100 kg of N per hectare (Deaker et al. 2004). However, N fertilizers are expensive, and chemical fertilization may promote soil pollution. In contrast, biofertilizers are gaining importance in sustainable agriculture. The term "biofertilizer" specifies that the fertilizer meets the nutritional requirements of a crop through microbiological means. In several countries, biofertilizers are synonymous with bacterial inoculants (Brahmaprakash and Sahu 2012).

A bacterial inoculant is a formulation that contains one or more beneficial bacterial strains or species in an easy-to-use and economical carrier material. Inoculants are the "vehicle" to transport living bacteria from the factory to living plants to produce the desired effects on plant growth (Bashan 1998). For legume plants, BNF can be used by inoculating legume seeds with rhizobia (Deaker et al. 2004). In Brazil, the use of inoculants for legume plants began in the 1950s (Freire et al. 1968), and the inoculation process currently saves approximately 11 billion dollars per year when considering only soybean crops (Hungria 2012). Although Brazil has a long tradition in research and production of inoculants for legumes, the studies of nitrogen fixation in *Azospirillum*-grass associations have only begun recently; thus, only a few commercial inoculants are available in the market (Bashan 1998).

Peat is the most frequently used carrier for the rhizobial inoculant industry because it has high water-holding capacity and large surface area, which support rhizobial growth and survival in large numbers (Smith 1992). However, a peat-based inoculant requires a significant amount of processing, such as mining, drying, milling, and neutralizing, before its use in a commercial production system. The formulation step is a crucial aspect for producing microbial inoculants and determines the success of a biological agent. Formulation typically consists of establishing viable bacteria in a suitable carrier together with additives that aid in the stabilization and protection of microbial cells during storage and transport and at the target (Brahmaprakash and Sahu 2012). The formulation should also be easy to handle and apply so that it is delivered to the target in the most appropriate manner and form and should also protect bacteria from harmful environmental factors and maintain or enhance the activity of the organisms in the field (Xavier et al. 2004).

A good quality inoculant should be composed of a superior carrier material. Smith (1992) suggested that the following features are characteristics of a superior

quality carrier material for microbial inoculants: high water-holding capacity, high water retention capacity, no heat production from wetting, nearly sterile, chemically uniform, physically uniform, nontoxic in nature, easily biodegradable, nonpolluting, nearly neutral pH (or easily adjustable pH), and supports bacterial growth and survival. Inoculants come in four basic dispersal forms (powders, slurries, granules, and liquids). The use of each type of inoculant depends upon market availability, farmers' choice, cost, and the need of a particular crop under specific environmental conditions (Arora et al. 2010).

These characteristics have prompted researchers to find new carrier materials, including clays, inorganic soils (Chao and Alexander 1984), compost (Iswaran et al. 1972), wheat bran (Jackson et al. 1991), spent agricultural waste material (Sadasivam et al. 1986), and spent mushroom compost (Bahl and Jauhri 1986). Apart from these materials, many other synthetic and inert materials, such as vermiculite (Sparrow and Ham 1983), perlite, ground rock phosphate, calcium sulfate, polyacrylamide gels (Dommergues et al. 1979), and alginate (Bashan 1986), have also been evaluated.

Liquid inoculant formulations are one solution to the problems associated with processing solid carriers. Liquid inoculant formulations may use various broth cultures amended with agents that promote cell survival in the package and after application to seeds or soil. Additives to liquid inoculant formulations should have a role in protecting the cells on seeds at high temperatures and during desiccation. Many types of biopolymers have been used for inoculant production due to their ability to limit heat transfer, good rheological properties, and high water activities (Mugnier and Jung 1985).

In recent years, the strong potential of biopolymers as inoculants has been studied (Freitas et al. 2003; Borschiver et al. 2008; Silva et al. 2009; Abd Elgadir et al. 2012). Biopolymers may be defined as polymers (proteins, nucleic acids, or polysaccharides) that are produced by living organisms (Borschiver et al. 2008). These polymers have demonstrated potential as bacterial carriers for microbial inoculants. These formulations encapsulate living cells, protect microorganisms against many environmental stresses, and gradually release the cells into soil in large quantities where the polymers are degraded by soil microorganisms.

Another recent possibility for the development of new inoculants or biofertilizers is the use of biofilm. Biofilm consists of microbial cells (algal, fungal, bacterial, and/or other microbial cells) in addition to an extracellular biopolymer (known as an extracellular polymeric substance) (EPS) produced by the cells, which provides structure and protection to the community. The formation of fungal-bacterial biofilms (FBBs) by bacterial colonization on biotic fungal surfaces provides enhanced metabolic activities as compared to monocultures. Incorporation of a nitrogen (N_2)-fixing rhizobial strain to FBBs to form fungal-rhizobial biofilms (FRBs) has been shown to improve potential biofilm applications in N-deficient settings and in the production of biofilm inoculants for biofertilizers and biocontrol in plants (Seneviratne et al. 2007). Biofilms attached to the plant roots of some crops help in the cycling of nutrients as well as in the biocontrol of pests and diseases, resulting in improved agricultural productivity (Seneviratne 2003). A developed FRB inoculant

has been shown to significantly increase N_2 fixation in soybean by approximately 30 % as compared to a conventional inoculant consisting only of rhizobia (monoculture inoculant) (Jayasinghearachchi and Seneviratne 2004). Reports have indicated that these symbiotic bacteria may have the potential to be used as PGPR with nonlegumes. Seneviratne et al. (2009) observed the heavy colonization of FBBs/FRBs on root hairs of rice (*Oryza sativa*), tea (*Camellia sinensis*), anthurium (*Anthurium andraeanum*), and wheat (*Triticum aestivum*). Such FRBs may act as "pseudonodules" by fixing N_2 biologically on the roots of nonlegumes.

Concluding Remarks

Microorganisms are potential tools for sustainable agriculture and are the trend for the future. The BNF process offers an economically attractive and ecologically sound means of reducing external nitrogen input and improving the quality and quantity of internal resources. 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. However, field experiments are needed to provide a better understanding of the biological efficacy for increased yields in crop systems. The availability of complete genome sequences and functional genomics of symbiotic microorganisms (bacteria and mycorrhizal fungi) will enhance the understanding of symbiosis in the plant family, and obtaining this knowledge is a major challenge for future research. Increased use of BNF is one of the major pathways to maintain or increase yield and to reduce the environmental footprint of agriculture, which may be used to address the current challenge of meeting the fast-growing worldwide demand for agricultural products.

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Chapter 11
Alleviation of Salt Stress in Legumes by Co-inoculation with *Pseudomonas* and *Rhizobium*

Dilfuza Egamberdieva, Dilfuza Jabborova, and Stephan Wirth

Contents

Abstract Numerous studies have shown that soil salinity decreases nodulation and dramatically reduces N_2 fixation and nitrogenase activity of nodulated legumes. Thus, the development of salt-tolerant symbioses is an absolute necessity to enable cultivation of leguminous crops in salt-affected soils. Dual inoculation of legumes with plant growth-promoting rhizobacteria (PGPR) and rhizobia has been reported to increase the number of nodules compared to those formed by a rhizobial strain alone. The production of IAA by *Pseudomonas* strains represents a beneficial mechanism that promoted enlargement of root system and thereby further enhanced nutrient uptake, nodulation, and shoot growth of leguminous plants. When PGPR are able to alleviate salt stress experienced by the plant, more nodules might develop into nitrogen-fixing ones, thereby enabling the plant to obtain part of its nitrogen from the atmosphere. Co-inoculation techniques could be a new approach to increase

D. Egamberdieva (✉) • D. Jabborova
Faculty of Biology and Soil Sciences, National University of Uzbekistan, Tashkent, Uzbekistan
e-mail: egamberdieva@yahoo.com

S. Wirth
Leibniz-Centre for Agricultural Landscape Research (ZALF), Institute for Landscape Biogeochemistry, Müncheberg, Germany

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_11, © Springer India 2013

the salt tolerance and yield of legumes used for the food and green manure production in salt-affected soils, providing supply of biologically fixed N at low cost.

Introduction

Salinity is a major concern for irrigated agriculture in arid and semiarid regions of the world (Vincent et al. 2006). In particular, secondary salinity developed from irrigation is widely responsible for reducing soil and water quality, limiting crop growth, and leading to the abandonment of agricultural land (Egamberdiyeva et al. 2007). Salt affects plant growth mainly through toxicity caused by the excessive uptake of salts, especially that of NaCl (FAO 2005). Soil salinity reduces plant growth and photosynthesis due to the complex negative effects of osmotic, ionic, and nutritional interactions (Shannon 1997; Shirokova et al. 2000). Salinity stress increases levels of ethylene that significantly inhibits shoot and root elongation and reduces plant height and overall growth (Ma et al. 1998; Klassen and Bugbee 2002).

Most legumes are rather sensitive to salinity, and only a few agronomical legumes can grow in salt-affected soils (Ashraf and McNeilly 2004). For example, two annual pasture legumes, messina (*Melilotus siculus*) and burr medic (*Medicago polymorpha*), can persist in soils with an electrical conductivity (ECe) up to 36 dS/m (Rogers et al. 2005). Soil salinity particularly disturbs the symbiotic interaction between legumes and rhizobia (Marcar et al. 1991). Numerous studies have shown that soil salinity decreases rhizobial colonization and nodulation and dramatically reduces N_2 fixation and nitrogenase activity of nodulated legumes (Singleton and Bohlool 1984; Zahran and Sprent 1986; Elsheikh and Wood 1995; Zahran 1999). Thus, the development of salt-tolerant symbioses is an absolute necessity to enable cultivation of leguminous crops in salt-affected soils (Velagaleti and Marsh 1989; Mayak et al. 2004). There is now increasing evidence that the use of beneficial microbes can enhance the resistance of plants to adverse environmental stresses, e.g., drought, salts, nutrient deficiency, and heavy metal contaminations (Glick et al. 2007).

In this chapter we describe (1) the effect of salinity on legume-*Rhizobium* symbioses, (2) the *Rhizobium-Pseudomonas* interactions, (3) their ameliorative and beneficial effects, and (4) the mechanisms involved in plant growth stimulation and alleviation of salt stress.

Effects of Salinity on Legume-*Rhizobium* Symbioses

Many studies reported the negative effects of soil salinity on crop yield and total nitrogen fixation of leguminous plants such as bean, chickpea, lentil, and soybean (van Hoorn et al. 2001). Similar results were observed by Mensah and Ihenyen (2009) on mung bean (*Vigna mungo* L. Hepper), where they observed decreases in percentage germination and seedling emergence with increases in salinity. The existence of inter- and intraspecific variability in the sensitivity of N_2 fixation to

salinity has also been reported in legumes (Serraj et al. 2001). Subbarao et al. (1990) observed that nodule initiation by *Rhizobium* was the most salt-susceptible aspect of pigeon pea than growth. Rhizobial species *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Mesorhizobium* lead to symbiotic interactions with legumes and result in root nodule formation. However, root nodulation in legumes is dependent on numerous soil and environmental factors, and very often the introduced *Rhizobium* has to overcome intense competition from native microorganisms that colonize the rhizosphere (Mishra et al. 2009). Salinity leads to a failure in the establishment of rhizobia in the rhizosphere, by reducing survival and proliferation of rhizobia in the soil and rhizosphere, or by inhibiting very early symbiotic events, such as root hair colonization (Singleton and Bohlool 1984; Hashem et al. 1998). Cordovilla et al. (1999) reported that *R. leguminosarum* formed an infective symbiosis with faba bean under saline conditions, and that N_2 fixation was more sensitive to salinity than plant growth. The reduction of N_2-fixing activity is usually attributed to a reduction in respiration of the nodules and leghemoglobin production (Delgado et al. 1994; Walsh 1995). An explanation for the reduction in symbiotic legume growth might be that the salt stress causes a failure of the infection and nodulation process. For example, according to Bouhmouch et al. (2005), salt inhibits the absorption of Ca, which reduces the growth of roots, root tips, and root hairs, thereby decreasing sites for potential rhizobial infection and further nodule development. Cordovilla et al. (1995) observed that the depressive effect of salt stress on N_2 fixation by legumes is directly related to the salt-induced decline in dry weight, N content in the shoot, and the salt-induced distortions in nodule structure (Zahran and Abu-Gharbia 1995).

According to Rekha et al. (2007), colonization of the inoculated bacteria in the rhizosphere largely depends on the availability of the empty niche and the capacity of competing with other microflora. The colonization of leguminous root hairs by rhizobial cells is fundamental for the establishment of the legume-*Rhizobium* symbiosis (Gulash et al. 1984). The very early symbiotic events, colonization and infection of root hairs by rhizobial cells, are especially sensitive to environmental stresses (Räsänen et al. 2003). A decrease in the number of rhizobial cells was demonstrated to occur in the root of soybean, common bean, and chickpea (*Cicer arietinum*) grown under salt stress (Zahran and Sprent 1986; Bouhmouch et al. 2005). Since the symbiotic performance of legumes depends upon the population size and survival of introduced rhizobia in the root, the improvement of their colonization in saline conditions is important to develop salt-tolerant symbioses (Velagaleti and Marsh 1989).

Plant Growth-Promoting Rhizobacteria

Beneficial rhizosphere bacteria are of two general types: those forming a symbiotic relationship with the plant and those that are free living in soil and root (Barriuso et al. 2005; Lugtenberg and Kamilova 2009). The use of plant growth-promoting rhizobacteria (PGPR) in improvement of crop yield started long time ago, and there are many reports where beneficial microbes can enhance plant growth, development,

nutrient uptake, and yield (Lugtenberg et al. 2001; Arora et al. 2008; Egamberdieva et al. 2010). Treatments with PGPR like *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Enterobacter*, *Pseudomonas*, *Burkholderia*, *Bacillus*, and *Serratia* increase germination percentage, emergence, root and shoot growth, total biomass of the plants, seed weight, grains, and yields (Mantelin and Touraine 2004; Joseph et al. 2007; Yasmin et al. 2007). Further studies also confirmed enhanced growth, nodulation, and yield of chickpea by *Rhizobium* (Carter et al. 1994; Elsheikh and Elzidany 1997; Akhtar and Siddiqui 2009; Khosravi et al. 2010).

The plant growth promotion activity of rhizobacteria is primarily related to its impact on root growth and morphology (Dobbelaere et al. 2001). Creus et al. (2004) reported that bacterial inoculation caused the production of lengthy root hairs, stimulated the production of lateral roots, and improved the root diameter and surface respectively. The ability of other PGPR species to improve growth, nodulation, and nitrogen fixation is documented for many legume species (Burdman et al. 2000; Tanimoto 2005; Egamberdieva et al. 2010).

Rhizobium-Pseudomonas Interactions

In the rhizosphere, a synergism between various bacterial genera such as *Bacillus*, *Pseudomonas*, *Arthrobacter*, and *Rhizobium* has been shown to promote plant growth of various plants such as peanut, corn, soybean, and maize (Dey et al. 2004; Ratti et al. 2001). Available reports indicate improved yield of legumes health, and nodulation when co-inoculated with PGPB, compared to inoculation with *Rhizobium* alone (Valverde et al. 2005; Egamberdieva et al. 2010; Yadegari and Rahmani 2010). In other studies the co-inoculation with *Pseudomonas* spp. and *Rhizobium* spp. enhanced nodulation and nitrogen fixation, plant biomass, and grain yield in various leguminous species including alfalfa (Bolton et al. 1990), soybean (Dashti et al. 1998), chickpea (Goel et al. 2002), and pea (Tilak et al. 2006).

There are several reports on the positive effects of co-inoculation of legumes with *Pseudomonas* and *Rhizobium* spp. A significant increase in N content of root and shoot of *Galega orientalis* was also observed after co-inoculation of *Pseudomonas trivialis* strain 3Re27 with *Rhizobium galegae* HAMBI 540 which significantly increased the N content of the roots by 20 % and of the shoots by 52 % compared to *R. galegae* HAMBI 540 alone. Shoot and root growth was also increased by co-inoculation of both strains (Egamberdieva et al. 2010). Improved mineral nutrition would explain the promotion of root and shoot growth (Burdman et al. 1997; Cakmakci et al. 2005). Similar results were observed by Khurana and Sharma (2000) and Siddiqui et al. (2001) where combined inoculation of *Rhizobium* and *Pseudomonas* increased nodulation, nitrogenase activity, growth, and yield of chickpea under greenhouse conditions. In other studies a greater number of nodules and dry weight was recorded in soybean and alfalfa when the co-inoculation with *B. japonicum* and *Pseudomonas* was observed by Rosas et al. (2006).

Alleviation of Salt Stress in Plants

The ameliorative effects of PGPR on plant growth under saline conditions have been shown for various plant species, such as tomato, pepper, canola, bean, and lettuce (Barassi et al. 2009; Kang et al. 2009; Egamberdieva 2009). Salt-stressed soybean plants had significantly decreased plant growth, photosynthesis, and mineral uptake with increasing salinity, and inoculation of salt-stressed plants with PGPR strains could alleviate salinity stress (Han and Lee 2005). These PGPR (e.g., *Rhizobium*, *Azospirillum*, *Pseudomonas*, *Flavobacterium*, *Arthrobacter*, and *Bacillus*) utilize osmoregulation, oligotrophic, endogenous metabolism, resistance to starvation, and efficient metabolic processes to adapt under dry and saline environments (Lugtenberg et al. 2001; Egamberdiyeva and Islam 2008). These bacteria, with a physiological adaptation and genetic potential for increased tolerance to drought, increased salt concentration, and high temperatures, could improve plant production in degraded sites. The inoculation of bean with bacterial strains *P. extremorientalis* TSAU20 and *P. chlororaphis* TSAU13 increased shoot length of bean significantly at 5.0, 7.5, and 10.0 dS/m up to 50 % (Egamberdieva 2011). The *Pseudomonas* strains *P. trivialis* 3Re27 and *P. extremorientalis* TSAU20 have an excellent root-colonizing capability and plant growth-promoting activity. They are also salt tolerant, capable of growing in 4 % NaCl, and able to alleviate salt stress in pea and soybean plants (Egamberdiyeva and Hoflich 2002; Egamberdiyeva et al. 2004; Egamberdieva et al. 2010). Both a gnotobiotic sand system test and the greenhouse experiment with low-fertilized potting soil demonstrated that the salt tolerance of *Galega officinalis* clearly improved when the plant was inoculated besides its own specific symbiont *R. galegae* sv. *officinalis*, with either of the two PGPR strains, *P. extremorientalis* TSAU20 or *P. trivialis* 3Re27 (Fig. 11.1) (Egamberdieva et al. 2013). In earlier studies Hasnain and Sabri (1996) showed that inoculation of wheat with *Pseudomonas* sp. stimulated plant growth by reducing plant uptake of toxic ions and increasing the auxin content. Heidari et al. (2011) also reported that plant growth, auxin and protein contents of *Ociumum basilicm* inoculated by

Fig. 11.1 The effect of *R. galegae* R1141 combined with *Pseudomonas* strain TSAU20 on nodulation of *Galega officinalis* (pot experiments, 0 and 50 mM NaCl)

Table 11.1 The length of roots and shoots, biomass of whole plants, and the number of nodules of soybean when seedlings were inoculated with *Bradyrhizobium japonicum* strains USDA110 alone and together with *Pseudomonas putida* TSAU1

Bacterial strains	Root[a] length (cm)	Shoot[a] length (cm)	Biomass (g)[b] weight	Nodule numbers
0 mM NaCl				
USDA110	11.7	20.6	0.086	6.3
USDA110+TSAU1	13.4*	23.4	0.100	8.0
50 mM NaCl				
USDA110	10.2	10.6	0.067	4.2
USDA110+TSAU1	12.4*	16.0*	0.088*	4.6
75 mM NaCl				
USDA110	9.0	8.2	0.053	3.0
USDA110+TSAU1	10.2	12.2*	0.084*	4.0

Plants were grown in the gnotobiotic sand system under salt stress for 3 weeks. Values represent means for six plants ($N=6$)

[a]cm

[b]g/plant

*Significantly different from plants inoculated with *B. japonicum* alone at $P<0.05$

Pseudomonas sp. under drought stress conditions increased compared to the control. The combined inoculation of *Azotobacter*, *Azospirillum*, *Pseudomonas*, and *Mesorhizobium* resulted in promotion of grain yield and biomass in chickpea (Rokhzadi et al. 2008). Parmar and Dadarwall (1999) also observed that co-inoculation of *Pseudomonas* and *Bacillus* sp. with *Rhizobium* strains enhanced the nodule weight, root length, shoot biomass, and total plant nitrogen in chickpea, when grown in sterilized jars or under pot culture conditions. We have observed that the co-inoculation of salt-stressed soybean with *B. japonicum* USDA110 and *P. putida* TSAU1 improved root and shoot length, dry weight, and nodulation compared to those plants inoculated with *B. japonicum* alone (Table 11.1).

Increasing the salt content decreased the ability of *B. japonicum* cells to colonize soybean roots, colony-forming units (CFU) counts decreased from $\log_{10}$ 3.9 CFU to $\log_{10}$ 3.5 CFU (Table 11.2). However, the co-inoculation of *B. japonicum* USDA110 with *P. putida* TSAU1 increased the number of rhizobial cells colonizing soybean roots. Competitive root tip colonization test showed that the *Pseudomonas* strain was a better colonizer than *B. japonicum* (Table 11.2). In other study we demonstrated that the colonization of *G. officinalis* root tips by *Rhizobium* cells increased almost twofold under saline conditions when the plants were inoculated besides *Rhizobium* with *Pseudomonas* strains (Egamberdieva et al. 2013). Such combined inoculation could also enhance formation of nodules on legumes grown in salinated potting soil. In addition, we observed that though salt stress decreased the proportion of big nitrogen-fixing nodules, enhanced nodulation achieved by dual inoculation compensated this decrease and the number of big nodules was duplicated compared to the plants inoculated with *Rhizobium* alone (Egamberdieva et al. 2013).

Table 11.2 The competitive root tip colonization of *B. japonicum* strain USDA110 and *Pseudomonas putida* TSAU1 in the rhizosphere of soybean

	Root colonization	
	Log CFU/1 cm root ± SD	
Bacterial strains	USDA110	TSAU1
0 mM NaCl		
USDA110	3.9 ± 0.06	
USDA110 + TSAU1	4.1 ± 0.08	4.2 ± 0.10
50 mM NaCl		
USDA110	3.7 ± 0.15	
USDA110 + TSAU1	4.0 ± 0.05	4.1 ± 0.10
75 mM NaCl		
USDA110	3.5 ± 0.19	
USDA110 + TSAU1	3.8 ± 0.20	3.9 ± 0.10

Plants were grown in the gnotobiotic sand system under salt stress for 3 weeks

Biomechanisms to Enhance Plant Growth

Mechanisms by which bacteria are able to promote plant growth and prevent damage caused by salinity include production of phytohormones like indoleacetic acid (IAA), gibberellic acid, cytokinins, and ethylene (Spaepen et al. 2009; Mishra et al. 2010), production of ACC-deaminase to reduce the level of ethylene in the roots of developing plants (Dey et al. 2004), asymbiotic nitrogen fixation (Ardakani et al. 2010), and production of exopolysaccharides (EPS) (Upadhyay et al. 2011).

Production of the auxin phytohormone indole-3-acetic acid (IAA) by bacterial inoculants might be responsible for the enlarged root system and number of infection sites prior to nodulation (Tanimoto 2005; Tilak et al. 2006). Rhizobacteria synthesize and release auxin as secondary metabolites because of the rich supplies of substrates exuded from the roots (Lugtenberg et al. 2001; Shahab et al. 2009; Egamberdieva and Kucharova 2009). Bacterial strains which belong to genera such as *Pseudomonas*, *Bacillus*, *Rhizobium*, and *Microbacterium* are among the most active IAA producers (Wang et al. 1982; Costacurta and Vanderleyden 1995; Mehnaz and Lazarovits 2006; Tsavkelova et al. 2007). The IAA that is secreted by bacteria, together with endogenous plant IAA, is taken by plant cells which can stimulate plant cell proliferation (Glick et al. 2007). The exogenous application of auxins to alfalfa (Gruodien and Zvironaite 1971) and groundnut (Srinivasan and Gopal 1977) promoted plant growth and nodulation. Earlier reports showed that *Rhizobium meliloti* associated with alfalfa produced 20 mg/ml of IAA (Williams and Singer 1990), whereas *Rhizobium leguminosarum* produced 2.0 mg/ml of IAA (Beltra et al. 1980). IAA produced by nodule bacteria is transported to other parts of the plant and might be involved in several stages of the symbiotic relationship (Wheeler et al. 1979; Hunter 1989).

In early studies, the depressive effect of salinity on plant growth was explained by decline in endogenous levels of hormones in the rhizosphere (Zholkevich and Pustovoytova 1993; Jackson 1997), whereas phytohormones released by rhizobacteria effect positively to seedling development (Frankenberger and Arshad 1995;

Afzal et al. 2005). Low concentration of pure IAA or low titer of IAA-producing bacteria enhanced root growth and nodulation (Remans et al. 2008), whereas high concentration of pure IAA or high titer of IAA-producing bacteria inhibited root growth and nodulation (Plazinski and Rolfe 1985). Bacterial IAA can also act as signal molecule in bacteria-bacteria communication (Spaepen et al. 2009). Another explanation for enhancement of nodule formation by the rhizobia in legumes might be the production of hydrolytic enzymes such as cellulases by root-colonizing *Pseudomonas* strains, which could make penetration of rhizobia into root hairs or intercellular spaces of root cells easier, leading to increased numbers of nodules (Sindhu and Dadarwal 2001).

Plant stress can be reduced by 1-aminocyclopropane-carboxylate (ACC) deaminase-producing bacteria (Glick et al. 1997). The ACC-deaminase enzyme can cleave the ethylene precursor ACC to α-ketobutyrate and ammonium and thereby lower the level of ethylene in developing or stressed plants (Glick 1995; Glick et al. 1998; Hontzeas et al. 2005). PGPR releasing the enzyme ACC-deaminase may decrease the ethylene level and enhance the survival of seedlings (Glick et al. 1998). It has been reported that PGPR strain *P. trivialis* 3Re27 was able to utilize ACC as N source indicating the presence of ACC-deaminase and increased salt tolerance of goats' rue, stimulating shoot and root growth under salinated soil conditions (Egamberdieva et al. 2013). Similar results were observed by Shaharoona et al. (2006) where co-inoculation of *Bradyrhizobium* with PGPR isolates strains possessing ACC-deaminase activity enhanced the nodulation in mung bean compared with inoculation with *Bradyrhizobium* alone. Arshad et al. (2008) observed that inoculation with PGPR containing ACC-deaminase was highly effective in removing the effects of water stress on growth, yield, and ripening of peas.

Conclusion

As discussed in this review, salinity decreases nodulation, reduces N_2 fixation and nitrogenase activity of legumes, and leads to a failure in the establishment of rhizobia in the rhizosphere by inhibiting very early symbiotic events. The co-inoculation of legumes with *Rhizobium* and PGPR *Pseudomonas* strains was able to alleviate salt stress of plants grown in salt-affected soils. The phytohormone auxin produced by root-colonizing bacteria plays an important role in alleviating salt stress in plants. Co-inoculation techniques could be a new approach to increase the salt tolerance and the yield of leguminous plants used for food and green manure production in salt-affected soils, providing supply of biologically fixed N at low cost. The future direction in research needs to include (1) the mechanisms involved in alleviation of salt stress in plants, (2) the potential competition between PGPR strains and indigenous soil microflora in the rhizosphere of plants grown under stressed environments, and (3) more research on the interaction between PGPR and rhizobia, as the latter are known to confer resistance to salt stress and drought while promoting growth of the host plant.

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Chapter 12
Potential of Rhizosphere Bacteria for Improving *Rhizobium*-Legume Symbiosis

Ijaz Mehboob, Muhammad Naveed, Zahir A. Zahir, and Angela Sessitsch

Contents

I. Mehboob • Z.A. Zahir (✉)
Institute of Soil and Environmental Sciences,
University of Agriculture, Faisalabad 38040, Pakistan
e-mail: zazahir@yahoo.com

M. Naveed
Institute of Soil and Environmental Sciences,
University of Agriculture, Faisalabad 38040, Pakistan

Bioresources Unit, AIT Austrian Institute of Technology GmbH,
Konrad-Lorenz-Strasse 24, 3430 Tulln, Austria

A. Sessitsch
Bioresources Unit, AIT Austrian Institute of Technology GmbH,
Konrad-Lorenz-Strasse 24, 3430 Tulln, Austria

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_12, © Springer India 2013

Abstract About 60 % of the earth's available nitrogen is fixed via biological nitrogen fixation (BNF). Being a major contributor to BNF, *Rhizobium*-legume symbiosis can provide well over half of the biological source of fixed nitrogen. Actually, *Rhizobium*-legume symbiosis results in the formation of nodules on legume roots where rhizobia fix nitrogen from the atmosphere. But nodulation and nitrogen fixation is a complex process and is dependent on the compatibility and potential of both partners of *Rhizobium*-legume symbiosis under variable soil and environmental conditions. Although, some selected efficient and effective traits of rhizobia and legumes have shown encouraging results, there is a need of consistent positive influence on nodulation and nitrogen fixation to maximize the growth and yield of legumes under variable conditions. Hence, the use of means capable of improving both the legume growth and the growth and function of symbiotic rhizobia is essential. Co-inoculation of *Rhizobium* species with favorably interacting traits of plant growth-promoting rhizobacteria (PGPR) is considered an applied, cost-effective, efficient, and environment-friendly approach to further improve legume growth and productivity under variable conditions because they can provide broad spectrum mechanisms of actions and improve reliability of inocula without genetic engineering. In addition, these PGPR when used in combination with rhizobia have also shown the strategies for dealing with stressful conditions like salinity, pH, temperature, drought, heavy metal, and pathogens which could further impose limitations on the capacity of *Rhizobium*-legume symbiosis. This chapter highlights various PGPR traits compatible with specific legume rhizobia and their phytostimulatory mechanisms contributing to augmentation in rhizobial growth and function for growth and yield enhancement of legumes under variable conditions.

Introduction

It is believed from over 100 years that rhizobia are symbiotic nitrogen-fixing bacteria that can improve the growth of legumes (Mehboob et al. 2009) under nitrogen-limiting field conditions (Freiberg et al. 1997) by forming nodules on the roots and producing ammonia by reducing the atmospheric nitrogen (a process known as biological nitrogen fixation). This clearly means that the amount of nitrogen fixed in this process depends upon the formation of successful nodules on the host roots and is a precondition for the potential increase of nitrogen fixation from the system. Though, the rhizobia usually occur in soils but often do not succeed to cause nodulation, because of

some unspecified kind of antagonism that discourages root colonization by the rhizobial strain (Jadhav et al. 1994). In general, the success of the process of nodulation and nitrogen fixation depends on the successful survival ability, competitiveness, and efficiency of the rhizobial strains involved. Within the soil, various factors like type and variety of host and bacterial strains, soil physical and chemical properties, temperature, light, and interaction with other rhizospheric microorganisms (Redmond et al. 1986; Lerouge et al. 1990; Polonenko et al. 1993; Prevost et al. 2003) also impose limitations on the process of nodulation and nitrogen fixation by affecting rhizobial function and growth, their initial steps of symbiosis, capability to fix nitrogen, and hence the vigor of the host legume. One way to achieve successful symbiosis and nitrogen fixation is to provide optimum conditions to rhizobial inoculants which are not possible under field conditions. So, conventionally, selection of persistent, effective, efficient, and competitive rhizobial strains is used to improve the process of nodulation and nitrogen fixation. But it has been noticed that the maximum potential productivity of legumes is not guaranteed by the use of superior introduced rhizobial strains (McLoughlin et al. 1985; Gupta et al. 1998), and there is a need to search more ways and means to improve growth and functions of rhizobia and their host for improvement in productivity of legumes. Hence, exploitation of beneficial rhizospheric bacteria referred to as plant growth-promoting rhizobacteria (PGPR) in combination with rhizobia has been found to be an interesting alternative and effective approach in recent years to achieve reliable and successful nodule formation and BNF (Janisiewicz 1996; Pankaj et al. 2011). This results in eco-friendly improved growth and productivity of legumes (Requena et al. 1997). The effect of PGPR in combination with rhizobia in improving legume growth and development may possibly be because of their variable characteristics such as increased production of nod-gene products inducing flavonoids by the legume host (Andrade et al. 1998), stimulation of root hair development (Garcia et al. 2004a), secretion of B vitamins by the PGPR enhancing rhizobial growth in the rhizosphere (Marek-Kozaczuk and Skorupska 2001), production of plant growth regulators (Spaepen et al. 2007), improved mineral uptake by mobilization of insoluble nutrients (Ahmad et al. 2008), and suppression of phytopathogenic organisms (Weller 2007). Moreover, investigations are also elaborating that the inhibitory effects of various types of abiotic and biotic stresses can be overcome by the use of appropriate plant growth-promoting rhizobacteria as co-inoculant with host respective rhizobia (Mishra et al. 2011). Finally, it could be inferred that the potential of plant growth-promoting rhizobacteria for improving *Rhizobium*-legume symbiosis under variable conditions is very promising. However, more studies on the rhizobia-PGPR interactions in the company of specific host legume are needed to identify the limiting factors of the association.

Rhizobium-Legume Symbiosis: An Overview

Symbiosis, a relationship among incongruent individuals of two or more different species, is recognized in such a way that may benefit both species. A plant of the family *Leguminosae* (or *Fabaceae*) whose characteristic fruit is a seed pod is called

legume, and the bacteria that fix atmospheric nitrogen (N_2) after becoming established inside the nodules on legume's root are called rhizobia. Generally, rhizobium-legume symbiosis is a kind of mutualistic relationship in which both the partners benefit. The rhizobia fix nitrogen from the atmosphere for plant uptake, and in turn the plant makes available carbon and energy to rhizobia. In fact, formation of *Rhizobium*-legume symbiosis is a very coordinated effort between the legume and the *Rhizobium* bacteria in the soil for exchange of molecular dialogue between both partners (Parniske and Downie 2003). This starts with the release of flavonoids into the rhizosphere by the leguminous plant (Redmond et al. 1986) that activates specific rhizobial receptor, *nod D*, which highlights the rhizobia from other nitrogen-fixing and endophytic bacteria. The *nod D* is divided into three groups according to their respective functions, i.e., regulatory, common, or host specific. The host-specific genes vary among the species in accordance with their function, copy number, and control mechanisms. Also, *nod D* varies in response to the type and quantity of host flavonoids (Spaink 2000). At least, one functional *nod D* is required in the initiation of nodulation (Schlaman et al. 1992). In response to plant flavonoids, the rhizobial *nod D* produces a protein "lipo-chitooligosaccharide or nodulation factors (nod factors)" which is the sensor that recognizes chemicals excreted by host plant roots (Russelle 2008). The nod factor in turn activates a set of plant genes, initially called nodulins (Geurts and Bisseling 2002), leading to induction of a number of morphological and biochemical changes which trigger the cortical cell division (Oldroyd and Downie 2008) and a nodule meristem is thus formed within the root, and the root hair growth is redirected to curl to the side where the bacteria are attached and deforms forming a "shepherd's crook" which serves to entrap/encapsulate the rhizobia. The encapsulated rhizobia initiate infections, inducing the plant to produce infection thread (a tube that facilitates the entry of rhizobia into deeper layers). The infection thread grows transcellularly through the root hair into the basal part of the epidermis cell and onward into the root cortex and reaches the nodule primordia where the rhizobia are delivered into plant cells converting the free-living *Rhizobium* bacteria to bacteroids. Another nodulation mode is infection through cracks in the root or "crack entry"; it is also present in many agronomically important crop plants. Once inside the nodule, rhizobia are released from the infection thread in a droplet of polysaccharide, or when it enters via crack entry, a plant-derived peribacteroid membrane quickly develops around this droplet via endocytosis. The entire unit of peribacteroid membrane-surrounded bacteroid is referred to as the symbiosome within which the bacteroids fix nitrogen by utilizing the enzyme nitrogenase to catalyze the conversion of atmospheric nitrogen (N_2) to ammonia (NH_3). Unfortunately, high amounts of ATP [16 mol of ATP to reduce each mole of nitrogen (Hubbell and Kidder 2009)] and oxygen reductant are needed to meet the demands of the enzyme, but at the same time, nitrogenase is oxygen sensitive. This is often referred to as the "paradox" of symbiotic nitrogen fixation (Schulze 2004). The job of bacteroids is made easier by contribution from their host plant which provides sugars from photosynthesis to make their ATP, whereas plant leghemoglobin supplies just the right concentration of oxygen to the bacteroids to satisfy their conflicting requirements.

For more than 100 years, this *Rhizobium*-legume symbiosis has been exploited widely by the scientists dealing with the mineral nutrition of plant (Burris 1994) because it is an efficient source of nitrogen (Peoples et al. 1995). Currently, this symbiosis is of great practical importance because it is not only environment friendly but also adds almost half the annual quantity of biological nitrogen fixation in soil ecosystem (Tate 1995). It also saves money because at least 70 million tons of N is added per year via legume symbiosis (Brockwell et al. 1995). As world's population is increasing on one hand and the resources supplying the nitrogen fertilizer are diminishing, hence there is a dire need to increase the efficiency of symbiotic relationship between legumes and rhizobia for ultimate increase in the amount of nitrogen to be fixed by this system. Moreover, the improvement in Rhizobium-legume symbiotic relationship is also essential to ameliorate several adverse environmental conditions which are limiting to the growth and activities of legumes and rhizobia.

Rhizosphere Bacteria and Legume Improvement: Mechanisms of Actions

Bacteria in the immediate vicinity of plant roots are called "rhizosphere bacteria or rhizobacteria." These have an encouraging effect on *Rhizobium*-legume symbiosis (Marek-Kozaczuk et al. 2000). Their competitive and effective traits could modify legume growth and productivity more profoundly if used in combination with rhizobia (Pankaj et al. 2011). A variety of mechanisms have been proposed in various investigations for the observed responses of symbiotic legumes to PGPR co-inoculation (Fig. 12.1) which are presented in the following subsections.

Creation of Additional Infection Sites

Roots are the initiation point for nodule formation. Hence, any stimulus that causes increase in root growth could result in more colonization sites for rhizobia (Fox et al. 2011). It has been recognized that growth and yield of leguminous plants could be enhanced by creating additional infection sites through inoculation of rhizobia in combination with beneficial rhizosphere bacteria (Plazinski and Rolfe 1985; Yahalom et al. 1987, 1990). For example, Tchebotar et al. (1998) observed stimulated formation of additional infection sites that were later occupied by rhizobia in a mixed inoculation with *Azospirillum lipoferum* and rhizobia. Also, Garcia et al. (2004a) observed significant effects on growth of soybean cv. Osumi as a result of co-inoculation of PGPR (i.e., *Pseudomonas fluorescens*, *Chryseobacterium balustinum*, and *Serratia fonticola*) with *Sinorhizobium fredii*. They explained that the availability of adequate sites for *Rhizobium* infection might

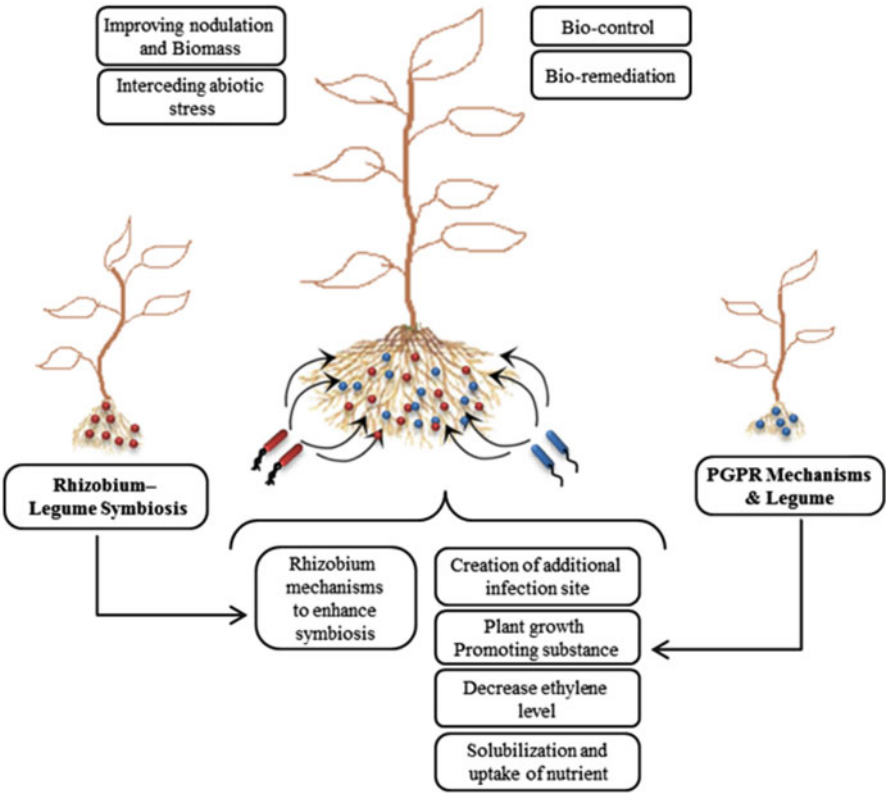

Fig. 12.1 Mechanisms due to simultaneous inoculation of PGPRs on *Rhizobium*-legume symbiosis

be the action mechanism. Likewise, Tilak et al. (2006) showed that PGPR in conjunction with efficient *Rhizobium* can also affect the growth and nitrogen fixation in pigeon pea by enhancing the occupancy of introduced *Rhizobium* in the nodule of the legume. Elkoca et al. (2008) reported significant increases in root dry weight and seed yield of chickpea as a result of dual inoculation of *Rhizobium* with *Bacillus subtilis* OSU-142 and *Bacillus megaterium* M-3 and supported the opinion strongly that increasing plant root growth added to the number of potential colonization sites. Estevez et al. (2009), while using *Rhizobium tropici* CIAT899 and *C. balustinum* Aur9 as co-inoculant of bean, reported that *C. balustinum* Aur9 might have improved rhizobial infection at initial stages by increasing root hair formation and infection sites which in turn might have added to increase the formation of nodule primordia and early nodule development. Similarly, Fox et al. (2011), while reporting enhanced rate of nodule initiation in *Medicago truncatula* cv. *Caliph* as a result of co-inoculation of *P. fluorescens* WSM3457 and *Ensifer* (*Sinorhizobium*) *medicae* WSM419, described enhanced root infection by the rhizobia. Srinivasan et al. (1997) also reported that enhanced early nodule initiation and increased density of root hair in *Phaseolus vulgaris* because of *B. megaterium*

S49 and *Rhizobium etli* co-inoculation was mediated by the increased number of attachment sites. Tsigie et al. (2011) reported that inoculation of soybean cultivar Pusa 22 with PGPR in combination with *B. japonicum* strains SB 271 significantly influenced nodule number and biomass. It was explained that the used rhizobacteria produced IAA which enhanced root hair formation. Finally, it was concluded that as root hairs are infection sites for rhizobia, hence it may be attributed that the observed enhancement in nodulation may be due to increased number of infection sites which may be the mechanism for enhancing the nitrogen fixation ability by soybean. It could be concluded from the discussion that PGPR as co-inoculant could add in the effectiveness of *Rhizobium*-legume symbiosis via enhancing infection sites but attempts are needed for further extensive studies.

Plant Growth-Promoting Substances

Beneficial rhizosphere bacteria can contribute in key processes of production of phytohormones and other plant growth-promoting substances. The use of such bacteria in agriculture can reduce the use of chemical fertilizers and support eco-friendly legume production. It has been investigated that some bacteria that live in the rhizosphere are competent to promote nodule formation and BNF when they are inoculated in combination with rhizobia (de Freitas et al. 1993; Zhang et al. 1996; Dashti et al. 1998) via systemic induction of secondary metabolites such as flavonoids (Andrade et al. 1998; Schultze and Kondorosi 1998), tabtoxinine-beta-lactam (Knight and Langston-Unkefer 1988), and B-group vitamins (Marek-Kozaczuk and Skorupska 2001) and release of phytohormones such as auxins, cytokinin, ethylene, and gibberellins (Garcia et al. 2004b).

Chebotar et al. (2001) reported that the high colonization activity of *P. fluorescens* 2137 and its possible production of a growth-promoting compound enhanced the nitrogen fixation of soybean co-inoculated with *B. japonicum* A1017 and *P. fluorescens* 2137. Zaidi et al. (2004) elaborated that the observed increased nodulation and yield of green gram as a result of *Pseudomonas striata* or *Penicillium variable* in mix inoculation with *Bradyrhizobium* sp. was likely to be due to the release of growth-promoting substances. Improved growth of clover via secretions of several B-group vitamins when co-inoculated with *P. fluorescens* strain 267 and *Rhizobium leguminosarum* bv. *trifolii* strain 24.1 has been described by Marek-Kozaczuk et al. (2000).

Studies have revealed that PGPR can increase nodulation of leguminous plants by improving the level of flavonoids in root exudates when used in combination with *Rhizobium*. For example, increased nodule weight, root length, shoot biomass, and total plant nitrogen in chickpea were observed when co-inoculated with *Pseudomonas* and *Bacillus* sp. with *Rhizobium* strains (Parmer and Dadarwal 1999). Similarly increased nodule number and plant dry weight of green gram was noticed when co-inoculated with *Pseudomonas* strains and *Bradyrhizobium* strain S24 (Malik and Sindhu 2008). Likewise, increased nodulation in *Vicia sativa* subsp. *nigra* (vetch) following co-inoculation with *R. leguminosarum* bv. *viciae* (Rlv) and wild-type strain (Sp7) or mutant strains of *Azospirillum brasilense* (napA$^-$, acdS$^+$,

and ipdC⁻) in pots, in pouches, and in hydroponic system (Star et al. 2012) via increased production of plant root flavonoids has been observed.

Valverde et al. (2006) speculated the production of phytohormones by *Pseudomonas jessenii* PS06 that increased nodule size, weight, N contents in shoot, and seed yield of chickpea when co-inoculated with *Mesorhizobium ciceri* C-2/2. Also, Pan et al. (2002) showed that PGPR when used in co-inoculation with rhizobia often enhanced overall plant physiology and growth through phytohormonal interactions between the rhizobia and plant. Dardanelli et al. (2008) evidenced the beneficial effect of co-inoculation of *A. brasilense* with *R. tropici* strain CIAT899, or *R. etli* ISP42 on *P. vulgaris* cv. Negro jamapa plant at the level of root development and nitrogen fixation due to the production of IAA by *Azospirillum*. Mishra et al. (2009a) indicated the highest and most consistent increase in nodulation, shoot dry weight and root fresh weight, and root volume of soybean by the production of indole-3-acetic acid when *Bradyrhizobium japonicum*-SB1 was co-inoculated with *Bacillus thuringiensis*-KR1. While studying on the effect of co-inoculation of *Pseudomonas* sp. with *Mesorhizobium* sp. *Cicer* strain Ca181 on the growth of chickpea, Malik and Sindhu (2011) suggested that IAA producing *Pseudomonas* strains have the potential usefulness in enhancement of nodulation and stimulation of plant growth. Garcia et al. (2004a) also reported significant effects of three PGPR on growth parameters of *Glycine max* cv. *Osumi* (soybean) when co-inoculated with *S. fredii* as a result of auxin effects produced by the PGPR. Cassan et al. (2009) reported that co-inoculation of *B. japonicum* E109 with *A. brasilense* Az39 promoted seed germination, nodule formation, and early development of soybean seedlings, which could be due to bacterial biosynthesis of IAA, gibberellic acid, and zeatin. Molla et al. (2001a) observed root growth stimulation of soybean via synthesis of indole lactic acid and gibberellic acid by *A. brasilense* when used in combination with *B. japonicum*. Likewise, Wani et al. (2007a) indicated that *Bacillus* spp. strain PSP 9 stimulated growth, nodulation, and yield of chickpea when used as co-inoculant with *Rhizobium* through the production of growth-promoting substances, e.g., auxins and gibberellins. In a study, Egamberdieva et al. (2010) reported that the production of IAA/cellulase by *Pseudomonas* strains might have contributed to increased number of nodules, shoot and root growth, and N content of root and shoot of fodder galega (*Galega orientalis* Lam.) when co-inoculated with *R. galegae* bv. *orientalis* HAMBI 540. It can be concluded that PGPR bump up *Rhizobium*-legume interactions, acting as "rhizobium helper bacteria." Thus, it is inferred that PGPR could further improve growth and symbiotic performance of legumes via production of plant growth-promoting substances if used in combination with rhizobia.

Biological Nitrogen Fixation

Number of nodules has been considered as a good measure in order to determine BNF potential of the inoculant strains. PGPR were found to enhance symbiotic nitrogen fixation potential of the inoculated rhizobial strains by increasing

nodulation in various studies conducted by different researchers (Yahalom et al. 1987; Zhang et al. 1996). Therefore, strains of PGPR which interact synergistically with rhizobia and produce better nodulation and BNF have received much attention in the recent past and are becoming a practical way to improve nitrogen availability in sustainable agricultural production system (Bai et al. 2002a; Abdel-Wahab et al. 2008).

Several PGPR have been reported to enhance ability of the native rhizobia to elicit nodulation (Remans et al. 2007; Castro-Sowinski et al. 2007; Mishra et al. 2009a). Barea et al. (2005) reported that the cooperative interactions between rhizobia and other plant root-colonizing bacteria are responsible for improvement of nodulation and nitrogen fixation in legume plants. Alagawadi and Gaur (1988) indicated that co-inoculation of *Bradyrhizobium* and certain PGPR can positively affect symbiotic nitrogen fixation by enhancing both root nodule number or mass and increasing the nitrogenase activity. Similarly, increase in nitrogen fixation and grain yield as a result of co-inoculation with PGPR and *Rhizobium*/*Bradyrhizobium* spp. have been shown in various legumes (Valverde et al. 2006; Yadegari et al. 2008; Elkoca et al. 2008). Similarly, Verma et al. (2012) demonstrated enhanced fixation of atmospheric nitrogen because of significant increase in nodule number and dry matter of chickpea as a result of co-inoculation of *P. fluorescens* BHUPSB06 and *Mesorhizobium* sp. BHURC02. Bai et al. (2002b) reported that the inoculation of soybean with PGPR at optimal dose (1×10^8 cells per seedling) and rhizobia increased nodule number, plant dry weight, and nitrogen fixation efficiency. Similarly, Wasule et al. (2003) have shown an increase in dry weight of soybean nodules when co-inoculated with *B. japonicum* and phosphate-solubilizing *P. striata*. Increase in nodule fresh weights and nitrogen fixation of soybean as a result of co-inoculation with PGPR/endophytic bacteria and rhizobia grown under greenhouse conditions has been reported (Atieno et al. 2012; Soe et al. 2012).

Likewise, Remans et al. (2008) reported that co-inoculation of common bean genotype DOR364 with *Azospirillum-Rhizobium* increased the amount of fixed nitrogen and the percentage of total N obtained by nitrogen fixation across different environments compared with *Rhizobium* treatment alone. Also, Burdman et al. (1997) reported significant increase in total nodule number and nitrogen fixation due to combined inoculation of common bean plants with *Rhizobium* and *Azospirillum* as compared to inoculation with *Rhizobium* alone. Figueiredo et al. (2008) reported that the *Paenibacillus polymyxa* DSM 36 when used in combination with *R. tropici* CIAT899 stimulated number of nodules of common bean which was translated into higher level of accumulated nitrogen. Rajendran et al. (2008) and Roseline et al. (2008) indicated that *Bacillus* sp. and *Azospirillum* sp. could enhance nodulation and nitrogen fixation if added together with the rhizobial inoculants in pigeon pea. Marek-Kozaczuk et al. (1996) and Derylo and Skorupska (1993) reported that *P. fluorescens* strain 267 improved symbiotic nitrogen fixation and growth of clover infected with *R. leguminosarum* bv. *trifolii* strain 24.1 under gnotobiotic conditions, while Chanway et al. (1989) reported that plant growth-promoting *Pseudomonas* strains in combination with rhizobial strains enhanced the nitrogen fixation and growth of western Canadian lentils and pea cultivars in field and laboratory

conditions. On the whole, the above discussion highlighted that PGPR in combination with rhizobia could act as effective stimulator of growth and yield of legumes by improving rhizobial fixation of atmosphere nitrogen.

Decreasing Ethylene Level (ACC Deaminase)

Ethylene is a plant hormone that is involved in the regulation of many physiological responses (Reid 1995). Many plant species require ethylene for seed germination. Usually, its rate of production rises during germination and seedling growth (Abeles et al. 1992). Generally, ethylene shows enhancement in root initiation and growth at low level, but higher levels can lead to suppression in root elongation (Shaharoona et al. 2006). Arshad and Frankenberger (2002) revealed that any factor/stimulus which changes the endogenous level of ethylene in a plant could result in modified growth and development of plant. The synthesis of ethylene in plants is directly related to the concentration of ACC (1-aminocyclopropane-1-carboxylic acid) (Machackova et al. 1997) which is the immediate precursor of ethylene, derived from methionine in plants (Yang and Hoffman 1984).

ACC deaminase is an enzyme present in certain microorganisms that can hydrolyze ACC into ammonia and α-ketobutyrate (Shaharoona et al. 2006). Hence, ACC-deaminase-containing rhizobacteria can decrease the amount of ACC, as well as ethylene, outside the germinating seeds which eliminates the potential inhibitory effect of higher ethylene concentrations (Glick et al. 1998). Therefore, it is highly likely that presence of PGPR containing ACC deaminase on the roots of legumes could repress endogenous production of ethylene at rhizobial infection stage and therefore could help nodulation and increase growth and yield. Moreover, Holguin and Glick (2001) demonstrated that the release of ACC deaminase by various PGPR in the rhizosphere could increase root elongation and plant growth by reducing ethylene synthesis.

Iqbal et al. (2012) reported improved nodule number, nodule dry weight, fresh biomass, grain yield, straw yield, and N content in grains of lentil as a result of lowering of the ethylene production via inoculation with PGPR strains of *Pseudomonas* spp. containing ACC deaminase along with *R. leguminosarum*. Similarly, Babar et al. (2007) also described that co-inoculation of chickpea with ACC deaminase containing strains of *Enterobacter* and *Rhizobium* improved the nodulation possibly by adjusting ethylene level in legumes. Shaharoona et al. (2006) reported that among the co-inoculants, rhizobacteria containing ACC deaminase hydrolyzed endogenous ACC into ammonia and α-ketobutyrate which suppressed the accelerated endogenous ethylene synthesis and hence eliminated the potential inhibitory effects of higher ethylene concentrations. Resultantly, the nodulation, root, and shoot growth of mung bean was promoted. Zahir et al. (2011) also suggested that *P. jessenii* containing ACC deaminase when used in combination with *Rhizobium* could exploit adjustment of ethylene levels as effective strategy for improving nodulation, growth, and yield of lentil. Similarly, Ahmad et al. (2011), while reporting increased effect of combined inoculation of PGPR strains containing ACC deaminase and *Rhizobium phaseoli* on

various growth and yield parameters of mung bean, explained that the observed increases in root and shoot could be due to lowering of ethylene levels by the used PGPR. Concisely, PGPR containing ACC deaminase could be used as successful co-inoculant because of having an effective strategy for improving growth and yield of legumes via adjusting ethylene level in legumes.

Solubilization and Uptake of Nutrient

Conventionally, modification in plant growth could be attained by direct application of nutrients or beneficial bacteria to seed (Linderman 1994). But as PGPR can effectively colonize plant roots and promote plant growth through increased mobilization of insoluble nutrients and subsequent enhanced plant uptake (Richardson et al. 2009; Adeseoye et al. 2010), this is a promising approach to minimize the use of chemical fertilizers and could sustain eco-friendly crop production (Requena et al. 1997). Therefore, application of dual and multiple mixtures of microbes has acquired an increasing interest in recent years (Bashan and de-Bashan 2005). It has been found that mixed inoculants give superior nutritional balance and improve uptake of N, P, K, and microelements by plants (Dobbelaere and Okon 2007) and may result in higher general plant health (Sindhu et al. 2002; Rosas et al. 2006). However, to ensure positive effect on symbiotic performance and plant growth, the use of competitive and effective PGPR and host specific rhizobia is a prerequisite (Egamberdieva et al. 2010). Usually, PGPR enhance the capacity of plants to obtain nutrients from the soil by either increasing the availability of nutrient by solubilizing insoluble nutrients or improving the root system (Bucio et al. 2007).

A number of studies have also indicated that dual inoculation with *Azotobacter* spp. or *Azospirillum* spp. and *Rhizobium* strains showed a synergistic effect on N uptake, nodulation, plant growth, and yield of soybean, clover, common bean, and peanut (Burns et al. 1981; Raverker and Konde 1988; Burdman et al. 1997). Rodelas et al. (1999) indicated that inoculation of *Rhizobium* in combination with some other plant growth-promoting rhizobacteria could manipulate nodulation and uptake of nutrient other than nitrogen. Gull et al. (2004) reported better nodulation, nitrogen fixation, and seed yield of chickpea co-inoculated with *Rhizobium* + phosphate-solubilizing bacteria (PSB) and attributed enhanced nutrient acquisition, especially phosphorus as a mechanism by which PSB bacteria integrate. Wani et al. (2007b) explained that the positive effect on the growth of chickpea plants inoculation with phosphate-solubilizing strain of *Bacillus* sp. and nitrogen-fixing *Mesorhizobium* sp. probably related to the increase in plant P uptake. Similarly, combined inoculation with *Rhizobium* and PSB has been reported to enhance growth and yield of chickpea plant by providing a more balanced nutrition to plants (Rudresh et al. 2005). Radwan et al. (2005) were also in agreement that PGPR that mobilize insoluble nutrients and thus enhance their uptake by the plants stimulated plant growth when *R. leguminosarum* and *P. aeruginosa* and *S. liquefaciens* were used as co-inoculants with *Vicia faba* plants grown in clean and sandy soil. Anandham et al. (2007) reported

significant increases in nodule number, nodule dry weight, and plant biomass of groundnut cv. ALR-2 as a result of co-inoculation of sulfur-oxidizing *Thiobacillus* sp. strain LCH and *Rhizobium* sp. strain TNAU14 having neither S nor thiosulfate oxidation property. The observed increases were attributed to the cumulative effects, such as increased supply of S and N as well as P and other insoluble nutrients in rhizosphere. Similarly, Elkoca et al. (2010) observed significant improvement in yields of common bean as a result of co-inoculation of *R. leguminosarum* with P-solubilizing *B. subtilis* or *B. megaterium*. They stated that *B. subtilis* was of particular importance having the potential to improve crop yields by providing a more balanced nutrition to plants as compared to sole inoculations. Mishra et al. (2012) reported increase in N and P uptake along with increase in nodulation, leghemoglobin content, total iron, and total chlorophyll content of lentil plant co-inoculated with *Pseudomonas* spp. and *R. leguminosarum*-PR1. They suggested a strong synergistic relationship between *Pseudomonas* sp. strain NARs1 and *R. leguminosarum-PR1*. The authors further described the nutrient uptake by the bacterized plantlets mainly to auxin production by the bacterium which stimulated root growth, thereby facilitating an increased uptake of nutrients from the soil. Zaidi et al. (2003) recorded improved N and P contents of grain and straw of chickpea grown in a sandy clay loam soil deficient in available phosphorus as a result of dual inoculation with *Rhizobium* sp. + *P. striata*, *Rhizobium* sp. + *P. variable*, and *Rhizobium* sp. + *Glomus fasciculatum* as compared to single inoculation. In another experiment, Zaidi et al. (2004) suggested that nodulation and nutrient uptake could be enhanced by the use of favorably interacting rhizospheric microorganisms in combination with rhizobia in order to get better yield of green gram. It has also been reported by Belimov et al. (1995) that combined inoculations with N_2-fixing and P-solubilizing bacteria were more effective than single inoculation possibly by providing more balanced nutrition for plants. Rudresh et al. (2005) have also prompted the use of combined application of *Rhizobium*, PSB, and *Trichoderma* spp. for improving the nutrient uptake, nitrogen fixation, growth, and yield of chickpea. Dual inoculation of *Azotobacter vinelandii* or *A. lipoferum* with *Rhizobium* strains showed a synergistic effect on N uptake, nodulation, and yield of soybean, clover, and peanut (Burns et al. 1981; Raverker and Konde 1988). Zaidi et al. (2004) and Sindhu et al. (1999) noted synergistic effect of dual inoculation of *Pseudomonas* spp. and *Bradyrhizobium* strain S24 on N uptake, nodulation, and plant dry weight of green gram. It can be concluded that PGPR enhance the capability of legumes to get nutrients from soil by either increasing the availability of nutrient or improving the root system when used in combination with rhizobia.

Production of Siderophores

Siderophores are iron-chelating metabolites that are able to correct the iron accessibility in the rhizosphere (Loper and Henkels 1999). Under iron stress conditions, the siderophore-producing rhizospheric microorganisms can provide iron to plants and improve their growth. It has been recognized that plants have receptors or channels

through which they can receive microbial siderophore and ferric reductase for unloading of iron from siderophores and to convert it into ferrous form (Budezikiewicz 1997; Crowley et al. 1998; Duffy and Defago 1999; Masalha et al. 2000).

Fuhrmann and Wollum (1989) reported enhanced nodulation by co-inoculation of a siderophore-producing *Pseudomonas* and *B. japonicum* strains USDA 110. Parmar and Dadarwal (1999), while studying stimulation of nitrogen fixation and induction of flavonoid compounds by rhizobacteria, showed a relationship between siderophore production and the level of flavonoid-like compounds in the root, with an increase in total plant nitrogen in chickpea. Likewise, Wani et al. (2007a) reported that the grain yield and uptake of nitrogen and phosphorus were significantly increased as a result of co-inoculation with *Mesorhizobium* and P-solubilizing *Pseudomonas* and *Bacillus* spp. They explained that the bacterial cultures produced considerable amount of siderophore which could have stimulated chickpea growth. Rajendran et al. (2008) used three *Bacillus* strains, NR2, NR4, and NR6, and reported growth promotion of pigeon pea with respect to increase in plant fresh weight, chlorophyll content, nodule number, and nodule fresh weight when co-inoculated with *Rhizobium* spp. strain IC3123. They indicated that siderophore-mediated interactions may be underlying mechanism of beneficial effect of the NR isolates on nodulation by IC3123. Gupta et al. (1998) reported increased nodule occupancy of *Bradyrhizobium* strains when used in combination with *Enterobacter* isolates producing siderophores, enabling the inoculant *Bradyrhizobium* strains to occupy successfully the nodulation sites. Hence, siderophore-producing PGPR strains can be exploited as co-inoculants with rhizobia to bring improvement in growth and development of legumes.

Biocontrol

Usage of rhizosphere microorganisms for biological control of soil-borne pathogens currently is of a considerable interest (Kloepper et al. 1989; Vargas et al. 2009) and has been considered a unique mechanism to protect crop from pathogen attack (Kumar et al. 1997; Hameeda et al. 2010). Kloepper (1993) indicated that most of the PGPR usually act as biological control agent when co-inoculated with rhizobia. Villacieros et al. (2003), while studying the colonization behavior of *P. fluorescens* and *Sinorhizobium meliloti* in the alfalfa rhizosphere, explained the usefulness of the strains for biocontrol. Pathak et al. (2007) observed that the combined effect of *Pseudomonas maltophilia* + *Mesorhizobium* + PSB was more beneficial as it reduced root rot incidence and positively affected nodule number, nodule biomass, and nodule occupancy as well as plant growth in chickpea cv. H208 which in turn increased seed productivity. Likewise, Sindhu and Dadarwal (2001) also reported that co-inoculation of *Mesorhizobium* with PGPR contributed to the suppression of plant disease by inhibiting the growth of phytopathogenic fungi and promoting nodulation. Hameeda et al. (2010) also reported reduced incidence of collar rot caused by *Sclerotium rolfsii Sacc*. in chickpea as a result of co-inoculation with *Pseudomonas* sp. CDB 35 and BWB 21 and *Rhizobium* sp. IC 59 and IC 76. Whereas, Kumar et al. (2001),

while studying the effect of co-inoculation of pea with plant growth-promoting fluorescent *Pseudomonas* and *Rhizobium*, observed that the strains were highly inhibitory to the investigated plant pathogens causing fusarium wilt. Also, Sindhu et al. (2002) observed that *Pseudomonas* strains used as co-inoculant with *Mesorhizobium* sp. *Cicer* inhibited the growth of chickpea pathogens, i.e., *Aspergillus* sp., *Curvularia* sp., *Fusarium oxysporum*, and *Rhizoctonia solani*. The production of antibiotics was found responsible for the antagonisms. Likewise, Tilak et al. (2006) observed significant increase in plant growth, nodulation, and nitrogenase activity due to dual inoculation with PGPR *Pseudomonas putida*, *P. fluorescens*, or *Bacillus cereus* and demonstrated that PGPR increased the nodule occupancy of *Rhizobium* by inducing systemic resistance (ISR) in host plant against plant pathogens. Improvements in legumes growth and yield could be sought via biocontrol of pathogens if PGPR are used in combination with rhizobia.

Increased Water-Use Efficiency

Co-inoculation has also been reported to improve the growth and yield of plants by increasing water-use efficiency (WUE) in plants under stress (Vivas et al. 2003). Ahmad et al. (2011) described increased water-use efficiency and relative water content by mung bean as a result of combined inoculation of *R. phaseoli* and *Pseudomonas* spp. They explained that the observed increase in water-use efficiency might be because of longer roots of plants which could have helped them to uptake comparatively more water from deeper soil under stressed conditions. Rosas et al. (2006) revealed positive effects on the growth of legumes including a superior uptake of water and nutrients, early nodulation, an increase in the number of nodules, and higher nitrogenase activity by the roots due to co-inoculation of leguminous seeds with rhizobia and phosphate-solubilizing *Pseudomonas* sp. Overall, improvement in water-use efficiency in leguminous plants co-inoculated with PGPR and rhizobia could be demonstrated as a benefit; however, work done so far on this aspect is scanty and there is a need to explore more number of effective combinations of PGPR and rhizobia.

Rhizosphere Bacteria and Legume Improvement: Co-inoculants

The use of microbial formulations consisting of a single microbial species has often resulted in non-consistent performances in agriculture; hence, emphasis has been on the application of co-inoculation of microbes (Bashan and de-Bashan 2005). In co-inoculation, the co-inoculants interact synergistically or function as “helper”

bacteria to improve the performance of other beneficial microorganisms. Although the potential to enhance nodulation, nitrogen fixation, and plant growth and yield of legumes by co-inoculation with PGPR and *Rhizobium* does exist, there is a need to fine-tune combinations of PGPR, *Rhizobium*, and host genotype used under particular environmental conditions (Remans et al. 2007, 2008). However, studies have shown that the growth-promoting ability of co-inoculants may be highly specific to the inoculant's strain specificity (Molla et al. 2001a), strain inherent potential (Medina et al. 2003) to certain plant species, cultivar, and genotype (Remans et al. 2008), while other studies have emphasized on cell density of applied inocula (Mishra et al. 2009a) or optimal inoculation dose (Bai et al. 2002b; Fox et al. 2011), strains effectiveness (El-Sawy et al. 2006) and composition of root exudates of host plant, and temperature variation or interaction of applied inocula with rhizospheric microflora predominant in the particular crop (Sindhu et al. 2002). Also, it has been reported that through the use of favorably interacting rhizospheric microorganisms as microbial inoculant, nodulation as well as N and P uptake and hence yield of legumes could be improved (Zaidi et al. 2003). Some co-inoculants, from a range of genera, improved legume growth and yield (for detail, see Table 12.1) when used in combination with rhizobia and are being reviewed individually in the following subsections.

Azospirillum *spp*.

A. lipoferum, *A. brasilense*, *Azospirillum amazonense*, *Azospirillum halopraeferens*, and *Azospirillum irakense* are the species of *Azospirillum* that have been identified so far for co-inoculation with *Rhizobium* (Tarrand et al. 1978; Magalhaes et al. 1983; Reinhold et al. 1987; Khammas et al. 1989). *Azospirillum* is a free-living atmospheric N_2 fixer and budding PGPR which could manipulate growth and yield of several leguminous crops upon inoculation (del Gallo and Fabbri 1991; Burdman et al. 1997). However, the cell density of *Azospirillum* has been described as a factor in co-inoculated legumes (Plazinski and Rolfe 1985; Yahalom et al. 1987). The key advantage and the principal ways of stimulating growth of legumes when *Azospirillum* was used as co-inoculant with rhizobia are reviewed in the following text.

The ability of *Azospirillum-Rhizobium* co-inoculation for improving nitrogen fixation and yield of legumes has been recognized (Groppa et al. 1998; Roseline et al. 2008). Okon et al. (1995) revealed that combined inoculation with *Azospirillum* could lead to increased susceptibility of roots to nodulation by *Rhizobium*, e.g., better nodulation has been reported in white clovers with a mixture of *A. lipoferum* and *R. leguminosarum* bv. *trifolii* (Tchebotar et al. 1998), in chickpea with *A. brasilense* and *Rhizobium* strains (Rai 1983), in faba bean and chickpea with *Rhizobium* and *Azospirillum* (Rodelas et al. 1999; Wani et al. 2007a), in pigeon pea with *Azospirillum*

Table 12.1 Effect of Rhizosphere bacteria for plant growth promotion in *Rhizobium*-legume symbiosis

Rhizosphere bacteria/ co-inoculants	Symbiotic bacteria	Growth conditions	Specific comments	References
***Cicer arietinum* L.**				
Pseudomonas fluorescens BHUPSB06	*Mesorhizobium* sp. BHURC02	Field conditions	The maximum increase in nodule number, dry matter, and nutrient content were recorded in co-inoculation of BHURC02 and BHUPSB06	Verma et al. (2012)
Pseudomonas sp. MRS13	*Mesorhizobium* sp. *Cicer* strain Ca181	Axenic experiment	The plant dry weights of co-inoculated treatments showed 1.10–1.28 times increase in comparison to *Mesorhizobium* inoculated plants alone	Malik and Sindhu (2011)
Bacillus sp., *Pseudomonas* sp. CDB 35 and BWB 21	*Rhizobium* sp. IC 59 and IC 76	Pot trial	Increase in shoot weight was 36 and 39 % by seed coating/priming with *Rhizobium* sp. IC 59 and *Pseudomonas* sp. CDB 35 when compared to control	Hameeda et al. (2010)
Pseudomonas strain 6N and PM-4	*Mesorhizobium ciceri*	Field trial	Co-inoculation with PM-4 strain increased nodule weight and grain yield up to 54 and 40 %, respectively, compared to control	Pathak et al. (2007)
Bacillus sp. strains CBS106, CBS127, and CBS155	*Mesorhizobium* sp. *Cicer* strain Ca181	Agar plate assay	*Bacillus* sp. inoculation increased shoot dry weight and nodule fresh weight up to 73 and 51 %, respectively, compared to *Mesorhizobium* treatments alone	Sivaramaiah et al. (2007)
Bacillus subtilis OSU-142, *B. megaterium* M-3	*Rhizobium* sp.	Field trial	Combined inoculation increased seed yield up to 30 % compared to control	Elkoca et al. (2008)

Azotobacter chroococcum A10, *Pseudomonas* PSB5, and *Bacillus* PSB9	*M. ciceri* RC4	Field trial	The dual inoculation with *M. ciceri* RC4 and *Bacillus* PSB9 increased the plant dry matter, nodule weight, and grain yield, i.e., 12, 47, and 17 %, respectively, compared to *M. ciceri* RC4 inoculation alone	Wani et al. (2007b)
Pseudomonas jessenii PS06	*M. ciceri* C-2/2	Greenhouse and field trial	The co-inoculation treatment ranked the highest in nodule number, nodule weight, and seed yield, i.e., 19, 12, and 52 % greater than the control under field conditions	Valverde et al. (2006)
Pseudomonas striata or *Penicillium variable*	*Rhizobium* sp.	Pot trial	The combined inoculation of *P. striata* and *Rhizobium* sp. increased plant dry matter, nodule number, and weight, i.e., 87, 181, and 188 %, respectively, compared to *Rhizobium* sp. treatment only	Zaidi et al. (2003)
Pseudomonas spp. strains MRS13, CRS55b, and CRS68	*Mesorhizobium* sp. *Cicer* strain Ca181	Pot culture conditions	The dual inoculation of strains CRS38 and Ca181 increased nodule weight and plant dry matter, i.e., 33 and 98 %, compared to Ca181 treatment alone	Sindhu et al. (2002)
***Glycine max* L.**				
Streptomyces sp. strain, P4	*Bradyrhizobium japonicum* USDA 110	Pot trial	Dual inoculation of USDA 110 and P4 showed the highest shoot N accumulation and seed weight among treatments	Soe et al. (2012)
B. subtilis	*B. japonicum*, 532c and RCR 3407	Greenhouse	Co-inoculants increased the nodule fresh weights by up to 4 g plant^{-1}	Atieno et al. (2012)

(continued)

Table 12.1 (continued)

Rhizosphere bacteria/ co-inoculants	Symbiotic bacteria	Growth conditions	Specific comments	References
Azospirillum canadense	*B. japonicum*	Controlled trial	The highest total biomass of soybean (root + shoot) was observed with the double association *Azospirillum* and *Bradyrhizobium* compared to control	Juge et al. (2012)
B. subtilis, *Klebsiella planticola*, and *Proteus vulgaris*	*B. japonicum*, *Rhizobium leguminosarum* bv. *viciae*	Field trials	Co-inoculation increased nodule dry weight, i.e., 26 % of soybean plants compared to *Rhizobium* inoculation alone	Tsigie et al. (2011)
Azospirillum brasilense Az39	*B. japonicum* strain E109	Hydroponic conditions	The Az39 and E109, in combination, showed the capacity to promote seed germination, nodule formation, and early development of soybean seedlings	Cassan et al. (2009)
Chryseobacterium balustinum Aur9	*Rhizobium tropici* CIAT899 and *R. etli* ISP42; *Ensifer fredii* SMH12 and HH103	Controlled trials	Soybean plants receiving double inoculation (*E. fredii* SMH12 and *C. balustinum* Aur9) showed better symbiotic performance, than with a single inoculation	Estevez et al. (2009)
Bacillus thuringiensis-KR1	*B. japonicum*-SB1	Growth pouch experiment	Co-inoculation with *B. thuringiensis*-KR1 provided the highest increase in nodule number, shoot weight, and root weight up to 73, 47, and 40 %, respectively, over rhizobial inoculation and control, under in vitro conditions	Mishra et al. (2009b)
P. fluorescens Aur6, *C. balustinum* Aur9 and *S. fonticola* Cell 4	*Sinorhizobium fredii* strain SMH12	Greenhouse trial	The most significant effects on growth parameters were found when inoculations with PGPR and *S. fredii* were at different times	Garcia et al. (2004b)

Serratia proteamaculans 1-102, *S. liquefaciens* 2-68	*B. japonicum*	Potted conditions	The co-inoculation of PGPR at their optimal dose increased nodule number, plant dry weight, and fixed nitrogen up to 38, 37, and 49 %, respectively, over control	Bai et al. (2002b)
Serratia proteamaculans 1-102, *S. liquefaciens* 2-68	*B. japonicum* strain 532C	Leonard jars experiment	Total nodule number, nodule dry weight, and specific nodule dry weight increased significantly (16, 107, 49 %, respectively) with co-inoculation of Sp7 and UPMR48 compared to UPMR48 inoculation alone	Bai et al. (2002a)
P. fluorescens 2137, *P. fluorescens* WCS365, *Azomonas agilis* 125, and *Azospirillum lipoferum* 137	*B. japonicum* A1017	Axenic conditions	Co-inoculation of *P. fluorescens* 2137 and *B. japonicum* A1017 increased the colonization of *B. japonicum* A1017 on soybean roots, nodule number, and acetylene reduction activity (ARA) at 10 and 20 days after inoculation	Chebotar et al. (2001)
Serratia proteamaculans 1-102, *S. liquefaciens* 2-68	*B. japonicum* USDA 110	Greenhouse conditions	The most stimulatory effect in term of fixed N (86 %) was observed with *S. proteamaculans* 1-102 plus *B. japonicum* USDA 110 treatment compared to control	Dashti et al. (2000)
***Lens culinaris* L.**				
B. subtilis, *K. planticola*, and *P. vulgaris*	*B. japonicum*, *R. leguminosarum* bv. *viciae*	Field trials	Co-inoculation increased nodule dry weight by 48 % of lentil plants compared to *Rhizobium* inoculation alone	Tsigie et al. (2011)

(continued)

Table 12.1 (continued)

Rhizosphere bacteria/ co-inoculants	Symbiotic bacteria	Growth conditions	Specific comments	References
Pseudomonas sp. strains (NARs1, PGERs17)	*R. leguminosarum*-PR1	Greenhouse trial	Co-inoculation increased the nodulation 27.5, leghemoglobin content 45.9, total iron 115.7, total chlorophyll content 21.3, N uptake 52.1, and P uptake 88.9 % over *R. leguminosarum*-PR1 alone	Mishra et al. (2011)
Pseudomonas jessenii, *P. fragi*, and *Serratia fonticola*	*R. leguminosarum*	Pot and field trials	Co-inoculation increased the dry nodule weight and grain yield up to 109 and 150 % under pot and up to 100 and 82 %, respectively, under field conditions compared to control	Zahir et al. (2011)
Bacillus thuringiensis-KR1	*R. leguminosarum*-PR1	Axenic and field trials	The enhancement in nodulation due to co-inoculation was 73.3 % compared to *R. leguminosarum*-PR1 treatment alone	Mishra et al. (2009a)
***Phaseolus vulgaris* L.**				
Agrobacterium sp. 10C2	*E. meliloti* RCR 2011 and A321	Controlled trial	Inoculation with *Agrobacterium* sp. 10C2 increased shoot dry weight with both strains and enhanced nodule number with strain 2011	Salem et al. (2012)
C. balustinum Aur9	*R. tropici* CIAT899 and *R. etli* ISP42; *E. fredii* SMH12 and HH103	Controlled trials	Co-inoculation improved nodule primordia formation when compared with single inoculation (*R. tropici* CIAT899)	Estevez et al. (2009)
A. brasilense strain Cd	*R. tropici* CIAT899 and ISP42	Axenic trial	Co-inoculation of *A. brasilense* and *R. tropici* ISP42 increased nodule number, shoot and root weight up to 10, 40, and 31 %, respectively, over control	Dardanelli et al. (2008)

Azospirillum sp.	*Rhizobium etli* CNPAF512	Field trials	Co-inoculation with *Rhizobium* and *Azospirillum* enhanced the N_2 fixation capacity from 1.8 to 22.4 kg ha^{-1} compared to single *Rhizobium* or no inoculation. Likewise, yield increase obtained by co-inoculation relative to inoculation with *Rhizobium* alone varied between 8 and 29 %	Remans et al. (2008)
Bacillus/*Paenibacillus* spp. strains DSM 13796, DSM 27, DSM 704, DSM 13411, DSM 24, Loutit (L), DSM 36, 65E180	*R. tropici* strain CIAT899	Pot trial	Beans co-inoculated with *R. tropici* (CIAT899) and *P. polymyxa* (DSM 36) had higher leghemoglobin concentrations, nitrogenase activity, and N_2 fixation efficiency compared to control	Figueiredo et al. (2008)
A. brasilense Sp245, *B. subtilis* LMG7135, *P. putida* UW4, *P. fluorescens* SBW25	*Rhizobium etli* CNPAF512	Pot trial	Enhance nodulation and plant growth under P deficiency by co-inoculation with Rhizobium and PGPR	Remans et al. (2007)
***Pisum sativum* L.**				
Pseudomonas sp. strain PGERs17	*R. leguminosarum*-PR1	Field trial	Co-inoculation significantly increased nodulation (156.2 %) and 57.1 % higher plant biomass over control	Mishra et al. (2012)
B. thuringiensis-KR1	*R. leguminosarum*-PR1	Axenic and field trials	The enhancement in nodulation due to co-inoculation was 84.6 % compared to *R. leguminosarum*-PR1 treatment alone	Mishra et al. (2009a)
Pseudomonas putida strain Å 313	*R. leguminosarum* bv. *viciae*	Gnotobiotic trial	Mixed inocula of A˚ 313 and rhizobia gave a higher proportion of small evenly distributed nodules when compared with a single rhizobial inoculation	Berggren et al. (2005)

(continued)

Table 12.1 (continued)

Rhizosphere bacteria/ co-inoculants	Symbiotic bacteria	Growth conditions	Specific comments	References
P. fluorescens strains	*R. leguminosarum* biovar *viciae*	Pot trial	Co-inoculation of the *P. fluorescens* and Rhizobium improved plant growth in terms of shoot height, root length, and dry weight, i.e., 24, 20, and 22, respectively, compared to control	Kumar et al. (2001)
***Vigna radiata* L.**				
Pseudomonas syringae, Mk1; *P. fluorescens*, Mk20 and Mk25	*Rhizobium phaseoli* (M6 and M9)	Pot trial	Co-inoculation increased the shoot fresh weight (145 %), root fresh weight (173 %), pod fresh weight (182 %), and total dry matter (269 %) over control	Ahmad et al. (2012)
Pseudomonas strains CPS63 and MPS78	*Bradyrhizobium* strain S24	Axenic and pot trials	Significant gains in plant dry weights, i.e., 2.0–3.06 times increase in comparison to uninoculated control plants, were observed upon co-inoculation	Malik and Sindhu (2008)
P. putida biotype A Q7 and *P. fluorescens* Q14	*Bradyrhizobium japonicum*	Pot trial	The combined inoculation increased total plant biomass and nodule weight up to 19 and 100 %, respectively, compared to *B. japonicum* alone	Shaharoona et al. (2006)
Pseudomonas striata	*Bradyrhizobium* sp. (vigna)	Pot trial	The dual inoculation increased nodule number, nodule weight, and grain yield, i.e., 46, 50, and 44 %, respectively, as compared to *Bradyrhizobium* inoculation alone	Zaidi et al. (2004)

***Medicago sativa* L.**				
Pseudomonas sp. FM7d and *Bacillus* sp. M7c	*Sinorhizobium meliloti* B399	Pot trial	Co-inoculation of *Pseudomonas* sp. and *Sinorhizobium* increased nodule weight and root and shoot weight up to 1.4-, 1-, and 1.3-fold compared to control	Guinazu et al. (2010)
***Medicago truncatula* L.**				
P. fluorescens WSM3457	*Ensifer* (*Sinorhizobium*) *medicae* WSM419	Glasshouse trial	Co-inoculated plants had enhanced rate of nodule initiation (25 %) over control	Fox et al. (2011)
***Cajanus cajan* L.**				
Bacillus megaterium strains NR2, NR4, and NR6	*Rhizobium* spp. IC3123	Axenic conditions	*Bacillus* sp. showed plant growth-promoting activity and improvement in *C. cajan* nodulation when co-inoculated with rhizobial bioinoculant IC3123 under N-free culture conditions	Rajendran et al. (2008)
A. chroococcum, *A. brasilense*, *P. fluorescens*, *P. putida*, and *B. cereus*	*Rhizobium* sp. AR-2–2k	Glasshouse trial	The dual inoculation of *Rhizobium* and *P. putida* increased plant biomass, grain yield, and nodule occupancy up to 37, 67, and 85 %, respectively, compared to control	Tilak et al. (2006)
***Arachis hypogaea* L.**				
Serratia marcescens	*Bradyrhizobium* spp.	Field trials	The combined inoculation increased nodule weight up to 178 % over control	Badawi et al. (2011)
Thiobacillus thiooxidans strain, LCH, SWA5, and SWA4	*Rhizobium* sp. strain TNAU14	Pot and field trials	Co-inoculation of *Thiobacillus* sp. strain LCH with *Rhizobium* under field condition enhanced pod yield by 18 %, compared to control	Anandham et al. (2007)

(continued)

Table 12.1 (continued)

Rhizosphere bacteria/ co-inoculants	Symbiotic bacteria	Growth conditions	Specific comments	References
***Galega orientalis* L.**				
Pseudomonas trivialis 3Re27 and *P. extremorientalis* TSAU20	*Rhizobium galegae* bv. *orientalis* HAMBI540	Pot trial	The *P. trivialis* 3Re27 was able to significantly increase nodule numbers (32 %) and nitrogen content (52 %) of the co-inoculated plants	Egamberdieva et al. (2010)
***Lupinus albus* L.**				
P. fluorescens Luc 1, *P. putida* Luc 2, *P. fluorescens* Luc 3, *P. putida* Luc 4, *B. thuringiensis* Luc 5	*B. japonicum* strain ISLU-21	Greenhouse trial	The co-inoculation of strain Luc 4 and *B. japonicum* increased the plant dry matter and grain N content up to 31 and 39 %, respectively, compared to control	Garcia et al. (2004a)
***Vicia sativa* L.**				
A. brasilense (Sp7)	*R. leguminosarum* bv. viciae (Rlv)	Growth pouches	Co-inoculation increased nodule weight and plant dry weight up to 32 and 14 %, respectively, compared to control	Star et al. (2012)
***Trifolium pratense* L.**				
P. fluorescens strain 267, 267.4	*R. leguminosarum* bv. *trifolii* strain 24.1	Agar medium	The dual inoculation of *P. fluorescens* and *Rhizobium* increased nodule number and shoot weight up to 119 and 145 %, compared to *Rhizobium* inoculation alone	Marek-Kozaczuk et al. (2000)

spp. and *Rhizobium* spp. (Deanand et al. 2002), and in soybean with *A. brasilense* Az39 and *B. japonicum* E109 (Cassan et al. 2009). Numerous other studies have also demonstrated the enhancement of nodulation and growth of a wide array of grain legumes because of positive interaction between *Rhizobium* species and bacteria of the genus *Azospirillum* (Iruthayathas et al. 1983; Plazinski and Rolfe 1985; Yahalom et al. 1987; del Gallo and Fabbri 1991; Hamaoui et al. 2001). Increased nodulation in legumes following inoculation with *Azospirillum* has been attributed to the secretion of nod-gene-inducing flavonoids by the roots (Burdman et al. 1996; Volpin et al. 1996).

Phytohormones and vitamins have also been coupled with the positive outcome of combined inoculation of legumes with *Azospirillum* plus *Rhizobium* (Plazinski and Rolfe 1985; Okon and Itzigsohn 1995). Growth of various legumes have been improved as a result of combined inoculation with *Azospirillum* and *Rhizobium* via enhanced production of plant hormones such as indole-3-acetic acid, gibberellic acid, and zeatin claimed as a mechanism for enhancement in rhizobial infection, nodule formation, and N_2 fixation activity (Hungria and Vargas 2000; Molla et al. 2001b; Dardanelli et al. 2008; Cassan et al. 2009). In addition to plant hormone, the production of siderophores has also been reported when *Azospirillum* was used in combination with *Rhizobium* and stimulated legumes growth (Khan et al. 2002; Wani et al. 2007b). Whereas, an increase in proton efflux due to the *Azospirillum*'s effects on the membrane activities, which resulted in enhancement of total mineral uptake of inoculated legume plants, has also been documented (Bashan 1998).

Azotobacter *spp.*

Azotobacter are free-living, aerobic N_2-fixing bacteria (Beijerinck 1901) which occur widely in agricultural soils of temperate regions with almost neutral pH and can be easily found in association with legume rhizosphere. Production of phytohormones and vitamins is a common feature of *Azotobacter* (Arshad and Frankenberger 1991; Martinez-Toledo et al. 1991), i.e., *Azotobacter chroococcum*, *A. vinelandii*, and *Azotobacter paspali* that associate with the rhizosphere of plants. Antifungal activity of *Azotobacter* strains is also common, and thus, inhibition of pathogenic fungi by these bacteria has been often discussed as a mechanism promoting plant growth (Brown 1974). Also, increases in nodulation; number of nodules; nodule weight; root/shoot length and weight; root dry weight; total nitrogen content; relative water content; total contents of N, P, K, Ca, Mg, Fe, B, Mn, Zn, and Cu; plant biomass; and yield of various legumes, i.e., lentil, urdbean, soybean, clover, chickpea, and peanut, have been investigated as the result of dual inoculation of *Azotobacter* and *Rhizobium* (Burns et al. 1981; Paul and Verma 1999; Chandra and Pareek 2002; Qureshi et al. 2009; Dashadi et al. 2011; Akhtar et al. 2012). However, significant improvement in *Rhizobium*-legume symbiosis by co-inoculant *Azotobacter* is dependent on specific combinations of microbial strains and plant cultivars used (Burns et al. 1981) as well as on appropriate cell numbers of the inoculants (Rodelas et al. 1999).

Bacillus *spp*.

Bacilli are spore-forming, Gram-positive, and rod-shaped bacteria. It is one of the most familiar soil bacterial groups that are commonly isolated from the plant's rhizosphere. A range of rhizospheric *Bacillus* species have been found to be beneficial in growth and yield enhancement of legumes. Phytohormones inducing stimulation of legumes as a result of mixed inoculation of *Bacillus* and *Rhizobium* have been reported in various investigations (Vessey and Buss 2002). Medeot et al. (2010) reported synergism between *Bacillus* and *Bradyrhizobium* in the rhizosphere to promote the growth and yield of legumes. The potential of *Bacillus* together with *Rhizobium* as promoter of growth and yield of various legumes via improving rhizobial colonization (Camacho et al. 2001), by providing a more balanced nutrition to plants (Tsigie et al. 2012), by increased number of infection sites (Srinivasan et al. 1997; Elkoca et al. 2008; Tsigie et al. 2011), by enhancing the nodule occupancy of introduced *Rhizobium* (Tsigie et al. 2011; Atieno et al. 2012), and by increasing the chance of changing nodulation competition between strains via altering the related root microflora in an environment of multistrain (Gupta et al. 1998) has been described. Moreover, the potential of *Bacillus* for improving *Rhizobium*-legume symbiosis by production of phytohormone indole-3-acetic acid (Srinivasan et al. 1996; Yuming et al. 2003; Mishra et al. 2009b), production of siderophore (Rajendran et al. 2008), biocontrol of diseases (Handelsman et al. 1990; Podile and Laxmi 1998; Vessey and Buss 2002), efficient uptake of nutrients (Stajkovic et al. 2011), and increased P availability (Singh et al. 2011) has also been investigated. Among the noted stimulatory effects of co-inoculation of some of the *Bacillus* strains on various legumes were enhanced root and shoot dry weight, length, surface area and number of roots, nodulation, nodule number and dry weight, nitrogen fixation, N % and total plant nitrogen, P contents in plants, plant height, branch plant^{-1}, growth, and yields (Parmer and Dadarwal 1999; Lian et al. 2001; Guinazu et al. 2010; Stajkovic et al. 2011; Singh et al. 2011).

Pseudomonas *spp*.

Pseudomonads are the most studied plant growth-promoting rhizobacteria and belong to Gram-negative genera of which the greatest numbers of strains are members of the fluorescent pseudomonads (Kloepper 1993). These rhizobacteria have huge potential in agriculture for use as biofertilizer and biocontrol agent and in bioremediation due to their plant growth-promoting ability, antagonistic activity, and degradation of pollutants (Ahmad et al. 2008). Several *Pseudomonas* strains, namely, *P. fluorescens* 2137, *P. fluorescens* P-93, *P. fluorescens* strain BHUPSB06, *P. trivialis* 3Re27, *Pseudomonas* spp. MRS13 or MRS16, *P. striata*, *P. putida*, *P. putida* strain M17 *Pseudomonas* LG, *Pseudomonas* sp. CDB 35 and BWB 21, *P. maltophilia*, *Pseudomonas* strains CPS63 and MPS, *Pseudomonas* sp. FM7d, *P. jessenii*

PS06, *P. striata*, and *Pseudomonas* sp. strain PGERs17, have been reported by various researchers. These are potential pseudomonads, having their role in improving length, surface area and dry weight of root/shoot, root respiration, a superior uptake of water and nutrients, nodulation, nodule fresh weight, nitrogenase activity, N_2 fixation, total N_2 content, plant dry weight, N and P uptake, and growth and yield of various legumes when used in combination with *Rhizobium* (Parmer and Dadarwal 1999; Goel et al. 2001; Kumar et al. 2001; Sindhu and Dadarwal. 2001; Deanand et al. 2002; Zaidi et al. 2003; Garcia et al. 2004a; Tilak et al. 2006; Malik and Sindhu 2008; Stajkovic et al. 2011; Zahir et al. 2011; Mishra et al. 2012; Verma et al. 2012).

Also, some species of *Pseudomonas*, namely, *P. fluorescens*, *P. putida*, *P. cepacia*, and *P. aeruginosa*, have been reported as potential biocontrol agents of several phytopathogens such as *Aspergillus* sp., *Curvularia* sp., *F. oxysporum*, and *R. solani*, and the production of siderophores as well as antibiotics were found to be responsible for the antagonism (Sindhu et al. 1999). But it has been reported that significant effects on *Rhizobium*-legume symbiosis by *Pseudomonas* could only be observed when certain combinations of microbial strains and plant cultivars are used (Burns et al. 1981). However, variations in effects could be attributed to differential behavior of the *Pseudomonas* strains to composition of root exudates, temperature variation, or to their interaction with rhizospheric microflora predominant in the particular crop (Sindhu et al. 2002).

Serratia *spp.*

Serratia could promote the growth of legumes through increasing photosynthesis and by production of a plant growth-regulating compounds which stimulate nitrogen fixation by invigorating overall plant vigor and growth, resulting in a subsequent increase in nitrogen fixation (Zhang et al. 1996; Dashti et al. 1997). Production of plant growth-promoting substances such as auxins, flavonoid-like compounds, and siderophore as a result of co-inoculation of *Serratia marcescens* with *Bradyrhizobium* has been reported by Badawi et al. (2011) which acted to enhance root proliferation and provided more infection sites to be occupied by rhizobia and played role in improving number and mass of nodule and yield of peanut. Also, improvement in the growth of legumes via solubilization of phosphate through mixed inoculation of *S. marcescens* with *Rhizobium* has been reported (Radwan et al. 2005). Whereas, stimulated soybean growth, nodulation, and nitrogen fixation via affecting signal exchange between plant and rhizobia as a result of co-inoculation of some *Serratia proteamaculans* strain along with effective rhizobia have been reported by Zhang et al. (1996) and Bai et al. (2002a). Enhanced ACC-deaminase activity and auxin production, P solubilization, and root colonization have also been attributed to better root elongation, nodulation, and consequently improved growth and yield of lentil as a result of co-inoculation of *S. fonticola* and *R. leguminosarum* (Zahir et al. 2011).

Enterobacter *spp*.

Enterobacter is the most abundant plant growth-promoting endophytic bacteria in legumes (Mishra et al. 2009a). Several reports have indicated increased grain yield and nodule occupancy of green gram when co-inoculated with *Bradyrhizobium* sp. It has been suggested that *Enterobacter* showed the effects by producing antibiotics and siderophores which might have inhibited other rhizospheric rhizobia and enabled the inoculant bradyrhizobial strains to occupy successfully the nodulation sites. Gupta et al. (1998) demonstrated that two strains of *Enterobacter* co-inoculated with two strains of *Bradyrhizobium* sp. increased nodule occupancy of the two rhizobial strains. Mirza et al. (2007) has reported the growth and yield promotion of chickpea by co-inoculation with phytohormone-producing *Enterobacter* strains and *Rhizobium*.

Actinomycetes

Actinomycetes are attractive because their secondary metabolites might be promising sources of novel antibiotics and growth regulators for other organisms (Matsukuma et al. 1994; Okazaki et al. 1995). Soe et al. (2012) reported highest shoot N accumulation, nitrogen fixation, and seed weight of soybean because of dual inoculation of *Streptomyces* strain P4 and *B. japonicum*. Tokala et al. (2002) observed that root colonization of *Streptomyces lydicus* WYEC 108 resulted in increased root nodulation frequency, size of nodules, and nodule dry weight of soybean possibly at the level of infection by *Rhizobium* sp. while demonstrating the positive effects of co-inoculation of actinomycetes and rhizobia on nodulation, nitrogen fixation, and disease control of *Pisum sativum* and soybean and reported that *Streptomyces* strain P4 was one of the effective actinomycetes which could be used in combination with selective root nodule bacterial strains for improved production of leguminous crops.

Rhizosphere Bacteria in *Rhizobium*-Legume Symbiosis: Agricultural Aspects

The need to maximize the capacity of *Rhizobium*-legume symbiosis is not only due to certain environmental biotic and abiotic stress factors which adversely affect this system but also because of economic and environmental concerns relating to the use of chemical fertilizers in agriculture. Although the application of efficient and effective rhizobial inocula to legumes is a well-recognized cost-effective and eco-friendly approach, it does not guarantee for consistent performance. Hence, application of competent and beneficial rhizosphere bacteria as “helper” bacteria or co-inoculant comes out as a mean capable of improving the performance of rhizobia and legumes for ultimate increase in the amount

of nitrogen to be fixed by this system in a number of ways which are elaborated in the following subsections.

Synergistic Effect of PGPR with Rhizobium *in Improving Nodulation and Biomass*

Only those rhizospheric bacteria, which have shown to enhance plant yield or health, are generally named as plant growth-promoting rhizobacteria (PGPR) (Kloepper et al. 1989). It has been recognized that certain PGPR when co-inoculated with rhizobia can modify nodule formation and BNF (Zhang et al. 1996). Rautela et al. (2001) were of the opinion that PGPR could help rhizobia in their survival through synergism resulting in an increase in their nodulation ability and nitrogen-fixing efficiency. Several mechanisms such as alteration in the composition of rhizospheric microorganisms; release of plant signaling molecules, siderophores, bacteriocins, and phytostimulatory hormones; and increasing accessibility of nutrients have been reported for such synergism in numerous studies which have shown beneficial responses on various legumes development (Burdman et al. 1996; Dashti et al. 1998), for example, enhancement in plant growth and yield due to inoculation with synergistically acted combination of *Azotobacter* and *R. leguminosarum* bv. *viciae* in faba bean (Rodelas et al. 1999), *P. striata* and *Bradyrhizobium* sp. in green gram (Zaidi et al. 2004), *Pseudomonas* and *Bacillus* sp. with *Rhizobium* sp. in black gram (Gunasekaran et al. 2004), *Rhizobium*/*Mesorhizobium* sp. and PSB in chickpea and pigeon pea (Khurana and Sharma 2000; Rudresh et al. 2005; Valverde et al. 2006: Sivaramaiah et al. 2007; Malik and Sindhu 2011; Reddy et al. 2011), PSB and *B. japonicum* in soybean (Mishra et al. 2009a), *Azospirillum* and *Rhizobium* in common bean (Remans et al. 2008), and *Pseudomonas* sp. 3Re27 or TSAU20 and *Rhizobium galegae* bv. *orientalis* HAMBI 540 of fodder galega (Egamberdieva et al. 2010). Similarly, synergistic role of *Pseudomonas* and *Bacillus* sp. with *R. leguminosarum* in lentil (Kumar and Chandra 2008; Zahir et al. 2011) compared to *Rhizobium* alone treatment clearly suggested that rhizobacteria can be used as microbial inoculants to improve nodulation and the productivity of legumes (for further detail, see Table 12.1).

Effectiveness of PGPR with Rhizobium *in Interceding Abiotic Stresses Affecting* Rhizobium-*Legume Symbiosis*

From an agricultural point of view the *Rhizobium*-legume symbiosis is considered the most important nitrogen-fixing interaction. However, various abiotic stresses can reduce the symbiotic effectiveness (Lerouge et al. 1990; Polonenko et al. 1993) by modifying molecular dialogue between rhizobia and legumes (Medeot et al. 2010).

Typical abiotic environmental stresses of nitrogen-fixing systems comprise drought, salinity, high soil temperature, acidity, heavy metals, and nutrient deficiency (Zahran 1999) leading to a chain of molecular, morphological, physiological, and biochemical changes, which negatively affect plant growth and productivity (Barka et al. 2006). Although, selection of site-specific, effective, and efficient symbiotic partners could guarantee the best possible performance of the *Rhizobium-legume* symbiosis, Mishra et al. (2011) stated that the efficiency of the symbiotic process could be further improved by the use of appropriate PGPR as co-inoculant with host-specific rhizobia. This property of PGPR has prompted the use of double or mixed inoculants to overcome environmental limitations on nitrogen fixation and improve crop production (Bai et al. 2003; Rudresh et al. 2005). Therefore, approaches investigated so far to overcome various kinds of abiotic stresses by the use of PGPR as co-inoculant with rhizobia to achieve optimal yields are reviewed in this section.

Rhizobia are sensitive to drought stress and may result in a significant decrease in N_2 fixation when faced with low soil-water content. But in a study, Figueiredo et al. (2008) reported that two strains of *P. polymyxa* when used in combination with *R. tropici* resulted in increased plant height, shoot dry weight, and nodule number of co-inoculated bean plants grown under drought stress. Moreover, from the results, they suggested that the observed results were due to the synergistic effect of the mixed strains. Dashadi et al. (2011) studied the effects of co-inoculation of *A. chroococcum* strain AGO11 and *R. leguminosarum* bv. *viciae* strain F46 on growth and growth indices of faba bean under water stress and reported that co-inoculation increased inoculation, nodule number, total nitrogen content, relative water content, and root dry weight of the inoculated plants. Additionally, it was demonstrated that combined inoculation of *Azotobacter* and *Rhizobium* enhanced water and nutrient uptake under water stress, consequently lessening the effect of shortage of water and improving some of the growth parameters of faba bean under water-stressed conditions.

Soil salinity can significantly reduce the uptake of nutrient by plants in saline soils (Grattan and Grieve 1999). Dardanelli et al. (2008) studied the effect of *A. brasilense* co-inoculated with *R. tropici* strain CIAT899 or *R. etli* ISP42 on *P. vulgaris* cv. Negro jamapa on the production of flavonoids and nod factor under salt stress. They reported that the co-inoculation clearly benefited the plant at the level of production of more flavonoid signals, nod-gene transcription, nod factor pattern, root development, and nitrogen fixation. It was further elaborated that the inoculation with *Azospirillum* favorably affected the production of more species of flavonoids in plants with *R. tropici* CIAT899 compared to those produced with *Rhizobium* alone at day 14 which caused activation of nod genes and may be due to IAA and other plant growth substances produced by *Azospirillum* in the rhizosphere, while Estevez et al. (2009) reported that *C. balustinum* Aur9 improved bean growth when used in combination with *R. tropici* CIAT899 compared with single inoculation with *R. tropici* CIAT899 under saline stress. They reasoned that the improvement might be by increasing root hair formation and infection sites. Moreover, bean plants receiving dual inoculation of *C. balustinum* Aur9 and *Ensifer fredii* SMH12

showed better symbiotic performance than with a single inoculation under saline stress suggesting that the effect of dual inoculation was strongly dependent on rhizobial species.

Another abiotic stress that may influence *Rhizobium*-legume symbiosis is the unavailability of adequate nutrients or nutrient stress. PGPR are promising components to enhance nutrient availability and uptake and to support the health of plants (Barea et al. 1998). Phosphate-solubilizing bacteria can increase P availability to plant by solubilizing insoluble phosphate, and this improved P nutrition can increase BNF and the availability of other nutrients because these bacteria can produce plant growth-promoting substances. For example, Remans et al. (2007) studied the effect of four PGPR strains on the symbiotic interactions between *Rhizobium* and common bean under deficient versus sufficient phosphorus supply. They reported that the effect of PGPR on nodulation and plant growth was dependent on plant P nutrition as co-inoculation enhanced nodule number, shoot, and root dry weight significantly under high P conditions compared with low P conditions where the same dual inoculation showed negative effect on these parameters. Singh et al. (2011) evaluated the effect of PSB in combination with *Rhizobium*, on nodulation, water-use efficiency, and growth and yield of chickpea. They further reported that combined inoculation of P-solubilizing bacteria and *Rhizobium* yielded significantly more nodules, more number of pods plant^{-1}, increased plant height, and more grain yield than *Rhizobium* alone.

Also, acid soils limit nodulation and N_2 fixation in many *Rhizobium*-legume symbioses by restricting *Rhizobium* survival and persistence in soils (Ibekwe et al. 1997). But Vijila and Jebaraj (2008) reported that combined inoculation of PSB *B. polymyxa* and PGPR *P. fluorescens* with *Rhizobium* increased nodulation, plant biomass, and grain yield of green gram than single inoculation with each of the inoculants under acid-stressed soils (pH 4.8). Release of phosphates from bound phosphates by PSB strains and production of growth hormones and siderophores were described to be the mechanisms by which PSB and PGPR helped the crop plants. Zhang et al. (1996, 1997) reported that PGPR strains of *S. proteamaculans* 1-102 or *S. liquefaciens* 2-68 with *B. japonicum* increased nodulation, nitrogen fixation, and plant growth of soybean plants grown at low root zone temperature of 15 and 25 °C. They concluded that improvement in plant growth, development, and physiological activities of soybean seedlings was due to direct effects of PGPR on overall physiology rather than specific effects on N_2 fixation.

Role of PGPR for Biocontrol in Rhizobium-*Legume Symbiosis*

Biocontrol is a procedure by which a pathogenic organism is restricted at low inoculum density or controlled or eliminated by beneficial organisms. Generally, PGPR avert the establishment of pathogen in the rhizosphere via antibiosis, siderophore production, and secretion of other hydrolytic enzymes (Bakker et al. 1991).

Usually, a single inoculant may sometimes result in inconsistent performance as it may not be active in all soil environments or against all pathogens that affect the host plant. On the contrary, mixtures of inoculants with different plant colonization patterns may be of use for the biocontrol of diverse plant pathogens via wider range of biocontrol mechanisms (Pierson and Weller 1994). Hence, development of mixtures of biocontrol agents is needed, as they may better adapt to the environmental changes that occur throughout the growing season and shelter against a broader array of pathogens.

Yuttavanichakul et al. (2012) used lytic protease enzyme and IAA producing strains of *B. megaterium* and *B. subtilis* in combination with *Bradyrhizobium* sp. TAL 173 to inoculate peanut. It was demonstrated that the co-inoculant inhibited the root rot disease of peanut caused by *Aspergillus niger* and increased the growth of peanut roots. They suggested that use of selected PGPR in combination with rhizobia could increase nitrogen fixation and lessen fungicide usage in peanut and offer a suitable technique for sustainable agriculture. Dutta et al. (2008) studied the effect of PGPR *B. cereus* strain BS03 and a *P. aeruginosa* strain RRLJ 04 on induction of systemic resistance against *Fusarium udum* wilt in pigeon pea, both individually and in combination with a rhizobial strain RH 2. They reported that plants with combination treatments of PGPR and *Rhizobium* survived longer than in individual and control treatments. Increased production of defense-related enzymes, namely, polyphenol oxidase (PPO), L-phenylalanine ammonia lyase (PAL), and peroxidase (POX), was also observed in co-inoculated plants. The results revealed that the combined use of PGPR and rhizobia was effective for induction of systemic resistance against fusarial wilt in pigeon pea. It was also noted that in vitro conditions production of β-1,3-glucanase, polymethyl galacturonase, and fusaric acid by the pathogen was drastically reduced in the presence of PGPR strains. Esteve de Jensen et al. (2002) found that combined application of *B. subtilis* with *Rhizobium* controlled bean root rot and improved plant growth. Samavat et al. (2011) described that seed treatment with *Pseudomonas* isolates and rhizobia along with rhizobial culture filtrates diminished the harshness of bean damping-off and significantly enhanced the growth of bean in comparison with the untreated control. As all the tested *P. fluorescens* and rhizobial isolates proved to be producers of IAA, siderophore, HCN, exopolysaccharides, and chitinase, hence, a number of mechanisms for the control of bean damping-off disease and growth improvement were speculated like production of chitinolytic enzymes, exopolysaccharides, IAA, and siderophore and induction of systemic resistance. Shweta et al. (2008) also reported suppression of charcoal rot in groundnut as result of beneficial effect of co-inoculation of *Pseudomonas* and rhizobia. Elkoca et al. (2010), while studying the effect of single, dual, and triple inoculation with *B. subtilis*, *B. megaterium*, and *R. leguminosarum* bv. *phaseoli* on nodulation, nutrient uptake, yield, and yield parameters of common bean, reported that dual and triple inoculation produced improvement in the measured parameters. They reported that *B. subtilis* was of particular importance since it was known to positively influence the ability of the plant to cope with pathogens often resulting in higher yield as well as having

the potential to improve crop yields by providing a more balanced nutrition to plants as compared to sole inoculations. Goel et al. (2000, 2002) have also indicated the stimulation of nodulation and plant growth in chickpea using *Pseudomonas* strains that were antagonistic to fungal pathogens (*Aspergillus* sp., *F. oxysporum*, *P. aphanidermatum*, and *R. solani*) in co-inoculation with *Mesorhizobium*. Also, this co-inoculation resulted in the formation of 68–115 % more nodules compared to single inoculation with *Mesorhizobium*. The beneficial effect on plant shoot dry mass was more pronounced with HCN-producing *Pseudomonas* strain. Sindhu et al. (2002) noted plant growth-promoting effect of *Pseudomonas* when used in combination with *Mesorhizobium* sp. *Cicer* strain on green gram and reported suppressive effect of *Pseudomonas* on the growth of plant pathogens *Aspergillus* sp., *Curvularia* sp., *F. oxysporum*, and *R. solani*. The productions of siderophores as well as antibiotics were found to be responsible for the antagonism. Similarly, nodule promotion and reduction in wilt incidence or root rot disease of chickpea in wilt sick soil due to *Pseudomonas* strains inoculation have been reported by Khot et al. (1996). Likewise, Siddiqui et al. (2001) reported that combined application of PGPR (*P. aeruginosa* and *B. subtilis*) and *Rhizobium* controlled root rot-root knot disease complex of mung bean and showed synergistic effect on symbiotic parameters and grain yield of mung bean.

Synergistic Effect of PGPR with Rhizobium *in Bioremediation*

Use of PGPR with *Rhizobium* in rhizoremediation is another interesting topic in contaminated zones. Selection and application of interactive tolerant beneficial bacteria having the ability to survive and colonize the rhizosphere may facilitate plants to perform better in contaminated soil (Vivas et al. 2006). Hadi and Bano (2010) have suggested the combined application of *Azotobacter* and *Rhizobium* both for phytoremediation purpose as well as for growth and biomass improvement of plants on metal-contaminated soils. Dary et al. (2010) studied the in situ multi-metal-contaminated soil reclamation ability of *Lupinus luteus* inoculated with mixture of metal-resistant PGPR (i.e., *Bradyrhizobium* sp. 750, *Pseudomonas* sp., and *Ochrobactrum cytisi*). They observed heavy metals (Cd, Cu, and Pb) accumulation mainly in lupines roots and confirmed the potential use of this plant in phytostabilization of metals. Furthermore, they reported that inoculation with consortium of PGPR showed additional increment in plant biomass to that produced by *Bradyrhizobium* sp. 750 inoculation. Finally, they suggested that *L. luteus* inoculated with mixture of metal-resistant PGPR is a useful strategy for in situ reclamation of soils contaminated with heavy metals. Engqvist et al. (2006), while evaluating the impact of mixture of PGPR, rhizobia, and AM fungi on the uptake of P and Cd and growth of various pea genotypes in Cd-polluted soil, reported higher seed and shoot biomass and Cd uptake but lower accumulation of P in seeds of plants grown in polluted soils compared with the plants grown in nonpolluted soil. They concluded that

for booming production of pea on cadmium-contaminated soils, most advantageous inoculations with beneficial microbes need active interaction among the microbial components and the plant under particular soil conditions. Lee et al. (2006) used engineered strains of *Pseudomonas* Pb2-1 in combination with *Rhizobium* strain 10320D to test their ability to bioremediate trichloroethene (TCE) from soil co-contaminated with both organic and heavy metal pollutants. They reported sixfold higher accumulation of cadmium by engineered co-inoculants than non-engineered strains in the level of 16 μM $CdCl_2$. They explained that these engineered bacterial combination increased the accumulation of Cd and removed the metal-induced inhibition on the degradation of TCE. Finally, they speculated that similar increments on TCE degradation will be observed when these engineered bacteria will be co-inoculated on to the roots of plants. Radwan et al. (2005) used two PGPR strains, i.e., *P. aeruginosa* and *S. liquefaciens*, in combination with two rhizobial strains of *R. leguminosarum* to test phytoremediation capacity of *V. faba* plants grown in oily desert area. They recorded that mixture containing one strain of PGPR and one of rhizobia improved total nodule weight per plant, nitrogen contents of shoots, plant fresh and dry weight, and plant height. Finally, they advocated that co-inoculation with PGPR and rhizobia improved the capacity of *V. faba* plants for phytoremediation of oily desert via increasing nitrogen fixation and plant growth. In another study, Radwan et al. (2007) clearly reported the utilization of hydrocarbons by PGPR and rhizobia themselves as a sole source of energy and carbon. Dashti et al. (2009) investigated consortia of bacteria (periphytic and endophytic which did not match the specific *Rhizobium* spp. of the host legume) associated with legume (*V. faba* and *Lupinus albus*) root nodules which showed potential to clean oily deserts. Furthermore, they elaborated that legumes with nodulated roots attenuated more oil from the surrounding water than nodule-free roots, confirming that the associated bacteria were oil utilizing and diazotrophic which means the nitrogen fixed by these bacteria was the only source of compound nitrogen used for oil degradation. They concluded and reported legume crop root host oil-utilizing diazotrophic bacteria other than rhizobia as useful means for bioremediation of oily desert soils deficient in nitrogen.

Future Perspectives and Conclusions

From above discussion, it is clear that PGPR strains of various bacterial genera have promising potential for use as co-inoculant with rhizobia to improve *Rhizobium-legume* symbiosis in a way that could harness to benefit sustainable increased production of legumes under diverse conditions. However, for successful and broader exploitation of PGPR as co-inoculant with rhizobia for enhancing *Rhizobium-legume* symbiosis and for discovery of more number of such co-inoculant PGPR, future research should concentrate on:

1. The molecular and genetic study of selected competitive, efficient, and effective co-inoculant PGPR
2. The study of microbe-microbe and microbe-plant signal exchange

3. The option of PGPR strains advantageous equally to legumes and rhizobia, especially with their wide variety and under variable environments
4. The understanding of mechanisms affecting interaction of PGPR strains with rhizobia and leguminous plants
5. The selection of co-inoculant PGPR strains having multiple mechanisms of actions
6. The identification and evaluation of more efficient combinations of PGPR and rhizobia, which enable inoculated legume plant to behave in a more competitive way and remediate soils when established in contaminated fields, and unraveling of the mechanisms underlying their success

Acknowledgement We are thankful to Hafiz Muhammad Haroon for his help in drawing Fig. 12.1.

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Chapter 13
Diversity of Plant Root Associated Microbes: Its Regulation by Introduced Biofilms

G. Seneviratne, N. Weeraratne, and U.V.A. Buddhika

Contents

Abstract Microorganisms use dormancy as a tactic to evade from unfavourable fluctuations of environment conditions, which results in a voluminous soil seed bank of coexisting species. This has now been well proven with the advent of molecular techniques. Sporadic resuscitation of the dormant microbes contributes to maintain ecosystem functioning. The interchange of dormant and active stages aids vast number of species to coexist whilst maintaining persistent populations amidst constant evolutionary pressure. This interchange is a response to dynamic biotic and abiotic factors in the soil environment. Amongst factors deciding this switch,

G. Seneviratne (✉) • U.V.A. Buddhika
Microbial Biotechnology Unit, Institute of Fundamental Studies,
Hantana Road, Kandy, Sri Lanka
e-mail: gaminis@ifs.ac.lk

N. Weeraratne
School of Agriculture and Wine Sciences, Faculty of Science, Charles Sturt University,
Locked bag 588, Boorooma Street, Wagga Wagga, NSW 2678, Australia

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_13, © Springer India 2013

host factor is well documented in the case of plant-associated microorganisms. In addition to the responsive interchange in the fluctuating environments, a spontaneous interchange takes place in stable environments, which is determined by quorum sensing (QS) that leads to emergence of subpopulations. This is theoretically known as "kin selection" or the promotion of species depending on the degree of genetic relatedness amongst the individual organisms. All in all, those mechanisms have resulted in a lesser number of individuals in active stage, due to ever-increasing adverse conditions imposed on the environment. This has caused to collapse sustainability in many ecosystems. However, recent research shows that if developed beneficial microbial communities in biofilm mode would be introduced to the soil, they can increase the emergence of soil microbial diversity, favouring surfacing of subpopulations of beneficial species. It is now evident that the biofilm actions break dormancy of the microbial seed bank for the increased resuscitation of the dormant cells.

Introduction

Rhizosphere is the place where rhizobacteria generally flourish and proliferate to do a lot of advantageous functions in soil–plant system. Genome sequencing has already been done by culture-independent techniques like metagenomic analysis for most plant-associated bacteria of a wide range of genera. Amongst them, *Pseudomonas* sp., Actinobacteria, *Bacillus* sp., and their different species perform different microbial actions for plant growth promotion and ecosystem sustainability. Diverse plant factors allow microbiota to thrive in the plant rhizosphere, which in turn support a healthy plant growth. Chemotaxis response of microbes towards plant's rhizodepositions creates suitable ecological niches, enabling to grow a wide range of rhizobacteria for better establishment in plant rhizosphere (Bais et al. 2006). A subset of the soil bacterial community typically resides in the rhizosphere, exploring a wide range of interactions such as parasitism, commensalism and symbiosis with the host plant by colonizing associatively on the root surface and in the endophytic compartments. These interactions are crucial in addressing the ecological consequences of root-colonized microbial diversity. Rhizosphere microbes generally take part in environmentally integral functions and unique roles for better plant growth and development. Production of plant growth regulators, particularly IAA, has been observed to have an important role not only in plant growth and development but also in suppression of plant pathogens (Navarro et al. 2006). In addition, detoxification of chemical toxins accumulated in the rhizosphere is an important task of the microbes. For instance, it has been observed that xenobiotics were degraded by fluorescent pseudomonas (Zablotowicz et al. 1995; Khan 2005). Further, the rhizobacteria play an important role in nutrient cycling. Generally, N_2-fixing bacteria either freely (e.g. *Azospirillum* sp., *Acetobacter* sp.) or endophytically (e.g. *Rhizobium* sp. and related genera) in plant specialised structures like nodules convert N_2 into plant-available forms (i.e. NH_4^+). Also, ammonifiers and nitrifiers convert organic N compounds into plant-available forms. Organic acid

production by rhizobacteria contributes to solubilisation of unavailable phosphates to make them available. Endophytic microbial diversity that is determined by the intrinsic bacterial community has been proposed to modulate plant ethylene levels by the bacterial ACC deaminase which cleaves ACC into ammonia and α-ketobutyrate (Hardoim et al. 2007). This also happens when plants are subjected to abiotic stresses like salt and toxins (Arshad et al. 2007; Cheng et al. 2007).

Generally, diversified root microbiota is influenced by biophysical and/or host-derived metabolic cues. Thereby, the rhizospheric community is shaped up to be compatible to the association. For example, similar bacterial communities were observed in several cultivars belonging to *Oryza sativa* sp. *indica*, whereas those belonging to subspecies japonica and aromatica showed divergent community structures (Hardoim et al. 2011). Similarly, out of the 513 endophytic isolates, genetic background of the hybrid poplar clones has been observed to be correspondent well with the endophytic community structure (Ulrich et al. 2008). The most abundant genera amongst the isolates were *Pseudomonas* and *Curtobacterium*, whilst *Sphingomonas* also prevailed amongst the clones. However, the host genotype was found to have a limited effect on the root endophyte profile. Root endophytes were reported to have originated most likely from the microbiota reservoir of coexisting species present in natural soil, and, therefore, they represent a subset of microorganisms present in the soil (Buée et al. 2009). Recent findings have found out the dependence of diversified microbiome on soil type (Lundberg et al. 2012). Soil type has shown a marked influence on the microbial population of maize rhizosphere (Chiarini et al. 1998). Indeed the rhizosphere microbial density and community structure have been reported to vary significantly amongst the different sampling sites.

In general, microbial cells which face unfavourable environmental conditions transform into dormant stages such as cyst, spores and akinetes and form microbial seedbed with vast number of microbial species (Teeling et al. 2012). Induction of dormant microbes to resuscitation is an interchange amongst microbes as a response to dynamic biotic and abiotic factors in the environment. Direct soil application of developed microbial communities in biofilm mode has been shown to increase microbial diversity in the agroecosystems through breaking dormancy of microbial seed bank (Seneviratne and Kulasooriya 2013). That contributes to strengthen biodiversity–ecosystem functioning relationship, which leads to agroecosystem sustainability. In this chapter, we first review dormancy of soil microbes and its ramifications on diversity, and then we discuss the role of developed microbial biofilms in reviving dormant microbes from microbial seed bank for enhancing root-associated microbial diversity.

Dormant Microorganisms and Microbial Diversity

Life in its many forms does not exist in all places on earth, as it is kept under control by unprecedented fluctuations in environmental conditions which are most of the time suboptimal for the growth and reproduction of a particular living organism. Microorganisms, however, have shown the adaptability to survive under extreme

environmental conditions where life is thought to be impossible, such as extreme cold (Margesin and Miteva 2011; Koh et al. 2012), acidic hot springs (Dean et al. 2005), complete darkness and high pressure (Orcutt et al. 2011), and higher concentrations of elements such as arsenic (Erb et al. 2012) and sulphur dioxide (Du Toit et al. 2005), which are known as toxic to life, as well as that make microorganisms the most adaptable living organisms on earth. This trait is attributed to the vast adaptability of microorganisms, owing to their small volume/mass ratio, relatively small amount of genetic material (~1,000 kbp). Thus, the trigger of previously non-transcribed genes activated to survive in a changed environment can be observed even within genetically homologous population, creating subpopulations, susceptible and resistant. Even though, all forms of microbes do not fit to all places despite their pandemism and fecundity. Amongst the individual cells of genetically homogenous populations, a significant phenotypic variation occurs, because under a particularly stable set of conditions, certain genes are expressed in a non-uniform manner across a population of genetically identical bacteria (Avery 2006; Dubnau and Losick 2006). Such phenotypic heterogeneity allows the fittest to survive, when the others chose to die, yet many more chose to stay undercover (Veening et al. 2008). The presence of multiple subpopulations in dormant cells is an emerging theory of survival mechanisms in stressful environments (Sachidanandham and Yew-Hoong Gin 2009). Microbes enter a reversible state of low metabolic activity, which is known as dormancy, as a common response to environmental stress (Guppy and Withers 1999). Dormancy is broadly defined as any rest period or reversible interruption of the phenotypic development of an organism (Sussman and Douthit 1973). Dormancy is one of such stress-induced phenotypic bistable forms (Dubnau and Losick 2006), in which microbial cells as a measure of preservance turn into a protected, nondividing, slow growing, nongrowing or unculturable state despite being alive (Balaban et al. 2004; Lewis 2007; Nichols et al. 2008). Presence of dormant or viable, but not culturable (VBNC), bacteria and their role in ecosystem function have long been in debate (McDougald et al. 1998; Edwards 2000; Barcina and Arana 2009), within the arguable paradox of whether the bacteria actually senesce and die (Bogosian and Bourneuf 2001; Nyström 2001). Within the last three decades, advanced microscopic techniques have shown the existence of dormant stages (Meyer and Dworkin 2007) and, most importantly, have highlighted the significance of such dormant forms in sustaining the diversity of life in ecosystems (Rappé and Giovannoni 2003).

Indications of Microbial Dormancy

The understanding that only a small fraction of microbes can be cultivated and studied using conventional culturing methods sheds light on the presence of "microbial diversity way beyond practical calculation" (Wilson 2001). The immense phenotypic and genetic diversity found in soil bacterial and fungal communities makes it one of the most difficult communities to study (Ovreas et al. 1998). Exploring

microbial diversity has been compared to exploring outer space, as soil harbour a largely unknown microbial universe, where "more than 10^{16} prokaryotes live in a tonne of soil compared to 10^{11} stars in our galaxy" (Curtis and Sloan 2005). However, similar to astronomers' efforts on deducing the celestial objects of the universe using mathematical inferences, the abundance distribution and total diversity have been decoded, using improved analytical methods, despite the complexity of soil bacterial communities, which confines effective measurement. It has been suggested that at least 99 % of bacteria observed under a microscope cannot be cultured by conventional cultural techniques (Borneman et al. 1996; Giller et al. 1997; Pace 1997; Torsvik et al. 1998; Trevors 1998). Those unculturable bacteria could be in a physiological state that eludes our ability to culture them. It is doubtful whether the culturable 1 % is representative of the entire population or phenotypically and genetically different from the rest 99 % (Rondon et al. 1999). It is estimated that 1,500,000 species of fungi exist in the world (Giller et al. 1997), though many fungi cannot be cultured by current standard laboratory methods (van Elsas et al. 2000).

In contrast to conventional approaches, molecular microbiology leads to a deeper understanding of the biodiversity of soil microorganisms, hence validating theoretical assumptions derived by mathematical modelling. A wide range of fluorescent dyes have been used for the detection of soil microorganisms and their viability, as they would bind to the cell membrane to show intact cell membranes, as well as to indicate the presence of metabolic products (Tippkötter 1990). Combining fluorescence staining techniques with soil thin section technology has allowed obtaining images of microorganisms in situ (Li et al. 2004). Electron microscopy has been very useful in studying dormant endospores of environment samples (Laue et al. 2007; Laue and Bannert 2010). Epifluorescence microscopy-based methods have been effectively used to quantify the levels of microbial metabolic activity and total microbial biomass not only of aerobic bacteria but also of diverse groups of anaerobic bacteria, allowing rapid quantification of total and active bacterial numbers in complex soil samples without enrichment or cell elution. The microscopic methods have shown significant bacterial populations in all soils examined, and the biomass estimates to be several orders of magnitude higher than those obtained by conventional culture-based techniques (Bhupathiraju et al. 1999). When compared to typical epifluorescence microscopy, confocal scanning laser microscopy has been able to show a significant heterogeneity in a microbial biofilm (Chalmers et al. 1997).

Furthermore, DNA analytical methods such as fluorescence in situ hybridisation (FISH) are being applied to improve knowledge regarding the spatial distribution of microbiota in the complex soil matrix, localisation and identification of soil microorganism diversity in relation to the specific properties of their microhabitats and the interactions between the soil structure and microorganisms (Eickhorst and Tippkötter 2008). The DNA heterogeneity employed to understand the size and complexity of metagenome resembled a genome that is 4,000 times as large as the genome of a single bacterium, corresponding to about 4,000 completely different genomes of standard soil bacteria (Torsvik et al. 1990). A reanalysis of reassociation kinetics for bacterial community DNA from pristine soil has shown that a power law best describes the abundance distributions and more than one million distinct

genomes occur in pristine soils, exceeding previous estimates by two orders of magnitude (Gans et al. 2005). Metagenomic analyses have been able to demonstrate the microbial diversity of unculturable microbes (Rondon et al. 2000).

Analysis of genes coding for SSrRNA (16S and 18S rDNA) became popular in the 1990s as a culture-independent exploration of the soil microbial diversity of many ecosystems and is still widely used (Barns et al. 1994; Borneman et al. 1996). Studies have been performed with oligonucleotide probes labelled with a fluorescent dye to detect specific 16S rRNA sequences of uncultured bacteria in natural samples and to microscopically identify individual cells in various complex microbial associations ranging from simple two-component bacterial endosymbiotic associations to highly complex marine and soil communities (Amann et al. 1995). Combining methods of reassociation of denatured DNA to measure total genetical diversity, PCR-denaturing gradient gel electrophoresis (DGGE) analysis of rRNA genes to enumerate dominating bacterial populations, hybridisation with phylogenetic group-specific probes and sequencing to get information about affiliation of the bacterial populations showed that the diversity of the total soil community could be at least 200 times higher than the diversity of bacterial isolates from the same soil, indicating that the culturing conditions select for a distinct subpopulation of the bacteria, whilst bacterial communities present in the environment may contain more than 10,000 different bacterial types (Torsvik et al. 1998). Use of PCR primers specific for fungal 18S ribosomal RNA genes for soil DNA followed by DGGE has been successfully employed to analyse dynamics of fungal communities in the soil (van Elsas et al. 2000). An increasingly popular molecular method of detecting unculturables is reverse transcriptase (RT)-PCR, which detects gene expression, by very short living bacterial mRNA. Continued gene expression by unculturable cells is considered as an excellent indicator of bacterial cell viability (Conway and Schoolnik 2003). Culture-independent approaches have been extremely beneficial to study microbial communities in extreme environments. Multiplexed pyrosequencing of the 16S rRNA gene to examine soil- and cactus-associated rhizosphere microbial communities in a desert biome revealed that vast majority of operational taxonomic units were rare and unique to either soil or rhizosphere communities and are highly localised (Andrew et al. 2012). A theoretical model proposed by Jones and Lennon (2010) observed that rare bacterial taxa were disproportionately active relative to common bacterial taxa. Previously non-reported isolates of unculturable soil bacteria have been cultured using improved culture media (Janssen et al. 2002). Owing to these efforts, it is now known that in general, less than 1 % of the bacterial species in the soil are currently known (Torsvik and Øvreås 2007). What is not known is if this 1 % is representative of the bacterial population (Torsvik et al. 1998) when the number of bacterial species described so far is about 5,000 (Pace 1999). Often, there is a discrepancy between direct microscopic counts and numbers of culturable bacteria from environmental samples, indicating that only a minor part of the diversity of microorganisms in nature is known at present, whilst implying a vast seed bank of dormant microbes, although it may be an overestimation. Meta-analysis of bacterial richness estimates from a variety of ecosystems has suggested that bacterial richness far exceeds the richness levels typically observed for plant

and animal taxa. When it was considered that the apparent diversity of bacterial communities is influenced by phylogenetic breadth and allometric scaling issues, the levels of microbial diversity may appear less astounding (Fierer and Lennon 2011). This kind of knowledge would shift the paradigms regarding the microbial community dynamics in the future.

Advanced microscopic techniques have revealed many different phenotypes of dormant microbes, reflecting evolutionary diversity of microbial dormancy. These structures are physical differentiations of vegetative cells to more hardy resting bodies such as endospores. Those produced by Gram-positive bacteria when nutrients are limited can survive without nutrients and resist ultraviolet radiation, desiccation, temperature extremes and chemical disinfectants and even have been revived, cultured and identified from the abdominal contents of extinct bees preserved for 25–40 million years in buried Dominican amber (Cano and Borucki 1995). Cysts produced by bacteria, protists and nematodes tolerate harsh conditions to a certain level, though much susceptible than endospores. Encysted resting spores (conidia) of fungi stay alive amidst detrimental biotic and abiotic stresses arising latent infections. In marine environment, microbes metamorphose to a dwarf form, as a response to nutrient depletion, and, thus, still are able to dominate the ecosystem (Humphrey et al. 1983). In suboptimal nutrient condition, *Mycobacterium* species formed dormant ovoid forms of low metabolic activity and unable to culture on standard media plates, which showed elevated resistance to antibiotics and heat and when resuscitated transformed into typical rod-shaped cells (Anuchin et al. 2009). Dormancy is a way of cryptobiosis by microorganisms, which could be anhydrobiosis, anoxybiosis, chemobiosis, cryobiosis and osmobiosis. In addition to morphological changes, microbes under stress have reduced concentration of nucleic acids especially RNA, functional cellular components of lipids, fatty acids and proteins though reserves are accumulated (Lebaron et al. 2001). Proteins are employed to protect genetic materials and outer cell wall. Receptivity of cell membrane gets retarded and cell wall thickens. Stoichiometry of important biological elements gets altered. Energy use is diverted from cell membrane to support ultracellular structures and functions during dormancy (Shleeva et al. 2011). Symbiosis is reported as another dormancy tactic used by enteric microbes *Salmonella enterica* and *Escherichia coli* O157:H7 (Gourabathini et al. 2008). Some bacteria like *Pseudomonas aeruginosa* elucidate a phenotypic switching to biofilm as a response to environmental stimuli such as antimicrobials (Häußler 2004).

Ramifications of Microbial Dormancy

Microbial communities are central to human health, agriculture and most of the Earth's geochemical cycles, as well as the sustainability of ecosystems. They are reservoirs for the discovery of new drugs and metabolic processes. Thus, as with any other resource, extent of microbial diversity is important. The ability to persist or disperse in dormant states through unfavourable environments results in a voluminous

seed bank of a diverse range of coexisting species, showing a greater resistance to collapse, thus enriching microbial diversity. On the other hand, the capability to disperse beyond otherwise unsuitable environments becomes an important factor, determining biogeography of microorganisms (Locey 2010). Dormancy and resuscitation patterns of microorganisms of an ecosystem as a response to various biotic and abiotic factors influence the temporal patterns in the distribution and abundance of microbial taxa (Jed et al. 2006). Such periodically recurring distinguishably dominant microbes may have a significant influence on the ecosystem function as well as resistance to environmental change during a given time. For example, a previously unrecognised spore-forming *Bacillus* species was isolated and revived from a brine inclusion within a 250-million-year-old salt crystal from an underground saltern (Vreeland et al. 2000).

Dormancy of microorganisms is a key strategy of survival, and it ensures omnipresence and gives them the ability to coexist and co-evolve with all plant and animal lives on Earth. However, this trait of microbes becomes detrimental to humans when they are pathogenic to human and economically important plants and animals. Since dormancy is used to evade destruction by common preventive methods and a mean of resistance, most effective microbial control methods become useless and the consequences might be in serious jeopardy. Significance of dormancy of bacterial pathogens is immense (Sardessai 2005). For instance, a pioneering study on survival and viability of nonspore-forming bacteria *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environments documented the existence of "viable but nonculturable" (VBNC) state of bacteria (Xu et al. 1982), which is characterised by the presence of viable cells that are still alive but unable to grow on routine laboratory media, on which they would normally grow and form colonies (Oliver 2000). It is very important to know whether these VBNC cells formed in response to environmental stress are in a state of dormancy or in a stage preceding cell death. It is understood now that the state of dormancy induced by environmental stress in these nonspore-forming bacteria is reversible and also implies that it is an orderly and spontaneous adaptation to evade adverse conditions (Sachidanandham and Yew-Hoong Gin 2009). Upon resuscitation, these VBNC cells recover both culturability and pathogenicity (Oliver 2000). A study on *Helicobacter pylori* indicated that spinach-associated *H. pylori* cells can remain viable and virulent despite their lack of culturability. The pathogen cells rapidly became non-detectable by plating, although mRNA transcripts were detected 6 days after the cells were introduced to the spinach (Buck and Oliver 2010). This apparent decease and resuscitation of microorganisms is interesting to be noted here, which is useful to explain the disease outbreaks recurring once in a century or millennia of the human history (Krause 1992), although it is not soil ecosystem. For example, *Mycobacterium tuberculosis* has shown very efficient dormancy and recurrence tactics despite the diverse means of controlling the disease (Taneja et al. 2010). Recurrence of tuberculosis has occurred after years through resuscitation of dormant cells (Pai et al. 2000). Under suboptimal conditions, *Mycobacterium smegmatis* showed formation of unculturable cells in stationary phase and resuscitation (Shleeva et al. 2004), and latent or the dormant phase of *M. tuberculosis* infections represented the VBNC state pathogen (Young et al. 2009). It is now clear that the VBNC state is a survival strategy in response to harsh environmental

conditions, and it amounts to an important reservoir of pathogens in the environment (Lleò et al. 2007). The list of pathogens and nonpathogens which express VBNC state is ever increasing (Oliver 2005).

Dormancy plays a major role in failure of plant disease control measures. Dormancy of pathogens infecting staple food crops has created devastating problems in the past, causing massive famines, because of their ability to create postharvest diseases and thus affecting food security; e.g. European, Irish and Highland famines in the 1840s were caused by *Phytophthora infestans*, a pathogen of potato and tomato late blight. *Ralstonia solanacearum* has been reported to have a VBNC state involved in long-term survival and plant infection (Grey and Steck 2001), which can be induced with low temperature (Imazaki and Nakaho 2008). *Xanthomonas axonopodis* pv. *citri* which causes the most severe form of citrus canker disease enters the VBNC state after copper treatment and retains its virulence (Del Campo et al. 2009). *Erwinia amylovora*, causal agent of fire blight in pome fruits and other rosaceous plants, also uses dormancy as a tactic for survival. Regardless of temperature and copper and inoculum dose, VBNC cells of *E. amylovora* have been reported to recover culturability and pathogenicity inside mature apples calyces under some storage conditions (Ordax et al. 2009).

Not only in disease aspect but also in the case of industries, ubiquitous nature of microbial growth can be a problem when undesired. For an example, petroleum industry may be concerned of petroleum-degrading microbial growth, since crude oil production can be adversely affected quality and quantity wise and specialised conditions will be required during drilling, recovery and storage. Although conventional heterotrophic plate counts failed to show significant microbial activity, bioremediation activity of hydrocarbon contaminant degradation in soil by unculturable microorganisms has been observed via epifluorescence microscopy and soil carbon dioxide and methane measurements (Bhupathiraju et al. 1999).

Other than adverse consequences, dormant microorganisms may also have beneficial impacts on nature. Natural ecosystems show variable resistance to invasions by alien species, and this resistance relies on the diversity of species in the system. Soil microbial diversity is a key factor that controls the extent to which bacterial invaders can establish (van Elsas et al. 2012). In nutrient-poor ecosystems, the proportion of dormant bacteria can be accounted for up to 40 % of taxon richness (Jones and Lennon 2010).

Sustaining Microbial Diversity Through Dormancy

Microbes become dormant to evade unfavourable environmental conditions, thus being members of a microbial seed bank to sustain a minimal metabolic activity and revive if future conditions would be favourable. These dormant individuals determine the dynamics of the future microbial communities. A theoretical model suggested by Jones and Lennon (2010) demonstrates that the structure of microbial communities is shaped by environmental factors that trigger dormancy.

This theory has been supported by empirical data and simulations which show that the composition of active communities across the landscape is coordinated by regional environmental cues and dormancy, whilst at local scales, active microbes are decoupled from the total community. It implies that recurring transitions to and from the seed bank may help maintain the high levels of microbial diversity that are observed in nearly all ecosystems (Jones and Lennon 2010).

Dormancy can be viewed as the metabolic flexibility to changing conditions and physiological tolerance of an organism, which makes it resistant to environmental disturbances. Awakening of a previously unknown or a rare organism can change the composition of the community and thereby potentially affects ecosystem processes. A study on the impact of disturbance on the composition of bacterioplankton communities showed that the composition of the disturbed communities had changed due to the recruitment of phylotypes present in the rare biosphere of the original community (Sjöstedt et al. 2012). When the members of the rare biosphere become abundant in a bacterioplankton community after disturbance, those bacteria might play important roles in maintaining ecosystem processes. Dormancy is a way of keeping a large population in an environment of seemingly finite resources. A study on the abundance and activity of rare bacterial taxa of an aquatic environment suggests that though abundance follows activity in the majority of the taxa, a significant portion of the rare community is active, with growth rates that decrease as abundance increases, meaning that the numbers of individuals are kept higher at an inactive stage (Campbell et al. 2011).

Native microorganisms in environmental samples such as soil have shown the ability to survive in chemically contaminated environment even without previous exposure to pollutants (Caliz et al. 2011). In a nutrient-poor micro-environment, various stresses such as toxic concentrations of metal ions are able to render the bacteria unculturable within a few days. When the bacteria are relieved of stress factor, resuscitation occurs whilst preserving their fitness, major virulence gene markers and specific phenotypes (Aurass et al. 2011).

Resuscitation of Dormant Microorganisms and Its Consequences

It is fair to say that as dormancy is coerced by environmental factors, resuscitation of dormant microorganisms is also influenced by the state of environment present. Therefore, diversity of microbial community varies widely depending on both abiotic and biotic factors. In the case of plant-associated microorganisms, the effect of host plant on the diversity of microbial community in the ecosystem is well documented. In some situations, the soil and in others the plant type is the key factor determining soil microbial diversity and complex microbial interactions in soil, including interactions between microorganisms and plants (Garbeva et al. 2004). As a result of plants' influence on the spatial distribution of soil bacteria, the number of bacteria in the rhizosphere is about twofold larger than in bulk soil (Campbell and Greaves 1990).

Some fungi such as arbuscular mycorrhizal fungi (AMF) require a host plant; thus, their distribution in soil is also clustered around plant species. In addition to different symbiotic relationships, allelopathic effect by plants on soil microorganisms affects the distribution of soil microorganisms. Allelochemicals naturally released by *Acacia dealbata* Link, an Australian tree legume, modified soil bacterial functional diversity in a pine forest, leading to a significant reduction in bacterial richness and diversity in the forest soil (Lorenzo et al. 2012).

However, according to Fierer and Jackson (2006), bacterial diversity, unlike plant and animal diversity, is largely independent of geographic distance and unrelated to site temperature, latitude and other physical variables. Use of high throughput, culture-independent technologies such as terminal restriction fragment length polymorphism (T-RFLP) has demonstrated the equal effects of both the host plant and the soil depth that have on the bacterial community structure and the dynamics with changes in plant cover and environmental conditions (Kuske et al. 2002). Multiplexed pyrosequencing of rhizosphere microbial communities of a desert biome showed that diverse microbial communities were shaped primarily by edaphic variables, particularly soil pH and carbon content, associated with geographic locations, whilst rhizosphere associations are secondary factors (Andrew et al. 2012). The difference in the diversity and richness of soil bacterial communities amongst different ecosystems at a continental scale is attributed to soil pH. Bacterial diversity is highest in neutral soils and lower in acidic soils; most acidic soils show the least diversity (Fierer and Jackson 2006). As soil is heterogeneous, it contains many different microhabitats that are suitable for microbial growth, where bacteria are highly aggregated in soils, existing in clumps or "hot spots" (Kirk et al. 2004).

The two main factors which determine soil microbial community diversity, structure and functions, exert their effects in a complex manner (Meliani et al. 2012). Some soils have certain selectivity on the growth of disease-causing fungi, generally referred to as disease-suppressing soils. Studies on mechanisms of soil biostasis/soil fungistasis suggest that specific components of the microbial community regulate the growth and development of fungal propagules to a certain extent, hindering emergence of fungi by withdrawal of nutrients from fungal propagules and production of fungistatic compounds (Garbeva et al. 2011). Regulation of resuscitation of one microorganism by another microorganism has been widely observed. The presence of a tiny proportion of viable cells at the beginning of resuscitation facilitates the recovery of the majority of the remaining (dormant) cells. This is known as "population effect". In this, recovery is due to the excretion of some factor(s) which promotes the transition of cells from a state in which they are incapable of growth and division to one in which they are capable of colony formation (Votyakova et al. 1994). In natural environments, most organisms live as a part of a community in which distinct cells work in harmony and communicate either by trading metabolites, by exchanging dedicated signalling molecules (e.g. QS molecules) or by competition for limited resources (West et al. 2007). Some biofilms have shown to be effective to promote the survival of microbes as dormant state, where they are protected from harsh conditions by extra polymeric substances of the biofilm. Beach sediment biofilms on supratidal sands have been favourable for the incorporation and

persistence of enterococci (Piggot et al. 2012). Cell-to-cell signalling helps biofilm populations of opportunistic pathogens to sustain in harsh environmental conditions such as antibiotics (Popat et al. 2012). Furthermore, a higher relatedness amongst the individuals in a community favours cooperative QS and hence leads to higher growth (Rumbaugh et al. 2012). Therefore, the growth of mixed populations offers great potential for dormant microorganisms to re-emerge. The presence of more beneficial microorganisms in the initial population of viable cells is likely to promote the resuscitation of more beneficial microbes by "kin selection".

Implications of Global Change on Soil Microbial Diversity

Stress of climatic change is a major driving force of evolution. The development of the soil ecosystem throughout 70,000 years of ecosystem progression promoted the development of distinctive microbial communities that were reminiscent of successional processes (Tarlera et al. 2008). The effects of climate change on living organisms have been shown primarily on regional and global scales. As soil bacteria are important contributors to nutrient cycling and hence primary productivity in ecosystems, changes in the composition of their population due to changes in environmental conditions may greatly affect the sustainability of sensitive ecosystems. Rapid responses of bacteria to sudden changes in their environment will heighten the importance of dormant microorganisms because it gives opportunity for subpopulations to emerge and spread fast. With the changing climate, rapid and high-intensity short-duration rainfalls are expected. Consequent rewetting of soil can lead to change of structure and composition of soil microbial community given the fact that different taxa respond in different speed to rewetting and subsequent CO_2 pulses from soil (Aanderud and Lennon 2011). The CO_2-induced resuscitation of the indigenous microbial community, resulting from the first rainfall after the dry summer in Mediterranean ecosystems, has been reported to be so large that the CO_2 released in a few days is comparable in magnitude to the annual net carbon exchange of many terrestrial ecosystems (Placella et al. 2012). This implies the influence of functional traits of microbes such as dormancy on the structure and function of microbial communities under dynamic soil moisture regimes, which might highly affect soil carbon dynamics in a global change context.

Manipulating environments to make conditions that relate to production, remediation and accommodation favourable to human is a practice as old as the human civilization. Though it is difficult to quantify the effect of land-use change and management practices on soil microbial community and their subsequent influence on soil function, molecular methods employed to monitor the effects of perturbations due to anthropogenic activities and pollution on microbial communities show that agricultural management, fish farming and pollution may lead to profound changes in the community structure and a reduction in the bacterial diversity (Torsvik et al. 1998). Different land-use practices employed to

improve soil properties such as soil organic carbon, total nitrogen, soil texture, pH and soil enzymes have indeed caused them to vary significantly across land-use regimes whilst consistent with shifts in soil microbial community structure and composition (Shange et al. 2012). The bacterial diversity of agricultural soils is rich in species compared to the forest soil which is phylum rich, yet shows more archaeal diversity (Roesch et al. 2007).

With the advancement of industries, pollution of soil with heavy metals, slowly degrading polymers and toxic hydrocarbons has rendered severe damages to the ecosystems around the world. Metal pollution of soil as a result of anthropogenic activities showed reduced soil microbial diversity by more than 99.9 % (Gans et al. 2005). In the future, soil will be a major sink for engineered metal oxide nanoparticles (ENPs). Nano-TiO_2 and nano-ZnO, two widely used ENPs, significantly altered the bacterial communities known to be associated with nitrogen fixation, methane oxidation and decomposition of recalcitrant organic pollutants and biopolymers including protein, indicating potential consequences to ecosystem processes, through effects on susceptible, narrow-functioning bacterial taxa (Ge et al. 2012). Revival of depleted microbiome to maintain ecosystem sustainability will be the challenge for novel biotechnological approaches in future. Biofilms made of beneficial microbes will be a competent candidate in the sense that self-generated diversity in biofilms acting as a form of biological insurance can safeguard the community in the face of adverse environmental conditions (Boles et al. 2004). Thus, if we can convert soil microbial community to a more biofilm mode, it will help protect the microbes from events like climate change.

Regulating Plant Root-Associated Microbial Diversity by Applying Developed Microbial Biofilms

This is a novel concept that is being experimented and introduced to mainly agriculture. Microbes being the focal point of the ecosystem can regulate all other biotic and abiotic components. Therefore, their application particularly in an effective mode like microbial biofilms can manipulate microbial diversity of the soil–plant system. Development and application of the biofilms and their actions in the rhizosphere are described below.

In Vitro Development and Application of Biofilms

Biofilms are complex multicellular and multispecies assemblies attached to each other and also to surfaces from self-secreted exopolysaccharides. They are ubiquitous in nature. In various ecosystems, they are stable and resist to various biotic and abiotic stresses to keep up their functions, especially via QS. There is an

increasing trend in studying microbial biofilms, since it provides efficient and effective symbiotically optimum functions. Biofilms have unique properties over mono- or mixed cultures of their resident microbes. Development of biofilms by incorporating N_2-fixing bacteria and rhizosphere fungi is a newly introduced biotechnology to biofertilizer use in agriculture (Seneviratne et al. 2008a). They are now known as biofilmed biofertilizers (BFBFs). The N_2 fixers maintain high cell densities on root hairs of nonlegumes, forming biofilms called pseudonodules fixing N_2 biologically (Seneviratne 2009). Growth enhancement of crop plants with the application of the BFBFs is attributed to different mechanisms. The colonization of biofilmed microbes in the rhizosphere provides an excellent metabolic cooperation to enhance the plant growth. The biofilm microbes release higher amounts of organic acids than their monocultures, and they enhance mineralization of soil nutrients in the rhizosphere (Seneviratne and Jayasinghearachchi 2005). Some of the organic acids are plant growth promoting hormones (Seneviratne et al. 2008b). Therefore, there is a great contribution of this technology to crop production in agriculture.

Biofilm Actions in Rhizosphere

Outcomes of plant–microbe interactions benefit plant growth in different ways as discussed above, and these interactions are significantly influenced by the conformation of adherent microbial populations in the soil–plant system. In addition, rhizodepositions help colonize microorganisms and biofilm formation (Walker et al. 2004). Initially, formation of microcolonies coordinates amongst the colonies via QS signalling for the development of multicellular assemblages (Whitchurch et al. 2002). It has been reported that the plant growth promoting rhizobacteria (PGPR) when found in multicellular assemblies like biofilms not only enhance plant growth but also protect plant from soilborne pathogens (Rudrappa et al. 2008). Importance of biofilm formation of rhizobacteria has been described in terms of plant growth and development and other beneficial biological functions in the micro-environment of plants (Ramey et al. 2004; Seneviratne et al. 2011). Developed microbial communities in biofilm mode have been observed to produce a higher number of diverse organic compounds that enhance availability of organic substrates to dormant microbial cells for resuscitation (Seneviratne and Kulasooriya 2013). Further, the production of secondary metabolites and other physiological and biochemical processes of bacteria are known to influence population density of microorganisms (Johnson et al. 2005; Barnard et al. 2007). Population density dependent cell-to-cell communication via QS is reported to allow resuscitating cells to break dormancy of other dormant cells (Lennon and Jones 2011). In biofilm formation, QS is a prerequisite, which helps establish the biofilm. Therefore, the role of the introduced biofilms in breaking dormancy of the microbial seed bank in this manner is also obvious. The resuscitation of soil microbial seed bank with the application of the biofilms in the form of BFBFs was experimentally demonstrated when a soil was applied with them in combination with some nutrients in the form of

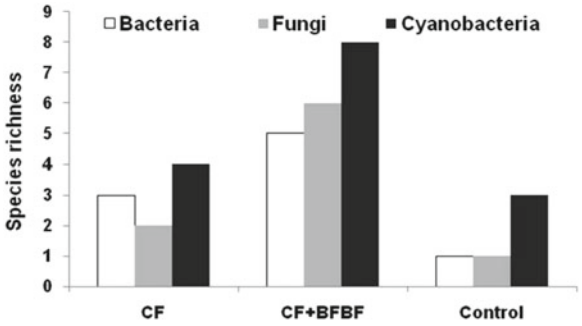

Fig. 13.1 Effect of developed biofilms in biofilmed biofertilizers (BFBFs) mode in combination with nutrients in the form of chemical fertilizers (CF) on increasing microbial diversity of bacteria, fungi and cyanobacteria. Application of CF + BFBF showed the highest species richness of the microbes in comparison to CF alone and the control with no amendments

chemical fertilizers (Fig. 13.1). In this study, it was clearly observed that the biofilm mode increased species richness and hence diversity of bacteria, fungi and cyanobacteria in comparison to chemical fertilizers alone application.

Issues related to dormancy of pathogens would not arise in this approach, because biocontrolling agents of common pathogens tend to naturally emerge, thus establishing a balance of the microbial diversity (Seneviratne 2012). These processes contribute to strengthen biodiversity–ecosystem functioning relationship (Langenheder et al. 2010), which leads to ecosystem sustainability (Tilman et al. 1996).

Conclusion

In this chapter, we discussed how the diversity of root-associated microbial community is moderated and the role of introduced community of microbes in the form of developed microbial biofilms in increasing the diversity of the microbial community. Vast majority of different microorganisms exist as dormant microbial cells in a voluminous seed bank in soil, since dormancy is a bet-hedging tactic employed by microorganisms to evade unfavourable circumstances. These dormant microbial cells could be resuscitated to become active cells by introducing beneficial communities of microorganisms in the form of developed biofilms which create favourable micro-environmental conditions, such as enhanced availability of a wide spectrum of organic substrates in soil. Quorum sensing (QS) that aids biofilm formation of rhizobacteria also supports spontaneous resuscitation of dormant microbial cells. Proportion of the active and dormant cells determines the levels of microbial diversity, ecosystem functioning and sustainability. Above processes are summarized and depicted in Fig. 13.2. Agricultural ecosystems which are heavily depleted in microbial diversity due to current agronomic practices could thus be restored with the application of the developed microbial biofilms. Accordingly, all

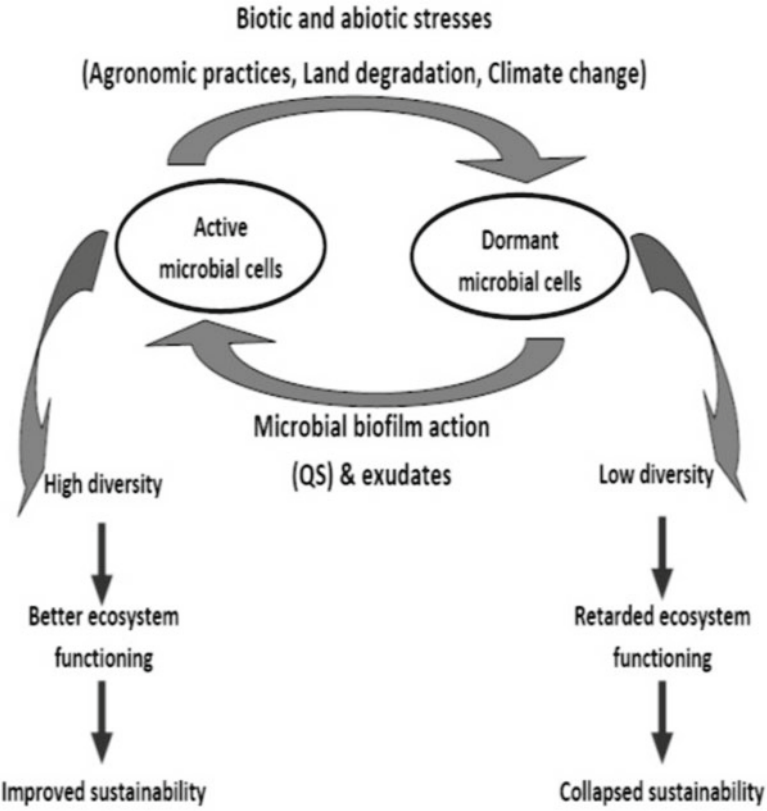

Fig. 13.2 The interchange of dormant and active stages of microbes in the soil seed bank. Transition of metabolically active cells into dormant cells is a response to biotic and abiotic stresses induced by the human and the environment. Developed biofilms switch dormant cells to become metabolically active cells, thus improving sustainability in agroecosystems through increased microbial diversity and ecosystem functioning

plant-associated biological functions steered by the diversified microbiome could be achieved in this manner, which should heighten the importance of community approach of soil microbial inoculations in agriculture, ultimately leading to sustainable agroecosystems.

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Chapter 14
Secondary Metabolites of *Pseudomonas aurantiaca* and Their Role in Plant Growth Promotion

Samina Mehnaz

Contents

S. Mehnaz (✉)
Department of Biological Sciences, Forman Christian College University, Zahoor Elahi Road, Lahore 54600, Pakistan
e-mail: saminamehnaz@gmail.com; saminamehnaz@fccollege.edu.pk

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_14, © Springer India 2013

Abstract Most of the fluorescent pseudomonads isolated from plant rhizosphere promote plant growth by direct and indirect mechanisms. These bacteria produce phytohormones and promote plant growth directly. In addition, they produce secondary metabolites which inhibit the growth of pathogenic bacteria and fungi and promote plant growth indirectly. Among fluorescent pseudomonads, *Pseudomonas aurantiaca*, a subspecies of *Pseudomonas chlororaphis*, is known to produce antibiotics with antifungal activity. Strains of *P. aurantiaca* have been isolated from sugarcane, soya bean, canola, soil, and municipal sludge in different parts of the world including North America, Europe, and Asia. These strains are reported to produce IAA, HCN, siderophores, phenazines, cyclic lipopeptides, pyoverdin, and quorum-sensing signaling compounds. Most of these strains have shown antifungal activity against several pathogenic strains of *Fusarium*, *Pythium*, *Colletotrichum*, *Rhizoctonia*, and *Sclerotium* sp. One of these *P. aurantiaca* strain SR1 has been proven as a plant growth promoter for several crops. In this manuscript, a review of all reported strains of *P. aurantiaca* and their growth-promoting abilities is presented. The main focus is on secondary metabolites and mechanism used by these metabolites to promote plant growth, with a suggestion that this bacteria can be used as a biofertilizer and a biocontrol agent in the near future.

Introduction

Biomolecules such as nucleic acids, proteins, and lipids are essential for the existence of life. These are primary metabolites – products of primary metabolism. With the passage of time, when organisms are getting mature, they start operating additional metabolic pathways to synthesize secondary metabolites – products of secondary metabolism. These compounds are not essential for normal life activities and produced in small quantities as compared to primary metabolites. Sometimes, they have a role in the defense against microorganisms or insects and pests. Some secondary metabolites are produced in response to the attack of a pathogen.

Bacteria produce secondary metabolites at the stationary phase of the growth. Most of these compounds are secreted in the growth medium and easily extractable. The biosynthesis of these compounds is dependent on the growth stage and growth conditions. Production of secondary metabolites can be increased or decreased by changing growth conditions and media compositions. Among bacteria, pseudomonads are well known for the production of secondary metabolites. These metabolites play a major role in the defense mechanism of the producer itself and also help to the plants with which they are associated. Among pseudomonads, secondary metabolites produced by fluorescent *Pseudomonas* spp. are well

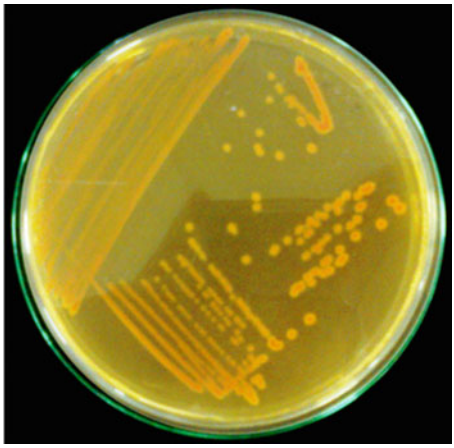


Fig. 14.1 *Orange* color colonies of *P. aurantiaca* PB-St2 on LB medium

studied. Isolation and identification of these metabolites and the genes involved in their biosynthesis have been characterized. Fluorescent pseudomonad species such as *Pseudomonas fluorescens*, *P. aeruginosa*, *P. aureofaciens*, *P. putida*, and *P. pyrrocinia* have been demonstrated to show antifungal activity with varying degrees of antagonism (de Weger et al. 1986). The antifungal/antibacterial activity of pseudomonads is traced back to the production of following metabolite classes: phenazines, 2-4-diacetyl phloroglucinol, pyrrolnitrin, pyoluteorin, cyclic lipopeptides (CLPs), and rhizoxin (Liu et al. 2007; Loper et al. 2008).

Phenazine-type antibiotics, heterocyclic nitrogen-containing brightly colored pigments, are especially active against lower fungi and most Gram-positive and Gram-negative bacteria. They play a vital role in biological control. In addition, some phenazines were shown to play a role in ecological fitness (Chin-A-Woeng et al. 2003). CLPs are also produced by several plant-associated *Pseudomonas* spp., including pathogenic *P. syringae*, *P. tolaasii*, *P. fuscovaginae*, *P. corrugata*, and *P. fluorescens*, and by multiple strains classified as antagonistic *P. fluorescens* and *P. putida* (Raaijmakers et al. 2006). CLPs are versatile molecules with antimicrobial, cytotoxic, and surfactant properties. For the antagonistic *Pseudomonas* spp., CLPs play a key role in antimicrobial activity, motility, and biofilm formation. In particular, the studies with viscosinamide produced by the antagonistic *Pseudomonas* strain DR54 provide several lines of evidence that CLPs are important constituents in the biological control of plant-pathogenic fungi (Thrane et al. 1999).

Recently, Peix et al. (2007) reclassified *P. chlororaphis* into three subspecies, namely, *P. chlororaphis*, *P. aureofaciens*, and *P. aurantiaca*. Previously these were treated as independent species of *Pseudomonas*. Production of secondary metabolites specifically phenazines is well known in all subspecies of *P. chlororaphis*. *P. aurantiaca* produces orange colonies, and this orange color is due to the production of phenazines, one of the secondary metabolites (Fig. 14.1). In this manuscript, the focus is *P. aurantiaca*, its secondary metabolites, and its role in plant growth promotion. Strains of *P. aurantiaca* have been isolated from all over the world

Table 14.1 List of the *Pseudomonas aurantiaca* strains, their host or source of isolation, country of origin, and references

Strain	Source	Country	References
S1	Municipal sludge	Belarus	Mandryk et al. (2007)
SR1	Soybean rhizosphere	Argentina	Rosas et al. (2001)
PB-St2	Sugarcane stem	Pakistan	Mehnaz et al. (2009)
BL915	Soil	Switzerland	Nowak-Thompson et al. (2003)
IB5-10	Coastal sand dune	Korea	Park et al. (2012)
DF200	Canola stubble	Canada	Fernando et al. (2005)

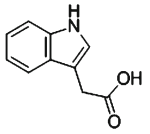
(Table 14.1). Researchers, who have isolated these strains, have reported the secondary metabolites production and their use as a biological control and a biofertilizer. In this manuscript, the information has been compiled.

Secondary Metabolites of *P. aurantiaca*

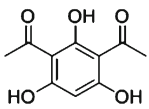
A complete list of secondary metabolites of *P. aurantiaca* which has been published up until now and their chemical structures are provided (Fig. 14.2). Studies involving the use of these strains as a biofertilizer and a biocontrol agent for different crops have also been included. More than 20 secondary metabolites have been included in this list. As the purpose of isolation and usage of these strains is different for every researcher, therefore the author could not find the production of all metabolites in all strains. It does not indicate that these strains are not capable to produce those secondary metabolites; rather these are not analyzed for this purpose. Most of the compounds included in this list are produced by an endophytic strain PB-St2, isolated by the author herself. Isolated PB-St2 has been thoroughly investigated for the production of secondary metabolites. Information about most of its secondary metabolites has been published separately (Mehnaz et al. 2009, 2013); some unpublished information have been included in this manuscript. Complete profile of PB-St2 secondary metabolites is not characterized yet. Name of the compound and the strains which are reported for its production are provided in the following text. Detailed information about these compounds, strains, and their biocontrol/biofertilizer activity can be found in the given references.

Indole-3-Acetic Acid (IAA)

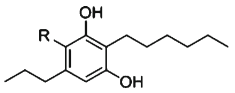
Auxins are the group of phytohormones that are well known for plant growth promotion. Among auxins, indole-3-acetic acid is commonly produced by plant growth-promoting rhizobacteria (PGPR). After nitrogen fixation, it is the second most important trait of PGPRs, responsible for direct growth promotion of



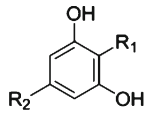
Indole-3-Acetic Acid (IAA)



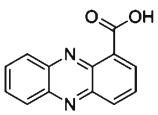
2, 4-Di Acetyl Phloro Glucinol (2, 4- DAPG)



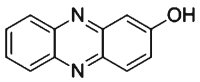
2-Hexyl, 5-Propyl Alkyl Resorcinol (HPR)



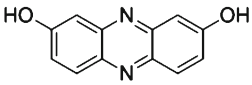
2, 5- Di Alkyl Resorcinol (DAR)



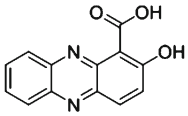
Phenazine-1- Carboxylic Acid (PCA)



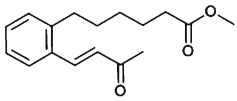
2- Hydroxy Phenazine



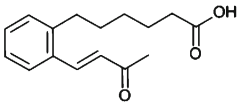
2, 8- Di Hydroxy Phenazine



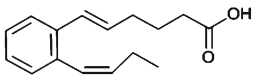
Hydroxy Phenazine -1- Carboxylic Acid



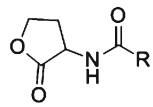
Lahorenoic acid A



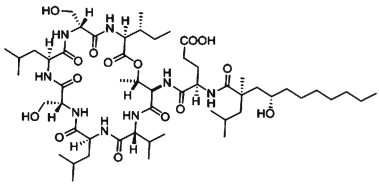
Lahorenoic acid B



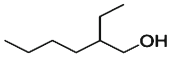
Lahorenoic acid C



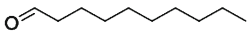
N-Acyl Homoserine Lactone (N-AHL)



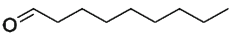
Viscosin/WLIP



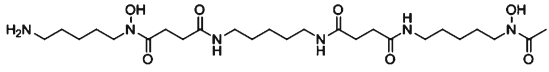
2-Ethyl-1-Hexanol



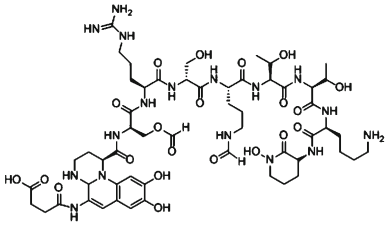
n-Decanal



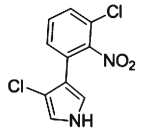
Nonanal



Hydroxamate type siderophore



Pyoverdin



Pyrrolnitrin

Fig. 14.2 Structure formulas of the compounds produced by different strains of *P. aurantiaca*

inoculated plants. Several species of *Pseudomonas* are known for its production, and among them, most commonly known are *P. putida* and *P. fluorescens*. IAA production at different rate is known among most of the strains of *P. aurantiaca* (Andres et al. 2011; Mandryk et al. 2007; Mehnaz et al. 2010).

2,4-Diacetylphloroglucinol (DAPG)

This antibiotic has wide antifungal, antibacterial, antihelminthic, nematicidal, and phytotoxic activity (Cronin et al. 1997; Raaijmakers et al. 2002). DAPG production by *P. aurantiaca* is reported in strain SR1 (Andres et al. 2011). The antibiotic was characterized by using thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and spectrometric techniques. Andres et al. (2011) reported the antifungal activity of this compound against phytopathogen *Macrophomina phaseolina*. Production of DAPG by SR1 in rhizosphere soil was also confirmed.

Alkylresorcinols (HPR and DAR) and Pyrrolnitrin

A systematic antifungal screening program of Syngenta natural products research group in Switzerland demonstrated that *P. aurantiaca* produces various antifungal compounds including 2-hexyl, 5-propyl alkylresorcinol (HPR). Nowak-Thompson et al. (2003) performed a detailed study on BL915, one of the *P. aurantiaca* strains, and reported the isolation of 2,5-dialkylresorcinol (DAR), an analogue of HPR. The authors characterized the biosynthetic pathway and gene cluster responsible for the production of this compound. BL915 was initially identified as *P. fluorescens*, and production of pyrrolnitrin by this strain was reported (Hill et al. 1994). Hill et al. (1994) characterized a gene involved in the synthesis of pyrrolnitrin and proved the strain as a strong biological control agent for *Rhizoctonia solani* (causes damping-off in cotton), due to pyrrolnitrin production as mutant strain could not inhibit the fungal growth.

$C_{18}H_{36}NO$ and $C_{20}H_{31}O_3$

P. aurantiaca S1 strain was isolated in Belarus, from municipal sludge containing cellulose and lignin. Mandryk et al. (2007) have isolated two compounds $C_{18}H_{36}NO$ and $C_{20}H_{31}O_3$ of mass 282.3 and 319.3, respectively, from this strain. These compounds were identified on the basis of QTOF-MS, and a proper name has not been assigned to them. $C_{18}H_{36}NO$ is a cyclic aromatic N-containing substance and corresponds to the new variety of pyo compounds (Leisinger and Margrafft 1979), but $C_{20}H_{31}O_3$ did not match with any reference compound in database. These compounds showed potential of being used as biological control agent against plant pathogens. Antibacterial activity against *P. syringae* pv. *glycinea* was shown by $C_{18}H_{36}NO$, and antagonistic activity against *Fusarium oxysporum* was observed by $C_{20}H_{31}O_3$. S1 strain also produced IAA and siderophores.

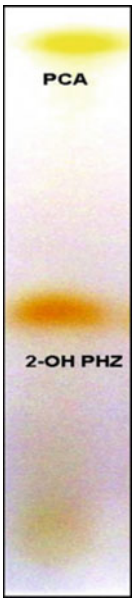


Fig. 14.3 Separation of two secondary metabolites phenazine-1-carboxylic acid (PCA) and 2-hydroxyphenazine (2-OH-Phz) from crude extract of *P. aurantiaca* PB-St2 by using *TLC*

Phenazine-1-Carboxylic Acid and 2-Hydroxyphenazine (PCA and 2-OH-Phz)

These compounds have been reported from two strains of *P. aurantiaca*, PB-St2 and IB5-10. PB-St2 was isolated from a stem of a local variety of sugarcane growing in Punjab, Pakistan (Mehnaz et al. 2009), and IB5-10 was isolated from a coastal sand dune in east coast of Korea. PCA and 2-OH-Phz are major secondary metabolites of PB-St2 (Fig. 14.3). PCA showed antifungal activity against *Phytophthora capsici*, *R. solani*, and *Pythium ultimum*, and 2-OH-Phz was active against *R. solani* (Park et al. 2012). Antifungal activity against *Colletotrichum falcatum* and antibacterial activity against human pathogen *Mycobacterium tuberculosis* have also been reported by PCA (Mehnaz et al. 2013).

2,8-Dihydroxyphenazine and 2-Hydroxyphenazine, 1-Carboxylic Acid (2,8-Di OH-Phz and 2-OH, 1-CA)

These compounds have been recently isolated from *P. aurantiaca* PB-St2 (Mehnaz et al. 2013). Calculated masses for 2,8-dihydroxyphenazine ($C_{12}H_9N_2O_2$) and 2-hydroxyphenazine, 1-carboxylic acid ($C_{13}H_8N_2O_3$) are 213.0664 and 240.0535, respectively. These are intermediate compounds, produced in the biosynthetic pathway of 2-OH-Phz and PCA (Chin-A-Woeng et al. 2003). 2,8-Di OH-Phz showed antibacterial activity against human pathogen *Bacillus cereus* and *Arthrobacter crystallopoietes* (Mehnaz et al. 2013). Production of these compounds is not reported from any other strain of *P. aurantiaca*.

Lahorenoic Acids A, B, and C

These compounds are ortho-dialkyl-substituted aromatic acids. These have been isolated from *P. aurantiaca* strain PB-St2 (Mehnaz et al. 2013). Structure formulas of these compounds are based on NMR data, and masses were calculated by ESI-MS m/z $[M+Na]^+$ and these are $C_{17}H_{22}O_3$ (297.2), $C_{16}H_{20}O_3$ (283.1), and $C_{16}H_{20}O_2$ (267.1) for Lahorenoic acids A, B, and C, respectively. Details about these compounds are available in Mehnaz et al. (2013). Antifungal activity of these compounds has not been checked yet. Searching database for structure formulas of these compounds ended up with some similarity with rubrenoic acid as a reference compound. As similarity with the reference compound was not 100 %, these compounds are named by the authors as Lahorenoic acid based on the name of the city of origin for strain PB-St2.

Viscosin/WLIP

Viscosin and WLIP (white-line-inducing principle) are CLP. CLPs produced by pseudomonads are composed of a fatty acid tail linked to a short oligopeptide, which is cyclized to form a lactone ring between two amino acids in the peptide chain. Viscosin is a cyclic lipodepsipeptide with structure formula $C_{54}H_{95}N_9O_{16}$. WLIP also has the same formula. Difference between the two compounds is that WLIP has D-leucine and viscosin has L-leucine. It is a major secondary metabolite of *P. aurantiaca* PB-St2 (Mehnaz et al. 2013). Production of viscosin has been reported by *Pseudomonas libanensis*, *P. fluorescens*, and other species of pseudomonads (Saini et al. 2008), and production of WLIP is reported by *Pseudomonas reactants* and *P. putida* (Mortishire-Smith et al. 1991; Rokni-Zadeh et al. 2012), but *P. aurantiaca* is not known previously for the production of viscosin or WLIP. Currently the author is working on experiments to make a final conclusion about its structure whether it is viscosin or WLIP. The role of lipopeptides in antagonism against viruses, bacteria, fungi, mycoplasmas, and oomycetes has been described in detail by Raaijmakers et al. (2010). Specifically the "antifungal activity" has been studied for many different CLPs and for a wide variety of plant and human-pathogenic fungi and yeast.

Nonanal, N*-Decanal, and 2-Ethyl, 1-Hexanol*

Pseudomonads are capable of producing organic volatile compounds, and their antifungal activity has also been demonstrated (Fernando and Lindermann 1994). Nonanal, *N*-decanal, and 2-ethyl, 1-hexanol are volatile organic compounds, and they showed antifungal activity against *Sclerotinia sclerotiorum*. Production and antifungal activity of these compounds have been reported by *P. aurantiaca*

strain DS200 (Fernando et al. 2005), an isolate from canola stubble. These compounds have been isolated from other species of pseudomonads as well, including *P. fluorescens* and *P. chlororaphis* (Fernando et al. 2005), but not from any other strain of *P. aurantiaca*.

HCN

It is a volatile antibiotic produced by several PGPRs. The compound inhibits the cytochrome oxidase of microorganisms. Cytochrome oxidase of HCN producers is resistant to cyanide and insensitive to HCN (Rudrappa and Baiss 2008). BL915, SR1, and PB-St2 strains of *P. aurantiaca* are reported as HCN producers (Gaffney et al. 1994; Mehnaz et al. 2009; Andres et al. 2011).

Siderophores

These are low molecular weight iron-binding molecules which have very high affinity for ferric ion. These molecules bind to the ferric ion, available in the rhizosphere, and make it unavailable to the pathogenic organism so these pathogens cannot proliferate. Some siderophore producers have a special mechanism to uptake the siderophore-iron complex. This complex binds to a specific receptor and then it is taken up by the producers themselves (O'Sullivan and O'Gara 1992). On the other hand, some plants have a special system to absorb the siderophore-iron complex and release it inside so plant can use this iron (Wang et al. 1993). In both ways, it helps to decrease the iron availability to phytopathogen and indirectly promotes the plant growth. Siderophore production is reported for S1, SR1, and PB-St2 strains of *P. aurantiaca* (Mandryk et al. 2007; Mehnaz et al. 2009; Andres et al. 2011). PB-St2 produces hydroxamate-type siderophores (Mehnaz et al. 2009). For other strains, the information about type or nature of siderophores is not available.

Pyoverdin

It is a yellow green, iron-chelating siderophore which fluoresce under UV, produced by fluorescent pseudomonads, under iron-deficient environment. Previously, it was known as fluorescein. The pyoverdin molecule has a quinoline chromophore, which is responsible for color, bound to a peptide chain and a dicarboxylic acid or a dicarboxylic amide. Production of this compound has been reported for several pseudomonads including *P. chlororaphis* and *P. aurantiaca*. PB-St2 produces the compound in enormous amount, and the gene involved in its biosynthesis has also been detected

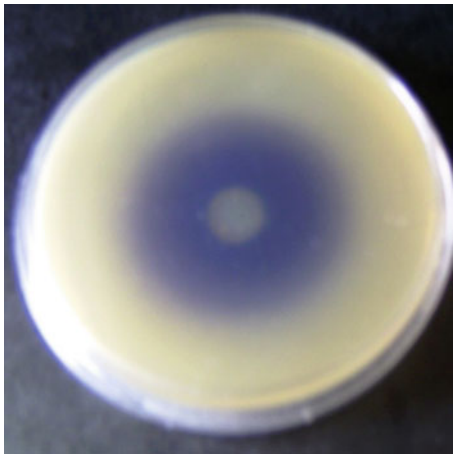


Fig. 14.4 Detection of quorum-sensing signaling compounds (AHL) produced by *P. aurantiaca* PB-St2 on LB medium containing AHL indicator strain *Chromobacterium violaceum* CV026

(unpublished results; communicated by S. Mehnaz). Isolation and characterization of pyoverdin in rest of the *P. aurantiaca* strains have not been reported or carried out. Involvement of pyoverdin (produced by *P. aeruginosa* 7NSK20) in suppression of damping-off of tomato plants, induced by *Pythium* sp., has been reported by Buysens et al. (1996).

Acyl Homoserine Lactones (AHL)

These are known as signal compounds which are responsible for the quorum-sensing (QS) mechanism. Many bacteria regulate the production of antifungal compound through quorum sensing. These molecules consist of a homoserine lactone ring linked via saturated or unsaturated acyl chain and with or without a keto or hydroxyl substituent at C3 position. Production of hexanoyl homoserine lactone (HHL) is reported in two *P. aurantiaca* strains, PB-St2 and B-162 (Fig. 14.4) (Feklistova and Maksimova 2008; Mehnaz et al. 2009).

Cyclo (L-Pro-L-Val)

Park et al. (2012) have isolated this compound from *P. aurantiaca* isolate IB5-10 and also reported its antifungal activity against *R. solani*. Production of this compound is reported in other bacterial strain, but it was always under discussion whether it is a natural product or an artifact. Mehnaz et al. (2013) have discussed this point in detail, and it has been proven as an artifact which is produced due to autoclaving of LB medium. Park et al. (2012) also cultivated IB5-10 in LB medium which creates the doubt about its production as a natural product of *P. aurantiaca*.

Role in Plant Growth Promotion

Direct Mechanisms

P. aurantiaca possesses several mechanisms, including the direct and indirect ones, to promote plant growth. *P. aurantiaca* is not a nitrogen fixer, but IAA production is known for all those strains which were assayed for auxins production. Phosphate solubilization is observed in SR1 and PB-St2 strains. 1-Amino, cyclopropane-1-carboxylate (ACC) deaminase enzyme has been detected in PB-St2. *P. aurantiaca* SR1 strain has been extensively studied for its growth-promoting activities through inoculation in different crops. Before going for long-term inoculation experiments, colonizing ability of this strain was studied in alfalfa, soybean, and wheat. Population density of this strain was in the range of 10^5 CFU/seed for these crops (Andres et al. 2011). Endophytic behavior of SR1 is also reported for several crops (Carlier et al. 2008; Rosas et al. 2005, 2009).

IAA Production

IAA production in SR1 was estimated, and it was noticed that production was maximum (11.7 μg/ml) in 24-h-old culture and later on it decreased. Production of IAA in PB-St2 was quantified by HPLC after 1-week growth. The amount was very low (0.15 μg/ml) and may be due to estimation after 7 days as it might be degraded in a week's time. After nitrogen fixation, IAA is considered as a major mechanism involved in plant growth promotion. IAA produced by root/rhizosphere-colonizing microbes is proposed to act in conjunction with endogenous IAA to stimulate cell proliferation and/or elongation and enhance the uptake of minerals and nutrients by plants, from the soil (Patten and Glick 2002; Suzuki et al. 2003). The growth of plants inoculated with IAA-producing bacteria is affected by the amount of IAA that the bacterium produces. Thus, bacteria facilitate plant growth by changing the hormonal balance of inoculated plant (Vessey 2003).

Phosphate Solubilization

Low levels of soluble phosphate can limit the growth of plants. Some bacteria solubilize phosphate from organic- or inorganic-bound phosphates and facilitate plant growth. Strains of genus *Pseudomonas* have the ability to solubilize insoluble inorganic phosphate (mineral phosphate) compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyl apatite, and rock phosphate (Rodriguez et al. 2006). Several enzymes, namely, phosphatases, phytases, phosphonatases, and Carbon-phosphorous (C-P) lyases, release soluble phosphorus from organic compounds in soil. C-P lyases cleave C-P links in organophosphonates. Release of phosphorus from mineral phosphate is related to the production of organic acids, such as gluconic acid (Rodriguez

et al. 2006). *P. aurantiaca* SR1 moderately solubilizes the phosphate (Rovera et al. 2008). This character was not detected in PB-St2 and neither reported for other strains of *P. aurantiaca*.

ACC Deaminase Production

ACC deaminase production is detected in PB-St2. Unfortunately this strain has not been used in plant experiments yet; however, presence of this enzyme, in addition to IAA production, makes it a good candidate for a biofertilizer. ACC deaminase-containing bacteria facilitate plant growth and development by decreasing endogenous ethylene level of host plant. These bacteria hydrolyze ACC (precursor of ethylene). The products of this hydrolysis, ammonia and α-ketobutyrate, can be used by the bacterium as a source of nitrogen and carbon for growth (Klee et al. 1991). In this way, the bacterium acts as a sink for ACC and thus lowers ethylene level in plants, preventing some of the potentially deleterious consequences of high ethylene concentrations (Saleem et al. 2007). Bacteria with ACC deaminase trait usually give very consistent results in improving plant growth and yield and thus are good candidates for biofertilizer formulation (Shaharoona et al. 2006). Several forms of stress are relieved by ACC deaminase producers, including effects of phytopathogenic bacteria, resistance to stress from polyaromatic hydrocarbons, heavy metals, salt, and drought (Glick et al. 2007).

Plant Growth Promotion due to Inoculation of *P. aurantiaca* SR1

P. aurantiaca SR1 has been inoculated in several crops, and growth promotion in these crops has been reported. Andres et al. (2011) inoculated alfalfa and soybean plants with *P. aurantiaca* SR1, in combination with *Sinorhizobium meliloti* 3Doh13 or *Bradyrhizobium japonicum* E109. It was observed that SR1 increased the length and dry weights of roots and shoots and dry weight of nodules of alfalfa plants in combination with *S. meliloti* 3Doh13 as compared to the plants inoculated with *S. meliloti* 3Doh13 alone. Similarly, increase in nodule numbers and dry weight of roots and shoots of soybean plants was observed with *P. aurantiaca* SR1 and *B. japonicum* E109, as compared to the plants inoculated with *B. japonicum* E109 but without SR1.

P. aurantiaca SR1 formulation promoted root development in wheat, sugarcane, and carob tree and root development and a higher number of nodules when co-inoculated in soybean and alfalfa, under greenhouse conditions (Rosas et al. 2005; Rovera et al. 2008). In order to evaluate its growth promotion effect in the field, *P. aurantiaca* SR1 was formulated as inoculant and applied on maize and wheat seeds at the sowing time. Low doses of phosphorous and nitrogen fertilizers were also added in the field. *P. aurantiaca* SR1 colonized the root system of both crops and persisted at appropriate population densities. Both crops produced higher yields with low fertilization doses as compared to

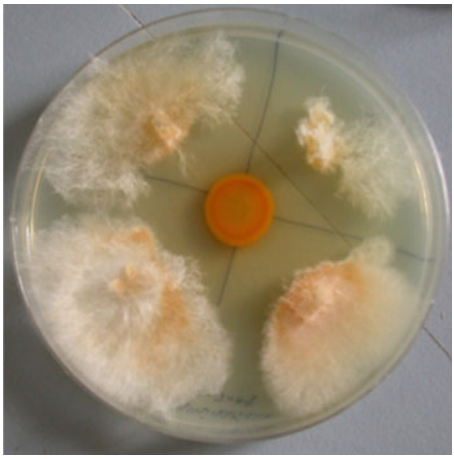


Fig. 14.5 Antifungal activity of *P. aurantiaca* PB-St2 against different strains of *C. falcatum*, a fungal pathogen of sugarcane, causal agent of red rot disease

conventionally applied fertilizer dozes. Growth promotion in SR1 inoculated can be due to involvement of more than one direct mechanism such as IAA production and phosphate solubilization, as strain is capable of performing both mechanisms.

Biological Control

The most common indirect mechanism of plant growth promotion due to bacterial inoculation is biocontrol activity of the bacteria. Fluorescent pseudomonads are known to suppress soilborne fungal pathogens by producing antifungal metabolites, by sequestering iron in the rhizosphere through release of iron-chelating siderophores making it unavailable to other organisms (Dwivedi and Johri 2003). These bacteria produce antibiotics including phenazines, chitinase enzyme, HCN, cyclic lipopeptides, and several other compounds which show antifungal and antibacterial activity against plant pathogens (Fig. 14.5). A list of pathogens against which *P. aurantiaca* showed antagonistic activity is provided in Table 14.2.

Root colonization by these bacteria not only increases their population density; it functions as delivery system of secondary metabolites. Bacterial action as a biocontrol agent involves two mechanisms: (1) inhibiting pathogen by action of their secondary metabolites and/or (2) inducing systemic resistance in host. In both cases, it is important that bacteria (capable of acting as biological control) should be able to compete with rhizospheric bacteria, establish itself in rhizosphere, and colonize the host plant roots. Higher bacterial population density is required if protection against pathogen is through secondary metabolites production, and comparatively, low population density works fine if the mechanism of systemic induced resistance is involved (Chin-A-Woeng et al. 2003).

Table 14.2 List of the pathogens against which antagonistic activity of *Pseudomonas aurantiaca* strains has been proved in bioassays

Strains	Pathogen	References
PB-St2	*C. falcatum* BF166, *C. falcatum* C01148, *C. falcatum* CP77400, *C. falcatum* SPF 234, *C. acutatum*, *C. coccodes* JAT2241, *C. lindemuthianum* 2221, *C. orbiculare* 2195, *Cylindrocarpon destructans* 1378, *Fusarium lateritium* 543, *F. graminearum* V20251, *F. graminearum* V14435, *F. graminearum* RS29B01, *F. graminearum* 212698, *F. oxysporum*, *F. oxysporum* 540, *F. oxysporum.lycopersici*. *FOL* 1835, *F. oxy. radicis-lycopersici* 1833, *F. solani* 1888, *F. solani* 1891, *F. solani* 1892, *Pythium aphanidermatum* 2102, *P. aphanidermatum* 2190, *P. capsici*	Mehnaz et al. (2010)
SR1	*R. solani*, *M. phaseolina*, *Pythium* spp., *S. sclerotiorum*, *Sclerotium rolfsii*, *Fusarium* spp., *Alternaria* spp.	Rosas et al. (2001)
S1	*F. oxysporum*, *P. syringae pv. glycinea*	Mandryk et al. (2007)
IB5-10	*P. capsici*, *R. solani*, *P. ultimum*	Park et al. (2012)
DS200	*S. sclerotiorum*	Fernando et al. (2005)

Competition for the Nutrients and Role of Siderophores

Competition for nutrients (carbon, nitrogen, iron, etc.) is one of the mechanisms through which biocontrol strains can reduce the ability of pathogens to proliferate in the soil (Fernando et al. 1996). Short-generation time, speed, and to which extent biocontrol bacteria can colonize the root system are considered important traits. Bacterial colonization of the root system is limited to a small part of the total available surface and probably corresponds to the most nutrient rich areas particularly the intracellular junctions between root epidermal cells (Chin-A-Woeng et al. 1997). If a pathogen does not have enough nutrients to survive and reproduce, it will be outcompeted.

The most well-known example of competition for nutrients is iron limitation. Iron is an essential growth cofactor for living organisms. For the soil microorganisms, availability of solubilized ferric ion in soils is limited at neutral and alkaline pH. Among bacteria, fluorescent *Pseudomonas* species are known to take up ferric ions through high-affinity iron chelators termed as siderophores that are released from bacterial cells under ferric limiting conditions. These siderophores binds with ferric ion and make siderophore-ferric complex which subsequently binds with iron-limitation-dependent receptors at the bacterial cell surface. The Ferric ion is subsequently released and active in the cytoplasm as ferrous ion. Bacteria producing high concentrations of high-affinity siderophores in the rhizosphere can inhibit the growth of fungal pathogens when the ferric concentration is low (Schippers et al. 1987). Siderophore-deficient mutants were found to be less suppressive to pathogens than the isogenic parental strain (Bakker et al. 1986).

Antagonistic Activity of Phenazines and Role of Signaling Compounds in Phenazine Production

Phenazines make a large family of heterocyclic nitrogen-containing brightly colored compounds with broad-spectrum antibiotic activity. These compounds have been known for their antifungal activity since long time; however, a limited number of phenazine derivatives have been evaluated in biocontrol. The mechanism for the action of phenazines in antifungal interactions is poorly understood. It is assumed that they diffuse across or insert into the membrane and act as a reducing agent, resulting in the uncoupling of oxidative phosphorylation and the generation of toxic intracellular superoxide radicals and hydrogen peroxide which are harmful to organisms (Mahajan et al. 1999).

The production of secondary metabolites depends upon internal factor of an organism and environmental conditions. Bacteria are known to regulate the production of antifungal metabolites through population density-dependent gene expression, known as "quorum sensing" (QS). QS adjusts bacterial physiology according to their environmental conditions and coordinates the behavior of entire bacterial population. Autoinducer signal molecules convey population density information from the neighbor sister cells to the cell. In Gram-negative bacteria, the most extensively studied signaling molecules belong to the class *N*-acyl, L-homoserine lactones (N-AHL) that regulate the range of compounds involving bioluminescence to virulence and secondary metabolite production. Phenazine-producing strains of *Pseudomonas* spp. when grown under lab condition show cell density-dependent production of phenazines. These bacteria produce enormous amount of phenazines in late exponential growth and early stationary phase and lag of phenazine production in early and mid-exponential growth phase (Chin-A-Woeng et al. 2003).

Phenazine production depends upon growth and environmental conditions as these factors affect the expression of those genes which are involved in their biosynthesis. The availability of certain carbon and nitrogen sources, major root exudates components, metal ions, and oxygen status affects the phenazine production (Chin-A-Woeng et al. 2000). Amount of autoinducers and subsequently phenazine production can vary according to the growth medium (Seveno et al. 2001). Some strains which produce more than one phenazine derivative increase or decrease the ratio of their production according to media composition, growth, and environmental conditions. Therefore, it is important to identify the most suitable environmental conditions for the production of phenazines of choice of interest and to work them effectively when applied as a biocontrol agent for plants.

Antagonistic Activity of Lipopeptides (LP)

Lipopeptides (LPs) are composed of a lipid tail linked to a short linear or cyclic oligopeptide. They are produced by fungi and various bacterial genera including *Pseudomonas*, *Streptomyces*, and *Bacillus*. LPs have received considerable attention

for its antimicrobial, cytotoxic, antitumor, immunosuppressant, and surfactant properties (Gross and Loper 2009). The proposed primary mode of action of LPs is pore formation in membranes, leading to an imbalance in transmembrane ion fluxes and cell death (Baltz 2009; Bender et al. 1999).

Antifungal activities have been reported for many LPs. LPs inhibit the fungal growth accompanied by increased branching and swollen hyphae. This growth-inhibitory effect is also due to decreased activities of esterases and mitochondria, changed organization of mitochondria, decreased intracellular pH, and decreased hydrophobicity of hyphae (Thrane et al. 1999). LPs from *Pseudomonas* spp. have significant impact on oomycetes of pathogens such as *Pythium* and *Phytophthora* spp. by their ability to lyse zoospores. This effect is well characterized for the LPs of viscosin group (Raaijmakers et al. 2010). The antiviral activity of LPs was already reported in 1951 by Group'e and colleagues (reviewed in Nybroe and Sørensen 2004) for viscosin against enveloped viruses.

For some plant pathogenic *Pseudomonas* strains, N-AHL-based quorum sensing was shown to be involved in cyclic LPs biosynthesis. In *P. fluorescens* strain 5064, the quorum-sensing signal was identified as *N*-3-acyl-hydroxyoctanoyl-HSL, and addition of culture extracts or the synthetic signal molecules restored viscosin biosynthesis in the mutants (Cui et al. 2005). For *P. putida* strain PCL1445, four N-AHLs were found to be associated with regulation of putisolvin biosynthesis (Dubern et al. 2006). For various other *P. fluorescens* strains, role of N-AHL-based quorum sensing was not observed in LP biosynthesis (Raaijmakers et al. 2010). It suggests that molecular and biochemical basis of QS-dependent regulation of LP biosynthesis may differ among species and strains.

Systemic Resistance Induced by Secondary Metabolites

The resistance caused by infection with a "pathogen" is known as "systemic acquired resistance" (SAR) (Hunt et al. 1996) and is associated with increased levels of salicylic acid (van Loon 1997) and the activation pathogenesis-related (PR) proteins (Gaffney et al. 1994). The plant defense mechanism induced by "nonpathogenic" biocontrol bacteria is known as "induced systematic resistance" (ISR) (van Loon et al. 1998). The ISR response requires jasmonic acid and ethylene production (van Wees et al. 1997); however, it can also be activated by lipopolysaccharides, siderophores, or flagella (Maurhofer et al. 1994; Leeman et al. 1995). It is also believed that ISR is associated with a closer contact between inducing agent and the host plant (van Wees et al. 1997). The production of the phenazine derivative pyocyanin was shown to be involved in ISR in tomato and bean against *Botrytis cinerea*. Its wild type, a salicylic acid or pyocyanin mutant of *P. aeruginosa* 7NSK2, was unable to induce resistance against *Botrytis cinerea* (Audenaert et al. 2001). It was hypothesized that the salicylic acid (precursor of pyochelin, a siderophore) was converted to pyochelin, and that pyochelin and pyocyanin act in concert to produce active oxygen species that cause cell damage, and this mechanism subsequently leads to induced resistance (Audenaert et al. 2001). Several LPs produced by nonpathogenic

Pseudomonas strains triggered defense responses in plants against pathogenic fungi and oomycetes. When tomato roots were treated with purified massetolide A of *P. fluorescens*, the leaves showed enhanced resistance to infection by *P. infestans* (Tran et al. 2007).

Conclusion

P. aurantiaca has been isolated from different parts of the world, from different sources including plants, soil, and sludge. All strains are known for the production of antibiotics, specifically phenazines. Other strains produce HCN, cyclic lipopeptides, siderophores, pyoverdin, protease, IAA, enzymes for phosphate solubilization, and several other secondary metabolites. Antifungal activity of almost every strain is reported against several important plant pathogens. Growth promotion of several crops is reported by inoculation of *P. aurantiaca* strain SR1. It has been formulated by the industry as an inoculant for its application in different countries. Several *Pseudomonas* strains have already been marketed as commercial biocontrol products, and one of them is "Cedomon" (BioAgri AB, Uppsala, Sweden), a seed treatment based on a *P. chlororaphis* strain, providing protection against seed-borne diseases in barley. This product is successfully marketed for more than 10 years in several European countries. "Mycotoxin" is an antifungal biopesticide formed by *P. aurantiaca* M-518 (Omel'yanets and Mel'nik 1987).

Considering the traits of *P. aurantiaca*, it can be suggested that after *P. putida* and *P. fluorescens*, another species of *Pseudomonas* can be used as a crop inoculant which can serve the purpose of biofertilizer and biofungicide. *P. aurantiaca* can promote plant growth by utilizing the direct and indirect mechanisms. Now there is a desperate need to carry out extensive field studies on all of these strains so as to evaluate their potential as a biofertilizer and biofungicide.

Acknowledgement Author is grateful to Dr. Rahman Shah Zaib Saleem (Department of Chemistry, School of Science and Engineering (SSE), Lahore University of Management Sciences (LUMS), Lahore, Pakistan) for the drawing of structures of secondary metabolites presented in Fig. 14.2.

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Chapter 15
Plant–Microbe Interaction: A Potential Tool for Enhanced Bioremediation

A.K. Marihal and K.S. Jagadeesh

Contents

Abstract A number of toxic and synthetic organic compounds are a problem worldwide because they can contaminate environmental soil through either local (e.g., industrial) or diffuse (e.g., agricultural) contamination. These pollutants have negative effects on environment as well as human health. In order to clean the polluted sites, the search for alternative methods for excavation and incineration resulted in the application of bioremediation techniques. Rhizoremediation is a specific form of phytoremediation and bioaugmentation that could be applied to

A.K. Marihal • K.S. Jagadeesh (✉)
Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad 580005, Karnataka, India
e-mail: jagsbio@gmail.com

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_15, © Springer India 2013

solve the problems encountered by the application of these techniques individually. During rhizoremediation, root exudates stimulate the survival and action of bacteria, which subsequently result in efficient degradation of pollutants. The root system of plants can help bacteria to spread through soil and penetrate even impermeable soil layers. The main contributors for rhizoremediation include endophytic and rhizospheric bacteria. These bacteria have potential for improving bioremediation of toxic organic compounds in contaminated sites. The efficiency of phytoremediation or bioaugmentation can be improved by inoculation of pollutant-degrading bacteria on plant seed.

Introduction

Environmental contamination due to anthropogenic and natural sources is increasing day by day because of increase in population, industrialization, and urbanization. The enigma for the public, scientists, academicians, and politicians is how to tackle the contaminants that jeopardize the environment. Advances in science and technology, since industrial revolution, have also increasingly enabled humans to exploit natural resources and cause damage to the environment. The ideal solution for pollution abatement is bioremediation, the most effective innovative technology to come along that uses biological systems for treatment of contaminants. The naturally occurring bacteria, fungi, or plants contribute to degrade or detoxify hazardous substances. The application of bioremediation has been growing partly for the past two decades because of better understanding of microbial processes and soil. An elaborate study should be made before applying the most effective technique on any soil that is polluted with toxic substances. Certain important parameters essential for bioremediation are nature of the pollutants, soil structure, and hydrogeology for movement of pollutants, nutritional status, and microbial composition (Kuiper et al. 2004). Bioremediation has several merits over physicochemical remediation and has obvious advantages like cost-effective, convenient, complete degradation of organic pollutants and no collateral destruction of the site material or its indigenous flora and fauna.

Although, this novel and recent technology is a multidisciplinary approach, its central thrust depends on microbiology. Rhizoremediation (plant and microbe interaction), which is the most evolved process of bioremediation, involves the removal of specific contaminants from waste products of contaminated sites by mutual interaction of plant roots and suitable microbial flora. The microbes present in the rhizosphere of plants during rhizoremediation contribute to the degradation of pollutants. The use of plants in conjunction with plant-associated bacteria offers much potential for bioremediation than using a plant alone. Toxic pollutants present in the environmental soil are degraded by plant-associated bacteria which can involve endophytic and rhizospheric bacteria. Endophytic bacteria which occur naturally in the internal tissues of plants are nonpathogenic, plant growth promoting, and benefit the plant host by producing a range of natural products, thus, enhancing the biodegradation of environmental soils (McGuinness

and Dowling 2009). Some of the reported genera include *Acetobacter*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, and *Pseudomonas* (Lodewyckx et al. 2002).

Microorganisms in the rhizosphere have marked influence on the growth of plants, while plant roots have direct effect on the surrounding microbial population. In the rhizosphere zone, microbial populations may benefit the plant by increasing recycling and dissolving mineral nutrients and synthesis of amino acids, vitamins, auxins, and gibberellins that stimulate plant growth (Atlas and Bartha 1998). The plants enter an amensal relationship with other plants when microorganisms release antagonistic substances in the rhizosphere. Microorganisms play a vital role through their ability to reduce phytotoxicity of contaminants, thereby, stimulating the degradation of the pollutants (Livhuwani 2009).

According to Licht and Isebrands (2005), phytoremediation includes the use of buffers, vegetation filters, in situ phytoremediation plantings, and percolation controlling vegetative caps. The degradation of toxic organic compounds by the use of endophytic and rhizosphere bacteria in combination with specific plants could offer an efficient, economic, and sustainable remediation for the twenty-first century.

Organic Compounds in Soil

Organic pollutants originate from diverse sources like anthropogenic pollutants (industrial chemicals), petroleum inputs, incomplete combustion of fuels, forest and grass fires, and biosynthesis of hydrocarbons by aquatic or terrestrial organisms. Because of their chemical structure, many synthetic organic compounds are extremely resistant to natural breakdown processes, and once released into the environment, they may persist for years and decades.

Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants. There are over 100 different PAH compounds. Morgan and Watkinson (1989) described the main input sources of PAH's pollutions which included leaches from old storage tanks, oil spills, domestic wastes, tanker leakages, and incomplete combustion of fossil fuels. They are rarely of industrial use, except for a few PAHs used in medicines and production of dyes, plastics, and pesticides. They are highly toxic to organisms due to their carcinogenic and mutagenic potential. Because of their low water solubility and hydrophobic nature, they tend to adsorb on and accumulate in sediments (Louisa 2010). In the beginning of the twentieth century, microbes were recognized for their ability to degrade oil components. Intensive studies have been done on PAH's degradation by microbes, and their ability was identified in a wide variety of bacteria, fungi, and algae (Kuiper et al. 2004). The hydrocarbon-degrading bacterial strains are mainly from the

genera *Pseudomonas*, *Sphingomonas*, *Burkholderia*, *Arthrobacter*, *Flavobacterium*, *Alcaligenes*, and *Nocardioides*. The phylogenetic diversity of hydrocarbon degraders is vast, with degraders found in most, if not all, branches of the microbial family tree.

Polychlorinated Biphenyls (PCBs) and Synthetic Organic Pesticides

PCBs are toxic synthetic aromatic compounds, known for their persistence and potential toxicity. Because of their toxicity, carcinogenicity, wide distribution, and slow biodegradation in the environment, PCBs are considered among the worst pollutants. PCBs are widely used hydraulic fluids, flame retardants, transformers, surface coatings, dielectric fluids in capacitors, adhesives, and dyes. Because of their toxicity, their manufacture was banned in the USA in 1978. There is evidence of the dispersal of these toxic synthetic organic compounds in natural environments and have been detected in polar bears in the Arctic (Skaare et al. 2002).

Other synthetic chlorinated organic pesticides of concern as contaminants of environmental soil include pentachlorophenol (PCP). PCP is used as a disinfectant, a fungicide, and a wood preservative. The products of PCP are also used as defoliants and general herbicides and hence toxic to plants. As a result of their manufacture, transport, storage, or use as a wood preservative, PCP can be released into the environment. Their extensive use in saw mills has led to groundwater contamination.

Volatile Organic Compounds (VOCs)

Benzene, toluene, ethylbenzene, and xylene (BTEX) compounds are a family of VOCs based on the benzene structure and are found in petroleum products. VOCs are vapors emitted by various solids or liquids, e.g., petrol, diesel, pesticides, paint, cleaning supplies, and adhesives, many of which have short- and long-term adverse health effects. Methyl tertiary butyl ether (MTBE), also a VOC, is used as a fuel oxygenate, i.e., a chemical containing oxygen that is added to fuels, especially petrol, to make them burn more efficiently. It can be a major contaminant of groundwater as a result of the widespread spillage or leakage of MTBE-containing petrol from underground storage tanks at petrol stations.

Heterocyclic Aromatic Hydrocarbons: Dioxins and Furans

Heterocyclic aromatic hydrocarbons include dioxins and furans. Dioxins are produced unintentionally by industry due to incomplete combustion, as well as during the manufacture of certain pesticides and other chemicals, metal recycling, and pulp and paper bleaching. Dioxins have also been found in automobile exhaust, tobacco smoke and wood and coal smoke, and in commercial mixtures of PCBs. Furans are

a group of 135 related heterocyclic aromatic hydrocarbons called polychlorinated dibenzofurans. Dioxins and furans do not dissolve in water and can attach to particles of soil, dust, and sediment. As a result, they can persist unchanged in the environment, mainly in soil and sediments, for years. A number of studies have been carried out on populations after accidental environmental exposure to high levels of dioxins and furans and have reported that chloracne, a skin disorder, was the most common human health effect (Panteleyev and Bickers 2006).

Explosives

Organic explosives including trinitrotoluene (TNT), hexahydrotrinitrotriazine or royal demolition explosive (RDX), and octahydrotetranitrotetrazocine or high melting explosive (HMX) can contaminate environmental soil.

Bioremediation Technologies

The process of bioremediation is the treatment of contaminants in the environment using metabolically potential microorganisms (including bacteria) and plants to degrade toxic contaminants to less toxic or nontoxic substances. Bioremediation is the permanent and cost-effective solution which may lead to complete mineralization of the pollutant. The clean up by physical and chemical methods would not be feasible for contaminants of lower concentrations while bioremediation can deal with such lower concentrations. Unfortunately, some major drawbacks still limit the application of these techniques, including the fact that the processes may take longer time (months or years) and are less predictable than the conventional methods.

The following are the strategies for bioremediation:

- Monitored natural recovery (MNR)
- Bioaugmentation
- Remediation of organic contaminants by PGPR/rhizoremediation

The strategy of bioremediation may be applied in combination, e.g., biostimulation and phytoremediation, where the use of plants enhances the activity of degrading microorganisms in their root system called as rhizoremediation.

Monitored Natural Recovery (MNR)

MNR involves leaving sediments in place and relying upon effective source control and ongoing natural processes like aquatic sedimentation and biological and chemical transformation to degrade or immobilize the contaminant in situ, thus reducing its bioavailability to environmental risks posed by contaminated sediments. MNR is

the only applied bioremediation strategy in sediment management. There are several physical, chemical, and biological processes identified which contribute to MNR (Magar 2001). The burial of contaminants by the natural deposition of clean sediments reduces surface sediment concentrations over time. Sorption to active compounds present in the sediment reduces contaminant mobility and bioavailability. Particle-bound contaminants may leave the site of contamination by erosion, transport, and dispersion, which removes them from the contaminated site but may increase contaminant concentrations in downstream areas.

Bioaugmentation

Certain microorganisms have specific catabolic abilities. By introducing such microorganisms into the contaminated environment, it may speedup or enable the degradation of pollutants in order to supplement the indigenous population. It is one of the methods to improve and enhance the transformation rate of degradation. Many microbes are described to have the genetic tools to mineralize recalcitrant pollutants such as PAHs, chlorinated aliphatics and aromatics, nitroaromatics, and long-chain alkanes (Cerniglia 1993). Two factors limit the use of added microbial cultures in a land treatment unit: (1) nonindigenous cultures rarely compete well enough with an indigenous population to develop and sustain useful population levels, and (2) most soils with long-term exposure to biodegradable wastes have indigenous microorganisms that are effective degraders, if the land treatment unit is well managed. Strains selected for remediation purpose should not account only for degradation abilities but also for ecological characteristics concerning adaptation to the habitat which have shown success. Three criteria were used to select strains in series of experiments aiming to develop bacterial inocula to treat spent metal working fluids in bioreactor: (1) the relative abundance of the source populations in the target habitat (waste), (2) tolerance to co-contaminants, and (3) the ability to degrade target contaminants. Choosing bioaugmentation as remediation strategy is viable if the limiting factor of biodegradation is the absence of relevant catabolic genes within the indigenous microbial community, and this lack of genetic information will be filled by the introduced strain.

Remediation of Organic Contaminants by PGPR/Rhizoremediation

Without the microbial contribution, phytoremediation alone may not be a viable technology for many hydrophobic organic pollutants (Chaudhry et al. 2005). In many of these studies, an important contribution to the degradation of pollutants is ascribed to microbes present in the rhizosphere of plants used during phytoremediation or of plants

which are emerging as natural vegetation on a contaminated site. This contribution of the rhizomicrobial population is referred to as rhizoremediation (Anderson et al. 1993; Schwab and Banks 1994). In some cases, rhizosphere microbes are even the main contributors to the degradation process. A plant can be considered to be a solar-driven biological pump and treatment system, attracting water and accumulating water-soluble pollutants in the rhizosphere leading to degradation or translocation of the pollutant (Erickson 1997).

The success of a plant species as the spot of rhizoremediation might depend on (1) highly branched root system to harbor large numbers of bacteria, (2) primary and secondary metabolism, and (3) establishment, survival, and ecological interactions with other organisms (Kuiper et al. 2004). Plant roots can act as a substitute for the tilling of soil to incorporate additives (nutrients) and to improve aeration (Kuiper et al. 2004). Various grass varieties and leguminous plants have shown to be suitable for rhizoremediation (Kuiper et al. 2004). *Populus* sp. and *Salix* sp. have been used successfully for rhizoremediation of PHC-contaminated soils probably due to introduction of oxygen into deeper soil layers through specialized root vessels, parenchyma (Zalesny and Bauer 2007).

The success of beneficial processes is based on the rhizosphere competence of the microbes, which is reflected by the ability of the microbes to survive in the rhizosphere, compete for the exudate nutrients, sustain in sufficient numbers, and efficiently colonize the growing root system (Kuiper et al. 2004). Usually, several bacterial populations degrade pollutants more efficiently than a single species/strain due to the presence of partners, which use various intermediates of the degradation pathway more efficiently (Kuiper et al. 2004). During rhizoremediation, the degradation of a pollutant has been reported as the result of the action of a consortium of bacteria. Root cells also secrete mucilage, a gelatinous substance that is a lubricant for penetration of root through the soil during growth. Soil microorganisms use this supply of nutrients and proliferate to form the plant rhizosphere (Anderson et al. 1993).

Although rhizoremediation occurs naturally, it can also be optimized by deliberate manipulation of the rhizosphere. This can be accomplished by using suitable plant–microbe pairs. These can be either combinations of plants and plant growth promoting rhizobacteria (PGPR) or combinations of plants and contaminant-degrading microbes. For example, a grass species combined with a naphthalene-degrading microbe protected the grass seed from the toxic effects of naphthalene, and the growing roots propelled the naphthalene-degrading bacteria into soil that would have been too deep to penetrate in the absence of roots (Kuiper et al. 2004). A convergence of phytoremediation and microbial bioremediation strategies led to a more successful approach to remediation of contaminants, particularly organic compounds. Microbe-assisted phytoremediation, with both naturally occurring microbes and deliberately stimulated via seed inoculation, has been investigated in the laboratory, greenhouse, and field (Banks et al. 2003; Chaudhry et al. 2005; Greenberg 2006). A variety of contaminant-degrading enzymes can be found in plants, fungi, endophytic bacteria, and root-colonizing bacteria. These include peroxidases, dioxygenases, P450 monooxygenases, laccases, phosphatases,

Table 15.1 Rhizoremediation of PCP using efficient PCP-degrading bacteria in maize plants

Sl. no.	Treatments		Percentage of PCP degradation (30 DAI)
1	T1	Soil without PCP control (control)	–
2	T2	Soil with PCP@50 ppm (uninoculated)	35.01
3	T3	Soil with PCP@ 50 ppm + inoculated with MAZ1	94.09
4	T4	Soil with PCP@ 50 ppm + inoculated with MAZ2	93.19
5	T5	Soil with PCP@ 50 ppm + inoculated with MAZ1 and MAZ2	95.87
6	T6	Soil without PCP + maize (control)	–
7	T7	Soil with PCP + maize (uninoculated)	45.29
8	T8	Soil with PCP + maize + inoculated with MAZ1	100
9	T9	Soil with PCP + maize + inoculated with MAZ2	100
10	T10	Soil with PCP + maize + inoculated with MAZ1 and MAZ2	97.34
SEM±			1.49
CD@1 %			6.68

Note: *DAI* days after inoculation

dehalogenases, nitrilases, and nitroreductases (Susarla et al. 2002; Chaudhry et al. 2005). Although there are some organisms that can completely degrade a specific organic contaminant (e.g., *Sphingobium chlorophenolicum* strain ATCC 39723 mineralized pentachlorophenol (Cai and Xun 2002)), individual species generally do not contain entire degradation pathways.

It has been shown that maize plants exhibited maximum tolerance to PCP compared to other plants tested and a number of predominant bacteria have been isolated from rhizosphere soils of these PCP-tolerant plants (Marihal et al. 2009a). When screened for PCP degradation potential, isolate MAZ2 identified as *Pseudomonas* sp. (accession no. HQ641258) by 16S rRNA gene sequencing showed complete degradation of PCP (50 ppm). It can be observed (Table 15.1 and Fig. 15.1) that the bacterial inoculation in PCP-polluted soil resulted in significant degradation of PCP. At 30 days after inoculation (DAI), it improved from mere 35.01 % degradation by strain UIC to 93.19 % degradation due to inoculation with MAZ2 strain. However, mixing of the strains MAZ1 and MAZ2 did not improve degradation in soil.

In case of phytoremediation of PCP, i.e., in the presence of the plant (maize), the degradation increased to 45.29 %. It further increased to 100 percent due to bacterial inoculations. The bacteria present in the rhizosphere of these tolerant plants have degraded PCP effectively and protected them against the PCP toxicity, suggesting that both plants and associated microbial communities play a significant role in attenuating the PCP toxicity (Fig. 15.2). The experiment has revealed that most of the PCP (50 ppm) was degraded completely within 30 days indicating that plants protect themselves from the phytotoxicity effects. They further showed that the PCP-degrading bacteria belonged to PGPR groups (Marihal et al. 2009b). They isolated as many as 27 PCP-tolerant rhizobacteria and 19 endophytic bacteria and checked them for plant growth promotional abilities such as phosphate solubilization,

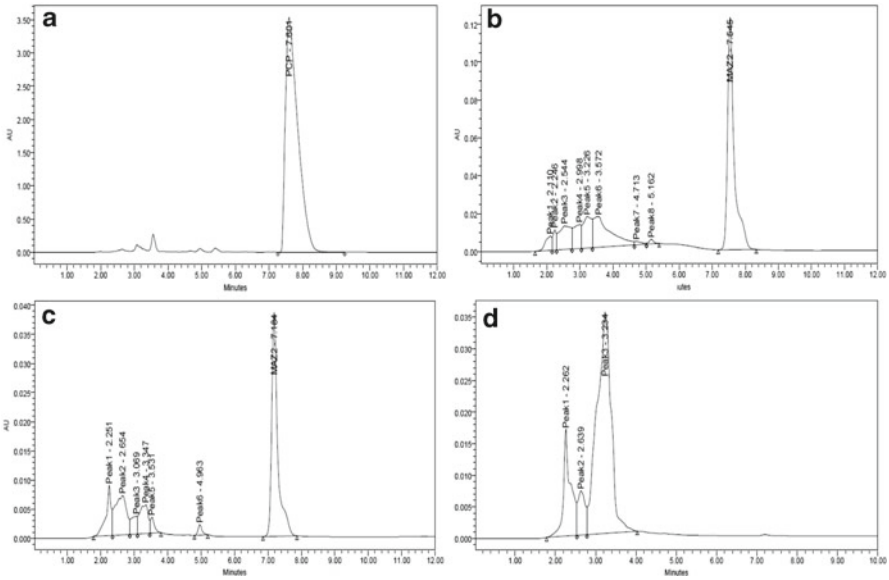

Fig. 15.1 HPLC profile of PCP degradation (**a**) standard PCP, degradation of PCP by isolate MAZ2 at (**b**) 10 DAI, (**c**) 20 DAI, and (**d**) 30 DAI

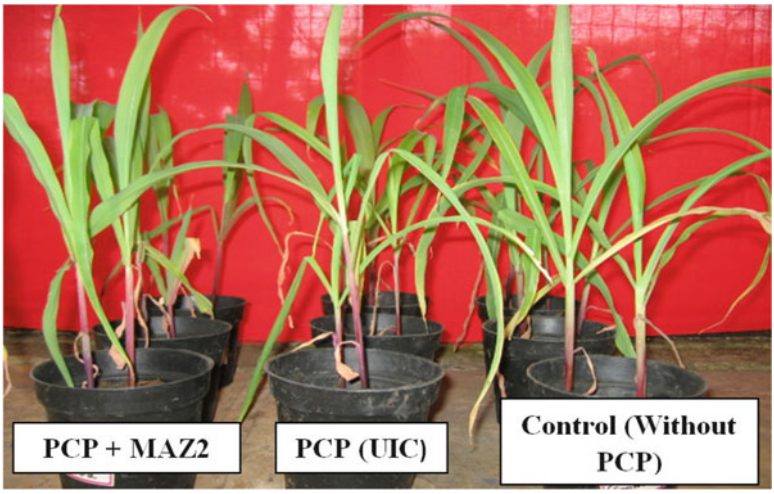

Fig. 15.2 Rhizoremediation of pentachlorophenol-polluted soil by maize plants

IAA production, antagonistic activity against plant pathogens, and HCN production (Tables 15.2 and 15.3) and observed that six rhizobacteria and five endophytic bacteria capable of PCP degradation exhibited all these four characters, indicating that these isolates could be used as both PGPR and bioremediating agents.

Table 15.2 Plant growth promotional activities of the PCP-degrading rhizobacterial isolates

Sl. no.	Code no. of the isolates	Plant growth promotional activities of the isolates			
		P-solubilization	IAA	HCN	Antibiosis
1	SOY1	–	–	–	+
2	SOY2	+	+	+	+
3	SOY3	–	+	+	+
4	SOY4	+	+	+	–
5	SOY5	+	+	+	–
6	SOY6	–	+	+	+
7	SOY7	+	–	+	+
8	SOY8	+	+	+	+
9	SUN1	+	+	–	+
10	SUN2	+	–	–	+
11	SUN3	+	–	–	–
12	SAFF1	–	+	+	+
13	SAFF2	+	+	+	+
14	SAFF3	–	+	+	+
15	SAFF4	–	–	–	+
16	SAFF5	–	–	+	+
17	SAFF6	–	–	+	+
18	SAFF7	–	+	+	+
19	SAFF8	+	+	+	+
20	GRN1	–	–	–	+
21	GRN2	+	–	+	+
22	GRN3	+	+	+	+
23	MAZ1	+	+	+	+
24	MAZ2	+	+	+	+
25	WHT1	+	+	+	–
26	WHT2	+	+	+	–
27	WHT3	+	+	+	–

Note: +, positive; –, negative

Interaction Studies

The interaction between plant and microbial communities is complex in the rhizosphere and evolves to the mutual benefit of both organisms. The rhizosphere sustains large microbial populations by secreting substances such as carbohydrates and amino acids through root cells and by sloughing root epidermal cells. The type of interaction between plants and soil microorganisms is influenced by the type of root exudates. Bacterial attachment to plant roots is an early step in plant root colonization. Initial approaches for identifying and studying genes involved in root colonization were based on the use of random or directed mutagenesis to isolate mutants impaired for colonization. Bacterial attachment has been extensively studied in rhizobacteria, and although the molecular basis is still not completely understood, the general mechanism seems to be mediated by surface proteins, capsular polysaccharides, flagella, and chemotaxis (Rodriguez-Navarro et al. 2007). According to studies on mounting evidence, plants are able to select the bacteria in their rhizosphere by different mechanisms including root architecture, the modification of soil conditions,

Table 15.3 Plant growth promotional activities of the PCP-degrading endophytic bacterial isolates

Sl. no.	Code no. of the isolates	Plant growth promoting substances by the isolates			
		P-solubilization	IAA	HCN	Antibiosis
1	SOY(root) 1	+	+	−	−
2	SOY(root) 2	−	+	+	−
3	SAF(root) 1	−	−	+	−
4	SAF(root) 2	+	+	+	−
5	SAF(root) 3	+	+	+	+
6	SAF(root) 4	+	+	+	+
7	SUN(root) 1	+	+	+	+
8	SUN(root) 2	+	−	−	+
9	SUN(root) 3	−	−	−	+
10	GRN(root) 1	+	−	+	−
11	GRN(root) 2	−	−	+	−
12	GRN(root) 3	−	+	−	−
13	GRN(root) 4	+	+	+	+
14	WHT(root) 1	+	+	+	−
15	SOY(leaf) 1	+	+	+	−
16	SOY(leaf) 2	+	+	+	+
17	SAF(leaf) 1	−	+	+	−
18	GRN(leaf) 1	−	−	−	−
19	WHT(leaf) 1	+	−	+	−

+, positive; −, negative

or exudation of specific compounds. Each plant exudes specific compounds, which are dependent on the plant's particular secondary metabolism. Some plants can promote the growth of bacteria that are able to degrade certain compounds, while others secrete toxic compounds that select for tolerant bacteria, and some plants are able to secrete hydrolases that degrade acyl homoserine lactones, thus inhibiting bacterial quorum sensing (Hartmann et al. 2009).

Successful colonization of rhizosphere depends not only on interactions between the plant and the microorganism of interests but also on interactions with rhizospheric microorganisms and the environment. Researchers are able to find the modifications in the bacterial communities after environmental perturbations including the introduction of plants or biodegradative bacteria, changes in temperature, or the addition of contaminants by molecular techniques such as denaturing or temperature gradient gel electrophoresis (Kielak et al. 2008). During the last 15 years, several techniques have been developed to follow seed and root colonization by bacteria that mainly include in situ hybridization assays using fluorescent probes and the visualization of bacteria that carry *luxAB* genes encoding bacterial luciferase, the green fluorescent protein, *gusA* gene encoding β-glucuronidase (GUS), or other reporter genes (Segura et al. 2009). These techniques have been used to illustrate that the introduced microorganisms are often unable to compete with indigenous microorganisms or are unable to establish in high numbers in the rhizosphere.

Root Microbe Communications

Interactions between plants and soil microbes are highly dynamic in nature and are based on co-evolutionary pressures. Consequently, it is not astonishing that rhizosphere microbial communities differ between plant species, between genotypes within species, and between different developmental stages of a given plant. At a community scale, microbial diversity in the soil has been linked to plant diversity, the increased plant biomass commonly observed with highly diverse plant communities, or the increased diversity of carbon substrates and signaling compounds provided by the plants (Shukla et al. 2011).

Many plants engage in interactions with rhizosphere and root-associated microbes to survive in toxic and nutrient-limited environment. Plants can be genetically modified to enhance plant–microbial signaling, or chemical interventions in the environment can serve the same purpose. The growing plant secretes a wide range of chemicals in root exudates to communicate with rhizospheric microbes (Abhilash et al. 2012). Certain evidences suggest that organic acids, amino acids, and phenolic compounds present in root exudates play an active role in root and microbe communication. Furthermore, many low molecular weight organic acids can act as chelating agents and can enhance the phytoavailability of pollutants. In the future, screening and isolation of efficient signaling molecules from the root zone to modify the rhizosphere community for enhanced remediation potential could be harnessed for improved bioremediation efficiency. Because plant growth is enhanced by the removal of pollutants from the rhizosphere, in combination with genetic manipulation, this approach could be used to enhance biomass production and remediation.

Approaches of Remediation

The rhizospheric bacteria are responsible for the elimination of the contaminants, while the roots are responsible for providing nutrients used by the microorganisms to proliferate. A strategy was developed to select pollutant-degrading rhizobacteria that live on or are close to the root so that they can use root exudate as their major nutrient source. Scientists have developed a system to efficiently enrich such bacteria by starting from a crude mixture of bacteria from grass roots and, subsequently, alternating between selecting for growth on the pollutant naphthalene and selecting for efficient colonization of grass roots. One of the resulting strains, *Pseudomonas putida* PCL1444, effectively utilized root exudate, degraded naphthalene around the root, protected seeds from being killed by naphthalene, and allowed the plant to grow normally. Mutants unable to degrade naphthalene did not protect the plants (Yanhong et al. 2009). Root exudates have the potential to increase the degradation of xenobiotics by the growth of soil microorganisms.

Improved degradation of high molecular weight polycyclic aromatic hydrocarbons (PAH) during phytoremediation has provoked examinations of controlling

plant/microbe interactions. A number of scientists established chemotaxis of PAH-degrading rhizosphere bacteria (*Pseudomonas alcaligenes*, *Pseudomonas stutzeri*, *and P. putida*) to naphthalene, phenanthrene, and root exudates. Fascinatingly, the bacteria were repelled by anthracene and pyrene. The attraction of competent bacteria to the root zone may improve bioavailability and increase PAH degradation in the rhizosphere. Subjugation of the phenanthrene-degrading activity of *P. putida* following exposure to root extracts and exudates recommended that enzyme induction may not occur during rhizodegradation of PAHs (Rentz et al. 2004).

Endophytic Bacteria and Phytoremediation

Plant-associated endophytes have been identified with potential for bioremediation. There are some reported cases of successful bioremediation using endophytic bacteria (Van Aken et al. 2004, 2005). They described a methylotrophic endophytic bacterium isolated from hybrid poplar trees (*Populus deltoides* × *Populus nigra* DN34) that was capable of degrading the explosives TNT, RDX, and HMX, mineralizing approximately 60 % of the RDX and HMX to carbon dioxide in approximately 2 months, suggesting that these endophytes may have potential for remediation of environmental soil containing these explosive nitroaromatic compounds. Endophytes isolated from hybrid poplar trees (*P. trichocarpa* × *P. deltoides* cv. Hazendans and Hoogvorst) growing on a BTEX-contaminated site in Belgium were shown to be capable of degrading VOCs (toluene and naphthalene) as well as a chlorinated organic herbicide (2, 4-D). Porteus-Moore et al. (2006) described 121 endophytic strains isolated from these hybrid poplar trees and identified 34 of these strains as having potential to enhance phytoremediation. Germaine et al. (2009) reported that when pea (*Pisum sativum*) plants were inoculated with *Pseudomonas* endophytes, isolated from hybrid poplars (*P. trichocarpa* × *P. deltoides* cv. Hoogvorst) and capable of degrading 2,4-D, the pea plants showed no accumulation of 2,4-D in their tissues and showed little or no signs of phytotoxicity when compared to uninoculated controls suggesting that these endophytes have potential for bioremediation of environmental soil contaminated with 2,4-D. Weyens et al. (2009) reviewed the benefits of using plant-associated endophytes in bioremediation and emphasized that although successfully applied in several laboratory-scale experiments, the large-scale field application of this technology is limited by a number of issues including (1) the levels of contaminants tolerated by plants, (2) limited bioavailability of organic contaminants, and (3) unacceptable levels of evapotranspiration of VOCs into the atmosphere. Despite the disadvantages associated with the use of plant-associated endophytic bacteria to degrade toxic organic compounds in environmental soil, it is clear that there is potential for these bacteria to make a significant contribution to sustainable bioremediation.

Enhanced Bioremediation by Genetically Engineered (GE) Microbes

Using biotechnology, bacterial strains can be engineered to produce specific enzymes capable of degrading toxic organic substances. Bacteria (rhizospheric and/or endophytic) can be engineered, via natural gene transfer or recombinant DNA technology, to produce specific enzymes capable of degrading toxic organic pollutants found in the environment. Genetic engineering of endophytic and rhizospheric bacteria for use in plant-associated degradation of toxic compounds in soil is considered one of the most promising new technologies for remediation of contaminated environmental sites.

The bacteria capable of degrading certain kind of organic pollutant, such as PCBs, have been isolated from a range of sites, and the pathways and encoding genes have also been well studied (Brazil et al. 1995). But most of these bacteria could not survive in the competitive conditions found in the rhizosphere. Several effective methods have been developed to improve the degradation efficiency and the tolerance of bacteria to contaminants in soils. Using biotechnology, bacterial strains can be engineered to produce specific enzymes capable of degrading toxic organic substances. Decontamination of contaminated sites by GE bacteria may be preferred over the conventional approaches because of the special attributes of microorganisms, involving designed metabolic pathways, to bioremediate the numerous mix environmental pollutants without producing toxic intermediates (Furukawa 2003). For some pollutants such as trichloroethylene (TCE) and PCBs, the molecular mechanisms of degradation have been clearly studied (Brazil et al. 1995).

However, until now the generation of GEMs has not developed into a widely used approach. The inability to improve the action of microbial consortia and the restriction of degrading only a few pollutants limit the use of GEMs. Several hybrid strains have been engineered in recent years by conjugative matings of appropriate organisms or by introduction of the *bph* genes into chlorobenzoate degraders, usually using a degradative pathway for chlorobenzoates via the corresponding chlorocatechols. By cloning and expressing the genes encoding enzymes for ortho- and para-dechlorination of chlorobenzoates in the biphenyl-degrading and chlorinated biphenyls co-metabolizing strain *Comamonas testosteroni* strain VP44, derivatives capable of growing on and completely dechlorinating 2- and 4-chlorobiphenyl were obtained (Hrywna et al. 1999).

Conclusion

Rhizoremediation is a specific type of phytoremediation which involves both plants and their associated rhizosphere microbes. It can occur naturally or can be actuated by deliberately introducing specific microbes. These microbes can be

pollutant degraders and/or can promote plant growth under stress conditions. Since initial research on phytoremediation showed greater promise as a cost-effective remedial strategy, considerable effort has been devoted to making the transition from the laboratory to commercialization. Treating the seeds with rhizobacteria has opened up new avenues in the area of rhizoremediation and can contribute to the restoration of polluted sites. However, not many reports on the utilization of this technique on a massive scale are available. Screening and selection of rhizobacterial strains with multiple beneficial traits will go a long way in the restoration of polluted agricultural soils.

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Chapter 16
Multifaceted Plant-Associated Microbes and Their Mechanisms Diminish the Concept of Direct and Indirect PGPRs

Naveen Kumar Arora, Sakshi Tewari, and Rachna Singh

Contents

Abstract It is an old saying that when we take from nature, we have to give back also; this give-and-take phenomenon leads to sustainability and is important for growth of a relationship. This is also applicable in plant–microbial world. The association of microbes with plants can be exploited and used to gain the benefits not only for the associated organisms but also for the ecosystem as a whole. When we view it in a holistic way, it is clear that multifaceted and diverse mechanisms of

N.K. Arora (✉) • S. Tewari • R. Singh
Department of Environmental Microbiology, School of Environmental Science,
BBA University, Lucknow 226025, India
e-mail: nkarora_net@rediffmail.com

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_16, © Springer India 2013

plant-associated microbes (PAMs) participate in promoting plant growth; protecting plant health; strengthening plant–microbe association under stress-, pollutant-, or contaminant-affected conditions; and protecting plants from the attack of phytopathogens through biological control. The multiple functions performed by microbes in the vicinity of plants (rhizosphere, phyllosphere, or other regions) are extremely interwoven and interlinked and are inseparable from each other. At present, the plant growth promoting rhizobacteria (PGPR) and mechanisms by which they function or help their respective host plant have been broadly classified into direct or indirect. However, the scenario is not as simple, plain, or should we say two-dimensional. Several PGPRs and the metabolites they produce can function in multiple ways in same or diverse conditions diminishing the concept of direct and indirect. Several examples discussed in this chapter dilute the boundary between direct and indirect and raise questions for the researchers to gather more knowledge on the intricately woven relationship and functions of the metabolites and mechanisms as a whole. A microbial metabolite in the rhizosphere cannot only perform a big role which is quite apparent but also several other functions which are less visible or obvious but are equally important. Several examples cited in the literature prove that the so-called direct mechanisms (like nutrient acquisition, phytohormone production, iron chelation, phosphate solubilization, and nitrogen fixation) also help the plant in other (indirect) ways and similarly the so-called indirect mechanisms (like antimicrobial metabolites for biocontrol and induced systemic resistance (ISR)) perform several different (direct) functions. Diverse mechanisms function simultaneously in the soil and do not work individually, strengthening the concept of universal and holistic approach.

Introduction

The principal goal of agriculture is the production of high-quality, safe, and affordable food for an ever-increasing worldwide population (Avis et al. 2008). The widespread use of chemicals has been a subject of public concern due to potential harmful effects on the environment, undesirable effect on nontarget organisms, and the possible carcinogenicity on living beings. However, the use of these synthetic chemicals during the last three decades has raised a number of ecological problems including destruction of plant tissues and reduction in crop yields, varying from 25 to 100 % resistant pest varieties (Nakkeeran et al. 2005). Root diseases are estimated to cause 10 – 15 % yield losses annually in the world (Dua and Sindhu 2012). Plant pathogens are the most important biotic agents causing serious losses and damages to agricultural products. These phytopathogens are needed to be controlled to ensure food, feed, and fiber production quantitatively and qualitatively. Indiscriminate use of chemical pesticides and fungicides leads to environmental pollution and causes serious effects on human health and nontarget organisms (Khokhar et al. 2012). Stimulated by increasing demand, and by the awareness of the environmental and

human health damage induced by overuse of pesticides and fertilizers (Gamalero and Glick 2011), worldwide agricultural practice is moving to a more sustainable and environmentally friendly approach. Henceforth, researchers have diverted their attention toward exploring the potential of beneficial microbes, for plant growth enhancement and protection measures, against phytopathogens.

Bacteria inhabiting the rhizosphere and beneficial to plants are termed as PGPR (Kloepper and Schroth 1978). A number of PGPRs included in the genera *Azospirillum*, *Azotobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Serratia*, and *Rhizobium* are reported to enhance plant growth. PGPRs are known to influence the growth, yield, and nutrient uptake by an array of mechanisms including both direct and indirect as plant growth promoters and biological control agents. The so-called direct mechanisms opted by PGPR include the provision of bioavailable phosphorus for plant uptake; nitrogen fixation; sequestration of iron by release of siderophores; production of phytohormones like auxins, cytokinins, and gibberellins; and lowering plant ethylene levels using ACC deaminase that accumulate during biotic and abiotic stresses (Mayak et al. 2004). Similarly, the so-called indirect mechanisms of PGPR include production of antibiotics, namely, 2,4-diacetylphloroglucinol (DAPG), phenazine, pyoluteorin, and pyrrolnitrin, against pathogenic fungi and bacteria; reduction of iron available to phytopathogens in the rhizosphere; synthesis of fungal cell-wall lytic enzymes; and also competition with detrimental microorganisms for sites (infection sites or space for colonization) on plant roots and induction of systemic resistance against various pathogens in plants (Ramamoorthy et al. 2001).

Microbes in the rhizosphere show all kinds of relationships with the host plant that can be mutualistic, commensals, parasitic, predators, and even neutral. These relationships are never static and keep on changing with time and conditions. PGPRs are in mutual relationship with the host plant, and a single PGPR can act in diverse manners. In fact, a particular mechanism can always act in various apparent and unapparent ways. For example, by providing nutrients to the plant, the direct PGPRs enhance the growth of their host thus increasing the crop yield. This is directly visible, but what is not noticeable is the fact that by providing nutrients particularly in the deficient conditions, these very direct PGPRs are also performing indirect functions (as presently classified). As the plant becomes healthy, its ability to combat pathogens is also enhanced. Hence, a direct PGPR is also helping the plant to combat phytopathogens; also, a healthy plant is better adapted or able to survive and grow in stress conditions. A nitrogen fixer and a phosphate solubilizer can help and protect plants in N-and P-deficient soil and promote plant growth. Several such examples will be discussed in this chapter which blurs the distinction between direct and indirect, making it just a theoretical concept and not a practical one. This demarcation and characterization has limited the mechanisms and research lines in realm. The chapter focuses on the heterogeneous diversity of microbes present in the plant ecosphere, addressing the significance of their cooperative activities and looking on multidimensional

working of PGPRs and metabolites secreted by them. Though these bacteria (PGPRs) are diverse in nature and do work discretely, in totality, all of them are working in tandem under a common regulon to enhance and promote plant growth and form associations to sustain themselves in the competitive soil environment.

Microcosmic and Diverse Plant Ecosphere

The diversity of the plant ecosphere is so varied that restricting it into direct and indirect PGPRs and then reviewing about it will give partial knowledge of the existing scenario. Since time immemorial, the plant scientists and microbiologists designated certain plains and spheres of plants into diverse habitats on the basis of the microbial load carried by them and named these habitats as rhizosphere (Barea et al. 2005), phyllosphere (Whipps et al. 2008) and spermosphere (Liu et al. 2012), and as a whole can be denoted as the plant ecosphere. Realistic and profound analysis using latest techniques reveals the fact that the plant ecosphere is very complex, and as far as research is concerned, it needs to be explored more and more. Although progress has been made in elucidating the structure and distribution of microbes in the plant ecosphere, much less is known of the functional consequences of the community as a whole or its composition vis-à-vis individual plants. The pattern of distribution of microorganisms in an ecosphere is not even but dynamic (Whipps et al. 2008). Many studies suggest that *Pseudomonas* form the most common of the dominant populations found in the rhizosphere of many different plant species like finger millet (*Pennisetum glaucum* L.), sunflower (*Helianthus annuus* L.), and maize (*Zea mays* L.) (Liu et al. 2012). Diversity of microbes may vary from plant to plant and from niche to niche, for example, wheat rhizosphere is dominated by *Azospirillum*, *Azotobacter chroococcum Beijerinckia*, *Klebsiella pneumoniae*, *Pseudomonas*, *Rhodobacter*, *Serratia*, and *Vibrio diazotrophicus* (Nelson and Mele 2007). *Cytophaga* and *Flavobacterium* constitute a dominant fraction of the rhizobacteria, in rye grass (Marilley and Aragno 1999), maize (Chelius and Triplett 2001) or shore pine (*Pinus contorta*; Chow et al. 2002), and cucumber (*Cucumis sativus*; Green et al. 2006). Kaiser et al. (2001) found dominant Bacteroidetes, *Chryseobacterium*, and members of the family *Oxalobacteraceae* in canola. In a study of rhizosphere soil of *Medicago sativa* and *Chenopodium album*, dominant bacteria found were *Variovorax*, *Acinetobacter calcoaceticus*, and *Arthrobacter ramosus*. Structural and functional characterization of rhizosphere of legumes has been focused primarily on symbionts such as *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Bradyrhizobium* (Tan et al. 2001). Several non-rhizobial asymbionts like *Klebsiella* have been identified consistently on the surfaces of roots and nodules of soybeans, alfalfa, and clover. Associations of *Azotobacter paspali* with roots of *Paspalum notatum* and *Beijerinckia indica* with roots of sugar cane have been observed in tropical soils (Raju et al. 1972). The unique association of *Azolla–Anabaena* symbiosis in N_2 fixation has been enormously explored (Ray et al. 1978). Many of the root-colonizing bacteria belonging to the genus *Pseudomonas*,

Azospirillum, and *Bacillus* also colonize internal tissues of plants and exist as endophytes (Berg and Hallmann 2006). Beside these rhizospheric inhabitants, other endophytic bacterial species with plant growth promoting attributes are *Phyllobacterium rubiacearum*, *Burkholderia solanacearum*, *Sphingomonas trueperi*, *Serratia plymuthica*, etc. *Burkholderia*, endophytic bacteria capable of nitrogen fixation, are found to be associated with the number of crops, also have the ability to degrade number of organic pollutants, and enhance plant growth in heavily contaminated soils (Zhang et al. 2000). Another endophyte *Methylobacterium* isolated from aspen roots has been found to be associated with the degradation of nitro-substituted explosives (Van Aken et al. 2004). Among endophytic fungi, some well-known examples are *Neotyphodium* sp., *Pestalotiopsis microspora*, *Guignardia* sp., *Acremonium* spp., and *Epichloe typhina* which confer a great resistance toward biotic and abiotic stresses. Mycorrhizal fungi form symbiotic associations with roots of most of the plants. According to estimates, nearly 80 % of all higher plant species can form mycorrhizal symbiosis, and of these arbuscular mycorrhizal (AM) association is the most common (Brundrett 2009). AM association is found widely associated with plants and involves fungi belonging to to the phylum Glomeromycota with 14 families and 29 known genera (Oehl et al. 2011). The term AM is derived from the fact that fungi of this group form arbuscules, structures through which fungal hyphae interconnect with the plasmalemma of root cells and exchange nutrients with the host plant. AM fungi now have been found in almost every habitat in which plants are able to grow such as in deserts, grasslands, tropical forests, and agricultural communities (Brundrett 2002).

PAMs occupying a particular rhizosphere have taken several million years to establish the symbiosis by the process of natural selection. Plant zone exudes a variety of organic compounds including sugars and simple polysaccharides, amino acids, organic acids, and phenolic compounds (Toal et al. 2000). Some of these compounds influence the growth and development of surrounding plants and soil microorganisms (Badri and Vivanco 2009). Certain exudes like flavonoids, enzymes, fatty acids, growth regulators, nucleotides, tannins, carbohydrates, steroids, alkaloids, polyacetylenes, and vitamins act as signals for microbial attraction or are used as carbon sources for microbial nutrition (Schulz and Dickschat 2007). The relationships established by PAMs their diversity, and structure depend on the biological and environmental factors that control the establishment and dynamics of microbial populations on the plant surface (Whipps et al. 2008).

Recent developments in rhizosphere soil metagenomics reveal that many hitherto unexplored bacterial groups are also present in the rhizosphere. Exploring these groups by unraveling their possible relationships with plants will start a new and fascinating area in rhizosphere research. Exploration of rhizosphere using the latest analytical tools may lead to novel leads about bacterial species composition in this microcosmic habitat. By applying cultivation-based methods, particular groups will be strongly selected for, depending on the type of medium used. Like in all other terrestrial habitats, the cultured bacterial fraction in the rhizosphere must be considered as a small subset of the total, uncultured community (Staley and Konopka 1985; Hugenholtz et al. 1998). Therefore, the use of cultivation-independent

techniques for recovery of bacterial groups from the rhizosphere must be considered as less biased. The main advantage of cultivation-based over cultivation-independent techniques is that bacterial isolates will be obtained that can be used for studying biotic interactions, e.g., with other microbial groups or with the plant host. However, this does not mean that the hidden diversity should not be explored. With the advancement of technology and tools of genomics and proteomics, the unknown treasure of the rhizosphere can be and has to be unearthed.

Non-disruptive in situ visualization techniques are already being used for detailed studies on the interactions of microorganisms within the rhizosphere. Improving these techniques, based on the use of confocal laser scanning microscopy and fluorescent proteins, will not only allow the simultaneous imaging of different populations of microbes in the plant ecosphere but also the temporal–spatial visualization of gene expression (Barea et al. 2005). Recently, high-throughput sequencing using sequencing-by-synthesis technology (pyrosequencing) was introduced as a new approach capable of better revealing the taxonomic diversity within microbial communities (Acosta-Martinez et al. 2008). Partial ribosomal amplification before pyrosequencing is an approach that can be used to describe precisely the microbial communities in environmental samples (Vartoukian et al. 2010). Despite the inherent bias in PCR and all molecular methods which are same for all analyzed samples, it is interesting to combine the selectivity of primer-based PCR with high-throughput sequencing technology. Also, the use of the bacterial tag-encoded FLX amplicon pyrosequencing method allows for mixed samples to be run on the same sequencing run and later binned (Dowd et al. 2008). Novel research is needed to improve immunofluorescence techniques to assess gene transfer in ecosphere environments without the need to cultivate microorganisms. Many traits of root colonization by rhizo-microbes have already been identified, but novel molecular approaches are being used to screen for new traits. It will be interesting to decipher the genes encoding proteins involved in transport or signal transduction pathways involved in colonization. An increase in current knowledge on quorum-sensing systems, such as those based on N-acyl-homoserine lactones, is important in understanding the ecodynamics of microbial populations and the cellular and molecular aspects of signaling processes in microbe–microbe interactions. Development in functional genomics, proteomics, and metabolomics will be useful to identify the genes and metabolites expressed in the plant ecosphere, while the use of promoters to drive gene expression specifically at the root–soil interfaces will allow the engineering of microorganisms for beneficial purposes (Barea et al. 2005; Vartoukian et al. 2010). The use of microbial inoculants must take into account the importance of retaining microbial diversity in ecosphere ecology and in achieving realistic and effective biotechnological applications. The improvement of molecular biology-based approaches will be fundamental for analyzing microbial diversity and community structure and to predict responses to microbial inoculation/processes in the environment. The consequences of the cooperation between microbes in the rhizosphere under field conditions will be important to assess their ecological impacts and biotechnological potential (Miller and Jastrow 2000). Despite the difficulty in selecting effective single multifunctional microbial inoculates, appropriate combinations of

mixed microbial inoculants could be recommended. New environmentally friendly, genetically modified, microbial inoculates produced commercially to protect plants from disease and to promote plant growth can serve as a boon in agricultural technology. These new products are expected to lead to a reduction in the use of biocides and chemical fertilizers (Barea 2000; Barea et al. 2005).

Metabolomic approaches are required for detecting the role of metabolites released by microbes and plants, performing manifold functions. A wide array of metabolites are released by microbes governing multidimensional mechanisms, performing similar and dissimilar functions that could be determined using gene modeling and protein modeling tools of bioinformatics. Similarly, a single metabolite can be secreted by a wide array of organisms in the rhizosphere or plant ecosphere, and holistic metabolomics and bioinformatics tools will be required to determine its complete role. Finally, this data will be useful for bioengineering of future bioinoculants providing plants with proper diversity and metabolite concentration. The holistic approach will be to study the regulation of microbial genes, proteins, and their metabolites under a common roof under a wider perspective in totality for future benefits. Availability of technologies for studying cooperative microbial interactions in the plant ecosphere guarantees a greater understanding of these processes, which will facilitate their successful applications in agricultural biotechnology of course to achieve this; the research has to look beyond the concept of direct and indirect mechanisms and study the microcosmos of rhizosphere as a whole.

Is There Anything Like Direct and Indirect PGPRs?

Increase in agricultural productivity to meet ever-growing food demands of human population is a matter of great concern for all countries. United Nations Economic and Social Commission for Asia and the Pacific (ESCAP) conducted a theme study in April 2009 entitled "Sustainable Agriculture and Food Security in Asia and the Pacific" in which the importance of revitalization of native soil systems for improved crop yield was emphasized (Sachdev and Cameotra 2013). Such revitalization processes can be carried out in an eco-friendly manner using various biological amendments. Many microorganisms found in rhizosphere (the soil under the influence of plant roots) share a mutualistic relationship with plants conferring marked beneficial effects. Several mechanisms are reported by which rhizobacteria help in plant growth promotion (Glick 1995; Zahir et al. 2004; Gamalero and Glick 2011). Hence, rhizosphere biology is considered to be the most intensive area of research in agriculture. Kloepper and Schroth (1978) introduced the term "rhizobacteria" for the soil bacterial community that competitively colonized plant roots and stimulated growth thereby reducing the incidence of plant diseases. PGPR can be defined as the indispensable part of rhizosphere biota that when grown in association with the host plants can stimulate their growth. PGPRs are seen as successful rhizobacteria, getting established in soil ecosystem due to their high adaptability in a wide variety of environments, faster growth rate, and biochemical versatility to metabolize a wide

range of natural and xenobiotic compounds. Cook (2002) considered PGPRs as the significant components in the management of agricultural practices with innate genetic potential.

The concept of direct and indirect mechanisms of PGPR (Kloepper et al. 1980; Glick 1995; Gupta et al. 2000; Castro et al. 2009) suggested that PGPR strains can promote plant growth or development either directly and indirectly. Direct methods include providing nutrients to the plant and growth enhancement by secretion of phytohormones, while indirect stimulation is basically related to biocontrol (Zahir et al. 2004; van Loon 2007). Several other important roles of PGPRs in maintaining plant health and tolerance to environmental stress have also been well recognized (Malhotra and Srivastava 2009).

Although sometimes, it is really very difficult to exactly signify the difference between the two mechanisms as the combined impact of these devices play a significant role in plant health protection and disease management (Bhattacharya and Jha 2012). These so-called direct and indirect mechanisms, however, hold well in theoretical and literal explanation only. In nature, all these mechanisms are interconnected and interwoven just like a web. In natural conditions, the compounds or metabolites released by microbes function in multifaceted and diverse manners. A single compound released by microbes can play diverse functions depending upon the conditions or may perform similar functions under diverse conditions depending upon the biotic and abiotic factors. Several PGPR strains of *Pseudomonas* show both direct and indirect mechanisms to enhance plant growth as well as act as biocontrol agents (Arora et al. 2001; Walsh et al. 2001; Weller 2007; Avis et al. 2008; Kraepiel et al. 2009; Khare and Arora 2010; Kim 2012). Jha et al. (2009) reported simultaneous phosphate solubilization potential and biocontrol ability by *Pseudomonas aeruginosa*, *Pseudomonas plecoglossicida*, and *Pseudomonas mosselii*. Similarly, some epiphytic *Pseudomonas* spp. can release surfactants that increase the wettability of leaf surfaces, making it easier for microorganisms to use water and increasing solubilization and diffusion of nutrients, thereby increasing substrate availability to epiphytic bacteria and display antagonistic activity against pathogens (Bunster et al. 1989). *Bacillus* species have been reported to promote the growth of a wide range of plants; however, they are also very effective in the biological control of many plant microbial diseases (de Freitas et al. 1997; Kokalis-Burelle et al. 2006). Similarly, bacteria in the genera *Streptomyces*, *Pseudomonas*, *Burkholderia*, and *Agrobacterium* suppress plant disease through production of antibiotics or siderophores and induction of systemic resistance and enhance plant growth by supplying nutrients (Tenuta and Beauchamp 2003).

Endophytes also perform several multiple works like on one hand facilitate nutrient uptake from soil and on the other hand assist in management of abiotic stress. Colonization of a plant by endophytes leads to altered physiology of the host due to enhanced nutrient uptake from rhizosphere (Jumpponen and Trappe 1998; Caldwell et al. 2000), production of phytohormones (Tudzynski and Sharon 2002), secretion of bioactive alkaloids and secondary metabolites (Schulz et al. 1995; Miller et al. 2002), induction of the host's defense system (Zhang et al. 2004),

production of siderophores and lytic enzymes, nitrogen fixation, and phosphate solubilization (Chernin and Chet 2002). Endophyte infection increases root hair length and branching which enhances water absorption (Malinowski et al. 1999). Endophyte-infected plants also accumulate more carbohydrates and fungal metabolites like mannitol and arabitol in host tissues, which are osmotically active compounds and thus contribute to drought tolerance (Hill et al. 1990; Richardson et al. 1992). Within their host, endophytes produce secondary metabolites and alkaloids which sometimes perform dual functions of protection as well as growth enhancement. This can be exemplified with the help of loline alkaloid production in grasses *Festuca* and *Lolium* spp. infected with fungus *Neotyphodium*. Loline alkaloids are water-soluble secondary metabolites which perform dual role in protection from insect pests and osmotic adjustment during drought stress to reduce its adverse effects (Bacon 1993). Similarly, other plant growth enhancing attributes of endophytes such as nitrogen fixation and phytohormone production also boost the resistance against pathogens by strengthening their host's fitness (Berg and Hallmann 2006). Moreover, endophytes also compete with other organisms including insects, pests, pathogenic bacteria, fungi, and nematodes for nutrients and shelter (Pal and Gardener 2006).

Certain mycoparasitic fungi play an important role in the growth and ecological fitness of plants. *Trichoderma*, a widely studied mycoparasite, is a common inhabitant of the soil and found in all climatic zones (Brewer et al. 1971; Danielson and Davey 1973). *Trichoderma* have high reproduction capacity and ability to survive under extremely diverse and unsuitable habitats which further magnifies their ecological roles. Regardless of their lifestyle, various plant growth promoting microorganisms employ similar biochemical and physiological mechanisms to maintain the fitness of their mutualistic partners and so do these antagonistic fungi. *Trichoderma* control the population size of various destructive fungal pathogens by producing inhibitory compounds such as antibiotics, toxic metabolites, and hydrolytic enzymes (Benitez et al. 1998). *Trichoderma* also influence plant growth by enhancing root growth and development, abiotic stress tolerance and enhanced nutrient uptake. An increase of up to 300 % in crop productivity is reported after the addition of *Trichoderma hamatum* or *Trichoderma koningii* (Chet et al. 1997). *Trichoderma* are known to produce cytokinin-like molecules and organic acids which are able to solubilize phosphates and other mineral complexes (Harman et al. 2004). The solubilization of insoluble minerals increase availability of minerals in soluble form and make it accessible to plant roots. There are innumerable such examples which support the fact that a single PGPR or metabolite secreted by it can function in multiple ways (Holguin and Bashan 1996; Arora et al. 2001; Dietrich et al. 2006; Pamp and Nielsen 2007; Zhang et al. 2007; Avis et al. 2008; Kraepiel et al. 2009; Fajardo and Martinez 2008; Arora et al. 2008; Khare and Arora 2011).

Hence, it is not generally correct to state whether a particular PGPR strain is working in a direct or indirect mode. Instead of having a myopic vision, a broader perspective is required for a more holistic approach and look into the functions of PGPRs as well as their metabolites in totality, as is occurring in the rhizosphere

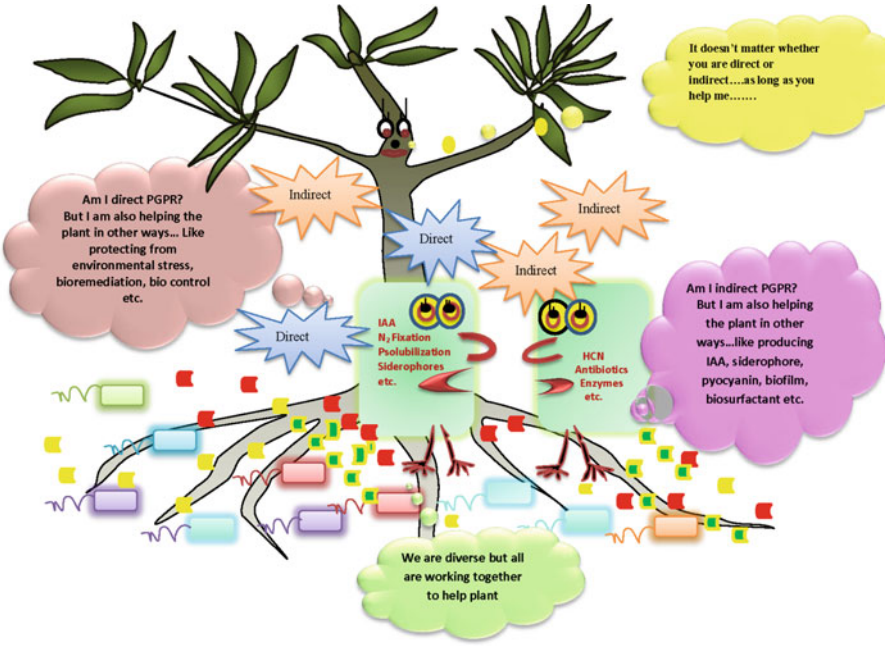

Fig. 16.1 Multifaceted microbes simultaneously performing diverse tasks blurring the distinction between direct and indirect

(Fig. 16.1). In this section, we discuss with examples how PGPRs and metabolite secreted by them can function in multiple ways, performing diverse functions in the rhizosphere for themselves and the host plant.

Biological Nitrogen Fixation (BNF)

Nitrogen fixation is a key process in which molecular nitrogen is reduced to form ammonia, which is the form of nitrogen that is used by living systems for the synthesis of many bioorganic compounds. Biologically fixed nitrogen could be directly absorbed by plants and used for the betterment of plant growth promotion. Annually, approximately 2.5×10^{11} kg NH_3 is fixed from the atmosphere by the process of BNF. All the nitrogen-fixing organisms are prokaryotes. Some of them live independently of other organisms—the so-called free-living nitrogen-fixing bacteria. Others live in intimate symbiotic associations with plants or with other organisms (e.g., protozoa) (Cheng 2008). By providing nitrogen (the most important limiting factor in any ecosystem), nitrogen fixers not only enhance the growth and productivity but result in a healthier plant which is much more capable of combating diseases and pathogens as well as able to survive under stress conditions. Some nitrogen fixers are also reported to possess the abilities to solubilize insoluble phosphates,

produce phytohormone, chelate iron, and suppress phytopathogens (Kukreja et al. 2004; Liba et al. 2006; Avis et al. 2008; Chen et al. 2011; Kannapiran and Ramkumar 2011; Keyeo et al. 2011).

Asymbiotic Nitrogen Fixation

A number of free-living soil bacteria have the capacity to fix atmospheric nitrogen, and the most common among these include *Azotobacter*, *Acetobacter*, *Azospirillum*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, and cyanobacteria (Baldani et al. 1997; Vessey 2003; Mirza et al. 2006). The associative effects of *Azotobacter* also include provision of soluble phosphate, growth-promoting hormones, vitamins, minerals, and improved water-holding ability (Rodriguez and Fraga 1999). *Azotobacter* are unique biofertilizers that maintain the N level in agricultural soil and synthesize the plant growth-promoting hormones such as indoleacetic acid (IAA) and gibberellins (Gauri et al. 2012). Exopolysaccharides (EPS) from *Azotobacter* can immobilize a variety of other rhizospheric bacteria to work as community that enables them to act as biofertilizers or antagonist to phytopathogens. Plant–microbe interactions and the interaction among the microbes are also enhanced due to EPS production (Mandal et al. 2007). A dual inoculation of *Azospirillum brasilense* and vesicular–arbuscular mycorrhizal fungi (VAM) increased the root biomass and the absorption of phosphorus by pearl millet, barley yield (Subba Rao et al. 1985), the number of colonization sites of VAM fungi in halophytic plants, and suppression of infection, suggesting the role of non-symbiotic nitrogen-fixing bacteria *Azospirillum* in nitrogen fixation, P solubilization, plant growth promotion, and disease suppression under salt stress (Holguin and Bashan 1996). The survivability of *Azospirillum* under hostile conditions holds promise for the future application of mixed inoculants of *Azospirillum* in salt-affected soils (Holguin and Bashan 1996). *Azospirillum* is capable of releasing signal molecules that enhance proton efflux from root and improves water and nutrient uptake from soil thereby encouraging the growth of microflora that assist in aggressive root colonization and biofilm formation, and this protects plant health and assists in disease management (Bashan 1993).

Symbiotic Nitrogen Fixation

Rhizobia are generally regarded as microbial symbiotic partners of legumes and are mainly known for their role in the formation of nitrogen-fixing nodules (Antoun and Prévost 2005). Rhizobia are a vast group of rhizobacteria with representatives that have proven plant growth promoting activities through nitrogen fixation. These bacteria can also produce plant growth regulators and solubilize organic and inorganic phosphates that also have a role in plant growth promoting activities (Antoun et al. 1998). In addition to their plant growth promoting effects, *Rhizobium* spp. have been increasingly associated with disease-suppressive effects (Elbadry et al. 2006; Huang et al. 2007; Huang and Erickson 2007; Siddiqui et al. 2007).

Disease suppression by *Rhizobium* spp. has been linked to direct inhibition of pathogen development (through competition or antibiosis) as well as indirect inhibition through the stimulation of plant defense mechanisms.

Another trait of *Rhizobium* spp. is their ability to produce iron-chelating siderophores and IAA (Arora et al. 2001; Sridevi and Mallaiah 2007). *Rhizobium* spp. has been studied more in depth where induced systemic resistance (ISR) is concerned (Avis et al. 2008). The presence of *Rhizobium* spp. activates the defense mechanisms of the plant when challenged with a pathogen through the production of plant defense compounds (phenolics, flavonoids, or other phytoalexins, in particular) (Andrade et al. 1998). The incorporation of nitrogen-fixing rhizobial strains to fungal–bacterial biofilms has been shown to improve potential biofilm formation in nitrogen-deficient environment (Seneviratne et al. 2007). The formation of biofilm results in aggregation of microbial cells in addition to an extracellular biopolymer, which provides structure and protection to the community. Biofilm attached to the plant roots help in cycling of nutrients thus promoting plant growth as well as in the biocontrol of pest and diseases, resulting in improved agricultural production (Seneviratne and Jayasinghearachchi 2003).

The effect of PGPRs in combination with rhizobia in improving legume growth and development may be possible because of increased production of nod gene products inducing flavonoids, stimulation of root hair development, secretion of vitamin B by PGPR enhancing rhizobial growth in rhizosphere, production of plant growth regulators, improved mineral uptake, mobilization of insoluble nutrients, and suppression of pathogens (Spaepen et al 2007; Weller 2007).

Rhizobia isolated from *Acacia*, such as *Sinorhizobium arboris*, turned out to be salt tolerant, capable of growing in 0.3–0.5 M (2–3 %) NaCl and promote plant growth, enhance nodulation, and provide protection under salt stress (Zahran et al. 1994; Kumar et al. 1999). About 1–10 % of rhizobial strains possess stress enzyme ACC deaminase (Duan et al. 2009); thus, it is possible to increase the nodulation efficiency of rhizobial strains that lack ACC deaminase by engineering them with rhizobia ACC deaminase genes (and regulatory regions). In fact, insertion of an ACC deaminase gene from *R. leguminosarum* bv. *viciae* into the chromosomal DNA of a strain of *Sinorhizobium meliloti* that lacked this enzyme dramatically increased both nodule number and biomass of host alfalfa plants (Ma et al. 2003). Because of political/regulatory considerations, genetically engineered strains of rhizobia may not currently be acceptable for use in the field; however, several commercial inoculant producers are already screening rhizobial strains for active ACC deaminase. Poonthrigpun et al. (2006) reported the role of *Rhizobium* in removal of pollutant (acenaphthylene) from petroleum-contaminated soil. Elimination of toxicity made the plant with easy accessibility of nutrient and promoted plant health, thereby increasing systemic resistance of the plant and protecting it from the attack of any foreign pathogens (Wehner et al. 2010).

Hence, it can be stated that multifaceted tasks are performed by nitrogen-fixing microbes, and their presence ensures not only supply of N_2 but also plant growth promotion, biological control, stress management, and removing toxicity of pollutant contaminated sites. These functions are both part of direct and indirect approaches.

Phosphate Solubilization

Phosphorus is another very important macronutrient that is reported to be a critical factor for many crop production systems, due to the limited availability in soluble forms in the soils (Xiao et al. 2011). Microbes present in the soil employ different strategies to make use of unavailable forms of phosphorus and in turn also help in making phosphorus available for plants to absorb. Microorganisms involved in the solubilization of insoluble phosphorus include bacteria, fungi, actinomycetes, and AM fungi (Khan et al. 2007; Wani et al. 2007a; Xiao et al. 2009). Phosphate-solubilizing microorganisms have attracted the attention of agriculturists as soil inoculums to improve plant growth and yield (Goldstein et al. 1999; Fasim et al. 2002; Young et al. 2003). PGPR included in the genera *Arthrobacter*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Rhodococcus*, and *Serratia* are beneficial to plant P nutrition and growth (Glick 1995). P-solubilizing microorganisms mediate soil processes such as exudation of soluble compounds, storage and release of nutrients and water, nutrient mobilization and mineralization by roots and microorganisms, soil organic matter decomposition, phosphate solubilization and nitrogen fixation, nitrification, denitrification and sulfur reduction, and detoxifying effect of heavy metal pollution (Ravikumar et al. 2007). All these processes promote the growth of the plant, enhance its nutritional status, and protect it from the attack of any foreign pathogens (Khan et al. 2007).

Phosphate-Solubilizing Bacteria (PSB)

PSB can benefit plant growth by several different mechanisms such as enhancing BNF, plant hormone production, and antagonizing pathogens (Misra et al. 2012). Son et al. (2006) have reported that the number of nodules, dry weight of nodules, yield components, grain yield, nutrient availability, and uptake in soybean were found to be enhanced by phosphate-solubilizing *Pseudomonas* spp. According to Afzal et al. (2005), inoculation of *Pseudomonas* and *Bacillus* species has resulted in increased phosphorus uptake followed by increased grain yield of wheat (*Triticum aestivum* L.). PSB inoculation had favorable effect on salinity stress tolerance of *Zea mays* L. under NaCl stress (Bano and Fatima 2009). Furthermore, symbiotic nitrogenous rhizobia, which fix atmospheric nitrogen into ammonia and export the fixed nitrogen to the host plants, have also shown phosphate solubilization activity. For instance, *Rhizobium leguminosarum* bv. *trifolii* (Abril et al. 2007), *R. leguminosarum* bv. *viciae* (Alikhani et al. 2007), and *Rhizobium* species nodulating *Crotalaria* species (Sridevi et al. 2007) improved plant growth by simultaneous solubilization of phosphates and BNF. PSB *Bacillus firmus* produces a phytohormone, IAA, and this bacteria is used for augmenting cultivation of rice (*Oryza sativa* L.) in acid soils (Datta et al. 1982; Sadaf et al. 2009). The role of PSB has been displayed in the management of abiotic stress like drought, chilling, alkalinity, acidity, salinity, calcium, and desiccation (Arora et al. 2012). Stress-tolerance potential of PSB isolated from acidic soils has been reported

that assists in plant growth promotion and disease suppression (Nautiyal 1999; Thakuria et al. 2004; Arora et al. 2012). Panhwar et al. (2012) reported diverse PSB included in the genera *Bacillus*, *Pseudomonas*, and *Rhizobium*, performing multiple tasks of plant growth promotion and disease suppression against phytopathogen *Rhizoctonia solani*. PSB have a high potential to be used for the management of phosphorus in P-deficient soils as well as disease suppression. Therefore, usage of environmentally friendly microorganisms is needed for plant growth promotion and disease control for sustainable agriculture (Panhwar et al. 2012).

Mycorrhiza

Mycorrhizae are the most important rhizosphere microorganisms with the capacity to solubilize phosphate and in an intricate and efficient manner provide it to the host plant. Under phosphorous-limiting conditions, mycorrhizal network helps host plants in allocating P and other nutrients in exchange for carbon (Pearson and Jakobsen 1993). The external hyphae of AMF extend beyond the P depletion zone and provide a wider physical exploration of undepleted soil to absorb nutrients. Moreover, mycorrhizal fungi also employ some biochemical mechanisms for P uptake such as production of extracellular phosphatases (Tarafdar and Marschner 1994), acidification of rhizosphere through proton efflux which mobilizes P (Rigou and Mignard 1994), and excretion of chelating agents like siderophores in acidic soils where P is bound with Fe or Al. While P uptake enhances plant growth, it also provides a bioprotective aspect. There is evidence that plants that took up larger amounts of nutrients through their AM fungal symbiont have an increased tolerance for pathogenic infections (Karagiannidis et al. 2002). Nitrogen uptake is also enhanced by mycorrhizal hyphal network which absorb N in the form of NO_3^- or NH_4^+ (Subramanian and Charest 1999). Phytohormone production by mycorrhizal fungi regulates the infection and establishment of nodulation by rhizobia. Gay and Debaud (1987) reported that P deficiency brings reduction in phytohormone production, nodulation, and nitrogen-fixing ability under drought stress. Production of cytokinin-like substances, gibberellic acid, and ethylene has also been reported by mycorrhizal fungi (Graham and Linderman 1980; Azćon-Aguilar and Barea 1982; Livingston 1991). Since phytohormones are important plant growth regulators, their production by fungal symbiont widely affects the physiology of the host plant by increasing their growth and fitness and by conferring tolerance to biotic and abiotic stresses. Mechanisms which help the host plant in enhanced nutrient uptake, protection to pathogens, and modifying their habitat also provide escaping mechanisms to survive under abiotic stresses. Among several mechanisms of drought stress tolerance are: enhanced water and nutrient uptake, alteration in soil–water retention properties, accumulation of antioxidant proteins, and osmotic adjustments (Allen 1982; Hardie 1985). The physical, chemical, and biological action of mycorrhizal hyphae and hyphal exudates affects the soil aggregation and thus increases the soil moisture retention properties (Oades and Waters 1991; Hamblin 1985). Another important mycorrhiza-mediated mechanism of drought tolerance includes

accumulation of antioxidant proteins which protect the plants from oxidative stress due to the generation of free radicals such as superoxide radicals or hydrogen peroxide (Smirnoff 1993). Mycorrhizal fungi also provide protection from metal stress by chelation of heavy metals. In addition to the cell-wall components, glomalin proteins produced by mycorrhizal fungi are efficient sequestering compounds for Pb, Mn, Fe (Chern et al. 2007), Cu, Cd, and Zn (Carnejo et al. 2008). Due to their outstanding capabilities of heavy metal chelation, mycorrhizal symbiosis is considered as an important component of habitats contaminated with industrial waste. Most of the functions performed by mycorrhizal fungi toward plant growth enhancement and tolerance to stress conditions rely on high degree of root colonization, biochemical modification of mycorrhizosphere, and extensive hyphal network which affect host fitness as well as maintain ecological balance by dissolution of insoluble and complex minerals from parent rock material of earth crust into soluble, biologically available form (Allen 1991; Rillig 2004).

A wide array of functions carried out by phosphate-solubilizing organisms cannot be covered by direct or indirect alone and have to be seen in a more complete manner.

Phytohormones

The phytohormones auxins, cytokinins, gibberellins, ethylene, and abscisic acid (ABA) play key roles in the regulation of plant growth and development (Salisbury and Ross 1992). *Azotobacter*, *Arthrobacter*, *Azospirillum*, *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Flavobacterium*, *Micrococcus*, *Agrobacterium*, *Clostridium*, *Rhizobium*, *Burkholderia*, and *Xanthomonas* (Miter et al. 2002; Tsakelova et al. 2006; Joo et al. 2009) are rhizobacteria that are known for IAA production (Gravel et al. 2007). IAA causes rapid establishment of root system advantageous for young seedlings as it increases the ability to anchor them to the soil and to obtain water and nutrients from soil. *Pseudomonas fluorescens* and *Pseudomonas chlororaphis*, two known biocontrol agents with growth-promoting ability, are reported to synthesize IAA and display antagonistic activity against pathogens (Kang et al. 2006). *Rhizobium* can equally produce growth-regulating phytohormones and solubilize organic and inorganic phosphates that would have a role in their plant growth promoting activities (Antoun et al. 1998). The role of IAA has now been explained in the suppression of phytopathogens such as *M. phaseolina* by the development of root system, providing nutrients and support to the infected plants (Arora et al. 2010). IAA when supplied to excised potato leaves eventually reduced the disease by *Phytophthora infestans* (Noel et al. 2001). In addition to stimulating plant growth as plant growth regulator, IAA can also stimulate ACC synthase to produce more ACC, which can be transformed into ethylene by ACC oxidase (Mayak et al. 2004). Conversely, the simultaneously produced ACC deaminase can hydrolyze ACC and inhibit ethylene production. As a consequence, the final effect on ethylene production or root growth depends on the balance of the IAA and the ACC deaminase produced in concert by *P. putida* (Arora et al. 2012). Some of the newly synthesized IAA is taken up by the plant and, in conjunction with the endogenous plant IAA,

can further stimulate plant cell proliferation and elongation (Arora et al. 2012). The combined effect of IAA and ethylene regulation in the rhizosphere has been reported for the increased growth of hydroponic tomatoes in the presence of *Trichoderma* (Gravel et al. 2007). Hence, it could be highlighted that IAA has multiple roles; it can serve as a plant growth stimulator, biocontrol agent, and stress regulator.

Siderophores

Microbial siderophores are well known for their ecological significance. They are known to directly promote plant growth by supplying iron (Loper and Henkels 1999). Bacteria belonging to the genera *Azotobacter*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Serratia*, etc., produce siderophores (Kloepper et al. 1980; Neilands 1995; Arora et al. 2001; West and Buckling 2002; Rajkumar et al. 2010; Saha et al. 2012). Generally, bacteria produce four types of siderophores, namely, hydroxamate, catecholate, salicylate, and carboxylate. These siderophores play an important role in the extracellular solubilization of iron from minerals or organic substances (Kloepper et al. 1980). Siderophore production in iron-stress conditions confers upon these organisms an added advantage, resulting in exclusion of pathogens due to iron starvation. Siderophores contribute to disease suppression by conferring a competitive advantage to biocontrol agents for the limited supply of essential trace minerals in natural habitats. Siderophores thus are involved both in plant growth promotion and health protection (Kraepiel et al. 2009). Siderophore-producing strains of *Penicillium chrysogenum* and *P. aeruginosa* assist in nodulation, nitrogen fixation, plant growth promotion, biological control, release of organic acids and in managing abiotic stress tolerance (Mahmod and Allah 2001). Loper (1988) reported the role of siderophore obtained from *Pseudomonas* in biological control of *Pythium ultimum* (a causal agent of damping off and root rot in many crops) and plant growth promotion. Arora et al. (2001) reported the role of siderophore-producing strain of *Rhizobium meliloti* in disease suppression of phytopathogen *M. phaseolina* and considerable improvement in seedling biomass and nodule weight. Kraepiel et al. (2009) reported multiple roles of siderophores by free-living nitrogen-fixing bacteria *Azotobacter vinelandii*. *A. vinelandii* is a diazotroph that excretes catechol siderophores that bind a variety of metals in addition to iron. At low concentrations, complexes of essential metals (Fe, Mo, V) with siderophores are taken up by the bacteria through specialized transport systems and result in nutrient cycling of the metals and promote plant growth. In the topsoil, metals are primarily bound to plant-derived organic matter; siderophores extract essential metals from natural ligands and deliver them to the bacteria. This process appears to be a key component of a mutualistic relationship between trees and soil diazotrophs, where tree-produced leaf litter provides a living environment rich in organic matter and micronutrients for nitrogen-fixing bacteria, which in turn supply new nitrogen to the ecosystem (Kraepiel et al. 2009). Interest in the pseudomonads has increased recently because of the possible use of siderophores as biopesticides (Fajardo 1997) and the possible use of pseudomonads in detoxifying chemical wastes through a wide range of

enzymatic metabolic activities (Jyothi and Rao 2009). Siderophores have also been reported to function as signal molecules helping in biofilm formation and even as antibiotic molecules that suppress the growth of phytopathogens and promote plant growth (Thomashow and Weller 1990; Iain et al. 2002; Dietrich et al. 2006; Maddula et al. 2008; Harrison and Buckling 2009; Khare and Arora 2011). Siderophores are thus one of the most diverse biomolecules secreted by PGPRs which perform a multitude of functions falling both under direct and indirect roles. A more collective approach is required to determine their complete role in the rhizosphere and for development of future inoculants.

Antibiotics

Antibiotics produced by PGPR include DAPG, phenazine-1-carboxylic acid, phenazine-1-carboxamide, pyoluteorin, pyrrolnitrin, oomycinA, viscosinamide, butyrolactones, kanosamine, zwittermicin A, aerugine, rhamnolipids, cepaciamide, ecomycins, pseudomonic acid, azomycin, cepafungins, and karalicin (Fernando et al. 2005). These antibiotics are known to possess antiviral, antimicrobial, antihelminthic, phytotoxic, antioxidant, cytotoxic, antitumor, and plant growth promoting activities (Kim 2012).

The main targets of these antibiotics are the electron transport chain (phenazines, pyrrolnitrin), metalloenzymes such as copper-containing cytochrome c oxidases (hydrogen cyanide), membrane integrity (biosurfactants), or cell membrane and zoospores (DAPG, biosurfactants) (Haas and Defago 2005; Raaijmakers et al. 2006). The recent work showed that DAPG-producing *Pseudomonas* spp. could colonize with about a 96% dominance ratio of total bacteria in rhizosphere and increase aggregation of soil particles and supply nutrients to plant thus assisting in plant growth promotion (Kim 2012). Thus, DAPG performs the dual function of plant growth promotion and disease management. A cascade of endogenous signals such as sensor kinases, N-acyl homoserine lactones, and sigma factors regulate the synthesis of antibiotics. The genes responsible for the synthesis of antibiotics are highly conserved. In addition to direct antipathogenic action, antibiotics also serve as determinants in triggering induced systemic resistance (ISR) in the plant system and contribute to disease suppression by conferring a competitive advantage to biocontrol agents (Bhattacharya and Jha 2012). Synergism between antibiotics and ISR may further increase host resistance to plant pathogens (Fernando et al. 2005).

PGPR *Bacillus amyloliquefaciens* is known for lipopeptide and polyketide production. It seems likely that the secretion of such compounds is important for biological control activity and plant growth promotion (Ongena and Jacques 2008). Additionally, it is now well established that lipopeptides can have multiple activities. In addition to their antibiotic activities, various lipopeptides have been shown to be involved in root colonization followed by biofilm formation and the induction of plant host resistance pathways. All of these activities contribute to the improvement of plant health and growth under different conditions (Ongena and Jacques 2008).

The antimicrobial activity of phenazine depends on the rate of oxidative–reductive transformation of the compound coupled with the accumulation of toxic superoxide radicals in the target cells (Hassett et al. 1992, 1995). Priming the seeds with phenazine-producing *P. chlororaphis* not only controlled seed-borne diseases of barley and oats but promoted growth and development of plants. Though phenazine plays a vital role in the management of soilborne pathogens, the chemotaxis and motility of the bacteria decides the antifungal action of the antibiotic producers (Hassett et al. 1992). The motile strain exerts antifungal action and displays aggressive rhizosphere colonization and promotes plant growth. A connection between phenazine and biofilm production has been established in *P. chlororaphis* (Maddula et al. 2008). Similarly, *P. aeruginosa* is reported to produce pyocyanin (a siderophore) which acts as antibiotic against phytopathogenic fungi thereby protecting the plants from infection and also act as signal molecules influencing EPS production leading to the development of biofilm by root-nodulating bacteria *Rhizobium* (Khare and Arora 2011). Formation of biofilms and root colonization is interlinked and regulated at different stages via diverse mechanisms. Henceforth, we can say that antibiotics perform multidimensional functions in protecting the plant from the attack of pathogens, triggering ISR, biofilm formation, root colonization, serving as signal molecules, influencing EPS production, causing nodulation, and promoting plant growth.

Induced Systemic Resistance (ISR)

Some PGPR can trigger the phenomenon of ISR which is phenotypically similar to systemic acquired resistance (SAR) which occurs when plants activate their defense mechanism in response to primary infection by pathogens. Activation of defense system protects the plant from being attacked by any foreign invader, thereby promoting growth of the plant (Kloepper et al. 2004). When appropriately stimulated, plants develop a state of enhanced defensive capacity that is called induced resistance (Van Loon et al. 1998). ISR can induce alterations to host physiology leading to an overexpression of plant defensive chemicals including pathogenesis-related (PR) proteins such as chitinases, peroxidases, superoxide dismutase phenylalanine ammonia lyase, phytoalexins, and polyphenol oxidase enzymes (Gamalero and Glick 2011).

ISR was discovered as a mode of action of disease suppression by PGPR *Pseudomonas* (Wei et al. 1991). Salicylic acid (SA) is a *Pseudomonas* metabolite that triggers induced resistance (Maurhofer et al. 1998; De Meyer et al. 1999). Most studies investigated the role of bacterially produced SA in induced resistance that functions as a signal molecule (Press et al. 1997; Audenaert et al. 2002; Ran et al. 2005). Interestingly, SA biosynthesis is often linked to the production of siderophores, like pyochelin in *P. aeruginosa* (Audenaert et al. 2002) or pseudomonine in *P. fluorescens* (Mercado-Blanco et al. 2001), and instead of excreting SA in the rhizosphere, these bacteria may well produce only the SA-containing siderophore.

Additional *Pseudomonas* traits that are involved in ISR include, an iron regulated N-alkylated benzylamine derivative (Ongena et al. 2005), the O-antigen of the lipopolysaccharides (LPS), and flagella (Van Peer and Schippers 1992; Van Wees et al. 2000; Meziane et al. 2005). The *Pseudomonas* metabolite DAPG (known as an antibiotic) demonstrated to effectively induce ISR in *A. thaliana* against *Peronospora parasitica* (Iavicoli et al. 2003) and against *P. syringae* pv. tomato (Weller et al. 2004). Hence, multiple genes are performing multiple functions to enhance ISR as a powerful tool for plant growth promotion and disease suppression.

Certain mycorrhizal fungi in roots of the host plant mediate the activation of the plant's defense system. Activated defense system responds quickly to any subsequent attack or penetration by pathogens. Defense reactions triggered by mycorrhizal colonization include increase in lignin deposition in the host's cell wall which is among the early defense responses and provide a strong physical barrier to restrict the pathogen attack (Dehne and Schoenbeck 1979). Other defense reactions include accumulation of callose and phenolic compounds (Cordier et al. 1998); production of hydrolytic enzymes such as chitinase, chitosanase, β-glucanase, and superoxide dismutase (Pozo et al. 2002); enhanced level of PR proteins (Liu et al. 1995); enhanced levels of jasmonic acid (JA) and SA which act as signaling molecules to activate plant defense response; accumulation of reactive oxygen species; and accumulation of phytoalexins (Morandi 1996). Most studies of systemic resistance have been carried out using fungal pathogens; however, this approach may also have potential in the control of bacterial pathogens such as *P. syringae* pv. *lachrymans*, the causal agent of bacterial angular leaf spot (Gamalero and Glick 2011).

Enzymes

Mechanisms by which rhizobacteria can also inhibit phytopathogens is the production of lytic enzymes like phosphatases, chitinases, β-glucanase, proteases, and dehydrogenase (Hayat et al. 2010). Primarily microbes release these extracellular enzymes for the initial degradation of high molecular weight substrates such as cellulose, chitin, pectin, and lignin and mineralize organic compounds to mineral N, P, S, and other elements (Mankau 1962; Walapora and Yoon 2012). These minerals act as a source of nutrient for the plant and serve as plant growth stimulants. The supply of nutrients to the plant helps in enhancing plant growth by development of resistance against foreign invaders and also results in increased root exudates and hence root colonization by PGPRs. The enzymatic degradation of cellulose and pectin by *Cellulomonas* and *Bacillus* provides *Azospirillum* with a usable C source to obtain energy for N_2 fixation (Khammas and Kaiser 1992). Glucanase-producing actinomycetes could significantly promote plant growth and also inhibit the growth of *Pythium aphanidermatum* (El-Tarabily et al. 2010). Several extracellular glucanases are involved in nodulation and EPS modification in rhizobia which regulates the process of nodule organogenesis and nitrogen fixation and promote growth of the plant. Similarly, AM fungi release enzymes

like phytases and acid phosphatases that mineralize organic P that participate in solubilizing insoluble P and make it readily available to the plant, thereby improving nutritional status of the plant and protecting the plant from being attacked by pathogens (Wani et al. 2007b).

Indigenous plant growth-promoting and disease-suppressing bioagents, *Pseudomonas*, are being used for production of lytic enzymes, namely, protease, chitinase, and β-1,3-glucanase. Microbial enzymes like proteases, elastase, subtilisin, and pronase also possess bacteriolytic properties against different Gram-positive and Gram-negative bacteria. Several studies have demonstrated the production of lytic enzyme by rhizospheric bacteria which are involved in the control mechanisms against plant root pathogens including *F. oxysporum* and *R. solani*. The soilborne fluorescent pseudomonads have received particular attention because of their capacity to produce a wide range of enzymes and metabolites (Kapoor et al. 2012).

Trichoderma control the population size of various destructive fungal pathogens by producing inhibitory compounds such as antibiotics, toxic metabolites, and hydrolytic enzymes (Benitez et al. 1998). Such antifungal metabolites include harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-pentyl-α-pyrone, massoia lactone, viridin, gliovirin, glisoprenins, and heptelidic acid (Vey et al. 2001). Synergistic action of hydrolytic enzymes and antibiotics results in a higher level of antagonism than that obtained by either mechanism alone (Howell 1998). A variety of contaminant degrading enzymes are released by fungi, endophytes, and root-colonizing bacteria which include peroxidase, dioxygenase, laccase, phosphatase, nitrilase, and nitroreductase (Gerhardt et al. 2008). These enzymes act upon the pollutant, mineralize it, and make unavailable nutrient easily available to the plant. Supply and availability of these macro- and micronutrients result in synthesis of amino acids, vitamins, and phytohormones that improve the nutritional status of the plant and enhance plant immunity protecting it from the attack of pathogens (Park et al. 2006). Enzymes released by PGPR are primarily implicated for biocontrol function but in fact are more for degradation of biopolymers providing nutrients to microbes and plants and resulting in mineral cycling (most important for sustainability of ecosystem).

Volatile Organic Compounds (VOC)

Volatile organic compounds (VOCs) are generally produced by the genus *Bacillus* including *B. amyloliquefaciens*, *B. cereus*, *B. mycoides*, *B. pumilus*, *B. sphaericus*, and *B. subtilis* (Bargabus et al. 2003; Kloepper et al. 2004; Lopez-Bucio et al. 2007; Gutierrez-Luna et al. 2010). VOCs act as plant growth regulating substances that affect other organisms, acting, for example, as attractants and/or repellents. Recently, some authors demonstrated that some PGPR can produce VOCs as signals that stimulate the growth of plants (Gutierrez-Luna et al. 2010). Volatile compounds produced by some *Bacillus* strains can also significantly impact plant growth and development. Groundbreaking work by Ryu et al. (2004) showed that the volatile compound 2,3-butanediol can be released by biocontrol and plant growth-promoting

strains of *Bacillus* that stimulate growth and production in *Arabidopsis thaliana*. Naznin et al. (2012) reported the production of VOCs known as 2-methyl-propanol and 3-methyl-butanol by a plant growth promoting fungus (PGPF) *Phoma* showed significant growth promotion of tobacco plant. VOCs like terpenes, jasmonates, and leaf components act as a signal molecules activating ISR or plant defense system thus protecting the plant from the attack of foreign invaders and strengthen host immunity (Ryu et al. 2004). Although signaling networks between plants and microbes have been extensively studied, their role in regulating plant growth and development is also explored (Naznin et al. 2012). The discovery that bacteria-produced VOCs trigger plant growth enhancement and ISR constitutes a novel mechanism for rhizobacteria–plant interaction (Ryu et al. 2004).

Some mechanisms can act in a distinct manner under a wide array of conditions, and each mechanism has multiple correlations that function simultaneously. Hydrogen cyanide (HCN), a VOC, is also reported to be produced by several PGPRs. HCN is commonly known for its role in biocontrol, but it has now been confirmed that regulation of HCN introduction is not that simple as it appears. The biosynthesis of the secondary metabolite HCN has been demonstrated in bacterial species, such as *P. aeruginosa*, *P. fluorescens*, and *Chromobacterium violaceum* (Castric 1975; Askeland and Morrison 1983; Knowles and Bunch 1986). Cyanogenesis is maximal during the transition from exponential to stationary phase (Askeland and Morrison 1983) and is influenced by several environmental factors including iron, phosphate, and oxygen concentrations (Knowles and Bunch 1986). Iron sufficiency is important for both HCN production and disease suppression (Keel et al. 1989; Voisard et al. 1989). Hence, production of HCN is closely linked with siderophore metabolism and genes (Blumera and Haas 2000).

Exopolysaccharides

PGPRs such as *Bacillus*, *Pseudomonas*, and *Rhizobium* synthesize a wide spectrum of multifunctional polysaccharides including intracellular polysaccharides, structural polysaccharides, and extracellular polysaccharides (EPS). Production of EPS is generally important in biofilm formation; root colonization and likewise can affect the interaction of microbes with roots appendages (Bianciotto et al. 2004). Effective colonization of plant roots by EPS-producing microbes helps to hold the free P from the insoluble one in soils and circulating essential nutrient to the plant for proper growth and development and protecting it from the attack of foreign pathogens (Upadhyay et al. 2011). Other innumerable functions performed by EPS-producing microbes constitute shielding from desiccation, phagocytosis, predation by protozoa, phage attack, antibiotics or toxic compounds (Ali et al. 2009), protection against stress (Upadhyay et al. 2011; Qurashi and Sabri 2012), attachment to surfaces (Tsuneda et al. 2003), plant invasion (Fraysse et al. 2003), and plant defense response in plant–microbe interactions (Kyungseok et al. 2008). EPS also have a role in cell recognition, in adhesion to surfaces, and in formation of biofilms, facilitating the colonization of soil ecosystem. Soil aggregation influences organic matter

storage, soil aeration, water infiltration, and mineral supply. Soil aggregation plays a significant role in fertility recapitalization and contributes to organic matter storage in soil (Cheshire et al. 1983; Benzing-Purdie and Nikiforuk 1989). EPS released by the *Rhizobium* assist in biofilm formation that enhances plant growth and provide protection from pathogens. Additionally, rhizobial polysaccharides are highly important in promoting plant growth, work as an active signal molecule during beneficial interactions, and provide defense response during infection process (Parada et al. 2006; Becker et al. 2005). Some PGPR-EPS can also bind cations, including Na^+, suggesting a role in mitigation of salinity stress by reducing the content of Na^+ available for plant uptake (Upadhyay et al. 2011). EPS produced by specific rhizobacteria can also elicit plant-induced resistance against biotic stress. For example, inoculation with the EPS-producing *Paenibacillus polymyxa* on peanut seeds significantly suppressed crown rot disease caused by *Aspergillus niger*, and the purified EPS from the PGPR *Burkholderia gladioli* induced resistance against *Colletotrichum orbiculare* on cucumber (Kyungseok et al. 2008).

Biosurfactants

Biosurfactants are widely exploited in areas related to agriculture for enhancement of biodegradation of pollutants and improve the quality of agricultural soil, plant growth promotion, and disease management. Biosurfactants have antimicrobial activities and increase plant–microbe interactions. Biosurfactants can replace the harsh surfactants presently used in pesticide industries (Scott and Jones 2000; Takenaka et al. 2007; Lima et al. 2011). Dusane et al. (2010) have recently reported that the biosurfactant (rhamnolipid) produced by *Pseudomonas* spp. regulates the process of quorum sensing (cell-to-cell communication). It is also reported that biosurfactants affect the motility of microorganisms and participate in signaling and differentiation as well as in biofilm formation (Kearns and Losick 2003; Van Hamme et al. 2006; Ron and Rosenberg 2011). Hence, these green surfactants are important parameters for microbes to achieve a beneficial association with the plant roots and improve the growth of the plant. Further, biosurfactants produced by rhizobacteria increase the bioavailability of hydrophobic molecules which may serve as nutrients (Sachdev and Cameotra 2013). Biosurfactants produced by soil microbes provide wettability to soil and support proper distribution of nutrients in soil, thus assisting plant growth promotion (Sachdev and Cameotra 2013). Apart from it, biosurfactants display activity against plant pathogens and therefore are considered to be promising biocontrol molecules for achieving sustainable agriculture (Nihorimbere et al. 2011).

Several evidences proved that strains of *Pseudomonas* sp. terminate the growth of pathogenic fungi *R. solani* (causes several plant diseases) and *P. ultimum* (causes damping off and root rot of plants) by production of dual functioning surfactant compounds tensin, viscosin, and viscosinamid (Andersen et al. 2003). *Colletotrichum gloeosporioides*, causative agent for anthracnose on papaya leaves, is reported to be controlled by biosurfactant-producing *B. subtilis* (Kim et al. 2010). A possible plant pathogen *P. aeruginosa* was reported to be inhibited by biosurfactant produced

by *Staphylococcus* sp., isolated from crude oil-contaminated soil (Eddouaouda et al. 2012).

Microbial biosurfactants thus play diverse roles in biofilm formation, plant pathogen elimination, promoting growth of plants, and protection from stress, oil spills, metal toxicity, and pollutants (Zhang et al. 2011).

Conclusion

Multifaceted and diverse mechanisms of PAMs participate in promoting plant growth; protecting plant health; strengthening plant–microbe associations in stress-, pollutant-, or contaminant-affected regions; and protecting plants from the attack of phytopathogens through biological control. A single metabolite can be secreted by diverse microbes in the rhizosphere and even by the host plant. For example, IAA has multiple roles; it can serve as a plant growth stimulator, biocontrol agent, and stress regulator and causes nodulation in legumes. Similarly, a single metabolite pyocyanin can perform a wide array of functions, as a signal molecule or an antibiotic or an iron chelator. Hence, same mechanism can act in a distinct manner under a wide array of conditions, and each mechanism has multiple correlations that function simultaneously. DAPG-producing *Pseudomonas* strains are reported to carry *hcn* genes for biosynthesis of the broad-spectrum biocide HCN, indicating an evolutionary linkage of the two metabolites (Duffy et al. 2004). Also, HCN expression and production by *Pseudomonas* is strongly dependent on iron availability and may act synergistically with siderophore (Keel et al. 1989). *Pseudomonas* produces DAPG, a phenolic compound with antibiotic properties, and a signal molecule that induces systemic resistance in plants, and stimulates root exudation and branching (Combes-Meynet et al. 2011).

A connection between phenazine and biofilm production has been established in *P. chlororaphis*. Similarly, *P. aeruginosa* is reported to produce pyocyanin (a siderophore) which acts as antibiotic against phytopathogenic fungi and also acts as signal molecules influencing EPS production leading to the development of biofilm by *Rhizobium*. There are numerous such examples almost for each and every mechanism and metabolite secreted in the rhizosphere which are discussed in this chapter (Table 16.1). This implies that a single metabolite can be governed by various genes or may also be by the genes for other metabolites as in the case of HCN and DAPG. This correlation can be decoded by the use of functional genomic, proteomic, and metabolomic tools. Without restricting to the direct and indirect concept, we have to go for extensive and comprehensive approaches so as to deduce the complete understanding of working of metabolite(s) or PGPR(s) or more broadly or appropriately the PAMs. While dealing with all these intersected examples, we cannot categorize that the metabolites or mechanism can be studied as direct or indirect. The literature also shows that selection of multifaceted microbes performing multiple tasks simultaneously under similar or diverse conditions can serve as a boon in reclaiming agricultural lands, disease management, and promoting yield, growth, and production of plants in an eco-friendly manner.

Table 16.1 Diverse roles played by different metabolites/mechanisms blurring the concept of direct and indirect

Mechanisms/metabolites	Microbes associated	Direct role	Indirect role	Mixed role	References
BNF	*Azospirillum* *Rhizobium* *Sinorhizobium* *Mesorhizobium* *Pseudomonas* *Azotobacter* *Cyanobacteria*	Phosphate solubilization, phytohormone production, iron chelation, signal molecules like flavonoids released that increases root colonization and nutrient uptake and enhances plant growth promotion	Suppressing the attack of phytopathogens by enhancing nutrient uptake and increasing the ISR of plants	EPS production, biofilm formation, bioremediation by offering protection against abiotic stresses	Holguin and Bashan (1996) Seneviratne and Jayasinghearachchi (2003) Avis et al. (2008)
Phosphate solubilization	*Bacillus* *Pseudomonas* *Rhizobium* *Serratia* *Mycorrhiza*	Mineralization of insoluble phosphate, nutrient cycling, increases IAA production, nodulation, easy accessibility of nutrient and enhances yield and production	Large amount of nutrient uptake increases tolerance for pathogenic infections and suppress disease incidence	Detoxify the effect of heavy metals, protection against abiotic stress, treatment of industrial waste, and bioremediation	Nautiyal (1999) Karagiannidis et al. (2002) Rillig (2004) Ravikumar et al. (2007) Panhwar et al. (2012)
Phytohormones	*Pseudomonas* *Rhizobium* *Bacillus* *Azotobacter*	Increase water and nutrients uptake from the soil, maintain hormonal balance, proliferation in cell division, differentiation, root elongation, nodulation phosphate solubilization, and promotes plant growth and development	Provide easy flow of nutrients and support to the plants, enhances plant immunity, prevent the plant from being attacked by foreign invaders, and suppression of phytopathogens	Mitigate the effect of abiotic stresses by immediate precursor ACC deaminase Function as stress regulators	Mayak et al. (2004) Kang et al. (2006) Arora et al. (2010)

Siderophores	*Azotobacter* *Bacillus* *Pseudomonas* *Rhizobium*	Plant growth promotion by nodulation, nitrogen fixation, release of organic acids, nutrient cycling of the metals, and enormous proliferation in growth and yield enhancement	Biological control of pathogens and disease suppression by acting as an antibiotic molecule and strong iron chelator	Management of abiotic stress, function as signal molecule, biofilm formation, EPS production, and biosurfactant production	Arora et al. (2001) Kraepiel et al. (2009) Khare and Arora (2011)
Antibiotics	*Pseudomonas* *Streptomycetes* *Bacillus*	Function as signal molecule, increases root colonization, soil aggregation, increases the supply of nutrient and water, and enhanced plant growth	Display diverse antimicrobial activities including antiviral, antifungal antihelminthic, antibacterial, cytotoxic, and antitumor	Triggering ISR in the plant system and contribute to disease suppression by conferring a competitive advantage to biocontrol agents	Fernando et al. (2005) Ongena and Jacques (2008) Bhattacharya and Jha (2012)
ISR	*Bacillus* *Pseudomonas* *Rhizobium* AM	Activation of defense system by eliciting signaling pathways (JA and SA) that help in promoting growth of plant	Activated defense system elicits hydrolytic enzymes that causes lysis, prevent the attack of foreign invaders, and prevent pathogenesis	Activation of ROS, phytoalexins that helps in stress management Synergism between antibiotics and ISR activates enzymatic action that prevents pathogenesis assist in bioremediation under abiotic stress	Maurhofer et al. (1998) Ran et al. (2005) Fernando et al. (2005)

(continued)

Table 16.1 (continued)

Mechanisms/metabolites	Microbes associated	Direct role	Indirect role	Mixed role	References
Enzymes	*Pseudomonas* *Rhizobium* *Bacillus* *Serratia* *Azotobacter*, etc	Nutrient cycling of polymers. Mineralize organic compounds to mineral N, P, and S. Minerals act as a source of plant nutrient and serve as plant growth stimulants	Lytic enzymes cause degradation of cell wall and kill pathogens and suppress disease incidence	Contaminant degrading enzymes assist in bioremediation of pollutants	Mankau (1962) Gerhardt et al. (2008) Kapoor et al. (2012)
VOC	*Pseudomonas* *Rhizobium* *Bacillus* *Azotobacter* *Azospirillum*	VOC act as a signal to stimulate siderophore metabolism and trigger plant growth and enhancement	VOC like terpenes, jasmonates, and leaf components act as signal molecules, activating ISR or plant defense system thus protecting the plant from the attack of foreign invaders and strengthen host immunity Provide protection from pathogens	Signal molecules released helps in biofilm formation, polysaccharide and surfactant production that assist in bioremediation	Ryu et al. (2004) Gutierrez-Luna et al. (2010) Naznin et al. (2012)

EPS	*Azotobacter* *Azospirillum* *Bacillus* *Burkholderia* *Pseudomonas* *Rhizobium*	Biofilm formation; root colonization, soil aggregation, circulating essential nutrients, increase plant–microbe interactions by adhesion, and plant growth promotion	Activates plant defense response and protect from pathogens	Provide shielding from desiccation, phagocytosis, predation by protozoa, phage attack, and antibiotics or toxic compounds and assist in mitigating the effect of stress	Kyungseok et al. (2008) Ali et al.(2009) Upadhyay et al.(2011)
Biosurfactants	*Azospirillum* *Bacillus* *Burkholderia* *Pseudomonas*	Strengthen increase in plant microbe interactions, biofilm formation, even distribution of nutrient	Antimicrobial activities that inhibit growth of pathogens	Emulsification activity helps in remove metal toxicity and assist in bioremediation	Zhang et al. (2011) Lima et al. (2011) Ron and Rosenberg (2011) Sachdev and Cameotra (2013)

Acknowledgements The authors are grateful to Vice Chancellor Babasaheb Bhimrao Ambedkar University, Lucknow, India, for their support.

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N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4, © Springer India 2013