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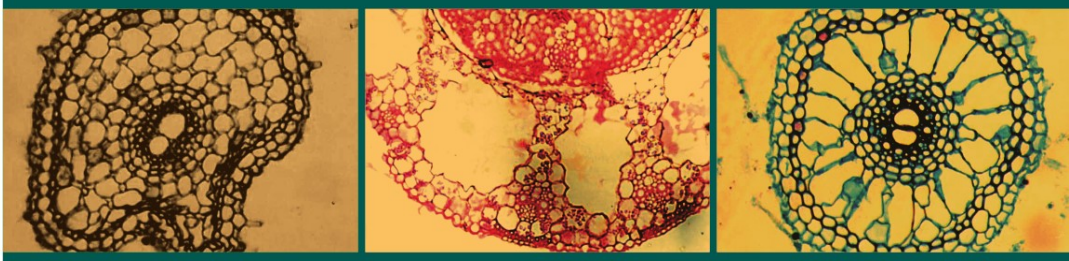

Plant Probiotics

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Editor

Bacteria in Agrobiology: Plant Probiotics

Springer

Editor
Dinesh K. Maheshwari
Gurukul Kangri University
Faculty of Life Sciences
Department of Botany and Microbiology
Haridwar (Uttarakhand)
India

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Cover illustration: Optical micrograph showing cross sections of intercellular colonization rice calli and regenerated plantlets by *A. caulinodans*: CS view of root uninoculated control; magnified cross section view of leaf colonized by *A. caulinodans* in regenerated rice plant; possible sites of infection and colonization of rice root (from left to right); see also Fig. 3.1 in "Endophytic Bacteria – Perspectives and Applications in Agricultural Crop Production", Senthilkumar M, R. Anandham, M. Madhaiyan, V. Venkateswaran, T.M. Sa, in "Bacteria in Agrobiology: Crop Ecosystems, Dinesh K. Maheshwari (Ed.)"

Background: Positive immunofluorescence micrograph showing reaction between cells of the rhizobial biofertilizer strain E11 and specific anti-E11 antiserum prepared for autecological biogeography studies; see also Fig. 10.6 in "Beneficial Endophytic Rhizobia as Biofertilizer Inoculants for Rice and the Spatial Ecology of this Bacteria-Plant Association", Youssef G. Yanni, Frank B. Dazzo, Mohamed I. Zidan in "Bacteria in Agrobiology: Crop Ecosystems, Dinesh K. Maheshwari (Ed.)"

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Preface

Bacteria are ubiquitous in nature: some of them are harmful but majority of them are beneficial to the plants. They comprise various attributes which directly and indirectly support plant growth and their fitness against adverse conditions of both abiotic and biotic in any given environmental system.

Coordinated interactions between microbes and plants are utmost important for their healthy association. Through this book we intend to provide a total of 18 chapters which signify the added advantages of bacteria, in general, and PGPR, in particular, in nutrient uptake and triggering defense responses of the plant against deleterious phytopathogens. Probiotics for plants exhibits multifarious functional characteristics beneficial in nature which lead to sustainable microbial complex ecosystem. Due to their diverse ecology, they exhibit multifarious functional characters beneficial in nature which lead to sustainable microbial complex ecosystem favorable to the host plants. Due to their probiotic nature and sometimes because of intimate association (example endophytes), they often serve as an alternative to fertilizers, herbicides, and chemical pesticides. A brief understanding of diversity, colonization, mechanism of action formulation, and application of such bacteria inoculants facilitate their contribution in the management of sustainable agroecosystem as exemplified by studying their responses on a plant model, *Arabidopsis*. Such bacteria have also been exploited in the improvement of quality of silk production. The probiotic nature of various group of bacteria found suitable candidates for combating fungal, bacterial nematode, and other diseases which are injurious to majority of plant besides conferring health benefits to above-ground plant parts and roots deep seated in soil. Some of the chapters highlight the impact of bacteria on soil structure and microbial community function that involved rhizosphere signals (molecules) apart from mediated systemic resistance for plants, potential for phosphorus nutrition application for microbial consortium, nitrogen fixation, and biofertilizer for eco-friendly low-input sustainable crop production. The book will benefit the teachers, researches students, and those interested in strengthening the subject of Agricultural Microbiology, Biotechnology, Plant Protection, Agronomy, and Environmental Sciences.

I wish to express my gratitude to all the subject experts who have provided their masterpiece work in giving the shape of the book. The work of several reviewers is

quite commendable in improvement of the scientific merits of this volume. Thanks are also due to my students, Dr. Abhinav, Rajat and Narendra, for their help. Due credit also goes to my wife, Dr. Sadhana Maheshwari, and son, Er. Ashish, for their continuous encouragement. I owe my special thanks to Dr. Jutta Lindenborn, Springer, Heidelberg, Germany, for her keen interest and facilitation of the process in bringing out the volume in its present form.

Haridwar, Uttarakhand, India Dinesh K. Maheshwari

Contents

List of Contributors

Subha Alam Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Emily Alff Department of Plant and Soil Sciences, University of Delaware, Newark, DE, USA; Delaware Biotechnology Institute, Newark, DE, USA

Jorge Angelini Dpto. Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto (UNRC), Río Cuarto (Córdoba), Argentina

Harsh Bais Department of Plant and Soil Sciences, University of Delaware, Newark, DE, USA; Delaware Biotechnology Institute, Newark, DE, USA, bais@dbi.udel.edu

Asghari Bano Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan, banoasghari@gmail.com

Ezékiel Baudoin IRD, UMR 113 CIRAD/INRA/IRD/SUP-AGRO/UM2, Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), Montpellier Cedex 5, France; Laboratoire Ecologie & Environnement (Unité associée au CNRST, URAC 32), Faculté des Sciences Semlalia, Université Cadi Ayyad, Marrakech, Marocco

Sandeep Bisht Department of Microbiology, S.B.S.P.G. Institute of Biomedical Sciences and Research, Dehradun, Uttarakhand, India

Archana Borah Biotechnology Division, North-East Institute of Science & Technology (CSIR), Jorhat, Assam, India

A. B. Cesari Departamento de Biología Molecular, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Río Cuarto (Córdoba), Argentina

Marta S. Dardanelli Departamento de Biología Molecular, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Río Cuarto (Córdoba), Argentina, mdardanelli@exa.unrc.edu.ar

Manoel de Araújo Teixeira Laboratory of Microbiology, University of Vale do Sapucaí – UNIVAS, Pouso Alegre, Minas Gerais, Brazil

Guilhem Desbrosses Laboratoire des Symbioses Tropicales et Méditerranéennes (UMR UM2/IRD/Cirad/SupAgro/INRA), Université Montpellier 2, Montpellier Cedex 05, France

A. M. Deshmukh Department of Microbiology, Dr B. A. Ambedkar Marathwada University Sub-Center, Osmanabad, Maharashtra, India

Basabrani Devi Biotechnology Division, North-East Institute of Science & Technology (CSIR), Jorhat, Assam, India

Armando Cavalcante Franco Dias Center for Nuclear Energy in Agriculture, University of São Paulo, São Paulo, Piracicaba, Brazil

Gaurav Dugar Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, Hauz Khas, New Delhi, India

Robin Duponnois IRD, UMR 113 CIRAD/INRA/IRD/SUP-AGRO/UM2, Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), Montpellier Cedex 5, France; Laboratoire Ecologie & Environnement (Unité associée au CNRST, URAC 32), Faculté des Sciences Semlalia, Université Cadi Ayyad, Marrakech, Marocco, Robin.Duponnois@ird.fr

Adriana Fabra Dpto. Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto (UNRC), Río Cuarto (Córdoba), Argentina, afabra@exa.unrc.edu.ar

Antoine Galiana CIRAD, UMR 113 CIRAD/INRA/IRD/SUP-AGRO/UM2, Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), Montpellier Cedex 5, France

N. S. Gangurde Department of Microbiology, P. S. G. V. P Mandal's, S. I Patil Arts, G B Patel Science and S. T. S. K. V. S Commerce College, SHAHADA, Maharashtra, India

M. B. García Departamento de Biología Molecular, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Río Cuarto (Córdoba), Argentina

W.F. Giordano Departamento de Biología Molecular, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Río Cuarto (Córdoba), Argentina

Reeta Goel Department of Microbiology, G.B. Pant University of Agriculture & Technology, Pantnagar, Uttaranchal, India, rg55@rediffmail.com

Rashi Gupta Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, Hauz Khas, New Delhi, India

Mohamed Hafidi Laboratoire Ecologie & Environnement (Unité associée au CNRST, URAC 32), Faculté des Sciences Semlalia, Université Cadi Ayyad, Marrakech, Marocco

Pablo Hardoim Department of Microbial Ecology, Centre for Ecological and Evolutionary Studies, Groningen University, Groningen, The Netherlands, phardoim@gmail.com

Md. Motaher Hossain Department of Plant Pathology, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur, Bangladesh

Fernando Ibñez Dpto. Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto (UNRC), Río Cuarto (Córdoba), Argentina

Noshin Ilyas Department of Botany, PMAS-Arid Agriculture University, Rawalpindi, Pakistan; Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan

Md. Tofazzal Islam Department of Biotechnology, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur, Bangladesh, tofazzalislam@yahoo.com

Yelena Kakoty Biotechnology Division, North-East Institute of Science & Technology (CSIR), Jorhat, Assam, India

Michel Lebrun IRD, UMR 113 CIRAD/INRA/IRD/SUP-AGRO/UM2, Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), Montpellier Cedex 5, France; Laboratoire Ecologie & Environnement (Unité associée au CNRST, URAC 32), Faculté des Sciences Semlalia, Université Cadi Ayyad, Marrakech, Marocco

Abhinav Aeron Department of Microbiology, Faculty of Life Sciences, Kurukshetra University, Kurukshetra, Haryana, India; Department of Botany and Microbiology, Gurukul Kangri University, Haridwar, Uttarakhand, India

Dinesh K. Maheshwari Department of Botany and Microbiology, Faculty of Life Sciences, Gurukul Kangri University, Haridwar, Uttarakhand, India, maheshwaridk@gmail.com

B. Meena Sugarcane Research Station, Tamil Nadu Agricultural University, Trichy District, Tamil Nadu, India, meepath@rediffmail.com

Rukshima Nesha Department of Botany, Aligarh Muslim University, Aligarh, India

Riitta Nissinen Department of Microbial Ecology, Centre for Ecological and Evolutionary Studies, Groningen University, Groningen, The Netherlands, R.M. Nissinen@rug.nl

Lok Man S. Palni GB Pant Institute of Himalayan Environment and Development, Almora, Uttarakhand, India

Anita Pandey GB Pant Institute of Himalayan Environment and Development, Almora, Uttarakhand, India, anita@gbpihed.nic.in

Piyush Pandey Department of Microbiology, Assam University, Silchar, Assam, India; Department of Life sciences and Bioinformatics, Assam University, Silchar, Assam, India

P. R. Patel Department of Microbiology, P. S. G. V. P Mandal's, S. I Patil Arts, G B Patel Science and S. T. S. K. V. S Commerce College, SHAHADA, Maharashtra, India

N. S. Paulucci Departamento de Biología Molecular, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Río Cuarto (Córdoba), Argentina

Yves Prin CIRAD, UMR 113 CIRAD/INRA/IRD/SUP-AGRO/UM2, Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), Montpellier Cedex 5, France

Anju Rani Department of Microbiology, G.B. Pant University of Agriculture & Technology, Pantnagar, Uttaranchal, India

M. S. Reddy Department of Entomology and Plant Pathology, Auburn University, Auburn, AL, USA, munagrs@auburn.edu

André Luís Braghini S Laboratory of Microbiology, University of Vale do Sapucaí – UNIVAS, Pouso Alegre, Minas Gerais, Brazil, biobragh@yahoo.com.br

Riyaz Z. Sayyed Department of Microbiology, P. S. G. V. P Mandal's, S. I Patil Arts, G B Patel Science and S. T. S. K. V. S Commerce College, SHAHADA, Maharashtra, India

Deepak Shantharaj Department of Plant and Soil Sciences, University of Delaware, Newark, DE, USA; Delaware Biotechnology Institute, Newark, DE, USA

G. D. Sharma Department of Life sciences and Bioinformatics, Assam University, Silchar, Assam, India

Shilpi Sharma Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, Hauz Khas, New Delhi, India, shilpi@dbeb.iitd.ac.in

Fo-Ting Shen Department of Soil and Environmental Sciences, College of Agriculture and Natural Resource, National Chung Hsing University, Taichung, Taiwan, Republic of China

Zaki A. Siddiqui Department of Botany, Aligarh Muslim University, Aligarh, India, zaki_63@yahoo.co.in

Neelu Singh Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Sonu Singh Department of Soil and Environmental Sciences, College of Agriculture and Natural Resource, National Chung Hsing University, Taichung, Taiwan, Republic of China

Anchal Sood Department of Microbiology, S.B.S.P.G. Institute of Biomedical Sciences and Research, Dehradun, Uttarakhand, India

Carla Spence Department of Biological Sciences, University of Delaware, Newark, DE, USA; Delaware Biotechnology Institute, Newark, DE, USA

Ashok K. Srivastava Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, Hauz Khas, New Delhi, India

Tania Taurian Dpto. Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto (UNRC), Río Cuarto (Córdoba), Argentina

Jean Thioulouse Université de Lyon, Lyon, France; CNRS, UMR5558, Laboratoire de Biométrie et Biologie Evolutive, Université Lyon 1, Villeurbanne, France

María Laura Tonelli Dpto. Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto (UNRC), Río Cuarto (Córdoba), Argentina

Bruno Touraine Laboratoire des Symbioses Tropicales et Méditerranéennes (UMR UM2/IRD/Cirad/SupAgro/INRA), Université Montpellier 2, Montpellier Cedex 05, France, touraine@univ-montp2.fr

Pankaj Trivedi Microbiology and Cell Science, Citrus Research and Education Center, University of Florida, Lake Alfred, FL, USA

Bala Gopalan Unni Biotechnology Division, North-East Institute of Science & Technology (CSIR), Jorhat, Assam, India, 0376-2370011/2370315bgunni@yahoo.com

Jan Dirk van Elsas Department of Microbial Ecology, Centre for Ecological and Evolutionary Studies, Groningen University, Groningen, The Netherlands, j.d.van. elsas@rug.nl

Fabrice Varoquaux Laboratoire des Symbioses Tropicales et Méditerranéennes (UMR UM2/IRD/Cirad/SupAgro/INRA), Université Montpellier 2, Montpellier Cedex 05, France

J. C. Vicario Departamento de Biología Molecular, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Río Cuarto (Córdoba), Argentina

Sawlang Borsingh Wann Biotechnology Division, North-East Institute of Science & Technology (CSIR), Jorhat, Assam, India

Chiu-Chung Young Department of Soil and Environmental Sciences, College of Agriculture and Natural Resource, National Chung Hsing University, Taichung, Taiwan, Republic of China, ccyoung@mail.nchu.edu.tw

Chapter 1
Probiotics for Plants: Importance of Rhizobacteria on Aboveground Fitness in Plants

Carla Spence, Emily Alff, Deepak Shantharaj, and Harsh Bais

1.1 Plants as Linking Agents for Aboveground and Belowground Interactions

Even though aboveground and belowground parts of plants are separated in space and are surrounded by different environments, they are still connected as one system, a fact that is sometimes overlooked. Well-studied crucial interactions include photosynthesis, respiration, water uptake, nutrient cycling, and the translocations of these products. For example, organic compounds synthesized during photosynthesis can end up in the soil in a variety of different ways and forms, influencing the microbial community living in the soil. In this section, we will focus on the interactions plants have with living organisms that can alter plant growth, productivity, and defense.

There are many components that effect on the overall fitness of plants. The interactions and reactions that occur with and within the shoots of plants depend on abiotic factors such as sunlight, water, temperature, atmospheric gases and wind, and biotic factors such as pollination, herbivory, and pathogen attack. The interactions of belowground parts of plants are dependent upon its surrounding environment, the rhizosphere. Soil chemistry and structure, the amount of water and nutrients, as well the vast number of soil-dwelling organisms that live there all play a role in root interactions. The soil surrounding plant roots is different from the shoot-surrounding atmosphere in that the roots are in constant contact with living

C. Spence
Department of Biological Sciences, University of Delaware, Newark, DE 19716, USA

Delaware Biotechnology Institute, 15 Innovation Way, Newark, DE 19711, USA

E. Alff • D. Shantharaj • H. Bais (✉)
Department of Plant and Soil Sciences, University of Delaware, Newark, DE 19716, USA

Delaware Biotechnology Institute, 15 Innovation Way, Newark, DE 19711, USA
e-mail: bais@dbi.udel.edu

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organisms. For example, there are roughly 5,000 operational taxonomic units (OTUs) of bacteria for every gram of soil (Schloss and Handelsman 2006), although this number is extremely variable depending on the soil environment and microenvironments.

Plants have different mechanisms of defense when confronted by insects or pathogenic organisms. When attacked aboveground, a plant's first line of defense is the physical barrier of their cuticle and epidermis. Once entry is achieved, either through mechanical wounding, use of enzymes, or through the stomata, other protection measures are taken. The plant can produce a number of secondary metabolites that are toxic to herbivores. Well-known examples include caffeine, menthol, nicotine, and capsaicin. It has also been shown that when being attacked underground by root herbivores, vertical communication from the roots to the shoots can help to inhibit survival of aboveground feeding herbivores through accumulation of phytotoxins in the leaves.

The plant can also release volatile organic compounds (VOCs) in response to insect herbivory that signals neighboring leaves to put up their defenses (Gershenzon 2007). This mechanism is more energy efficient than in-plant vascular signaling. These signals can also be picked up by neighboring plants and used as a cue that herbivory is occurring nearby (Arimura et al. 2010). Baldwin and Schultz (1983) were the first to report this phenomenon in poplar and sugar maple trees. These compounds also attract carnivorous predators and parasitoids that are natural enemies of these herbivores, forming a tri-trophic relationship and an indirect defense mechanism (Dicke and van Loon 2000). For example, when moth larvae damage cotton plants, the plants release volatiles that attract predatory wasps. Plants also have basal and adaptive immune responses that help with resistance toward microbial pathogens. These responses will be discussed in later sections.

Underground, roots use similar defense responses to deal with local stresses. Plants use a method of warfare against neighboring plants called allelopathy, in which they release phytotoxins from their roots that are harmful to other plants into the environment (Steenhagen and Zimdahl 1979; Bais et al. 2003). This strategy has been used to describe a possible reason for the invasiveness of exotic plants. For example, invasive populations of the common reed *Phragmites australis* release gallotannin into the soil that is metabolized by native plant and microbial communities and converted to gallic acid, a form that is toxic to native *Phragmites australis* (Bains et al. 2009). There are now only remnant native populations surviving along the Atlantic Coast of North America. This interaction shows how plants and microorganisms have a mutualistic relationship in which the plant provides food for the microbes and the microbes in turn help increase the plants noxiousness and abundance.

The compounds that are exuded by plant roots can be either byproducts of metabolism or synthesized for the purpose of being secreted, and do not necessarily have to be harmful to other plants. Recruitment of beneficial bacteria through root chemical secretion and chemotaxis has been shown not only to increase plant growth, but also to activate plant defenses and increase immunity throughout the whole plant (Rudrappa et al. 2008a, b). When under attack by a foliar pathogen,

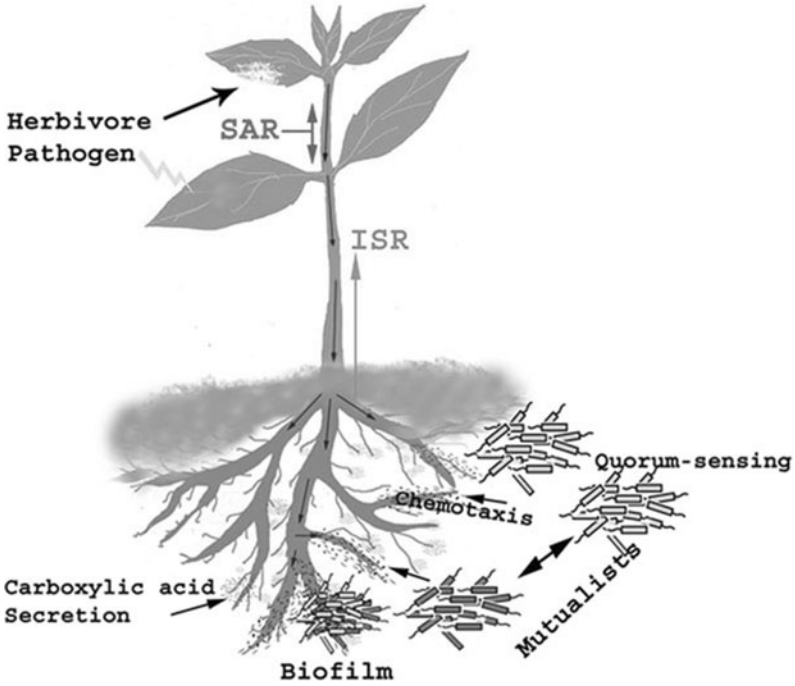

Fig. 1.1 A schematic depicting the long distance intraplant signaling to recruit rhizobacteria belowground under aerial pathogen infection regime. The schematic also represents the events belowground leading to microcolony and biofilm formation by rhizobacteria attracted to the carboxylic acid secretion from aerially infected plants. The schematic illustrates the attachment of rhizobacteria on roots that may induce systemic resistance response in plants against the invading pathogens

long distance intraplant signaling from shoots to roots stimulates recruitment of beneficial bacteria and biofilm formation on the roots, setting off innate defense responses (Rudrappa et al. 2008a, b). Induction of systemic resistance (ISR) by rhizobacteria sends signals back up the plant to confer aboveground resistance to the pathogen (Fig. 1.1). This shows how the interactions within a plant and with their surrounding environments, both abiotic and biotic factors as well as spatially separated aboveground and belowground interactions, are all connected and can influence one another.

The beneficial bacteria that live in the rhizosphere can be categorized as either plant-growth-promoting rhizobacteria (PGPR) or biological control agents (BCA). As their names suggest, the direct effect of PGPR is the promotion of plant growth, while the direct effect of BCA is to control soil-borne pathogens, which in turn improves plant productivity. There is evidence, however, that PGPR and BCA can have secondary effects on plants equal to the direct effects produced by the other (Avis et al. 2008). For example, *Sinorhizobium* species (PGPR) are most well known for their primary role in nitrogen fixation and, therefore, improved plant

growth; however, *Sinorhizobium* species have also been linked to suppression of disease by stimulating increased production of plant defense compounds, leading to ISR and pathogen control (Avis et al. 2008).

The role of humans in improving agriculture relies on understanding these complicated relationships. The use of beneficial bacteria (and other microorganisms) can be exploited to increase plant health and crop yield, while decreasing the cost and harm of fertilizers, pesticides, and other synthetic chemicals. Since variation is a key to biological success, the natural mechanisms that plants have evolved to respond to a wide variety of stresses are extremely variable and robust. Understanding these plant systems as a whole is necessary for improving food production for a burgeoning world population.

1.2 Rhizobacteria, a Novel Source in Enhancing Plant Growth

The rhizosphere has a large impact on plant growth, not only due to the level of the nutrients it contains, but also due to other organisms that inhabit the rhizosphere. Rhizobacteria, referring to bacterial population that are found in the soil surrounding plant roots, can have a variety of effects on the plant. Of particular interest are the PGPRs. For thousands of years, the ability of different types of soil to promote plant growth has been known. As early as 30 BC, farmers noticed that fields in which legumes had been grown were more fertile. We now know this is due, at least in part, to PGPR that form a symbiotic relationship with roots and provide nitrogen to the plant. Virgil mentions this in the poem *Georgics* (Chew 2002). Around the same time, Theophrastus reported mixing different types of soils to enhance plant growth, and with modern day insight, this may have been to diversify the microbiome (Tisdale and Nelson 1975).

In more recent history, specific bacteria have been marketed as a way to enhance plant growth. The bacteria are often applied directly to the seed coat. Once the seed has been planted, the bacteria must be able to compete with native soil microbes and efficiently colonize the plant roots (in most cases) in order to promote plant growth. For instance, the ability of *Epicoccum nigrum conidia*, a PGPR, to adhere to the surface of peaches is positively correlated with its ability to stimulate growth and control brown rot (Larena et al. 2010). For over 100 years, *Sinorhizobium* and *Bradyrhizobium* have been marketed to enhance plant growth. Additionally, bacteria have been marketed that are able to minimize the presence of pathogens in the soil. *Pseudomonas*, *Bacillus*, *Streptomyces*, and *Agrobacterium* are the most commonly used genera for this purpose (Bloemberg and Lugtenberg 2001).

The type of PGPRs present in the soil, how they promote plant growth, and why they interact with plants are some of the questions currently being investigated. Some PGPRs inhabit the rhizosphere without directly associating with plant roots. The most likely mechanism of growth promotion by these bacteria is through secretions. However, most PGPRs that have been identified bind directly to the plant root, facilitating communication. Some PGPRs have been found to be

endophytic, and therefore go inside root cells. Although rare, a few PGPRs have been found that do not enter into cells, but rather occupy spaces between root cells (An et al. 2001). Of the bacteria that form direct associations with plant cells, it is unclear if there are particular root regions where colonization is more likely to occur. One piece of evidence that would indicate specificity in the binding site is that *Kuvvera ascorbata* is able to colonize the upper portion of canola roots, but not the lower portion near the root tip (Ma et al. 2001). It is also important to note that there is evidence of arbuscular mycorrhizae which can also directly or indirectly promote plant growth (For a review, see Bethlenfalvay 1993 and Bothe et al. 2010).

The ways in which PGPRs promote plant growth also differ. Generally, PGPRs work as biofertilizers, phytostimulators, biocontrol agents, or they are a combination of the three (Vessey 2003). Biofertilizers promote plant growth by directly or indirectly supplying necessary nutrients to the plant (Glick 1995; Rodriguez and Fraga 1999). Biofertilizers are the most broad and well-understood category of PGPRs. In most soils, the limiting nutrients are nitrogen or phosphorous, but iron is also an important element that PGPRs can provide. Nitrogen is primarily made available to the plant by specific PGPRs that are able to fix atmospheric nitrogen into ammonium, which can be used by the plant. The bacteria enter the root hairs and form nodules, where they become differentiated to fix atmospheric nitrogen. This relationship is symbiotic, as the plant benefits from the source of nitrogen that is given in exchange for plant carbon sources. The symbiotic relationship between legumes and nitrogen fixing bacteria is well studied (For a recent review, see Downie 2010). Although less common, another subset of PGPRs increase the nitrogen supply available to plants without entering the plant, and this is not host specific and not well studied (Carvalho et al. 2010).

Phosphorous can also be a limiting factor to plant growth. Soil contains a generous amount of phosphorous, however it is mainly insoluble. Plants can only utilize phosphorous in the monobasic ($H_2PO_4^-$) or dibasic (HPO_4^{2-}) forms which are rarely found in the soil. Phosphate solubilizing bacteria convert insoluble phosphate into the soluble monobasic or dibasic forms using phosphatases or organic acids (Kim et al. 1998).

Another variation on the biofertilization theme is when a nutrient is found at a sufficient level in the soil, but the plants are inefficient at taking up the nutrient and can benefit from PGPRs that facilitate the uptake. One example is siderophore secretion by PGPRs. Soil contains adequate amounts of iron for plant growth, but it is in the form of Fe^{3+} which is insoluble. Plants can only acquire the more soluble form, Fe^{2+}. Siderophores secreted by PGPRs can bind the insoluble iron and carry it into plant cells (Bar-Ness et al. 1992).

The second main group of PGPRs is phytostimulators which promote plant growth directly, most often by secreting growth hormones. Indole-3-acetic acid (IAA) is the most common plant auxin which promotes division, enlargement, and initiation of root growth (Wickson and Thimann 1960). Larger roots allow the plant to be in contact with a larger area of the soil and provide an opportunity to absorb more nutrients for growth. *Azospirillum, Vibrio, Streptomyces,* and *Psuedomonas* as well as other rhizobacteria have been shown to secrete IAA, and in most studies this

was linked to plant growth promotion (Guiterrez et al. 2009; Narayana et al. 2009). Similarly, cytokinins also promote cell divisions, cell enlargement, and tissue expansion in plants and are secreted by some PGPRs (Spaepen et al. 2009). However, cytokinin secretion by bacteria is not always beneficial to plants. *Agrobacterium tumefaciens* also secretes cytokinins, but does so in such a way to cause plant tumors from unregulated growth (Hwang et al. 2010).

Biocontrol is an indirect way that PGPRs can promote plant growth. When this phenomenon was first discovered, it was termed the "suppressive soil" effect. Biocontrol involves competition between PGPRs and pathogens for nutrients. Effective biocontrol PGPRs can reduce the levels of pathogens in the soil by reducing the nutrients available to pathogens. This is sometimes called niche exclusion. This type of PGPRs may also trigger the plant's induced systemic resistance (ISR) without causing harm, which will prime the plant for a pathogen attack. This enables the plant to be more resistant if a pathogen does invade. Other PGPRs produce antifungal metabolites (AFMs) that will inhibit fungal pathogens from harming the plant (Whipps 2001).

Although the different groups of PGPRs have been discussed separately, it is often the case that a particular PGPR acts as a combination of biofertilizer, phytostimulator, and/or biocontrol agent. One example is *Burkholderia*, which promotes the growth of maize. *Burkholderia* inhibits the growth of *Fusarium*, a fungal pathogen to maize, which is a biocontrol mechanism. In addition, it can also stimulate maize growth directly by producing siderophores when there are low levels of soluble iron in the soil, which is a biofertilization mechanism (Hernandez-Rodriguez et al. 2008). Another study examined the ability of six different bacterial strains to promote growth in maize, and several of them used multiple mechanisms to do so, such as biofertilization and phytostimulation (Marques et al. 2010). In addition to combinations of the previously mentioned mechanisms, there are also other potential mechanisms for improving plant fitness that are not as well understood. It is thought that beneficial bacteria are able to assist plants in adapting to and coping with environmental changes such as drought (Compant et al. 2010). Furthermore, there is evidence that combining PGPRs such as different *Pseudomonas* species, or *Psuedomonas* and *Bacillus*, can be even more beneficial than a single PGPR (Saravanakumar et al. 2007). Also, it seems that although there are some general PGPRs such as *Bacillus* that seem to promote growth in a variety of plants, there is evidence that the plant microbiome is very specific. Even different cultivars can have different microbes in the rhizosphere. In the case of potato, there is a very diverse community of endophytic bacteria that are specific to their cultivar of potato (Manter et al. 2010). Plants may recruit beneficial bacteria via their root exudates, such as the case of *Arabidopsis thaliana* secreting malate, which recruits the beneficial, *Bacillus subtilis* (Rudrappa et al. 2008b).

An important question is why are some bacteria plant growth promoting while others are pathogenic? Interestingly, there are many similarities between the two groups. For example, nitrogen fixing bacteria that enter root hairs have a sym plasmid that encodes a type three secretion system (Gottfert et al. 2001). *Pseudomonas fluorescens*, also a PGPR, has a type three secretion system as well (Rainey 1999).

What makes this interesting is that the majority of plant pathogens use similar type three secretion systems to secrete elicitors that ultimately harm the plant. The next section examines the way in which pathogens and PGPRs are able to trigger the plant's defense responses.

1.3 Induction of Systemic Resistance by Rhizobacteria

ISR and systemic acquired resistance (SAR) are initiated in a very similar manner. When the process is initiated by a PGPR the resulting plant response is termed ISR, whereas if initiated by a pathogen it is termed SAR (Van Loon et al. 1998). The plant defense response can also be triggered by nonliving factors such as salicylic acid, ethylene, dichloro-isonicotinic acid (Sticher et al. 1997), or benzothiadiazole (Gorlach et al. 1996). In both ISR and SAR, the plant recognizes a MAMP (microbe associated molecular pattern, or more specifically in the case of pathogens, a pathogen associated molecular pattern or PAMP) (Van Loon 1997) which starts a cascade of signal transduction events (see Hammerschmidt 2009). In brief, the ISR/SAR begins with PAMP triggered immunity (PTI). In response, pathogens secrete elicitors that result in effector triggered susceptibility (ETS). The next phase is dependent on specificity between pathogen and host. The host will recognize an effector using NB-LRR proteins which results in effector-triggered immunity (Jones and Dangl 2006).

At the start of ISR, a signaling cascade will be initiated, in which the plant begins the process of protecting itself against further infection. In the case of pathogens, the end result could be necrosis of plant tissue (Cameron et al. 1994), whereas PGPRs do not induce the defenses to this extremity. PGPRs prime the defense system, making the plant more resistant to disease but do not cause harm.

Going into more detail, the ISR is triggered by MAMPs found on the surface of PGPRs, such as lipopolysaccharide (LPS), flagellin, exopolysaccharide, peptidoglycan, or in the case of fungi, chitin, or zymosan. If a plant is resistant to the microbe, localized responses will occur. One of the first plant responses is an oxidative burst (Lamb and Dixon 1997). In many cases, the host cells will not be able to tolerate this environment and will die (Kombrink and Schmelzer 2001) thus blocking the infection from spreading to the rest of the plant. Infected cells as well as nearby cells will begin to produce compounds to thicken the cell walls, preventing entry of additional microbes. These cells will also begin making chemicals such as phytoalexins to inhibit or kill microbes (Kuc 1995). Next, the expression of pathogenesis-related (PR) proteins will also be upregulated in cells near the infection site and throughout the plant if the defense response has been initiated by a pathogen (Heil and Bostock 2002). There are a variety of PR (pathogen response) proteins with diverse functions that have been identified in a variety of plants. Van Loon and van Kammen (1970) thought that PR proteins were only expressed in diseased plants, but later were able to find PRs expressed in a wide variety of healthy plants (Van Loon and Van Strien 1999). PGPRs typically stimulate a jasmonic acid (JA)

response pathway, which triggers the ethylene response pathway. Upregulation of PR1 is then triggered. With SAR, pathogens usually trigger the salicylic acid (SA) pathway, which also leads to upregulation of PR1 which in turn leads to the expression of additional PR proteins (Bent and Mackey 2007). The PR1 protein is an important component of defense response, and it is the point where different signaling pathways converge (Ton et al. 2002a). It is believed that JA signaling results in defenses more specific to herbivores rather than microbes (Creelman and Mullet 1997). However, there is a lot of cross talk between the signaling pathways, and in *Arabidopsis* it has been shown that different signaling pathways (JA, SA, or ethylene) can result in resistance against some types of pathogens but not others, yet there is a lot of overlap in which a signaling pathway will be effective in eliciting resistance against several pathogens (Ton et al. 2002b). SA is not necessary for ISR signaling, but some rhizobacteria secrete SA near plant roots which triggers defense similar to what occurs in the SAR signaling cascade (Pieterse et al. 2001). When ISR and SAR are both triggered, disease resistance is even stronger (van Wees et al. 2000). Additionally, once a plant is elicited by a PAMP/MAMP, it is more responsive to exposure to other PAMPs/MAMPs as shown with *Arabidopsis* and flagellin recognition (Zipfel et al. 2004). Recently, evidence of a novel pathway unrelated to SA signaling or PR proteins was shown in rice. Yasuda et al. (2009) showed that *Azospirillum* sp. B510 enhanced rice resistance to bacterial pathogen *Xanthomonas oryzae* and fungal pathogen *Magnaporthe oryzae* without elevated SA or PR.

Since induction of a defense response results in increased resistance, it is interesting to consider the consequences of consistently anticipating an infection. If ISR must be induced, there will be times when the plant does not have enhanced resistance, as compared to situations where plants are always resistant. Higher levels of SA are seen in plants after ISR has been triggered. Plants unable to accumulate SA (such as the *Arabidopsis* nahG mutants) are not able to produce an ISR response (Delaney et al. 1994). Some cultivars of rice naturally have higher SA levels, but these cultivars tend to have less evolutionary fitness. They do not reproduce as well as other cultivars (Silverman et al. 1995). Similarly, *Arabidopsis* plants with salycilate synthase, an enzyme that is more efficient at making SA, are more resistant to disease but have more difficulty in reproducing (Mauch et al. 2001). *Arabidopsis* that overexpress proteins that are upregulated in the ISR are often much smaller plants and are also less efficient at reproducing (Heil and Baldwin 2002). Mechanistically, the reduced size could be due to the plants utilizing their nutrients for defense rather than for growth or reproduction. Resistance is important when plants are at an elevated risk of infection; however, there is not always the threat of infection, and using resources toward defense when there is no threat present may be a wasteful strategy. The better strategy may be to induce defenses when necessary. There is a short delay while the ISR is being induced, but this may be more advantageous than constitutively expending resources on defense (Heil 2001). Heil et al. (2000) showed that in the absence of pathogens, plants that initiate ISR do not have enough nutrients. Also, proteins involved in other normal cellular functions may be degraded so that the component could be used for defense proteins. When ISR is initiated, as much as 10% of a cell's proteins are PR proteins

(Heil and Bostock 2002). Beneficial rhizobacteria seem to "prime" defense responses through JA signaling. In effect, the entire defense response is not mounted, but the plant is able to mount the defense much more quickly after infection with a pathogen, reducing disease (Van der Ent et al. 2009) Examining the balance between cost and benefits of an induced defense response is currently an area of active research (Walters and Heil 2007).

There are numerous examples of rhizobacteria triggering ISR to enhance a plant's ability to resist disease. Fluorescent *Pseudomonas* species aid eucalyptus in its resistance against bacterial wilt (Ran et al. 2005). Pepper plants are more resistant to *Xanthomonas* infection after being "primed" with *Bacillus cereus* (Yang et al. 2009). In tomato, resistance is induced via phytoalexins and the LOX pathway after treatment with *Pseudomonas putida* (Akram et al. 2008). In tea plants, pretreatment with a *Pseudomonas* and *Bacillus* mix induced ISR and prevented blight as effectively as a chemical fungicide. Plants treated with the PGPRs had higher levels of antimicrobial enzymes such as peroxidase, chitinase, and beta-1, 3-glucanase. Furthermore, the tea yield was increased in plants that were treated with PGPRs (Saravanakumar et al. 2007). Tobacco is more resistant to blue mold after treatment with PGPRs, even in plants with mutations in the SA pathway, indicating that the defense response triggered by PGPRs is independent of SA (Zhang et al. 2002).

ISR is an evolutionarily conserved mechanism used in a variety of monocotyledons and dicotyledons (Heil and Bostock 2002), emphasizing its importance to evolutionary fitness. The priming of the ISR by PGPRs could be additionally advantageous in halting disease progression early in the infection. This would allow the plant to focus its resources on growth rather than recovering from disease and therefore constitutes an indirect mechanism of plant growth promotion.

1.4 Consequences of Aboveground Biotic Stress on Rhizobacterial Recruitment

Evidence shows that stress to the aerial portions of the plant can stimulate rhizodeposition of chemoattractants to enhance colonization by PGPR. Rhizodeposition is the release of carbon compounds from living plant roots into the surrounding soil; it is a ubiquitous phenomenon (Jones et al. 2004, 2009). Rhizodeposition results from two different processes: (1) leakage of compounds over which the plant exerts little control; (2) exudation of specific compounds with a specific function and over which the plant exerts control. Rates of exudation vary widely among species and environmental conditions (Lambers et al. 2009).

Colonization of microbes is influenced by plants due to root exudates (Bais et al. 2006). Plants collectively produce a diverse array of $\geq$100,000 compositionally different secondary metabolites, each with different functions in the rhizosphere (Bais et al. 2004, 2006). ISR and SAR signaling in plant resistance to pathogens and

insect herbivores involve the connections among phytohormone signaling networks that regulate stress response (Bostock 2005). Research on root–herbivore and root–microbe interactions emphasizes the influence of belowground tissues on aboveground physiology and resistance. *Pseudomonas fluorescens* FPT9601-T5 and WCS417 were found to trigger ISR in *Arabidopsis* against *Pseudomonas syringae* pv. tomato. Using an Affymetrix Gene Chip probe array containing approximately 22,800 genes, Wang et al. (2005) detected 95 and 105 genes that were up- and downregulated, respectively, in leaves of soil-grown plants that had been root dipped in a suspension of the bacteria as compared to plants whose roots were not pretreated with bacteria.

Rhizobacteria chemotaxis toward root exudates is an important colonization characteristic. In tomato, rhizodeposition of amino acids (L-leucine) and dicarboxylic acids recruit *P. fluorescens* WCS365 (DeWeert et al. 2002). Inoculation of *Arabidopsis thaliana* leaves with the foliar pathogen *Pseudomonas syringae pv.* tomato (*Pst*DC3000) induced malate excretion in roots (Rudrappa et al. 2008b). The study revealed that *Pst*DC3000-infected shoots relay chemical signal(s) underground through root malate secretion, resulting in specific chemotaxis to recruit rhizobacteria *Bacillus subtilis* strain FB17. Furthermore, a dramatic effect on the root transcriptome was found adding increasing evidence for the existence of a defensive shoot–root–shoot loop in plant-defense reactions (Erb et al. 2009).

Effective plant defense may be due to an ability of the host to regulate PGPR biocontrol functions by modulating the composition of root exudates. When plants invaded by a pathogen are inoculated with PGPR, the amount of organic acids in exudates may increase and stimulate the growth of bacteria and encourage increased antibiotic production by the bacteria (Kamilova et al. 2006). Additionally, some plants (including the legumes, pea, and alfalfa) regulate their PGPR functions by exudating specialized signals from the roots which mimic the bacterial "quorum sensing" regulators required for root colonization and antifungal activities (Teplitski et al. 2000). These observations suggest that improvement of biocontrol functions in root–PGPR associations may be achieved via manipulations of the bacterial genotypes and host genotypes.

1.5 Conclusion

The research focus is currently to unravel and utilize the previously underestimated role of roots in aboveground defenses. The identification of shoot–root and root–shoot signals and their regulatory mechanisms will be crucial to understanding this flow of information from aboveground to belowground and back. Signals which regulate the exchange of information between roots and shoots will facilitate future efforts for a better understanding of root metabolism and its plasticity. Elucidation on root–shoot cross effects might harbor potential applications in plant protection.

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Chapter 2
Bacterial Inoculants for Field Applications Under Mountain Ecosystem: Present Initiatives and Future Prospects

Pankaj Trivedi, Anita Pandey, and Lok Man S. Palni

2.1 Introduction

During the past few decades, agricultural production has increased on account of the use of high yielding varieties and chemical fertilizers and pesticides, to supplement nutrition and control phytopathogens, respectively. Although the use of chemicals has several advantages, such as ease of handling and predictable results, several problems related to the continuous addition of chemical fertilizers to the soil have also been recognized, such as deleterious effect on soil ecology, high irrigation needs, as well as effect on human health (Pandey and Kumar 1989; Gloud 1990; Harman 1992). Excessive use of chemicals and change in traditional cultivation practices have caused deterioration in the physical, chemical, and biological health of cultivable land (Paroda 1997). Therefore, the health of a wide range of agricultural production systems, in the wake of shrinking land resources and diminishing biological potential of the soil, and overall biological wealth need to be suitably addressed. There is no simple or single solution to these complex ecological, socioeconomic, and technological problems, facing those engaged in promoting sustainable advances in agricultural biotechnology (Swaminathan 1999).

The agricultural policy in India has undergone a major change in recent years to meet the increased demand of food through diversification and emphasis on sustainable production systems. The latter is a consequence of problems associated with the nonjudicious use of fertilizers and pesticides, as well as low purchasing

P. Trivedi
Microbiology and Cell Science, Citrus Research and Education Center, University of Florida, 700-Experiment Station Road, Lake Alfred, FL 33850, USA

A. Pandey (✉) • L.M.S. Palni
GB Pant Institute of Himalayan Environment and Development, Kosi-Katarmal, Almora, Uttarakhand 263 643, India
e-mail: anita@gbpihed.nic.in

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power of the marginal farmers. The objective in coming decades is to optimize soil productivity (inclusive of stressed soils), while preserving its capacity to function as a healthy medium. In this context, there is a strong case for using microorganisms for improved plant performance in integrated plant management systems. The use of soil microorganisms, which can stimulate plant growth, will form part of environmentally benign approach for nutrient management and sustaining the ecosystem functions. This will ensure that the nature is not exploited in the production process, instead it is harmonized so that the entropy of environment decreases and sustainability in agricultural production is promoted (Purohit 1995; Sinha 1997).

2.2 Plant-Growth-Promoting Rhizobacteria

The rhizosphere (from the Greek, rhizos-meaning root) was first described as a region around the root, which is directly affected by the root system (Hiltner 1904). The rhizosphere harbors a multitude of microorganisms that are affected by both biotic and abiotic stresses. Among these are the dominant rhizobacteria that live in close vicinity to the root or on its surface and are important for soil health and plant growth (Curl and Truelove 1986; Sylvia and Chellemi 2001; Johri et al. 2003; Tilak et al. 2005). Some bacteria from the rhizosphere can affect plant growth, both positively and negatively; the term "plant-growth-promoting rhizobacteria" (PGPR) is used to describe strains of naturally occurring soil bacteria that possess the capability to stimulate plant growth (Kloepper and Schroth 1978). Current trend in agriculture is focused on reducing the use of pesticides and inorganic fertilizers on one hand and accelerating the search for alternative ways to sustainable agriculture on the other (Smit et al. 2001). The use of PGPR inoculants as biofertilizers and/or antagonists of phytopathogens provide a promising alternative to chemical fertilizers and pesticides.

Plant growth promotion by rhizobacteria can occur either directly or indirectly (Glick 1995; Persello-Cartieaux et al. 2003). There are several ways through which plant-growth-promoting bacteria affect plant growth directly (Kloepper et al. 1989; Lynch 1990; Glick 1995), e.g., by fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus (P), production of siderophores that solubilize and sequester iron, or production of plant growth regulators (hormones) that enhance plant growth at various stages of development. Indirect growth promotion occurs when PGPR promote plant growth by overcoming growth restricting conditions (Glick et al. 1999). This can happen directly by the production of antagonistic substances (Thomashow and Weller 1988; Weller 1988; O'Sullivan and O'Gara 1992) or indirectly through the induction of resistance against pathogens (Van Peer et al. 1991; Glick 1995; Leeman et al. 1996).

2.3 Prospects and Challenges of Using Microbial Inoculants in the Mountain Ecosystem with Reference to Indian Himalayan Region

The Indian Himalayan Region (IHR) includes ten states namely, Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Sikkim, Arunachal Pradesh, Meghalaya, Nagaland, Manipur, Mizoram, and Tripura, as well as the hill regions of two states—Assam and West Bengal. IHR occupies a special place in the mountain ecosystems of the world. The mountain agro-ecosystems are characterized by difficult terrain, inadequate infrastructure, fragility, inaccessibility and marginal societies, lack of irrigation, severe top soil erosion, and overall external inputs to the system. Agricultural production in the mountains is, to a large extent, influenced by low organic matter, soil moisture status, and colder conditions. Therefore, the hill agriculture is, by and large, a low input, low production and subsistence but a sustainable system. Use of synthetic chemicals for increasing plant produce and/or for disease management is largely uneconomical and does not fit within the framework of "organic farming" adopted by various hill states of India. The prospect of improved agriculture by use of microbial inoculants as biofertilizers or as biological control agents may prove to be particularly rewarding in this less intensive, low-input agricultural system of the mountain regions of IHR, all through the tropical, subtropical, and alpine zones.

Extreme environmental conditions are not uncommon, and the microbial diversity of such areas is of particular interest because of the superb adaptability of the native microbes. Due to slow growth rate and difficulty of handling, relatively very little attention has been paid to cold adapted psychrophiles or psychrotolerant microbes. The cold adapted microbes possess various plant growth promotional abilities that can be utilized for increased plant production especially in the Himalayan region. In fact, the major objective in studying the microbial communities from the colder regions has been to select suitable microbial inoculants for use in the mountains (Pandey et al. 2004, 2006a, b). While some success has been achieved in this direction, there is an immense scope for the development of hitherto lesser studied and novel bacterial species as microbial inoculants for the colder regions of IHR.

Development of biofertilizers for the mountain regions of IHR is a challenging task. The average minimum temperature in these regions during winter (*rabi* season) varies from 3.5°C to subzero levels depending upon the location. During winters, snowfall is quite common in the upper reaches and the ground remains frozen from a couple of days to several weeks, and the soil temperature also dips and averages from 2°C to 10°C, depending on the location. Such extremities of temperature are deleterious for the survival and functioning of the introduced mesophilic microorganisms. Pandey et al. (1998) have reported that the ability of nonnative, introduced bacterial strains to colonize roots and survive in soil is often limited and thus results in the frequently observed reduction of expected plant growth promotion. As a consequence, the selection and use of PGPR should be carried out keeping the adaptation capability of the inoculants to a particular plant

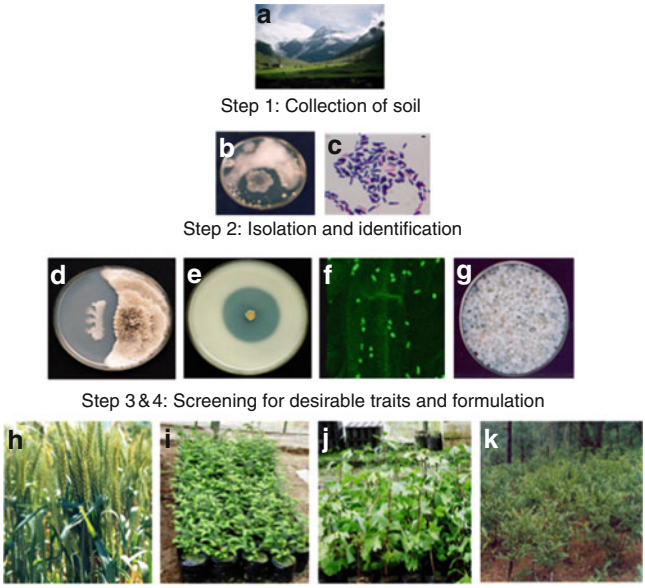

Fig. 2.1 Stepwise schematic representation of steps for the development of microbial inoculants: (**a**) a representative site in IHR for collection of soil samples; (**b**, **c**) isolation and identification of microbes, respectively; (**d**–**f**) screening for desirable traits such as biocontrol, phosphate solubilization, and root colonization (confocal laser scanning microscopy), respectively; (**g**) product formulation (alginate beads containing bioinoculant); (**h**–**k**) field application on appropriate plant species such as wheat, *Taxus baccata*, *Ginkgo biloba*, and tea, respectively

and soil in the rhizosphere ecosystem. Isolation, identification and characterization of microorganisms, screening for desirable characters, and selection of efficient strains leading to the formulation and production of inoculum and field testing are major steps for making use of this microbe-based technology (Fig. 2.1). Selection of an efficient PGPR requires understanding the dynamics and composition of the microbial communities colonizing the rhizosphere and its characterization with reference to plant growth promotion.

In the following sections of this chapter, an attempt will be made to highlight the significant achievements with reference to the development of microbe-based biofertilizers for use in the mountain regions of IHR, along with a brief discussion on new and emerging PGPR related technologies which have potential for application.

2.3.1 Exploration of Microbial Diversity for the Selection of PGPR

The IHR presents a unique ecological niche, where microbes have evolved and adapted to the prevailing edaphic and climatic conditions. Considering the inherent

opportunities their location offers, clearly the efforts put to unravel and utilize the potential of beneficial microorganisms have been disproportionate. With the surging awareness and demand for safe food, it is necessary to develop region-specific microbial inoculants. A study using three strains of *Azotobacter chroococcum* and two of *Azospirillum brasilense* was carried out on farmers' fields at two elevations, representing subtropical (1,200 m altitude) and temperate (1,900 m altitude) climates, in a watershed in Sikkim (Pandey et al. 1998). Statistically significant increase in plant growth and grain yield, and other yield attributing characters were observed in maize, at the subtropical site. Contrary to this, bacterial inoculations were found to be ineffective at the temperate site. The bacterial inoculants in the cited study were initially isolated from the tropical areas, and their ineffectiveness to promote plant growth at the higher altitude site was probably due to the inherent inability of the introduced bacteria to survive at lower temperatures. This study clearly indicated the need for isolation of native beneficial rhizobacteria, selection, and further development of inoculants for use at the higher elevations of IHR. Since then, various studies have been initiated to explore the microbial diversity of IHR. Various species of bacteria, mostly belonging to *Pseudomonas* and *Bacillus*, have been characterized for their beneficial properties (Fig. 2.2).

The research group at GB Pant Institute of Himalayan Environment and Development, Almora has contributed substantially toward the exploration of microbial diversity of IHR with a view to harness their potential to increase agricultural production in the hills (Pandey et al. 2004, 2006a, b and references therein). This has resulted in the isolation of various novel PGPR from the Garhwal and Kumaun regions of Uttarakhand, north-eastern regions in Sikkim and Arunachal Pradesh, and Himachal Pradesh in IHR. A number of bacterial species, mostly belonging to genus *Bacillus* and *Pseudomonas*, have been isolated and characterized from the rhizosphere of various plants of IHR (Pandey et al. 2006a, 2011). The isolates have been maintained in a culture collection of "high-altitude microbes" at 4°C in agar slants and at −20°C in glycerol stocks. Various strains from the culture collection have already been characterized and developed as microbe-based formulations (Trivedi et al. 2005a).

Recently, a research group at the Vivekananda Institute of Hill Agriculture, Almora has also made progress in elucidating the microbial diversity of PGPR, especially from the north-western parts of Uttarakhand (Mishra et al. 2008, 2009a, b; Selvakumar et al. 2007, 2008a, b, 2009a, b, 2010). Various novel psychrotrophic and psychrotolerant bacteria have been isolated and characterized for their beneficial properties (Mishra et al. 2011). Research carried out at the Institute of Bioresource Technology, Palampur have helped elucidate the microbial diversity of the cold desert region of trans-Himalaya in Lahaul-Spiti, and other parts of Himachal Pradesh. Various species of *Pseudomonas* have been isolated from the rhizosphere of Seabuckthorn (*Hippophae rhamnoides* L.) growing in the cold deserts of Lahaul and Spiti (Gulati et al. 2008, 2009, 2010; Vyas et al. 2009, 2010). Diversity of fluorescent *Pseudomonas* in the rhizosphere of tea (*Camellia sinensis* Linn.), gladiolus (*Gladiolus hortulanus* L.H. Bailey), carnation (*Dianthus caryophyllus* Linn.), and black gram (*Vigna mungo* Linn.), collected from different locations of Himachal Pradesh, have also been

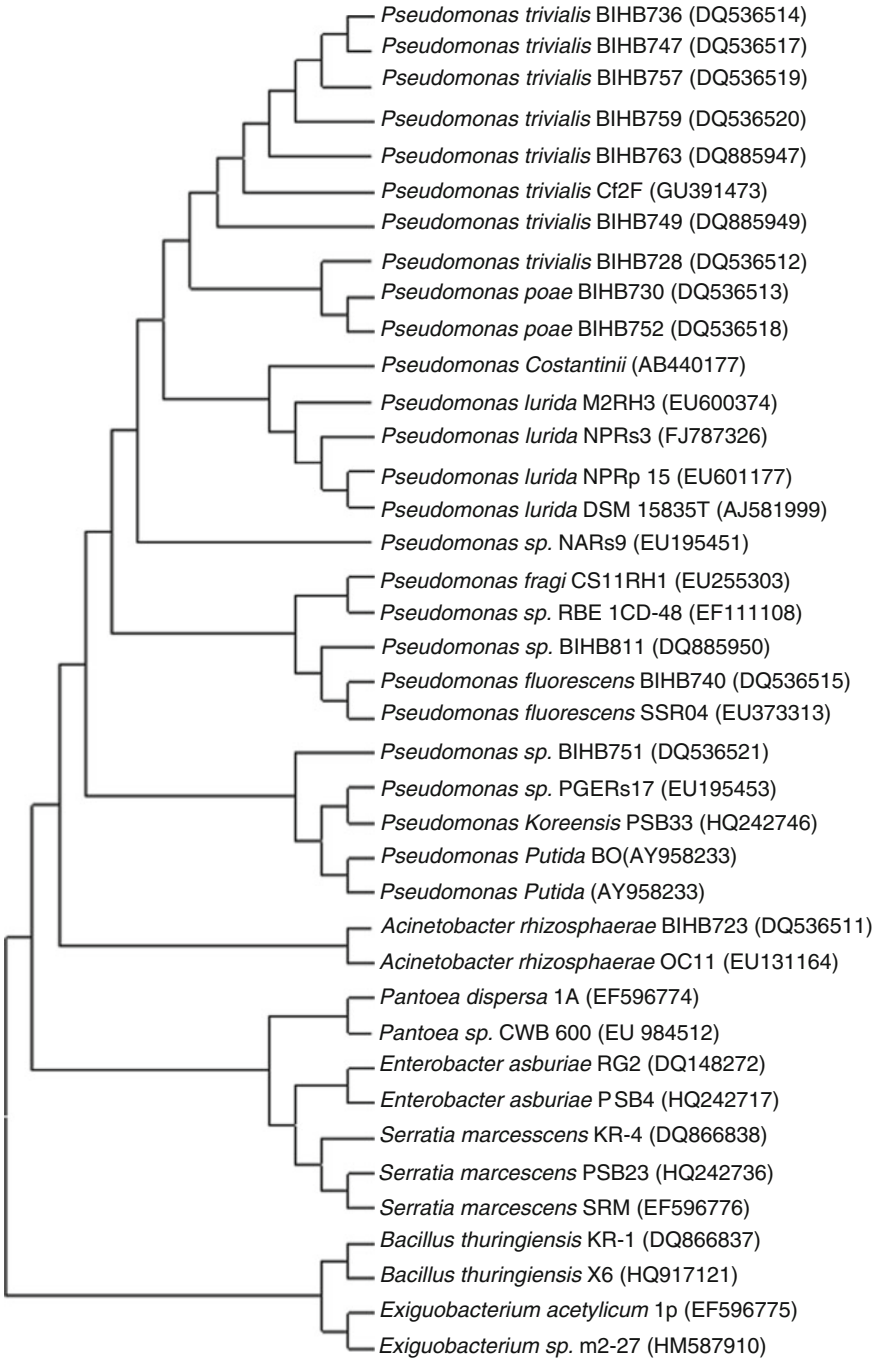

Fig. 2.2 Phylogenetic tree based on 16S rDNA gene sequence of beneficial bacteria isolated from the IHR along with their nearest relatives

described (Ajit et al. 2006; Verma et al. 2007; Shanmugam et al. 2008). The work done at GB Pant University of Agriculture and Technology (GBPUAT), Pantnagar has also contributed substantially in the exploration of *Pseudomonas* sp. from the Himalayan region, with a view to elucidate the molecular mechanisms of biocontrol and cold adaptability of novel bacterial strains (Sharma and Johri 2003; Tripathi et al. 2005a, b; Sharma et al. 2007). A repository of cold tolerant isolates of fluorescent *Pseudomonas* from the Grahwal region of Uttarakhand has been established at the Ranichauri Hill campus of GBPUAT (Negi et al. 2005).

2.4 Mechanisms of Plant Growth Promotion

Bacteria usually show one or many plant growth promoting mechanisms as listed below:

2.4.1 Fixation, Mobilization and Uptake of Nutrients

Nutrients are extremely important and directly influence growth, yield, and quality of crops. Soil microorganisms can provide nutrients to plants through the fixation of atmospheric N_2 and or by enhancing nutrient mobilization/uptake through their biological activities such as mineralization, and through the production of siderophores, organic acids, and phosphatases.

2.4.1.1 P-Solubilization

Among the rhizosphere bacteria that exert a positive influence on plant growth, P solubilizers occupy a prime position due to the essential requirement of phosphorus in plant nutrition. Though the soil P reserves are adequate for sustaining crop growth, they are mostly found in the fixed forms, thereby rendering them unavailable for plant uptake. The soils in IHR are generally acidic in nature and contain low moisture content and organic matter. The applied water soluble phosphatic fertilizers are rapidly fixed in the unavailable form(s) which accounts for the low phosphate use efficiency of crops grown in this region (Pal 1998). Therefore, microbe-mediated solubilization of soil phosphate reserves and the addition of low quality mineral rocks have been advocated as possible means of sustainable agriculture in IHR. There has been some interest on P-solubilization at cold temperatures by natural psychrophilic strains (Das et al. 2003). This has prompted the search for novel psychrotolerant bacterial strains that possess the ability to make available insoluble sources of P at temperatures unfavorable for the optimal performance of solubilization mechanisms.

Pseudomonas corrugata, a soil isolate, initially obtained from a temperate location in Sikkim (Himalaya), was examined for its tricalcium phosphate (TCP) solubilizing ability along a wide temperature range, from psychrophillic to mesophillic (Pandey et al. 2002b). The P-solubilization by indirect petridish assay was in conformity with the results of direct broth-based estimation. While the maximum solubilization was found to occur at 21°C, the bacteria solubilized more phosphate at 4°C than at 28°C. Similarly, P-solubilizing ability of *P. putida* was also estimated along a temperature range (4–28°C), and maximum activity (247 mg ml^{-1}) was recorded at 21°C after 15 days of incubation (Pandey et al. 2006b). Determination of P-solubilization by *P. lurida* M2RH3 at three incubation temperatures revealed a steady increase in the soluble P levels across the incubation temperatures, coupled with a steady drop in pH of the culture supernatant till 14th day of incubation (Selvakumar et al. 2010). At 4°C, which was the lower temperature extreme for its growth, *Serratia marcescens* strain SRM (MTCC 8708) solubilized 28.0 mg ml^{-1} phosphate in NBRIP broth (Selvakumar et al. 2008a, b). *Pseudomonas* sp. PGERs17 (MTCC 9000) was also found to solubilize P at various temperatures (Mishra et al. 2008). Gulati et al. (2008) screened various *Pseudomonas* strains from the cold desert region of Lahaul and Spiti, which can solubilize various sources of insoluble P such as TCP, Mussoorie rock phosphate, Udaipur rock phosphate, and North Carolina rock phosphate. Cold tolerant species of *Pantoea dispersa* and *Exiguobacterium acetylicum* were able to effectively solubilize P at lower temperatures (Selvakumar et al. 2008b, 2010).

The process of P-solubilization commences with the decrease in the pH of the medium suggesting the role of organic acids in the P-solubilization mechanism. Nineteen P-solubilizing fluorescent *Pseudomonas* strains belonging to *P. fluorescens*, *P. poae*, *P. trivialis*, and *Pseudomonas* spp. produced gluconic acid, oxalic acid, 2-ketogluconic acid, lactic acid, succinic acid, formic acid, citric acid, and malic acid in the culture filtrates during the solubilization of various rock phosphates (Gulati et al. 2008; Vyas and Gulati 2009). The strains differed quantitatively and qualitatively in the production of organic acids during solubilization of phosphate from the substrates. *P. corrugata* produced gluconic and 2-ketogluconic acid during the growth at lower temperature (Trivedi and Sa 2008). Vyas et al. (2010) reported the detection of gluconic, citric, and isocitric acids during the TCP solubilization by *Rahnella* sp.

2.4.1.2 Siderophore Production

The availability of iron for microbial assimilation in various microenvironments, such as the rhizosphere, is extremely limiting. Consequently, to survive in such environments, organisms secrete iron-binding ligands (siderophores), which can bind the ferric iron and make it available to the host microorganisms. The role of such iron chelating siderophores in plant growth promotion is well established (Katiyar and Goel 2004). While siderophores are mainly implicated in the biological control of plant pathogenic fungi, their role in iron nutrition of plants

still remain unclear. Sharma and Johri (2003) studied the production and regulation of siderophores by fluorescent *Pseudomonas* strain GRP3A. Among various media tested, standard succinate medium (SSM) promoted maximum siderophore production of 56.59 mg l^{-1}. *Acinetobacter rhizosphaerae*, *Pantoea dispersa*, *Rahnella* sp., *Bacillus megaterium*, and various *Pseudomonas* spp. were found to form halo in CAS agar, indicative of siderophore production at lower temperature (Negi et al. 2005; Tripathi et al. 2005b; Pandey et al. 2006b; Selvakumar et al. 2008b, 2010; Trivedi et al. 2008; Trivedi and Pandey 2008a; Gulati et al. 2009; Mishra et al. 2009a; Vyas et al. 2010).

2.4.1.3 Production of Plant Growth Regulating Substances

Plant growth regulating substances are naturally occurring organic compounds that influence various physiological processes in plants such as cell elongation and cell division. They perform these functions at concentrations far below the levels at which nutrients and vitamins normally affect plant processes. It is now well established that the majority of soil microorganisms can produce plant growth regulating substances, including phytohormones (auxins, gibberellins, cytokinins, ethylene, and abscisic acid) and enzymes (Frankenberger and Arshad 1995; Glick 1995; Khalid et al. 2006) and form one of the major mechanisms of plant growth promotion by PGPR.

Among the plant growth promoting substances, bacterial production of indole-3-acetic acid (IAA) has a cascading effect on plant development, due to its ability to influence root growth, which in turn affects the nutrient uptake and ultimately the plant productivity. IAA production by cold tolerant bacteria has received little attention in the past, but recently several studies focused on developing microbial fertilizers for the mountain regions have described IAA producing ability of various strains from the Himalayan region. Selvakumar et al. (2008b), observed higher levels of IAA production by *P. dispersa* growing at 4°C. *Pseudomonas* sp. strain PGERs17 produced 1.38, 8.33 and 13.15 μg ml^{-1} of IAA after 3 days of incubation at 4, 15, and 28°C, respectively (Mishra et al. 2009a). Cold tolerant strain of *Rahnella* sp. produced IAA, indole-3-acetaldehyde, indole-3-acetamide, indole-3-acetonitrile, indole-3-lactic acid, and indole-3-pyruvic acid in tryptophan-supplemented nutrient broth (Vyas et al. 2010). *E. acetylicum* produced 10.04 μg ml^{-1} day^{-1} of IAA in tryptone supplemented media at 4°C (Selvakumar et al. 2009b). *Bacillus megaterium* has also reported to produce IAA at various growth temperatures in tryptophan supplemented medium (Trivedi and Pandey 2008b).

Some PGPR can influence plant growth by altering the synthesis of endogenous phytohormones through production of specific enzymes. Among these enzymes, bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase plays a significant role in the regulation of a gaseous plant hormone, ethylene, and thus influences the growth and development of plants (Arshad and Frankenberger 2002; Glick 1995). ACC deaminase activity of only a few isolates of Himalayan origin has been

described. Vyas et al. (2010) reported ACC deaminase activity of *Rahnella* sp., isolated from trans Himalaya. *A. rhizosphaerae* strain BIHB 723 was also found to possess ACC deaminase activity at lower temperature (Gulati et al. 2009). Trivedi et al. (2007b) reported a chromate reducing strain of *Rhodococcus erythropolis* showing ACC deaminase activity at different chromate concentration.

2.4.2 Biological Control

Bacterial antagonism toward phytopathogenic fungi is known to be mediated by a variety of compounds of microbial origin, *viz.* antibiotics, enzymes, siderophores, hydrogen cyanide (HCN), catalase, bacteriocins, toxic substances, volatiles, and others.

Pseudomonas corrugata showed antagonistic activity against *Alternaria alternata* and *Fusarium oxysporum* (Trivedi et al. 2008). The production of volatiles was reported as an antagonistic factor, although the strain also produced a variety of hydrolytic enzymes. Chaurasia et al. (2005) reported an efficient antagonistic strain of *Bacillus subtilis*, originally isolated from the rhizosphere of established tea bushes. The strain was found to cause structural deformities in six pathogenic fungi under in vitro culture conditions. This effect was attributed to the production of diffusible and volatile antifungal compounds. The bacterial strain successfully restricted the growth of all the test fungi in dual cultures and induced morphological abnormalities such as mycelial and conidial deviations. The inhibitory effect caused by volatiles was greater than that by diffusible compounds. Similarly, *B. megaterium* also produced diffusible and volatile compounds that inhibited the growth of two phytopathogens viz. *A. alternata* and *F. oxysporum* (Trivedi and Pandey 2008a). Various species of *Bacillus* isolated from the tea rhizosphere showed antagonistic activity in plate-based assays against 12 test fungi, which included nine minor (saprophytes) and three major pathogens of tea (Pandey et al. 1997).

P. putida exhibited antifungal activity against phytopathogenic fungi in petri dish assays and produced chitinase, ß-l,3-glucanase, salicylic acid, siderophore, and HCN (Pandey et al. 2006b). Four rhizospheric strains of *P. fluorescens* (Pf) showed antagonistic activity against *Fusarium solani* f. sp. *pisi* (causal agent of root rot in pea; Negi et al. 2005). In a liquid culture-based assay, Pfs could inhibit the growth of *F. solani* f. sp. *pisi* by 60–100%, suggesting that their secondary metabolites were sufficient to antagonize the target pathogen. Mode of inhibition of *F. solani* f. sp. *pisi* by Pf-102 and Pf-103 was fungistatic, while that of Pf-110 and Pf-173 was lytic. Sharma et al. (2007) reported various strains of *Pseudomonas* showing in vitro antimycelial activity against three zoosporic fungi, causing pre- and postdamping off of various plants. The authors also provided evidence of the involvement of rhamnolipids causing the lysis of plasma membrane of zoospores of fungi as an active component involved in antagonism. Ajit et al. (2006) established the role of chitinase in the antagonism exhibited by fluorescent

pseudomonads against the causal agent of vascular wilt of carnation, *F. oxysporum* f. sp. *dianthi*. A strain of *Pseudomonas* sp. capable of possessing HCN and siderophore production at 4°C was shown to exhibit inhibitory activity against several phytopathogenic fungi using three different bioassays (Mishra et al. 2008).

2.4.3 Root Colonization

Root colonization by specific rhizosphere/rhizoplane microorganisms is basic to the process of biological control of plant pathogens and enhancement of plant productivity (Kloepper et al. 1989). The key elements for colonization include ability to survive following inoculation of the target (e.g., seed), multiplication in the spermosphere (region surrounding the seed) in response to seed exudates, attachment on the root surface, and colonization of the developing root system (Nelson 2004). The inconsistency observed in PGPR performance (Lethbridge 1989) may result from a number of factors, the most likely reason may be difference in ability to establish and survive (by the introduced bacteria) (Burr et al. 1978; Harris et al. 1989). The ability to colonize plant roots and develop rhizosphere competence are prerequisites for any plant growth promoting agent(s) under consideration.

The presence of "introduced" bacteria in the rhizosphere of various plant species of the Himalayan region has been confirmed through antibiotic markers. The use of genetic markers such as intrinsic resistance to various antibiotics is simple and rapid method of strain identification and enumeration of the introduced bacterial isolates that exhibit resistance to selective antibiotics (Josey et al. 1979; Kluepfel 1993). Using such markers, Trivedi et al. (2005b) confirmed the effective rhizosphere colonization of tea by *B. subtilis*, *B. megaterium*, and *P. corrugata*, thereby suggesting a close bacterial–plant association with beneficial effects on plant growth. These three bacteria were also studied for colonization and survival in the rhizosphere of maize using field and pot experiments conducted for 3 consecutive years under rainfed conditions of the Himalayan region (Kumar et al. 2007). The three bacterial inoculants showed good rhizosphere competence giving high inoculum numbers ($\log_{10}$ 11.13–11.34 cfu g^{-1}). The rifampicin mutant of *A. rhizosphaerae* and *Rahnella* sp. effectively colonized the pea rhizosphere without adversely affecting the resident microbial populations (Gulati et al. 2009; Vyas et al. 2010). Rhizoplane population of fluorescent pseudomonads was reportedly maintained at a critical level (5.3 cfu) upto 30 days of soybean growth, followed by a steep decline (Tripathi et al. 2005a). Effective colonization of maize rhizosphere was observed for different carrier-based formulations of *B. subtilis, P. corrugata,* and *B. megaterium* (Trivedi et al. 2005a; Trivedi and Pandey 2008a).

Using molecular markers such as green fluorescent protein (gfp) or fluorescent antibodies, it is possible to monitor the location of individual rhizobacteria on the root using confocal laser scanning microscopy (CLSM). *P. corrugata* was tagged by gfp marker and its efficiency to colonize the root and leaves of wheat growing under greenhouse conditions at 21°C was assessed by CLSM. The results showed

that *P. corrugata* effectively colonized both the root (Fig. 2.1f) and leaf samples of wheat growing at lower temperature (unpublished records).

2.4.4 *Effect of PGPR Application on the Native Microbial Communities*

An important aspect of colonization is the ability to compete with indigenous microorganisms already present in the soil and rhizosphere of a developing plant. Various studies have shown that the ecologically competent bacteria can effectively establish in a plant environment as compared to nonnative strains. Further, the effect of a particular strain to promote plant growth is also dependent on its interaction with other beneficial microorganisms present in the vicinity of target plants. Several reports from IHR have shown that the effects of seed inoculation on plant growth may be due to stimulation of already existing PGPR in and around roots. A majority of nonfluorescent pseudomonads isolated from the rhizosphere or rhizoplane of two important hill crops did not resemble the antibiotic resistance pattern of the inoculated bacteria (Pandey et al. 1999). This indicated that the observed plant growth promotion, due to introduced bacteria, was largely through stimulation of native microbial communities of the rhizosphere/rhizoplane. Besides plant–microbe compatibility, the original habitat of the bacterial isolates was also important for the establishment and subsequent effect on plant growth. Pandey et al. (1998) have observed a two- to fivefold increase in population of actinomycetes (a group known to fix nitrogen), following inoculation of maize with *Azotobacter chroococcum* and *Azospirillum brasilense* at the Mamlay watershed in Sikkim. Bacterial inoculation of tea rhizosphere resulted in the stimulation of the native bacteria, actinomycetes, and a group of bacteria capable of growing on N-free medium while suppressing the rhizospheric fungal population (Trivedi et al. 2005b). Kumar et al. (2007) have also attributed the positive effect of bacterial inoculation on growth of maize through the stimulation of native microflora. In addition to the increase in number of beneficial microorganisms, bacterial inoculation also resulted in increased mycorrhizal colonization of highland rice varieties (Trivedi et al. 2007a). Inoculation of *Lens esculenta* by *B. subtilis* has been reported to enhance the efficacy of *Rhizobium*–legume symbiosis (Rinu and Pandey 2009). In this study, authors also made interesting observations on colonization of arbuscular mycorrhizal (AM) fungi and other endophytes in lentil roots due to bacterial inoculation. The endophytes in the root samples were filamentous with round spores growing along with the epidermal tissues. In some root samples, these were observed in vascular bundles as well. In the control root samples, percent colonization of AM was 52% and that of other endophytes was 56%, whereas in inoculated root samples, the corresponding values were 2% and 78%, respectively. There was a significant increase in the endophytic fungal colonization with

concomitant decrease in AM fungal colonization in *B. subtilis* NRRL B-30408-inoculated plants.

2.5 Role of PGPR in Agriculture

PGPR represent an essential component of biofertilizer technology to improve the productivity of agricultural systems in the long run (Bashan 1998; Nelson 2004). Many PGPR show great promise as potential inoculants for agricultural uses and environmental protection and play critical role in maintaining the sustainability of agroecosystems. However, the current use of PGPR in agriculture is somewhat poor despite numerous reports on their proven performance under laboratory conditions. PGPR possess the ability to colonize and establish an ongoing relationship with plants, resulting in better root growth, more biomass, and a substantial increase in crop yield. In this context, significant effects of PGPR have been observed on various agricultural crops, including legumes, cereals, and noncereals, and some other important plant species. Furthermore, the indirect impact of PGPR on increasing water and nutrient use efficiencies has also been observed (Ahmad et al. 2008; Arshad et al. 2008). The potential uses and benefits of PGPR in improving the overall performance of plants have been discussed in the following sections.

2.5.1 Greenhouse Trials

Nethouse-based inoculation trials using *B. subtilis* and *P. corrugata* revealed increase in shoot length and stem girth of four different tea clones (Trivedi et al. 2005b). Gulati et al. (2009) reported significant increase in growth of pea, chickpea, maize, and barley when inoculated with *A. rhizosphhaerae* BIHB 723, initially isolated from a Himalayan cold desert. Various endophytes isolated from the roots of leguminous vine *kudzu* have been reported to positively influence the growth and nutrient uptake in wheat (Selvakumar et al. 2008a). Seed bacterization with *P. lurida* has been reported to positively influence the growth and nutrient uptake of wheat in pot culture conditions growing in controlled cold temperatures (Selvakumar et al. 2010). Seed bacterization with *E. acetylicum* and *P. dispersa*, positively influenced the growth and nutrient uptake parameters of wheat seedlings in glasshouse studies at suboptimal cold conditions (Selvakumar et al. 2008b, 2009b). The P-solubilizing bacterial treatments with *P. trivialis* BIHB 745, *P. trivialis* BIHB 747, *Pseudomonas* sp. BIHB 756, and *P. poae* BIHB 808 resulted in significantly higher or statistically at par growth and the total N, P, and K content over single super phosphate treatment in maize (Vyas and Gulati 2009). These treatments significantly affected pH, organic matter, and N, P, and K content of soil. Seed bacterization of wheat by *Serratia marcescens* strain SRM resulted in greater enhancement of root growth, as compared with shoot growth

(Selvakumar et al. 2007). An increase of 17.3%, 18.6%, and 26.7% in the uptake of N, P, and K, respectively, was observed as a result of in the bacterized treatment over uninoculated controls. Seed bacterization with an isolate of *Pseudomonas* sp. enhanced the germination of wheat seedlings grown at 18 ± 1°C by 20.3% (Mishra et al. 2008). Bacterized seeds also resulted in 30.2 and 27.5% higher root and shoot length, respectively, compared to uninoculated controls. Inoculation of two important hill crops, *Amaranthus paniculatus* and *Eleusine coracana* by *P. corrugata* positively influenced plant growth and the nitrogen content of various plant parts (Pandey et al. 1999).

Mishra et al. (2011) accessed the bioassociative effect of two cold tolerant *Pseudomonas* spp. with *Rhizobium leguminosarum*-PR1 on plant growth and nutrient uptake of lentil under greenhouse conditions. Co-inoculation resulted in significant increases in vegetative growth, nodulation, leghaemoglobin content, P and Fe uptake, and chlorophyll content as compared to single inoculation. Similar positive responses were observed in pea and lentil when endophyte *B. thuringiensis*-KR1 was co-inoculated with *Rhizobium leguminosarum*-PR1 (Mishra et al. 2009b). Siderophore producing *Pseudomonas* strain GRP3A caused plant growth promotion of mung bean (*Vigna radiata* L. Wilzeck) under iron limited conditions (Sharma and Johri 2003). The germination was improved in treatments with strain GRP3A and maximum germination (87%) was recorded in 10 μM Fe^{3+} + GRP3A treatment. Iron chlorosis (pale green, particularly between the veins) was visualized predominantly in leaf lamina of untreated and no iron, 2.5 μM and 5.0 μM Fe citrate treated plants, whereas chlorotic patches did not appear in GRP3A + iron (different concentrations) treated plants. However, some patches were observed in no iron treatment. The plant growth promotion potential of *Pseudomonas* sp. strain PGERs17 and NARs9 was determined by a pot assay under nonsterile soil conditions at suboptimal temperatures (Mishra et al. 2009a), and the bacterized wheat seedlings recorded higher seed germination, root, and shoot lengths as compared to uninoculated controls.

In an experiment, a mixture of *Pythium ultimum*, *P. arrhenomanes*, and *F. graminearum* was introduced in soil; maize seed inoculated with one of the two strains of *P. corrugata* (1 or 7) was sown in pots containing such soil (Pandey et al. 2001). The bacterial inoculation resulted in significant disease suppression as well as growth promotion of seedlings. The plant growth promotion and antifungal properties of *P. putida* (B0) were demonstrated through a maize-based bioassay under greenhouse conditions (Pandey et al. 2006b). The bacterial inoculation resulted in significant increment in plant biomass and stimulated bacterial but suppressed fungal counts in the rhizosphere.

A novel application of PGPR technology has been in the biological hardening of tissue culture (TC) raised plants. A major limitation in the large scale application of TC technology is high mortality experienced by TC raised plants during or following laboratory to land transfer, mainly due to extreme differences between the in vitro and ex vitro environment (Pandey et al. 2002a). Apart from various abiotic factors, one major cause of mortality of such "aseptically" raised plants is their sudden exposure to the soil microbial community. Efficient antagonistic PGPR have been

used for biological hardening to increase the survival, as also to augment overall plant growth of micropropagated plants. Three PGPR viz. *B. megaterium*, *B. subtilis*, and *P. corrugata* were used for the biological hardening of TC raised plantlets of an important medicinal herb, *Picrorhiza kurrooa* (Trivedi and Pandey 2007b). The bacterial isolates antagonized the fungal spp. postulated to cause death of micropropagated plants in plate-based assays and positively influenced survival and growth parameters in greenhouse trials. These three bacterial isolates had earlier been used to enhance survival of micropropagated tea plants in rainy, winter, and summer seasons (Pandey et al. 2000).

2.5.2 Field-Based Experiments

There is increasing evidence to show that microbial inoculation of seeds may benefit plant growth through a number of mechanisms (Lynch 1990). However, recovery of inoculants from roots has been variable, which may be an important factor influencing yield in inoculation experiments (Kloepper et al. 1989). The inoculated microorganism may not always successfully survive and persist in the rhizosphere. This contributes to the inconsistence results of PGPR applications in different geographical regions (Burr et al. 1978; Weller 1988; Bashan and Levanony 1990; Nautiyal et al. 2000). The contributing factors may include the colonization potential of the inoculum, the nature of maximum population achieved by the introduced strain in the rhizosphere (Bennett and Lynch 1981), the method of inoculum preparation and delivery system used (Kommedahl and Windels 1981), and the prevailing environmental conditions (Burr et al. 1978; Suslow and Schroth 1982). Table 2.1 summarizes the response of promising PGPR that have been developed as carrier-based formulations and evaluated in field-based trials in mountain regions.

Trivedi et al. (2007a) reported significant increase in grain yield of a local landrace of rice (*dudil*) in field trials using charcoal-based formulations of *B. megaterium*, *B. subtilis*, and *P. corrugata*. Bacterial inoculation also resulted in higher values of P in shoots and grains of inoculated plants. Field results of 3 years showed that inoculation of maize by *P. corrugata* resulted in significant increase in plant yield and N, P, and K contents of root and shoot (Kumar et al. 2007). Field-based experiments were conducted to evaluate the PGPR abilities of *B. subtilis* NRRL B-30408 for growth of lentil at a mountain location in IHR in two consecutive years (Rinu and Pandey 2009). A positive influence of bacterial inoculation on plant biomass and other yield related parameters was observed in both years. Significant increase in nodule numbers and leghaemoglobin concentration indicated an enhancement in the efficiency of the *Rhizobium*–legume symbiosis due to bacterial inoculation. Based on the results of this field study, inoculation with suitable plant growth promoting rhizobacteria, instead of dual inoculation, is suggested as a better option for improving yield and related attributes of a primary dietary legume such as

Table 2.1 Field applications of PGPR in the mountain conditions of IHR

Bacterial strain(s)	Beneficial properties	Test plant	Inoculation response	Reference
Azotobacter chroococcum, Azospirillum brasilense	Nitrogen fixation, phytohormones production	Maize	Seed inoculation resulted in statistically significant and improved plant performance and nutrient content at a subtropical site. Bacterial inoculations stimulated beneficial native microflora. No positive effect of bacterial inoculation was observed at temperate locations	Pandey et al. (1998)
Pseudomonas sp. strains GRP_3, Pen-4, PRS_1, WRS-24	Biocontrol	Soybean	Severity of foliar anthracnose was significantly reduced in disease susceptible variety ps1042. Compared to nonbacterized control, dry root weight was improved	Tripathi et al. (2005a)
Rahnella sp.	P-solubilization, Production of ACC deaminase, IAA, ammonia, siderophore	Pea	Microplot testing of the inoculums at two different locations showed significant increase in growth and yield	Vyas et al. (2010)
Acinetobacter rhizosphaerae BHIB 723	P-solubilization, Production of ACC deaminase, IAA, ammonia, siderophore	Pea	A significant incremental increase in shoot length, dry weight and yield	Gulati et al. (2009)
Pseudomonas corrugata, Bacillus subtilis, B. Megaterium	P-solubilization; Production of IAA, ammonia, siderophore, HCN; biocontrol	Rice	Increase in yield and P-content of grains and shoot. Bacterial inoculation resulted in stimulation of native microbial community, including mycorrhizae	Trivedi et al. (2007a)
Pseudomonas sp. strains FQP PB-3, FQA PB-3 and GRP_3	P-solubilization; Production of siderophore, rhamnolipid, ACC deaminase, HCN, IAA; Biocontrol	Chili and Tomato	Significant increase in shoot and root length in two different seasons. The bacterial treatments significantly increased the activity of peroxidase and phenylalanine ammonia lyase in plant tissues. The suppression efficacy for post-emergence damping-off was less compared to pre-emergence damping-off although still significant	Sharma et al. (2007)

Pseudomonas fluorescens strain Pf-102, Pf-103, Pf-110, Pf173	P-solubilization, production of siderophore; Biocontrol	Pea	Increase in germination (%), seedling length, root and shoot length, fresh and dry weight, vigour index and mortality	Negi et al. (2005)
Bacillus subtilis	P-solubilization; Production of IAA, ammonia, siderophore, HCN; biocontrol	Lentil	Positive influence of bacterial inoculation on plant biomass and yield related parameters were observed for two consecutive years. The significant increase in growth and nodule numbers as well as leghaemoglobin and protein concentration of nodules indicated enhanced efficiency of the *Rhizobium*–legume symbiosis	Rinu and Pandey (2009)
Pseudomonas corrugata	P-solubilization; production of IAA, ammonia, siderophore, HCN; biocontrol	Maize	Inoculations resulted in significant increase in grain yield, root–shoot ratio and nutrient content for three consecutive years. Recommended *P. corrugata* as suitable bioinoculant for maize fields of temperate climate grown under rainfed conditions	Kumar et al. (2007)

lentil. Field testing of *A. rhizosphaerae* and *Rahenella* sp. with pea also showed significant increment in plant growth and yield (Gulati et al. 2008; Vyas et al. 2010).

Based on in vitro screening for plant growth promoting and antimycelial activity against three zoosporic pathogenic oomycetes, seven bacterial isolates were selected for field trials on tomato (*Lycopersicon esculentum* Mill.) and chile (*Capsicum annum* L.) in a Central Himalayan location (Sharma et al. 2007). The efficacy of the isolates to promote plant growth and health were evaluated under natural and artificial disease-infested sites in both winter and wet seasons. Seed bacterization with bacterial strains reduced pre-emergence damping off by 60–70% at two sites, with and without previous history of fungicide use, during winter season, and to a lesser extent (20–40%) in warmer wet season. In another experiment similar strains enhanced peroxidase and phenylalanine ammonia-lyase activities in chile and tomato (Sharma et al. 2007). Modulation of the enzymatic activities after bacterial inoculation protected the plants against invasion of *Pythium* and *Phytophthora* spp., causal agents of damping-off in nurseries. Tripathi et al. (2005a) have used various strains of fluorescent pseudomonads for seed bacterization of soybean (*Glycina max* L.), dry weight of soybean was reportedly improved by bacterial inoculation. Also the severity of foliar anthracnose caused by *Colletotrichum dematium* was significantly reduced in bacterial treatments in case of soybean variety PS 1042. Cold tolerant fluorescent *Pseudomonas* isolates reduced the mortality of pea in *F. solani* f. sp. *pisi* (causal agent of root rot in pea) infested field and increased the vigor index in two separate experiments (Negi et al. 2005).

2.5.3 Formulation of Microbial Inoculants

A number of steps are involved in developing effective PGPR-based biofertilizers for achieving consistent results in terms of crop productivity under field conditions. The survival and maintenance of activity in both rhizosphere and non-rhizosphere soils is important for the success of any inoculation protocol. To facilitate introduction of high cell numbers and increase survival of microorganisms in soil, carrier-based preparation of microbial inoculants with right formulation is a prerequisite (Bashan 1998). Similarly, the viability of such preparations under storage for some time is also important for commercialization of this microbe-based technology. Most formulations of PGPR used in IHR have utilized charcoal or similar inert carrier material (Kumar et al. 2007; Tripathi et al. 2005a; Trivedi et al. 2007a; Mishra et al. 2009a; Rinu and Pandey 2009; Vyas et al. 2010). Negi et al. (2005) mixed the bacterial suspension with talc powder containing 1% carboxymethyl cellulose to prepare formulations of biocontrol PGPR. Recently, formulations based on polymers have been developed and evaluated for their potential as bacterial carriers (Trivedi et al. 2005a; Trivedi and Pandey 2007a, 2008a, b). These formulations encapsulate the living cells, protect the microorganisms against many environmental stresses, and release them into soil gradually but in large

quantities, when the polymers are degraded by soil microorganisms, usually at the time of seed germination and seedling emergence. Trivedi et al. (2005a) used five different formulations of *B. subtilis* and *P. corrugata* to improve the growth of maize. Best results in terms of overall plant growth parameters and number of bacteria present in the rhizosphere were observed with alginate-based formulations. Similar growth promotion effects were obtained in various test plants when alginate-based formulations of *B. megaterium* and *P. putida* were applied (Trivedi and Pandey 2007a, 2008a, b). Alginate-based formulations can be dried and stored at ambient temperatures for prolonged periods, and thus offer consistent batch quality and defined environment for the bacteria, and can be manipulated easily according to the specific needs of different bacteria (Trivedi et al. 2005a; Trivedi and Pandey 2008a, b). Bacterial isolates entrapped in alginate retained beneficial properties even after 3 years of storage (Trivedi and Pandey 2008a). These inoculants can be amended with nutrients to improve the short-term survival of bacteria upon inoculation, which is essential to the success of the inoculation process, especially with associative PGPR.

2.6 New Technologies and Their Potential in PGPR Related Research

2.6.1 Genome Sequencing

As of May 2010, 1,072 complete published bacterial genomes have been reported in the Genomes Online Database and another 4,289 bacterial genome projects are known to be ongoing (www.genomesonline.org). A common requirement of studies on bacterial genetics is the de novo determination of complete genome sequence of a species or strain of interest. Using Sanger technology, the "best practice" protocols for achieving the complete sequencing of a bacterial genome involve generation of sequencing libraries with different sized inserts, sequencing the genome to several fold depth from these libraries, and then using assembly tools to infer a draft sequence. This draft sequence, often in many fragments, is then refined by additional sequencing to span gaps, before a final assembly is produced, ready for annotation. Next generation sequencing technologies such as Roche GS FLX^{TM}, Illumina Genome Analyzer, and Applied Biosystems SOLiD^{TM} system make it possible to obtain millions of reads in a single run. "Next generation technology" speeds up (and reduces the cost of) the first draft, the data generation part of the bacterial genome sequencing process. It is relatively easy to generate many-fold coverage of genomes. As the next generation technologies do not include a step that involves cloning in bacterial vectors, coverage is usually more even and is little affected by different AT/GC proportions in the genome. Genome sequencing of novel strains from IHR will help in understanding the mechanism of cold tolerance and will hopefully result in the identification of the subset of genes

necessary for rhizosphere colonization and/or production of plant-growth-promoting substances.

2.6.2 Molecular Analysis of Microbial Communities

Although the above-mentioned studies related to bacterial diversity in IHR provided valuable insights on the microbe–microbe interactions in the rhizosphere, they suffer a major drawback in that all these have relied on culture-based techniques. It has now been emphasized that the understanding of factors involved in plant–microbe interactions has been hindered by inability to culture and characterize diverse members of the rhizosphere community and to determine how the community varies with plant species, plant age, location on the root, and soil properties. Phenotypic and genotypic approaches are now available to characterize the rhizobacterial community structure. Methods that rely on the ability to culture microorganisms include standard plating methods on selective media, community level physiological profiles (CLPP) using the BIOLOG system (Garland 1996), phospholipid fatty acid (PLFA; Tunlid and White 1992), and fatty acid methyl ester (FAME profiling; Germida et al. 1998). Culture-independent molecular techniques are based on direct extraction of DNA from soil and 16S-rRNA gene sequence analysis, bacterial artificial chromosome, or expression cloning systems. These provide new insight into the diversity of rhizosphere microbial communities, the heterogeneity of the root environment, and the importance of environmental and biological factors in determining community structure (Smalla et al. 2001). These approaches can also be used to determine the impact of inoculation of plant-growth-promoting rhizobacteria on the rhizosphere community.

Upcoming high throughput techniques such as microarray-based profiling of bacterial community and pyrosequencing are promising and add in the understanding of microbial communities and their interactions with plants. Microarray technology is a powerful, high throughput experimental system that allows the simultaneous analysis of thousands of genes at the same time (Trivedi and Wang 2012). Originally developed for monitoring whole-genome gene expressions, microarrays have been used for other purposes such as genome-wide mutational screening for single nucleotide polymorpisms and the distribution of species and strains in natural microbial communities. Several types of microarrays have been developed and evaluated for bacterial detection and microbial community analysis. Recently, this technique was adapted to elucidate the structure (Phylogenetic oligonucleotide microarrays) and functions (Functional gene arrays; FGA) of microbial community. Phylogenetic oligonucleotide microarrays use a conserved marker such as the 16S rRNA gene as probe template and are used to compare the phylogenetic relationship of microbial communities in different environments. The most comprehensive phylogenetic microarray, called "PhyloChip," has been developed by Gary Andersen's Laboratory at Lawrence Berkeley National Laboratory (Brodie et al. 2006; DeSantis et al. 2007). Manufactured by Affymetrix,

the high-density oligonucleotide microarray containing 500,000 probes is a reliable tool for comprehensive identification, detection, and quantification of all known prokaryotic 16S rRNA gene sequences (bacterial and archea). The PhyloChip targets the variation in the 16S rRNA genes of over 30,000 database sequences totaling almost 9,000 distinct taxonomic groups. Functional gene arrays are designed for key functional genes that code for proteins involved in various biogeochemical processes and may also provide information on the microbial populations controlling these processes. The most comprehensive FGA available is the GeoChip, which targets 10,000 genes involved in the nutrient cycling, metal reduction and resistance, and organic contaminant degradation (He et al. 2007).

Pyrosequencing has broken the barrier of sequence limitations in the study of bacterial diversity. The ability to generate megabases of sequences in a few hours allows intense exploration of species in any environmental sample (Edwards et al. 2006). Using these steps, an extremely low amount of reaction mixture is required, allowing upscaling of the process. The latest version of instruments can produce around 300,000 reads with approximately 200–400 bp in about 5 h using this process. The technique has been used to describe bacterial communities in different environments. The deep ocean biosphere was described by pyrosequencing of samples collected at different depths (Sogin et al. 2006), and of soil bacterial communities (Roesch et al. 2007) were similarly investigated. Both studies showed that the diversity of organisms was extremely high, and although 30,000 sequences were obtained, the complete description of species and sequences in both the environments remains to be completed. The authors showed that while the great majority of species were described, there remained the "rare biosphere tail" that could not be completely explored, even with the high amount of sequences. Although the microbial diversity associated with plants seems to be less diverse, particularly in respect of the endophytes, future applications of the technique have promise.

2.6.3 Reporter Genes and Plant–Microbe Analysis

One of the challenges in microbial ecology is trying to correlate the behavior of microorganisms in the laboratory with that in their natural environment. Many promising microorganisms have been isolated and marketed as biofertilizers; however, their effects on crop yield fluctuate from crop to crop, site to site, and from season to season, depending on the survival of the introduced microorganisms on seed, roots, and in the soil (Poi and Kabi 1979; Chanway et al. 1989; Nowak 1998; Khalid et al. 2004). To make effective utilization of microbial inoculants, accurate and reliable methods for monitoring the fate of introduced PGPR in the rhizosphere/rhizoplane are required to enhance their efficacy under field conditions. A variety of bacterial traits and specific genes contribute to the process of rhizosphere colonization, but only a few have been elucidated (Lugtenberg et al. 2001). These include motility, chemotaxis to seed and root exudates, production of pili or fimbriae,

production of specific cell surface components, ability to use specific components of root exudates, protein secretion, and quorum sensing. As the expression of genes is related to the cues present in the environment, it can be inferred that different plant species, growth conditions, interaction with other microbes, and soil properties will affect the genes related to rhizosphere colonization. This is particularly important in view of by the unique climatic conditions present in the colder Himalayan regions. The generation of mutants altered in expression of rhizosphere colonization traits can aid our understanding of the precise role of each gene in the colonization process (Persello-Cartieaux et al. 2003). Progress in the identification of new, previously uncharacterized genes can be made using nonbiased screening strategies that rely on gene fusion technologies. These strategies employ reporter transposons (Roberts et al. 1998) and in vitro expression technology (IVET; Rainey 1999) to detect genes expressed during colonization. Combining the gfp labeled strains with an rRNA-targeting probe can facilitate monitoring the metabolic activity of a rhizobacterial strain in the rhizosphere (Sorensen et al. 2001).

2.6.4 *Proteomics and Its Applications in the Analysis of Plant–Microbe Interactions*

The term "proteome" describes the "protein complement of the genome" and thus proteomics is best described as the large scale analysis of proteins. In the postgenomic era, annotated genes simply offer the list of genes without providing detailed insights into their expression and/or functional significance. Proteomics can be exploited as a powerful tool to understand the complex patterns of expression of genomes with respect to different environmental niches in which the bacteria adapt and survive. It also has the ability to complement genomics by characterizing their gene products and responses to a variety of changing biological and environmental factors.

Current methods of proteomic analysis commonly involve the solubilization of protein samples and their subsequent separation by 2D gel electrophoresis. Proteins are stained by Coomassie brilliant blue, and spots are excised and identified using mass spectrometry. To minimize the limitations associated with the process, gel free proteomic systems are also under development. Proteomic analysis has been used to identify proteins expressed in plant–microbe interactions such as those involved in nitrogen fixing symbiosis. Morris and Djordjevic (2006) used the proteome analysis to identify proteins that are involved in the early stages of nodulation between the subterranean clover cultivar Woogenellup and the *R. leguminosarum* bv. *trifolii* strains ANU843 (induces nitrogen-fixing nodules) and ANU794 (forms aberrant nodules that fail to develop beyond an early stage). Proteins found to be differentially expressed were involved in symbiosis and stress-related functions. The specific induction of alpha-fucosidase by ANU794 was found to be responsible for nodulation failure phenotype of strain ANU794.

Time-course analysis of root protein profiles was studied by two-dimensional gel electrophoresis and silver staining in the model plant *Medicago truncatula*, inoculated either with the arbuscular mycorrhizal fungus *Glomus mosseae* or with the nitrogen fixing bacterium *Sinorhizobium meliloti* (Bestel-Corre et al. 2002). Various proteins involved in the molecular events occurring in plant root symbioses were identified. Availability of various plant genomes have given major thrust in using proteomic in plant–microbe interactions. Peck et al. (2001) have used "directed proteomics" approach to identify proteins that are rapidly phosphorylated in response of *Arabidopsis* cells to microbial elicitors. A number of new pathogen and elicitor responsive proteins in the suspension cultured rice cells inoculated with blast fungus have been identified. Additional recent advances that allow parallel analysis of plant–microbe interactions using both transcriptional and proteins can also be taken up for the elucidation of plant bacterial interactions.

2.6.5 Metabolomics and Its Applications in the Analysis of Plant–Microbial Interactions

Metabolomics has been defined as the quantitative complement of all low molecular weight molecules present in the cells in a particular physiological or developmental state. Metabolomic studies have been shown to provide certain advantages over the other "omic-type" data mining technologies such as transcriptomics and proteomics. Indeed, examining metabolites or changes in the metabolome can play an important role in the integrative approach for accessing gene function and relationship to phenotypes. Metabolomics approach has been utilized in the analysis of plant–microbe interactions. Narasimhan et al. (2003) coined the term "rhizosphere metabolomics" for the study of secondary metabolites secreted by resident microbial populations. Using this approach they demonstrated the enhanced depletion of PCBs using root-associated microbes, which can use plant secondary metabolites such as phenylpropanoids. Metabolomic techniques have a huge potential to be applied for strategies aimed at improving competitive abilities of biofertilization and biocontrol strains.

2.7 Conclusion

Use of chemical fertilizers has received preference over biological fertilizers as they can be used irrespective of the environment, easy of storage and transport, and wide ranging applications. The use of biological fertilizers has gained attention mainly due to the (1) environment friendly nature of bioinoculants and (2) hazards associated with the chemical fertilizers. The practical use of biological fertilizers is well below its full potential, mainly due to nonavailability of suitable inoculants.

In view of the ecological specificity associated with the naturally occurring microorganisms, concerted efforts from various research laboratories are required for developing microbial inoculants for a specific set of climatic conditions, such as those prevailing in the mountain region of IHR. The chance of obtaining a successful inoculant in the end will be greatly enhanced when ecological principles are applied throughout the procedure of development of microbial inoculants. However, before PGPR can contribute the desired benefits, scientists need to learn more and explore ways and means for their better utilization in the farmers' fields. Future research should focus on managing plant–microbe interactions, particularly with respect to their mode of action and adaptability to conditions under extreme environments. Furthermore, certain issues need to be addressed, such as how to improve the efficacy of biofertilizers, what should be an ideal and universal delivery system, how to stabilize these microbes in the soil systems, and how to control/facilitate nutritional and root exudation aspects in order to get maximum benefits from PGPR application. Biotechnological and molecular approaches should help in the development of more understanding about the mode of PGPR action leading to successful plant–microbe interaction. Furthermore, proper guidelines for the production and commercialization of biofertilizers should be framed in order to popularize the use of such bioagents for maintaining the sustainability of agro-ecosystems across the globe, taking due care of required safety measures associated with the use of live cultures.

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Chapter 3
Potential Use of Soil Microbial Community in Agriculture

Noshin Ilyas and Asghari Bano

3.1 Introduction

One of the major challenges for the twenty-first century is sustainable crop production. Plant associated microorganisms fulfill important ecosystem functions for plants and soils (reviewed in Smith and Goodman 1999). This includes the effects of plant-associated microorganisms on plant health and growth; they enhance stress tolerance, provide disease resistance, aid nutrient availability, and uptake and promote biodiversity (Morrissey et al. 2004). Furthermore, plant-associated microbial communities show, due to specific secondary metabolism and morphology, a certain degree of specificity for each plant species (reviewed in Berg and Smalla 2009). This knowledge has yet to be exploited in agricultural biotechnology. However, over the past 150 years, research repeatedly demonstrated that bacteria and fungi have an intimate interaction with their host plants and are able to promote plant growth as well as to suppress plant pathogens (Whipps 2001; Thakore 2006; Ehlers 2006).

Low agricultural production efficiency is closely related to a poor coordination of energy conversion which, in turn, is influenced by crop physiological factors, the environment, and other biological factors, among which soil microorganisms play key role. The soil and rhizosphere microflora can accelerate the growth of plants and enhance their resistance to disease and harmful insects by producing bioactive

N. Ilyas
Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan

Department of Botany, PMAS-Arid Agriculture University, Rawalpindi, Pakistan

A. Bano (✉)
Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan
e-mail: banoasghari@gmail.com

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substances. These microorganisms maintain the growth environment of plants and may have secondary effects on crop quality.

3.2 Soil Microbial Community

Soil microbial communities mediate many biogeochemical processes that are central to ecosystem functioning, including carbon (C) mineralization to CO_2, nitrogen (N) cycling, and trace gas production and consumption. The potential for rapid microbial growth and the high degree of diversity and genetic exchange in microbial systems has led to a common assumption that microbial activity play important role in the processes involved in ecosystem like nutrient transfer and transformation (Balser et al. 2002). Soil microbes play key roles in ecosystems: they drive major biogeochemical processes and contribute to the maintenance of plant productivity and species richness on Earth. Soil microbes regulate plant productivity via a variety of mechanisms. Positive effects of microbes on plant productivity are most common in nutrient poor ecosystems where they enhance the supply of growth limiting nutrients such as N and P to plants. In such situations, up to 90% of P and N are provided by mycorrhizal fungi and N-fixing bacteria, pointing to their importance in regulating plant productivity. Negative effects of soil microbes on plant productivity can also occur when they act as pathogens, compete with plants for nutrients, or transform nutrients into forms that are inaccessible to plants (Heijden et al. 2008).

Emphasis has been laid upon seeking ways to quantitatively link microbial community composition and soil processes to understand the microbial diversity and ecosystem functioning (Waldrop and Firestone 2004).

All natural soils contain vast populations of microscopic plants and animals present in a state of dynamic equilibrium and changing balances. It has been estimated that within the top 1–3 ft of soil as much as 17,000 lbs. fungi and 40 lbs. bacteria exist per acre. All the soil microorganisms compete with each other for food and space. Any change in environmental conditions such as food supply, temperature, moisture, oxygen supply, etc., can result in alternations which cause one or many types of soil microbes to become temporarily dominant over the others (Muntean et al. 2004). Microbial communities are central in controlling how terrestrial ecosystems respond to global climate change. Plants generally respond to elevated CO_2 with increased root production and exudation (Korner 2000). The majority of fungi and bacteria present in soils are considered to be beneficial to higher plants by (a) direct association with roots (mycorrhizae, nodule forming bacteria); (b) breakdown and release of minerals from organic matter present in the soil resulting in essential element availability increases to higher plants; (c) parasitizing harmful or disease causing microorganisms; or (d) suppressing growth, reproduction, or activity of harmful disease causing microorganisms through other interactions such as chemical inhibition. Harmful fungi and bacteria that parasitize higher plants are generally present in all native soils as well.

3.3 Plant-Growth-Promoting Rhizobacteria

The plant-growth-promoting rhizobacteria (PGPR) may induce plant growth promotion by direct or indirect modes of action (Lazarovits and Nowak 1997). Direct mechanisms include the production of stimulatory bacterial volatiles and phytohormones, lowering of the ethylene level in plant, improvement of the plant nutrient status (liberation of phosphates and micronutrients from insoluble sources; nonsymbiotic nitrogen fixation), and stimulation of disease-resistance mechanisms (induced systemic resistance). Indirect effects originate for example when PGPR act like biocontrol agents reducing diseases, when they stimulate other beneficial symbioses, or when they protect the plant by degrading xenobiotics in inhibitory contaminated soils (Jacobsen 1997). Based on their activities, Somers et al. (2004) classified PGPR as biofertilizers (increasing the availability of nutrients to plant), phytostimulators (plant growth promoting, usually by the production of phytohormones), rhizoremediators (degrading organic pollutants), and biopesticides (controlling diseases), mainly by the production of antibiotics and antifungal metabolites.

3.3.1 Azoarcus

Azoarcus has recently gained attention due to its great genetic and metabolic diversity. It has been split into three different genera (*Azovibrio, Azospira,* and *Azonexus*) (Reinhold-Hurek and Hurek 2000). The most distinctive characteristic of these genera, which particularly differentiates them from other species, is their ability to grow in carboxylic acids or ethanol instead of sugars, with their optimum growth temperature ranging between 37°C and 42°C. *Azoarcus* is an endophyte of rice and is currently considered the model of nitrogen-fixing endophytes (Hurek and Reinhold-Hurek 2003).

3.3.2 Bacillus

Ninety-five percent of Gram-positive soil bacilli belong to the genus *Bacillus*. The remaining 5% are confirmed to be *Arthrobacter* and *Frankia* (Garbeva et al. 2003). Members of *Bacillus* species are able to form endospores and hence survive under adverse conditions; some species are diazotrophs such as *Bacillus subtilis* (Timmusk et al. 1999), whereas others have different PGPR capacities, as many reports on their growth promoting activity reveal (Kokalis-Burelle et al. 2002)

3.3.3 *Pseudomonas*

Among Gram-negative soil bacteria, *Pseudomonas* is the most abundant genus in the rhizosphere, and the PGPR activity of some of these species has been known for many years, resulting in a broad knowledge of the mechanisms involved (Patten and Glick 2002). The ecological diversity of this genus is enormous, since individual species have been isolated from a number of plant species rhizosphere in different soils throughout the world. *Pseudomonas* species show high versatility in their metabolic capacity. Antibiotics, siderophores and volatiles such as hydrogen cyanide are among the metabolites generally released by Pseudomonads (Charest et al. 2005). These metabolites strongly affect the environment, both because they inhibit growth of other deleterious microorganisms and increase nutrient availability particularly P for the plant.

3.3.4 *Rhizobia*

Strains from this genus may behave as PGPR when they colonize roots of nonlegume plant species in a nonspecific relationship. It is well known that a number of individual species may release plant growth regulators, siderophores and hydrogen cyanide or may increase phosphate availability, thereby improving plant nutrition (Antoun et al. 1998). An increase in rhizosphere populations has been reported after crop rotation with nonlegumes (Yanni et al. 1997), with this abundance benefiting subsequent crops (Lupwayi et al. 2004). The best known and most exploited symbiotic N_2-fixing bacteria belonging to family Rhizobia ceae include the genera such as *Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium, and Azorhizobium*. The N_2-fixing capability of Rhizobia varies greatly depending on the host plant species. Therefore selection of best strains must take Rhizobia host compatibility into consideration for the production of suitable Biofertilizers (Rajendra et al. 2005). Legumes (such as beans, soybean, and chickpea) inoculation is an old practice that has been carried out especially when local/resident rhizobial population in the soil are low.

3.3.5 *Frankia*

Frankia is the genus of N_2-fixing actinomycetes capable of fixing N_2 similar to rhizobial symbiosis. All the host of this type of symbiosis can habitat the marginal land and are implicated in reclamation of saline soil, deserted land. They are the good contributor of nitrogen in forests.

3.3.6 Cyanobacteria

Cyanobacteria are ecologically important in N_2-fixing organisms especially in rice cultivation. *Anabaena azollae* is a symbiotic heterocystous nitrogen-fixing bacteria. Cyanaobacteria which lies in fronds in the pores of the *Azolla* (Balachandar et al. 2005). Contribution of the cyanobacteria to total N uptake by rice seedlings was assessed using free-living and immobilized (Balachandar et al. 2005).

Exploitation of *Azolla* as a biofertilizer is a practical possibility in flooded soil condition. It is used as both green manure before planting and intercropped with rice after planting. The latter practice has wide adaptability and is more economical as Azolla decomposes within 2 weeks releasing about 67% of its N. The increase in grain yield due to *Azolla* green manuring is reported to 0.5–2 tonnes per hectare. Thereby, cyanobacteria should be seriously considered as biofertilizer with a great potential (especially immobilized) resulting in higher heterocyst frequency growth, nitrogenase activity, and increased ammonia excretion. They also have increased availability of micronutrients such as iron and manganese in the soil (Balachandar et al. 2005; Mishra et al. 2005; Rajendra et al. 2005).

3.3.7 Azospirillum

Azospirillum genus includes spirally curved bacteria which not only lives in rhizosphere of grasses but can also enter root cortex. It is an associative microaerophilic nitrogen fixer which not only colonizes root mass and fixes N_2 in close association with plant in an environment of low O_2 tension (Deshmukh 1998).

The positive aspect of this inoculant is to produce PGP substances in addition to fixing the nitrogen and has ability to differentiate into cyst under stress that enables its persistence for a long time in field condition. *Azospirillum* bears great promise as a growth-promoting nitrogen-fixing biofertilizer. It has been recorded that *Azospirillum* inoculation may be used as biofertilizer for wheat and rice thereby reducing use of urea N by approximately 20% (Mishra et al. 2005; Rajendra et al. 2005). Along with its growth promoting properties its commercial production is inexpensive as inoculum produced can be applied as peat formulation which can be directly utilized in field research and agricultural applications.

3.3.8 Acetobacter

Acetobacter is nonsymbiotic (microsymbiont) bacteria which is mostly associated with sugarcane crop. It grows inside the root as well as stem to some extent and fixes atmospheric nitrogen and benefits the crop. It is rather difficult to isolate the organism and grow artificially on a large scale (Guller et al. 2005;

Deshmukh 1998). The potential of it as a biofertilizer will depends on finding suitable techniques to grow it quickly in laboratory. If this is done successfully we may save huge quantity of chemical nitrogenous fertilizers.

3.3.9 *Klebsiella*

Klebsiella fix N_2 under anaerobic condition.These organisms have flexibility to grow under both aerobic and anaerobic environment. In the laboratory, *Klebsiella* spp. has shown to reproduce nitrogen under microaerophilic condition (Rajendra et al. 2005).

3.3.10 *Azotobacter*

Azotobacter is a group of free-living nitrogen-fixing bacteria. Sufficient research work has been carried out on the role of *Azotobacter* spp. in sugarcane cultivation.

The result in general has indicated that application of *Azotobacter* at the rate of 5 kg per hectare helps in reducing nitrogen dose by 50 kg per hectare with increase in yield of crop by 5–10%. The mechanism by which plants inoculated with *Azotobacter* derive its benefits such as increased biomass, nitrogen uptake is attributed to increase in nitrogen input by BNF development and branching of roots, production of plant growth hormones, vitamins, and enhancement of uptake of nitrate, ammonium orthophosphate, potassium, and iron improved water status of the plant and secretion of antifungal compounds (Guller et al. 2005; Rajendra et al. 2005)

3.3.11 *Mycorrhizal Fungi*

Mycorrhizal fungi form mutualistic symbiosis with a wide variety of plants roots. Three general types of Mycorrhizae are known such as Ectomycorrhizae, Endomycorrhizae and Endoectomycorrhizae (Dubey and Maheshwari 2010).

3.3.12 *Vesicular Arbuscular Mycorrhiza (or AM Fungi)*

They are obligate symbionts that occur in 90% of vascular plants. It improves plant growth and enhances the uptake of more nutrients generally unavailable to host plant especially “P” from soil. It also increases the water uptake or alters physiology

to reduce stress response to soil drought. It can also withstand high temperature, increase heavy metal tolerance of plants, as well as make the plant less susceptible to root pathogen.

3.4 Plant–Microbe Interaction

All plant associated microenvironments, especially the rhizosphere, are colonized in high abundances by microbes (Berg et al. 2005). When testing microbial isolates from plant associated habitats, between 1% and 35% showed antagonistic capacity to inhibit the growth of pathogens (Berg et al. 2002, 2006). The proportion of isolates which express PGP traits is much higher in general and was found up to two-thirds of the cultivable population (Cattelan et al. 1999; Fürnkranz et al. 2009).

Plant growth promotion can be achieved by the direct interaction between beneficial microbes and their host plant and indirectly due to their antagonistic activity against plant pathogens (Fig. 3.1).

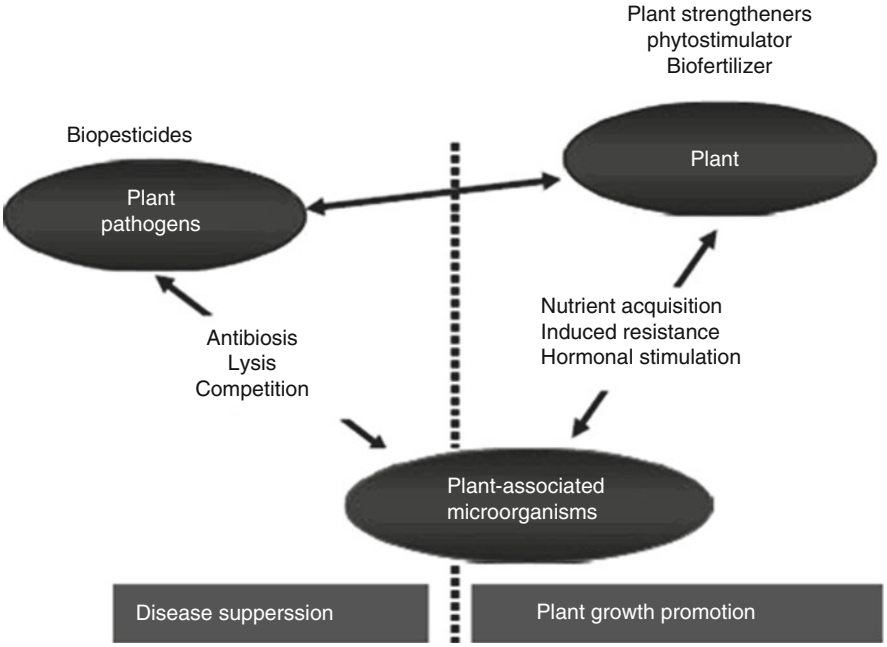

Fig. 3.1 Plant–microbe interactions promoting plant growth and health: mode of action and potential use in biotechnological applications (Berg and Smalla 2009)

3.5 PGPR Using Indirect Mechanisms

The list of indirect mechanisms used by PGPR is substantial (Maheshwari 2010). Some have been included here, with the most relevant being discussed in detail:

3.5.1 Free Nitrogen-Fixing PGPR

Biological nitrogen fixation is a high-cost process in terms of energy. Bacterial strains capable of performing this process do so in order to fulfill their physiological needs and thus little nitrogen is left for the plant's use. However, growth promotion caused by nitrogen-fixing PGPR was attributed to nitrogen fixation for many years, until the use of nitrogen isotopes showed additional effects. This technique showed that the benefits of free nitrogen-fixing bacteria are more due to the production of plant growth regulators than to the nitrogen fixation (Bedmar et al. 2005).

3.5.2 Plant Growth Regulations

Azotobacteraceae is the most representative of bacterial genera able to perform free nitrogen fixation. Various reports describe the benefits of *Azotobacteraceae* on several crops (Derylo and Skorupska 1993). According to data provided by the FAO (1995), amounts of nitrogen supplied to soil are low; Bhattacharya and Chaudhuri (1995) reported that the amount ranges between 20 and 30 kg per hectare per year. *Azotobacter* is the genus widely used in agricultural trials. As previously suggested, the effect of *Azotobacter* and *Azospirillum* is attributed not only to the amounts of fixed nitrogen but also to the production of plant growth regulators (indole acetic acid, gibberellic acid, cytokinins, and vitamins), resulting in additional positive effects to the plant (Rodelas et al. 1998). Application of inoculants in agriculture has resulted in notable increases in crop yields, especially in cereals, where *Azotobacter chroococcum* and *Azospirillum brasilense* have been proved quite effective. These two genera include strains capable of releasing substances such as vitamins and plant growth regulators, which have a direct influence on plant growth (Rodelas et al. 1998). According to González and Lluch (1992), the production of these substances by *Azotobacter* strains is seriously affected by nitrogen availability, which affects auxin and gibberellin production; but when nitrate is available, auxin release is impaired while gibberellin synthesis is enhanced. As mentioned above, the amount of nitrogen from free fixation available to the plant is low because it is used efficiently by the bacteria. Three strategies have been proposed to address this low-yield problem (1) glutamine synthase bacterial

mutants, (2) formation of paranodules, and (3) facilitating the penetration of plant tissues by nitrogen-fixing bacterial endophytes that enhance colonization in a low competition niche.

3.5.3 Siderophore-Producing PGPR

Iron is an essential nutrient for plants. Iron deficiency is exhibited in severe metabolic alterations because of its role as a cofactor in a number of enzymes essential to important physiological processes such as respiration, photosynthesis, and nitrogen fixation. Iron is quite abundant in soils but is frequently unavailable for plants or soil microorganisms since the predominant chemical species is Fe^{3+}, the oxidized form that reacts to form insoluble oxides and hydroxides inaccessible to plants or microorganisms. Plants have developed two strategies for efficient iron absorption. The first consists of releasing organic compounds capable of chelating iron, thus rendering it soluble. Iron diffuses toward the plant where it is reduced and absorbed by means of an enzymatic system present in the cell membrane. The second strategy consists of absorbing the complex formed by the organic compound and Fe^{3+}, where the iron is reduced inside the plant and readily absorbed. Some rhizosphere bacteria are able to release iron-chelating molecules to the rhizosphere and hence serve the similar function to that of plants. Siderophores are low molecular weight compounds, usually below 1 kDa, which contain functional groups capable of binding iron in a reversible way. The most frequent functional groups are hydroximates and catechols, in which the distances among the groups involved are optimal to bind iron. Siderophore concentration in soil is approximately around 10^{-30} M. Siderophore-producing bacteria usually belong to the genus *Pseudomonas*, the most common being *Pseudomonas fluorescens*, which release pyochelin and pyoverdine. Rhizosphere bacteria release these compounds to increase their competitive potential, since these substances have an antibiotic activity and improve iron nutrition for the plant (Glick 1995; Pandey et al. 2005). Siderophore-producing rhizobacteria improve plant health at various levels: they improve iron nutrition, inhibit growth of other microorganisms with release of their antibiotic molecule and hinder the growth of pathogens by limiting the iron available for the pathogen, generally fungi, which are unable to absorb the iron–siderophore complex (Arora et al. 2001; Gupta et al. 2001)

3.5.4 Phosphate-Solubilizing PGPR

After nitrogen, phosphorous is the most limiting nutrient for plants. However, phosphorous reserves, although abundant, are not available in forms suitable for plants. Plants are only able to absorb the soluble forms, that is, mono- and dibasic phosphate. Besides inorganic forms of phosphorous in soil, the phosphorous present

in organic matter is of considerable importance. The organic forms of phosphorous are estimated to comprise between 30 and 50% of total soil phosphorous. This reservoir can be mineralized by microorganisms, making it available to the plant as soluble phosphates. Many bacteria from different genera are capable of solubilizing phosphate and include *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aerobacter*, *Flavobacterium*, *Chryseobacterium,* and *Erwinia.*

Bacteria use two mechanisms to solubilize phosphate (1) releasing organic acids that mobilize enzyme phosphorous by means of ionic interactions with the cations of the phosphate salt and (2) by releasing phosphatases responsible for releasing phosphate groups bound to organic matter. Most of these bacteria are able to solubilize the Ca–P complex, and there are others that operate on the Fe–P, Mn–P, and Al–P complexes. Generally, these mechanisms are more efficient in basic soils. Results with PGPR capable of solubilizing phosphate are sometimes erratic, probably due to soil composition, and to demonstrate their effect they have to be inoculated in soils with a phosphorous deficit and stored in insoluble forms. Inoculations of these types of PGPR sometimes improve plant growth, and sometimes they are completely inefficient. Without doubt, knowledge of their mechanisms and ecology in the rhizosphere will improve their use in sustainable agriculture (Gyaneshwar et al. 2002).

3.5.5 Microbial Signals in Plant Growth and Development

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. Additional signals from microbes have been found to play a role in plant morphogenetic processes, including the *N*-acyl-L-homoserine lactones (AHLs) and volatile organic compounds (VOCs). AHLs belong to a class of bacterial quorum sensing signals from Gram-negative bacteria such as *Pseudomonas*. These compounds enable bacterial cells to regulate gene expression depending on population density. Very recently, it was found that AHLs can be recognized by plants, alter gene expression in roots and shoots, and modulate defense and cell growth responses. In another study, it was found that bacterial volatiles such as acetoin and 2, 3-butanediol, produced by certain PGPR, can be used for plant–bacteria communication and also trigger plant growth promotion triggers (Ortíz-Castro et al. 2009).

3.6 Remediation of Heavy Metals by PGPR

The heavy metal contamination has great effects on the microbial communities in soils in several ways (1) it may lead to a reduction of total microbial biomass (Fliessbach et al. 1994); (2) it decreases numbers of specific populations

(Chaudri et al. 1993); or (3) it makes shifts in the microbial community structure (Gray and Smith 2005). Sandaa et al. (1999) suggested that the presence of even small amounts of heavy metals caused a substantial reduction in the total bacterial diversity.

Due to the sensitivity and the sequestration ability of the microbial communities to heavy metals, microbes have been used for bioremediation (Umrania 2006). Although, microbial communities in metal-polluted bulk soils have been studied, there is little information published on the composition of microbial community in the plant rhizosphere growing in soils highly polluted with heavy metals (Dell'Amico et al. 2005). The rhizosphere, with high concentration of nutrients exuded from the roots, attracts more bacteria than in the bulk soils (Penrose and Glick 2001).

3.7 Microbial Community as Biopesticides

Development of pathogen resistance, increasing costs of pesticides and consumer demand for pesticide-free products have increased the need for alternatives for chemicals in agriculture (Compant et al. 2005) of which biological control is an option. The U.S. environmental protection agency (EPA) defines biopesticides as "certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals." Microbial-based products sold for plant disease control must be registered as biopesticides by EPA. The EPA recognizes three major classes of biopesticides: microbial biopesticides, plant incorporated protectants, and biochemical pesticides. In 2005, it was reported that microbial biopesticides for the control of plant diseases represented 1% of the total agricultural chemical sales (Fravel 2005). Currently, a total of 83 microorganisms are registered as active ingredients with EPA for the control of several diseases and pests. Moreover, novel organisms with biocontrol potential are still being identified (Benítez et al. 2004) with a potential to become a formulation which growers can use. The most economically successful microbial active ingredient of biopesticides is *Bacillus thuringiensis* as plant incorporated protectant for the insect pest control.

Revelations about the mechanisms of PGPB action open new doors to design strategies for improving the efficacy of biocontrol agents. Identification of key antimicrobials produced by superior agents, such as 2, 4-diacetylphloroglucinol, can be exploited for streamlining strain discovery by targeting selection of new isolates that carry relevant biosynthetic genes. Determination of the role of edaphic parameters favorable for disease suppression, particularly those that stimulate antibiotic production and activity, can be exploited by targeting inoculants for soils that are more likely to support biocontrol. Along this same line, biotechnology can be applied to further improve strains that have prized qualities (e.g., formulation ease, stability, or otherwise exceptionally suited to plant colonization) by creating transgenic strains that combine multiple mechanisms of action. For example, transforming the 1-aminocyclopropane-1-carboxylic acid deaminase gene,

which directly stimulates plant growth by cleaving the immediate precursor of plant ethylene into *P. fluorescens* CHAO, not only increases plant growth but can also increase biocontrol properties of PGPB (Compant et al. 2005)

3.8 Biocontrol Mechanisms

The mechanisms that lead to biological control include antibiosis, nutrient or niche competition, induction of systemic resistance, and predation or parasitism. However, the importance of each mechanism is determined by the physical and chemical state of the rhizosphere or phyllosphere (Andrews 1992).

3.8.1 Antibiosis

Some microorganisms are able to produce a broad collection of antibiotics and some antibiotics are produced by several bacteria. For example, pyrrolnytrin is produced by *Burkholderia* and *Pseudomonas* species (Raaijmakers et al. 2002), and has shown activity over *Rhizoctonia solani, Botrytis cinerea, Verticillium dahliae,* and *Sclerotinia sclerotiorum* (Ligon et al. 2000). The contribution of antibiotics to biological control of plant diseases can be confirmed by genetic studies in which the genes involved in the biosynthesis can be mutated and restored (Keel et al. 1992) and/or by assessment of in situ production of the antibiotics (Thomashow et al. 2002).

3.8.2 Competition

Perhaps nutrient competition happens in the majority of interactions between microorganisms, which may be responsible to certain extent to all biocontrol agents. An example of nutrient competition is the production of siderophores by certain bacteria. Siderophores are 1,500-Da molecules that sequester iron (III) from the rhizosphere, and once the iron is sequestered the siderophore is used exclusively by the microbe that produced it and by certain plants (Datnoff et al. 2007). Because this iron supply can only be used by the microbe that produced it, it limits the availability to other microbes and therefore, pathogen growth is suppressed (Kloepper et al. 1980). Among the bacteria capable of producing siderophores are *Streptomyces* spp., *Erwinia herbicola, E. amylovora, E. carotovora, Sinorhizobium meliloti, R. leguminosarum, A. tumefaciens, E. chrysanthemi, Pseudomonas spp.,* and *B. japonicum* (Datnoff et al. 2007).

3.8.3 Induced Systemic Resistance

Induced systemic resistance (ISR) is the type of plant-mediated systemic resistance that is stimulated by rhizobacteria (Van Loon et al. 1998). The bacterial factors involved in ISR induction comprise antibiotics, flagella, lipopolysaccharides, siderophores, and salicylic acid (Bakker et al. 2003). Biocontrol mediated by ISR is assessed by evaluating reduction on pathogen growth and disease development when the pathogen and biocontrol organism are not in contact (Siddiqui and Shaukat 2003). Among the bacteria that promote ISR are *P. putida, Serratia marescens, Serratia plymuthica,* and *P. fluorescens* over fungal, bacterial, and viral pathogens (Van Loon et al. 1998).

3.8.4 Parasitism

Parasitism is an association in which one organism receives benefits from the other while the other is harmed (reviewed by Pal and Gardener 2006). *Pasteuria penetrans* is an obligate parasite on nematodes, therefore, reducing nematode's reproduction and juvenile infections on root (reviewed by Siddiqui and Mahmood 1999). Actinomycetes parasitize fungal and oomycete spores and/or hyphae; however, this mechanism is known to be strongly related to production of lytic enzymes (i.e., chitinases, cellulases, amylases, and β-1, 3 glucanases) (El-Tarabily and Sivasithamparam 2006).

3.8.5 Biofilm

Among the diverse soil microflora, PGPR mark an important role in enhancing plant growth through a range of beneficial effects. This is often achieved by forming biofilms in the rhizosphere, which has advantages over planktonic mode of bacterial existence. Beneficial PGPR play a key role in agricultural approaches through quorum sensing in their biofilm mode. The in vitro production of biofilmed PGPR can be used to give increased crop yields through a range of plant growth mechanisms. They can be used as biofertilizers through improved N_2 fixation and micro- and macronutrient uptake. Further, higher levels of plant growth with PGPR have been observed due to their production of plant growth regulators and their abilities to act as biocontrol agents, which are carried out by the production of antibiotics and other antimicrobial compounds. The microbial inoculant industry would also benefit greatly by developing biofilmed PGPR with N_2-fixing microbes. Biofilmed PGPR can be manipulated to achieve results in novel agricultural endeavors and hence is as an area which needs a deeper probing into its potential (Seneviratne et al. 2011).

3.9 Abiotic Stress Tolerance

Rhizosphere bacteria help plants tolerate abiotic stress. Drought stress limits the growth and productivity of crops, particularly in arid and semiarid areas. Co-inoculation of lettuce (*Lactuca sativa* L.) with PGPR *Pseudomonas mendocina* and arbuscular mycorrhizal fungi (*Glomus intraradices* or *G. mosseae*) augmented an antioxidant catalase under severe drought conditions, suggesting that they can be used in inoculants to alleviate the oxidative damage elicited by drought (Fig. 3.2). Investigations into how drought stress affects plant hormone balance revealed an increase in abscisic acid (ABA) content in the leaves, indicating that the reduction of endogenous cytokinin levels magnifies ABA content, eliciting stomata closure. The cytokinin–ABA antagonism might be the result of metabolitic interactions because they share a common biosynthetic origin. Inoculation of wheat with isolates from water-stressed plants induced tolerance to water stress in inoculated plants. *Azospirillum* spp. isolated from moisture stressed conditions improved tolerance to water stress, and thus they can be inoculated to promote plant growth on stressed sites, such as semiarid and arid regions. The isolates from water stressed condition had less production of IAA, GA, and t-zr than isolates from water-stressed/arid field area but have higher ABA/t-zr ratio and ABA is the phytohormone which gives tolerance to plant under water stress, and thus they can be used as biofertilizer (Ilyas and Bano 2010). Soil salinity in arid regions is frequently an

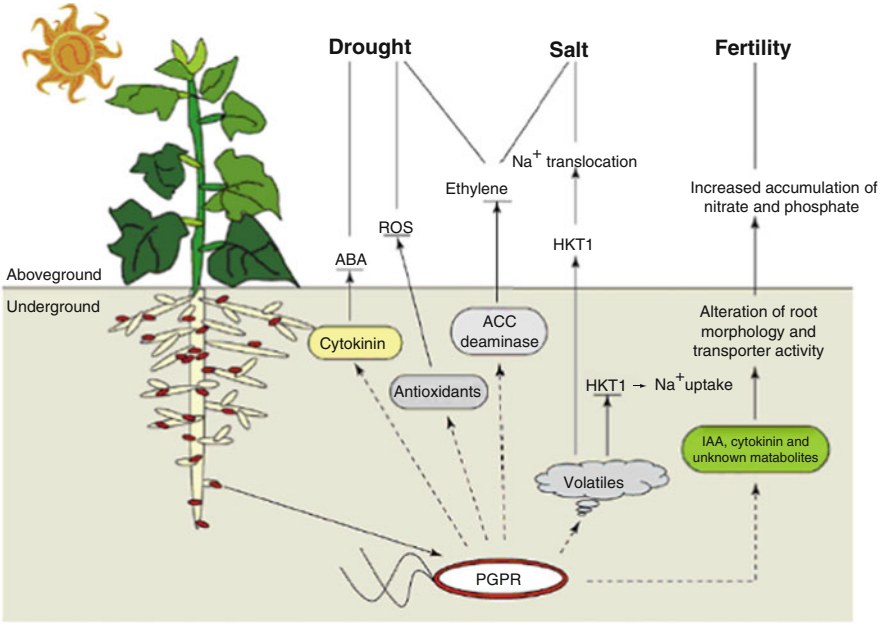

Fig. 3.2 IST elicited by PGPR against drought, salt, and fertility stresses underground (root) and aboveground (shoot) (Yang et al. 2009)

important limiting factor for cultivating agricultural crops. Although, many technologies have been implicated in the improvement of salt tolerance, only PGPR-elicited plant tolerance against salt stress has been previously studied. PGPR-elicited IST can aid the growth of crops in environmentally unfavorable conditions. In a study carried by Naz et al. (2009), it was shown that strains isolated from Khewra salt range of Pakistan exhibited their tolerance when tested on saline media simulated by rhizosphere soil filtrate. Noteworthy, the isolates produced ABA in a concentration much higher than that of previous reports. Furthermore production of proline, shoot/root length, and dry weight was also higher in soybean plants inoculated with these isolates under induced salt stress. Similarly, isolated *Pseudomonas* sp. particularly *Pseudomonas stutzeri* Khsr3 responded well as bio-inoculant on *Zea mays* under NaCl stress and are good phosphate solubilizer too. This is proved best alternative to vegetate saline fields by producing growth-promoting substance and will help the plant in the uptake of phosphate meant for better growth of plants and allow a better exploitation of natural soil resources (Naz and Bano 2009).These results suggested the tolerance of PGPR to salt stress. More investigations into the mechanisms by which PGPR elicit tolerance to specific stress factors should improve the use of IST in agriculture by enabling the optimization of microbial mixtures for the production of specific bacterial determinants (e.g., cytokinin, antioxidants, ACC deaminase, VOCs, and IAA) (Yang et al. 2009) Microcapsules of rhizobacteria consists of a cross-linked polymer deposited around a liquid phase, where bacteria are dispersed and microparticles based on the distribution of particle size, morphology, and bacterial load (Nakkeeran et al. 2005).

Plant-growth-promoting rhizobacteria (PGPR) are associated with plant roots and augment plant productivity and immunity; however, recent work by several groups shows that PGPR also elicit so-called induced systemic tolerance to salt and drought. Some PGPR strains produce cytokinin and antioxidants such as catalase, which result in ABA accumulation and ROS degradation, respectively. Degradation of the ethylene precursor ACC by bacterial ACC deaminase releases plant stress and rescues normal plant growth under drought and salt stresses. The volatiles emitted by PGPR downregulate hkt1 expression in roots but upregulate it in shoot tissues, orchestrating lower Na^+ levels and recirculation of Na^+ in the whole plant under high salt conditions. Production by PGPR of IAA or unknown determinants can increase root length, root surface area, and the number of root tips, leading to enhanced uptake of nitrate and phosphorous. PGPR might also increase nutrient uptake from soils, thus reducing the need for fertilizers and preventing the accumulation of nitrates and phosphates in agricultural soils. A reduction in fertilizer use would lessen the effects of water contamination from fertilizer runoff and lead to savings for farmers.

3.10 Characteristics of a Successful PGPR for Formulation Development

To develop a successful PGPR formulation, rhizobacteria should possess:

(a) High rhizosphere competence
(b) High competitive saprophytic ability
(c) Enhanced plant growth
(d) Ease for mass multiplication
(e) Broad spectrum of action
(f) Excellent and reliable control
(g) Safe to environment
(h) Compatible with other rhizobacteria
(i) Should tolerate desiccation, heat, oxidizing agents, and UV radiations (Jeyarajan and Nakkeeran 2000)

3.11 Conclusion

The effects of PGPR need to be fully exploited with respect to phytohormone production and induction of ISR. The commercial application of these microbes is imperative for economic uplift of the country to cut down the cost of fertilizers and biopesticides. Microbial production and stability of stress hormone ABA need to be looked in more detail for the tolerance of crop plants exposed to abiotic stresses.

Azoarcus capable of growing at high temperature and with less C requirement may be a good candidate for improving plant growth in marginal lands.

The Frankia–actinorhizal symbiosis also needs to be investigated for wider application in agriculture economy of the country. The quantification of antibiotics produced by certain PGPR need to be explored further to combat the diseases increasing with the changing climate scenario.

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Chapter 4
Impact of Application of Biofertilizers on Soil Structure and Resident Microbial Community Structure and Function

Shilpi Sharma, Rashi Gupta, Gaurav Dugar, and Ashok K. Srivastava

4.1 Biofertilizers

Microorganisms can exert beneficial effects on plants directly by enhancing crop nutrition or indirectly by reducing damages caused to plants by pathogens, pests, and frost. They can be used in the form of bioinoculants in agriculture as amendments to promote plant health, in induction of plant growth by stimulating plant hormone production (Bashan and Holguin 1997), as well as to initiate systemic acquired resistance of crop species to several common pathogens.

Broadly, microorganisms in the form of bioinoculants include biofertilizers, biocontrol agents, and organic decomposers. Biofertilizers are living cells of beneficial microbial isolates that provide necessary nutrients (nitrogen, phosphorous, etc.), excrete growth-promoting compounds, and provide resistance to a variety of diseases that culminates to enhanced yield and production. This category of microorganisms includes symbiotic or free-living dinitrogen fixing bacteria, mycorrhizal fungi, phosphate solubilizers, and bacteria stimulating root development through the production of compounds such as phytohormones, thereby improving mineral nutrients and water uptake. Biocontrol agents are living microbial isolates or their metabolic products that protect the plant against a variety of diseases. The third component of bioinoculants are the organic decomposers that include certain fungal species, bacterial genera, and actinomycetes that hasten the decomposition of organic compounds and make available nutrients held as organic matter.

S. Sharma (✉) • R. Gupta • G. Dugar • A.K. Srivastava
Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110016, India
e-mail: shilpi@dbeb.iitd.ac.in

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4.2 Impact of Bioinoculants on Ecosystem Processes

Despite large scale application of biofertilizers in agriculture since last few decades, information on their ecology and colonization behavior is poorly understood. Additionally, the way in which they interact with plant and resident community and the mechanisms involved in real/field applications is still a matter of curiosity among scientists. One of the major factors determining the efficacy of the biofertilizer in natural system is the indigenous microflora present in rhizosphere. This highly competitive community, with diverse species, may affect the survival and plant growth-promoting (PGP) properties of the biofertilizer (Van Veen et al. 1997). Bacterization of seeds and seedlings, or soil amendments may lead to changes in the structure of the indigenous microbial community, which is important with regard to the safety of introduction of bacteria into environment (Kozdroj et al. 2004). The alterations in soil microbial structure and function, as an effect of application of microbial biofertilizer, could either be through direct or indirect means, not only on target organisms but also on nontarget populations.

Before the release of biofertilizers in the environment, assessing their nontarget effects on the population of resident microorganisms is crucial to estimate the impact they would have on rhizosphere functioning and thereby on the ecosystem. Nontarget effects can be defined as effects of the introduced biofertilizer on organisms other than the target organisms or on biogeochemical cycles. Loss of native species from the rhizosphere is undesirable as there is a possibility of disruption of microbial processes that are essential to general soil ecosystems functioning. Likewise, immediate effects or cumulative long-term effects will cause environmental concern. The duration of effects is an important parameter in environmental risk analysis, as generally large temporary fluctuations can be accepted (Winding et al. 2004). Also, the loss of certain bacterial species may not lead to changes in functioning because of the phenomenon of bacterial redundancy wherein other groups of bacteria may perform similar functions in soil (Roesti et al. 2006). Therefore, in depth examination of effect of biofertilizer is needed before modifying agricultural practices.

This chapter is an attempt to critically evaluate the nontarget effects of biofertilizers reported till date. Effects observed both on the soil structure and microbial communities have been incorporated as the former would translate to an effect on the latter as well.

4.3 Effect on Soil Structure

Investigators have analyzed the impact of biofertilizers on the soil fertility. Direct contribution of N and P to the soil pool by N fixing and P solubilizing biofertilizers, respectively, seems to be the major factor contributing to betterment of nutrient status in soil. This direct increase of nutrients in soil results in enhanced uptake of the same by plants and their subsequent growth promotion. Also, enhancement of

soil organic matter content, as a result of application of biofertilizers, has contributed to the betterment of soil status.

When studying the effect of a combination of *Azospirillum brazilense* or *Rhizobium* species, *Bacillus megaterium*, and *Glomus fasciculatum* on microbial biomass, C, N, and P under sorghum and chickpea, Saini et al. (2004) observed an increase in the soil nutrient status as compared to the control soil. The soil was a Typic Ustochrepts, clay loam in texture with pH 8.4, microbial biomass C, N, P (mg g^{-1}), 9.60, 5.37, and 2.25, respectively, and *Azospirillum* population of (cfu g^{-1}) 24.0. The increase on application of biofertilizers was maximum for 50% recommended fertilizer along with biofertilizer. This was in strong correlation with N and P uptake of plants thereby pointing to the beneficial role of microorganisms in the uptake of N and P by both the crops studied. Comparison of the same at three different durations revealed that biomass C, N, and P was greater after 30 days than 60 days and at the time of harvest. With crop aging, there was corresponding decline in biomass C, N, and P. An additive effect of the microbial inoculants viz. *Azospirillum* in sorghum; *Rhizobium* in chickpea; and *Bacillus* and *Glomus* for both the crops was observed over the use of single or double inoculation with performance of multi-inoculation being the best. The increase in yield due to inoculation could be attributed to factors such as nitrogen contribution either by the inoculated strain or carry-over effect from previous crops; the positive effect of inoculation in combination with organic matter, i.e., farm yard manure; the interactive effect of two or more organisms and increased uptake of phosphorus due to the effect of phosphate solubilizing bacteria; or better uptake under vesicular-arbuscular mycorrhiza (VAM) treated plots (Saini et al. 2004). Recently, Kumar et al. (2009) reported growth enhancement of sesame with application of rhizospheric microorganism amended with reduced dose of chemical fertilizers. A long-term effect of *Pseudomonas aeruginosa* GRC_1 on yield of subsequent crops of paddy after mustard seed bacterization has also been reported (Deshwal et al. 2006).

Wu et al. (2005) evaluated the effect of four biofertilizers both bacteria and fungi viz. *Glomus mosseae* or *Glomus intraradices* with or without *Azotobacter chroococcum*, *Bacillus megaterium*, and *Bacillus mucilaginous* on soil properties and growth of *Zea mays*. The basic properties of the soil were as follows: pH 5.46, organic matter content 1.08%, total N 0.062%, total K 7,408 mg kg^{-1}, total P 1,090 mg kg^{-1}, available P ($NaHCO_3$-extractable) 2.78 mg kg^{-1}, and water-soluble K 13.43 mg kg^{-1}. Comparison of application of two types of biofertilizers was made with control soil, soil treated with chemical fertilizer and also with organic fertilizer. Soil properties like organic matter content and total N in soil were improved significantly by the microbial inoculants. The AM fungus, *Glomus mosseae*, had a higher root infection rate in the presence of bacterial inoculation. However, high infection of the AM fungus was observed to strongly inhibit P solublizer *B. megaterium* and K solubilizers *B. muculeginous*. A combination of bacterial fertilizer and AM fungi increased the organic matter content by 75% as compared to uninoculated soil. A significant correlation between organic matter content and plant dry biomass was observed suggesting organic matter content in the rhizosphere was mainly influenced by plant growth, especially root exudates,

through root metabolism and physiological activities. This greenhouse study also indicated that reducing the recommended amount of biofertilizer application by half had similar effects when compared with organic fertilizer or chemical fertilizer treatments. Nutrient deficiency in soil resulted in a larger population of N fixing bacteria and higher colonization of AM fungus (Wu et al. 2005).

In a study performed to evaluate the PGP effects of two *Bacillus* strains OSU-142 (N_2-fixing) and M3 (N_2-fixing and phosphate solubilizing), applied either individually or in combinations on organically grown primocane fruiting raspberry plants, showed that total N, available P, K, Ca, Mg, Fe, Mn, Zn contents, and pH of soil were significantly affected as a result of bacterial inoculations. Available P contents in soil was observed to be increased in all the treatments reaching a maximum of 5.36 kg P_2O_5 Da^{-1} by M3 and of 4.71 kg P_2O_5 Da^{-1} by OSU-142 + M3 treatments (1.55 kg P_2O_5 Da^{-1} at the beginning of the study). Soil pH reduced from 6.7 to 6.0 by OSU-142, to 5.6 by M3, and to 5.7 by OSU-142 + M3 applications. The lowering of soil pH mainly occurs due to production of organic acids and enzyme phosphatases. Applications of plant-growth-promoting rhizobacterial (PGPR) strains also had significant effects on K, Ca, Mg, Fe, Mn, and Zn contents of the soil earlier. Decrease in pH and increased mineralization of organic complex were believed to be the two parameters contributing to increased availability of mineral nutrients in soil (Orhan et al. 2006).

In a pot experiment, Nisha et al. (2007) observed that biofertilizer consisting of three indigenous cyanobacterial isolates when applied to an organically poor (0.35% organic carbon and 0.06% nitrogen) semi-arid clay–loam soil attained significant increase in total organic carbon (TOC), total nitrogen (TKN), and $PO_4^{3-}P$ during the middle (reaching a maximum of 50%, 15%, and 45%, respectively, as compared to untreated samples). However, the effects diminished at later stages of the experiment. Higher TOC in treated soils can be attributed to autotrophic nature of the cyanobacteria, which synthesize and add organic matter to soil. The biofertilizer improved C and N mineralization by promoting soil microbial activities and narrowed down C: N ratio. Effect of biofertilizer on cation exchange capacity of the soil became evident corresponding with time. Application of cyanobacterial biofertilizer led to decline in bulk density and increase in water holding capacity of soil. The native strains showed remarkable potential for improving structural stability, nutrient status, and productivity of the soil under limited water regime. The authors attributed better soil aggregation in biofertilizer treated soils to the production of polysaccharides by the algae. The study exhibited that despite earlier belief of requirement of high soil moisture for favorable growth of cyanobacteria, even under limited water regime, the biofertilizers performed quite well.

Yadav et al. (2009) in a field experiment observed the integrated effect of bioinoculants (*Gluconacetobacter diazotrophicus* and *Trichoderma viride*), farm yard manure (FYM) and fertilizer N on sugarcane rhizosphere, crop yield and N economy for two crop cycles in the middle Indo-Gangetic plain region. An enhancement in soil microbial C and N was observed probably due to more available N present in the soil. Microbial carbon was further enhanced in treatments of FYM/bioinoculants. This treatment further resulted in enhanced uptake of N, P, and

K in sugarcane at all the levels of fertilizer N, which was mainly due to increased nutrient availability in the rhizospheric soil as soil organic C and available N, P, and K content as a result of application of bioinoculants/FYM. The betterment of soil nutrient status (in terms of N supplementation) by the addition of biofertilizers was calculated to result in saving of huge amounts of N fertilizer application with only marginal decline in the sugarcane yield.

4.4 Effect on Microbial Community Diversity

The effect biofertilizers have on microbial community diversity has been majorly studied using culture-dependent techniques. As such methods suffer from the limitation that less than 1% of soil microorganisms are culturable (Sharma et al 2005), efforts are being directed toward analysis using cultivation-independent techniques. DNA and lipids have been reliably used as molecular markers to assess the changes in inoculated samples as compared to control soil. Additionally, enzymatic assays give an idea of the functional diversity of the system. A summary of different approaches employed in such risk and efficacy assessment studies of biofertilizers has been presented in Table 4.1. An understanding of these effects as part of ecosystem processes is essential for obtaining the maximum benefit for plant growth and health in the context of sustainable soil–plant system.

Employing both culture-dependent and -independent approaches, there have been certain reports of no observable changes in the bacterial community dynamics as a result of application of biofertilizers when compared to untreated samples. Even group-specific primers, instead of universal bacterial primers, did not reveal

Table 4.1 Approaches to analyze impact of biofertilizers on microbial community diversity in soil

S. No	Methodology employed	Remarks	References
1.	Culture-dependent	–	Pandey et al. (1998), Wu et al. (2005), Mishra et al. (2008)
2.	Culture-independent DNA based Enzymatic assays Fatty acid analysis Catabolic diversity	Fingerprinting techniques like RISA, ARDRA, DGGE employed for profiling of microbial community	Marschner and Timonen (2005), Herschkovitz et al. (2005a), Baudoin et al. (2009)
		Estimation of enzymes like phosphatase, esterase, chitinase, trehalose, dehydrogenase, nitrogenase	Vazquez et al. (2000), Aseri et al. (2008), Zarea et al. (2009)
		Fatty acid extraction and analysis using gas–liquid chromatography	Gagliardi et al. (2001), Kozdroj et al. (2004)
		Use of different substrates like carbohydrates, carboxylic acids, amides, followed by measurement of CO_2	Dabire et al. (2007), Duponnois et al. (2005)

any changes in community profiles of bacterial members. Lottmann et al. (2000) monitored the effect of rifampicin resistant mutants of two antagonistic plant-associated bacteria for seed tuber inoculation of transgenic T4 lysozyme expressing potatoes, transgenic control potatoes, and nontransgenic parental potatoes. The T4 lysozyme tolerant *Pseudomonas putida* QC14-3-8 was originally isolated from the tuber surface (geocaulosphere) of T4 lysozyme producing plants and showed in vitro antibacterial activity towards the bacterial pathogen *Erwinia carotovora* ssp. *atroseptica*. Denaturing gradient gel electrophoresis (DGGE) profiling of amplicons of 16S rRNA genes of the whole bacterial community did not show differences between the inoculated and noninoculated potatoes at any sampling time. Neither of the introduced strains became a dominant member of the bacterial community. No differences were apparent even when group-specific primers (α-Proteobacteria, β-Proteobacteria, or Actinomycetales) were employed. However, fungal communities have been found to be more sensitive to the introduction of biofertilizers. Glandorf et al. (2001) conducted field experiments to compare the effects of a wild-type biocontrol strain, *P. putida* WCS358r, on the fungal rhizosphere microflora of wheat with the effects of two of its genetically improved derivatives constitutively producing phenazine and a noninoculated control. It is interesting to note that both the wild and modified strains did not lead to any significant differences in the metabolic activity of the soil microbial population, soil nitrification potential, cellulose decomposition, and plant yield. However, transient changes in the composition of the rhizosphere fungal microflora, that last a longer time for the modified strains than for the unmodified strain, could be observed as earlier reported by Zhao et al. (2005). They studied the effect of two commercial biofertilizers, G (five bacterial species related to *Pseudomonas* sp., *Enterococcus* sp., *Lactobacillus* sp., *Streptococcus* sp., and *Bacillus mucilaginous*) and Y (two bacterial species related to *Lactobacillus* sp. and *Bacillus* sp.) on the microbial community dynamics and cellulolytic activities in an agricultural soil over a 50 days amendment period. A DNA-based PCR-TGGE (temperature gradient gel electrophoresis)-cloning approach was followed. While a significant shift in PCR-TGGE profiles of fungal members could be observed, the bacterial profiles were largely unaffected, with 90% similarity coefficients.

Using the same bacterial inoculants inconsistent results have been reported, e.g., Pandey et al. (1998) performed a field experiment using three strains of *Azotobacter chroococcum* and two strains of *Azospirillum brazilense*. During the middle of growing period there was two- to fivefold increase of actinomycetes and a group of bacteria able to grow on N-free medium. This suggested that the observed effect of seed inoculation on plant growth may in part be due to stimulation of already existing PGPR in and around roots. On the other hand, Herschkovitz et al. (2005a) did not observe any difference in bacterial community profiles generated with amplicons of 16S rRNA gene (using universal bacterial primers) from control and *A. brazilense* (seedling treatment) in a pot experiment. This inconsistency indicates the involvement of more than one factor in the entire process. In another related experiment, they further increased the resolution of their study by using group specific primers (*Actinobacteria*, *Bacteroidetes*, *α-Proteobacteria*, *Pseudomonas*,

and *Bdellovibrio* spp.). No significant effect could still be observed in the treated rhizospheres which led to the authors concluding *A. brazilense* to be a "safe" biofertilizer (Herschkovitz et al. 2005b). However, using a different isolate of the same genus (*Azospirillum lipoferum* CRT1), plant system, and analysis approach Baudoin et al. (2009) reported a shift in structure of indigenous rhizobacteria of field-grown maize which lasted for atleast 1 month.

Also with respect to application of pseudomonads as biofertilizers inconsistency has been reported as far as their effects on resident communities are concerned. While De Leij et al. (1995) reported the pseudomonads population to be sensitive (decrease in population) to exogenous application of PGPR strains, Mahaffee and Kloepper (1997) did not detect differences in the size and structure, at the generic level, of the bacterial communities from both the rhizosphere and internal root tissues of field-grown cucumber plants inoculated with either a wild-type strain of *P. fluorescens* or its bioluminescent derivative in two successive years. A transient reduction and change in the structure of the population of cucumber root resident pseudomonads by a biocontrol strain of *P. fluorescens* and its antibiotic overproducing derivative was observed in a controlled environment by Natsch et al. (1997). These perturbations were, however, less pronounced than those naturally occurring during plant growth. Blouin-Bankhead et al. (2004) in a study with wild and genetically modified *P. fluorescens* strains reported effect on bacterial community structure in wheat rhizosphere. Comparison of T-RFLP profiles revealed the recombinant strain resulting in microbial shift as compared to untreated samples. Similar to reports by Natsch et al. (1997), the changes reported were also transient, of small magnitude and not reproducible in multiple cycles. Walsh et al. (2003) reported almost equal percentage of nodules yielding rhizobial isolates in *P. fluorescens* F113Rif treatment in red clover crop and its control. However, it appeared that the genetic diversity of rhizobia was significantly less in the inoculated treatment. The treatment had no effect on the percentage of rhizobia sensitive to 2, 4-diacetylphloroglucinol (Phl; the antimicrobial metabolite produced by *P. fluorescens* F113Rif). A significant modification in bacterial community profile was observed by Roesti et al. (2006) when employing a PCR-DGGE approach to study the bacterial community structure in rhizosphere and rhizoplane in Indian wheat fields in response to a consortia of two PGPR strains of Pseudomonad spp. (used either alone or in combination with AMF or AMF alone). The type of PGPR consortium had more impact on bacterial community structure than the presence of AMF. This was explained by the fact that high density inoculation of PGPR strains on the seeds may have shifted the bacterial community equilibrium at early stages of plant growth. This shift did not lead to any adverse effect on grain quality and growth and yield thereby indicating that a negative effect caused by shift in bacterial community equilibrium was overcome by beneficial effect of bioinoculant. Alternatively, the shift in equilibrium may have favored the growth of beneficial populations.

Certain biofertilizers have a direct effect on the microbial communities depending on their inherent characteristics. Robleto et al. (1998) assessed microbial diversity changes in the rhizosphere of bean plants (using cultivation-independent approach) following inoculation with bacterial strains that differ only in the ability to produce

a narrow-spectrum peptide antibiotic, trifolitoxin. Trifolitoxin production dramatically reduced the diversity of trifolitoxin-sensitive members of α-subdivision of the class *Proteobacteria* with little apparent effect on most microbes. On the other hand, the toxins and cells of *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) introduced in Sardinian soil did not appear to affect the number of indigenous bacteria. Btk mostly persists as spores and so had no effect on the indigenous bacterial population (Vettori et al. 2003).

The mode of inoculation has been shown to be another important parameter determining whether or not the application would result in observable changes in microbial communities. Ciccillo et al. (2002) inoculated *Burkholderia ambifaria* MCI 7 (formerly *B. cepacia* MCI 7) into the rhizosphere of maize plants by either seed adhesion or incorporation into soil. As far as the bacterial community structure is concerned (amplification of 16S rRNA and subjecting the amplicon to ARDRA), *B. ambifaria* MCI 7 affected the indigenous microflora of treated plants according to the application method: seed treatment brought about an abrupt decrease in bacterial diversity, whereas incorporation into soil increased bacterial diversity. Moreover, changes in bacterial diversity were limited to r-strategist bacteria. Depending on the method of inoculation the bioinoculant, *B. ambifaria* MCI 7 can act as both a PGPR and a deleterious rhizobacterium. Growth of r-strategist microorganisms (*Bacillus* spp., *Pseudomonas* spp.) have been reported to be stimulated on inoculation with AM fungus *G. intraradices* (Dabire et al. 2007). Rivera-Cruz et al. (2008) in a mesocosm study analyzed the effectiveness of different carriers of bacterial consortium (strains of *Azospirillum*, *Azotobacter,* and P-solubilizer bacteria) for soil cultivated with banana. The biofertilizer prepared on banana waste (BW) enhanced the density of P-solubilizer bacteria, the concentrations of available P and foliar P to a greater extent than that of biofertilizer prepared on poultry manure. Phosphate solubilizers are able to degrade agro-wastes of a lignocellulosic nature, such as BW, and excrete organic acids, which increases the concentration of phosphorus in solution by mechanisms involving chelation and exchange reactions (Vessey 2003). Rivera-Cruz et al. (2008) observed the population density of phosphate-solubilizing bacteria to be higher in the soil amended with the bacterial consortium inoculated on BW. Dehydrogenase activity was significantly higher in the soils amended with the biofertilizers, both biofertilizers significantly stimulated hydrolases related to the cycle of N (urease and protease-BAA activities), P (phosphatase activity), and C (β-glucosidase activity). However, the proportion of increase differed with the type of carrier.

The plant system selects a specific rhizospheric community which is determined largely by its root exudates. Plant-dependent effect of biofertilizer has also been reported using the same biofertilizer, e.g., field inoculation of alfalfa with a *luc*-tagged *Sinorhizobium meliloti* was found to cause changes in the structure of the rhizosphere bacterial population from the plant host, *Medicago sativa*, but not in the rhizosphere of nontarget plant, *Chenopodium album*, which is the dominant weed present in the field plots (Schwieger and Tebbe 2000). An increase in the number of rhizobia (including the inoculant strain) was observed, while the numbers of the most dominant species, *Acinetobacter alcoaceticus*, and numbers of pseudomonads decreased. However, the effect of plant on the rhizospheric community was larger

Table 4.2 Effect of biofertilizers on microbial community diversity and soil

Bioinoculants	Host plant	Effects observed	Reference
Three AM fungi (*G. mosseae, G. intraradices, G. deserticola*); two PGPR bacteria (*B. pumillus, B. licheniformis*)	*Medicago sativa*	• *B. liceniformis* enhanced leucine • *B. liceniformis* + *G. intraradices* enhanced both leucine and thymidine • Maximum ergosterol with *B. pumillus* + *G. mosseae* • Maximum chitin with *B. licheniformis* + *G. intraradices*	Medina et al. (2003)
Azospirillum brasilense, Azotobacter chroococcum, G. fasciculatum, G. mosseae	*Punica granatum*	• Enhanced activities of dehydogenase, alkaline phosphatase and nitrogenase, and hydrolysis of fluorescein diacetate	Aseri et al. (2008)
Azospirillum brasilense + AM consortia (*Glomus* spp. *and Gigaspora* spp.)	*Panicum maximum*	• Maximum number of AM spores and *Azospirillum* cfu in soil • Increased AM infection in roots	Mishra et al. (2008)
Pseudomonas monteilii HR13 *Pisolithus* sp., *Scleroderma* sp. *G. intraradices*	*Acacia* sp.	• Stimulated ectomycorrhizal and endomycorrhizal colonization of *Acacia* root systems	Duponnois and Plenchette (2003)
G. intraradices	*Acacia holosericea*	• Increase in fluorescent Pseudomonads and ketoglutarate catabolizing microbes	Duponnois et al. (2005)
G. intraradices, G. mosseae	*Zea mays*	• Increase in alkaline phosphatase activity and rhizospheric pH	Marschner and Baumann (2003)
Azospirillum brasilense Sp245, *Pseudomonas fluorescens* WCS365, *Trichoderma harzianum* T12, AMF (*G. mosseae, G. deserticola*)	*Zea mays*	• Decreased *Azospirillum,* fluorescent *Pseudomonas*, and total bacterial population • Total saprophytic fungi increased • Increase or decrease in Esterase and Trehalose activity • Increased Phosphatase and Chitinase activity	Vazquez et al. (2000)
G. intraradices, Sinorhizobium terangae ORS 1009, *Mesorhizobium plurifarium* ORS 1096	*Acacia tortilis* spp. *raddiana*	• Increase in the competitiveness of ORS 1009 • Improved root nodulation	Andre et al. (2003)
G. mosseae	*Trifolium alexandrinum* *T. resupinatum*	• Increase in nitrogenase activity and microbial biomass	Zarea et al. (2009)
Ectomycorrhizal fungus *Paxillus involutus*	*Pinus sylvestris, Picea abies, Betula pendula*	• Increase in detection frequency of 1.1c crenarchaeotal 16S rRNA genes	Bomberg and Timonen (2009)
G. mosseae	*Sorghum bicolor*	• Increase in microorganisms number and water stable soil aggregate stability	Andrade et al. (1998)

than that observed by the inoculation. Table 4.2 is a compilation of other reports on effects observed after inoculation with biofertilizers in different systems.

4.5 Conclusions and Perspectives

Effects of biofertilizers on nontarget members of the food web in soil and rhizosphere have been identified to a certain extent. Most studies report measurable changes but the magnitude of these effects and significance to ecological functions remains to be fully characterized. It is still an open question as to which species within the indigenous microflora are selected during competition with biofertilizers. Which of them are beneficial or deleterious to the plant host? Attempts to answer such questions should be made while designing further studies and trials with introduced microbial inoculants into soil.

Analysis of nontarget effects on community structure has been performed with culture-dependent and culture-independent methods, including both genetic and physiological assays. Assessment of microbial community structures by both plating and cytochemical analyses may represent a suitable approach to study the impact of bacterial fertilizers. However, with the advent of newer technologies, application of these in studying the impact of biofertilizers on resident microbial community and soil functioning still needs to be carried out. Employing the technique of quantitative PCR and microarray to target specific genes in biogeochemical cycling would pinpoint the step that is influenced by the artificial introduction of microbial inoculants in soil. The additional advantage of such techniques is high throughput. Despite DNA being a reliable marker to assess community diversity and its potential, recent successful reports on mRNA extraction from soil is motivating enough to apply the same for such risk- and efficiency-testing studies with biofertilizers. Further, mRNA-based approach would reflect the actual functional diversity at any particular time point in the system under investigation. Besides using traditional techniques, methods with higher resolution need to be applied for multidimensional analysis. As observed in certain studies, changes went undetected when universal bacterial primers were used. However, group-specific primers revealed significant effect of biofertilizers on the same.

Though non target effects of biofertilizers are often small and transient, effects extending beyond the growth season have also been observed. The soil system has been found to be resilient to perturbations caused by introduction of biofertilizers. Also redundancy in the community allows for the system to stabilize relatively fast. The extent of effect on the resident communities depends on various factors, including soil characteristics, mode of application of biofertilizer, other environmental conditions etc. Very few studies have been performed on a larger time scale. This is crucial to draw conclusions on the risk and efficacy of the inoculant. Clearly, more nontarget effect studies are required on the marketed biofertilizers using combined approach and for longer durations before assigning a bioinoculant as "safe" for commercial purposes.

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Chapter 5
The Impact of Mycorrhizosphere Bacterial Communities on Soil Biofunctioning in Tropical and Mediterranean Forest Ecosystems

Robin Duponnois, Ezékiel Baudoin, Jean Thioulouse, Mohamed Hafidi, Antoine Galiana, Michel Lebrun, and Yves Prin

5.1 Introduction

Mycorrhizal fungi constitute a key functional group of soil biota that greatly contribute to productivity and sustainability of terrestrial ecosystems. These are ubiquitous components of most of the ecosystems throughout the world and are considered key ecological factors in governing the cycles of major plant nutrients and in sustaining the vegetation cover (van der Hejden et al. 1998; Requena et al. 2001; Schreiner et al. 2003). Two major forms of mycorrhizae are usually recognized: the arbuscular mycorrhiza (AM) and the ectomycorrhizas (ECMs). AM symbiosis is the most widespread mycorrhizal association type with plants that have true roots, i.e. pteridophytes, gymnosperms and angiosperms (Read et al. 2000).

R. Duponnois (✉) • E. Baudoin • M. Lebrun
IRD, UMR 113 CIRAD/INRA/IRD/SUP-AGRO/UM2, Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), TA A-82/J, Campus International de Baillarguet, Montpellier Cedex 5, France

Laboratoire Ecologie & Environnement (Unité associée au CNRST, URAC 32), Faculté des Sciences Semlalia, Université Cadi Ayyad, Marrakech, Marocco
e-mail: Robin.Duponnois@ird.fr

J. Thioulouse
Université de Lyon, 69000 Lyon, France

CNRS, UMR5558, Laboratoire de Biométrie et Biologie Evolutive, Université Lyon 1, 69622 Villeurbanne, France

M. Hafidi
Laboratoire Ecologie & Environnement (Unité associée au CNRST, URAC 32), Faculté des Sciences Semlalia, Université Cadi Ayyad, Marrakech, Marocco

A. Galiana • Y. Prin
CIRAD, UMR 113 CIRAD/INRA/IRD/SUP-AGRO/UM2, Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), TA A-82/J, Campus International de Baillarguet, Montpellier Cedex 5, France

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They affect about 80–90% land plants in natural, agricultural and forest ecosystems (Brundrett 2002). ECMs are found with trees and shrubs, gymnosperms (Pinaceae) and angiosperms, and usually result from the association of homobasidiomycetes with about 20 families of mainly woody plants (Smith and Read 2008). These woody species are associated with a larger (compared to the AM symbiosis) diversity of fungi, comprising 4,000–6,000 species, mainly Basidiomycetes and Ascomycetes (Allen et al. 1995; Valentine et al. 2004). The benefits of mycorrhizal symbiosis to the host plant have usually been considered as a result of closed relationships between the host plant and the fungal symbiont. However, the hyphae of these symbiotic fungi provide an increased area for interactions with other soil microorganisms by enhancing the development of the host plant root systems.

Plant roots influence the soil microbial community in the narrow zone of soil called the rhizosphere (Hiltner 1904). In the rhizosphere, root exudates and organic breakdown products provide a specific ecological niche for microbes with chemical and physical characteristics (concentration and forms of nutrients, soil structure, moisture and pH) that differ from those recorded in the bulk soil (Timonen and Marschner 2006). Hence the density and activity of microorganisms are generally higher in the rhizosphere than in the bulk soil (Lynch 1990). Since plant roots in natural conditions are mycorrhizal and it is well known that the fungal symbiosis modifies root functions, microbial communities associated with mycorrhizas differ from those of the non-mycorrhizal plants and of the surrounding soil (Garbaye 1991; Garbaye and Bowen 1987, 1989). Hence the rhizosphere concept has been enlarged to include the fungal component of the symbiosis to give the term "mycorrhizosphere" (Rambelli 1973; Linderman 1988). The mycorrhizosphere is the zone influenced by both the root and the mycorrhizal fungus. It includes the soil surrounding individual fungal hyphae that has been named "hyphosphere" (Johansson et al. 2004). Mycorrhizal fungi act as a bridge connecting the rhizosphere to the bulk soil and, through an active development of extraradical mycelium into the soil, the mycorrhizosphere extends root–fungal interactions with soil microbial communities (Whipps 2004; Leake et al. 2004). Interactions within the mycorrhizosphere microbial community are of special interest because some microorganisms associated with mycorrhiza may complement mycorrhizal activities (Toro et al. 1996). More recently, Frey-Klett et al. (2005) have proposed that the ectomycorrhizal symbiosis could be considered as a microbial complex where the fungal symbiosis has a direct effect on plant growth (nutritional and hormonal mechanisms) but also an indirect positive effect via a selective pressure on bacterial communities resulting, for instance, to a higher abundance of phosphate-solubilizing fluorescent pseudomonads in the hyphosphere. It has been previously demonstrated that P-solubilizing bacteria can interact synergistically with mycorrhizal fungi and improve the phosphorus nutrition of the host plant (Muthukumar et al. 2001).

To date, there is little information on the mechanisms controlling interactions between mycorrhizal fungi, soil bacteria and plant roots in the mycorrhizosphere. Although these interactions can influence the fungal symbiont itself and the plant and soil nutrient cycling processes, knowledge of these impacts and their

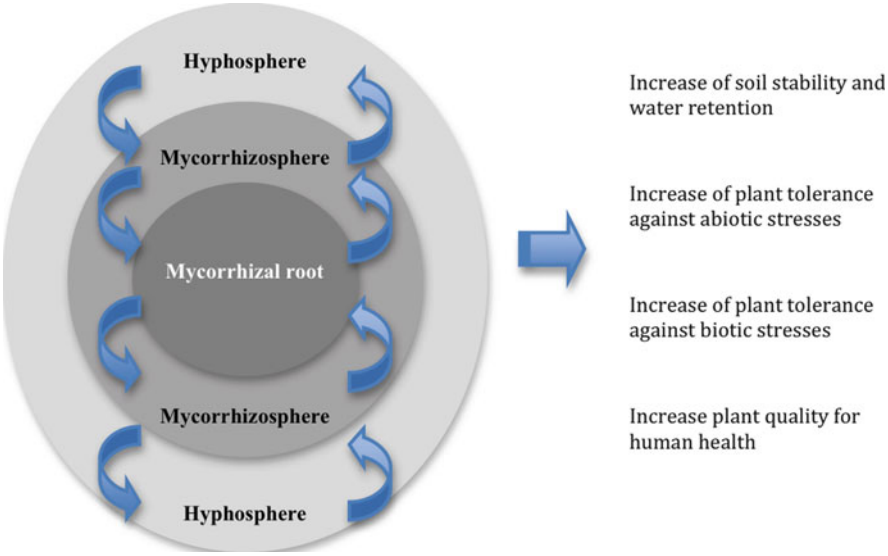

Fig. 5.1 The Mycorrhizosphere trophic complex and its role as an ecosystem service provider

consequences on soil biofunctioning and ecosystem productivity remains poorly understood (Fig. 5.1). On one hand, some soil bacteria can act as mycorrhization helper bacteria (MHB) by improving the establishment of the mycorrhizal symbiosis and on the other hand, mycorrhizal fungi can have an impact on the structure and functional diversity of bacterial communities (Assigbetse et al. 2005; Artursson et al. 2005). The purpose of this chapter is to outline the mycorrhizosphere interactions between ectomycorrhizal fungi associated with forest tree species and soil microflora of potentially synergistic properties that lead to stimulation of plant growth. By focussing on the ectomycorrhizal symbiosis associated with Tropical and Mediterranean tree species, we will review the global effects of ectomycorrhizal symbiosis on the functional diversity of soil microflora and in particular, the interactions between ectomycorrhizal fungi and some plant-growth-promoting rhizobacteria (i.e. rhizobia, fluorescent pseudomonads). It is well known that ectomycorrhizal fungi improve the phosphorus uptake of their associated host plant and enhance the plant development (Read and Perez-Moreno 2003). This ectomycorrhizal effect on plant growth has been mainly ascribed to the fact that the extramatrical mycelium increased the abilities of the host plant to explore a larger volume of soil than roots alone and to uptake nutrients from a greater surface area through different biological processes (Smith and Read 2008). Phosphorus (P) is one of the most essential macronutrients required for the growth and development of plant (Illmer and Schinner 1992) and occurs in various organic and inorganic forms not directly assimilable by plants and soil microorganisms. In degraded areas, the first objective of the controlled mycorrhization processes is to improve reforestation in areas presenting a loss or

reduction of major physico-chemical and biological soil properties (Requena et al. 2001) and more particularly severe phosphorus deficiencies. This review aimed to state the interactions between ectomycorrhizal fungi and soil microflora leading to a sustainable microbial complex with high efficiency against phosphorus mobilization and transferring phosphorus from the soil organic matter (SOM) or from soil minerals to the host plant.

5.2 Soil Microbial Processes Involved in Phosphorus Mobilization from Soil Organic Matter and Soil Minerals

Ectomycorrhizal fungi enhance the capacity of the host plants to mobilize P from soil inorganic and organic forms through different biological processes that are summarized below.

5.2.1 Mobilization of P from Soil Organic Matter

Soil organic matter contains a wide range of complex molecules such as inositol phosphate, nucleotides and phospholipids (Criquet et al. 2004). Soil microbes can degrade P-compounds through their capacity to produce a wide range of extracellular and surface-bound enzymes leading to the release into the soil of smaller organic compounds that provide potential sources of P for plants, ectomycorrhizal fungi and other soil microorganisms (Nahas et al. 1982; Haas et al. 1992). Phosphatase activities (acid and alkaline phosphatase) release orthophosphate ions (Pi) that are the unique form of P easily assimilable by soil microorganisms and plants (Rao et al. 1996). Enzymes can be free in the soil solution or bound to soil colloids, to humic substances, to living and dead microbial cells or to plant roots. Acid phosphatase activity in the rhizosphere may also be due to plant roots (Goldstein et al. 1988; Coello 2002), bacteria (Palacios et al. 2005; Boyce and Walsh 2007) and fungi (Yoshida et al. 1989; Weber and Pitt 1997; Bernard et al. 2002). The secretion of acid phosphatases is induced by Pi-deficient conditions for all the organisms studied (Goldstein et al. 1988; Bernard et al. 2002). Most of the studies performed in laboratory conditions with known substrates showed that these enzymatic activities are generally not substrate specific, except for phytases (Wyss et al. 1999), and are able to release Pi from different phosphorylated substrates. Phosphatase activity has often been used as a general indicator in measurements of biological activity (Joner and Johansen 2000). Ectomycorrhizal fungi differ in their physiological capacities to acquire and transfer nutrients to a range of plant hosts (Abuzinadah and Read 1989; Dighton et al. 1993; Bending and Read 1995). It has been suggested that ectomycorrhizal fungi contribute to organic P mobilization

from soil solution through the production of extracellular acid phosphatase (Quiquampoix and Mousain 2005). This beneficial effect (enhancement P nutrition of the host plant) is generally attributed to the development of external hyphae into the soil resulting in higher of soil volume exploited compared with nonmycorrhizal roots (Louche et al. 2010).

5.2.2 Mobilization of P from Soil Minerals

Ectomycorrhizal fungi have the potential ability to mobilize and translocate essential plant nutrients from minerals (Landeweert et al. 2001). Weathering processes of minerals (transformation of rock-forming primary minerals into dissolved compounds and secondary mineral residues into the biological environment) result from the activity of plant roots and their associated microbiota (rhizosphere bacteria and fungi). Plant root exudates and root-associated microorganisms affect the stability of minerals through the production of organic acids, phenolic compounds, protons, siderophores and polysaccharides (Barker et al. 1997; Drever 1994; Drever and Vance 1994). Soluble organic acids affecting mineral weathering range from low to high molecular weight such as humic substances but low molecular weight (LMW) organic acids are considered to be the main agents of mineral dissolution because of their dual acidifying and complexing properties (Ochs 1996; Barker et al. 1998). Numerous studies have shown that ectomycorrhizal fungal strains could dissolve minerals by excreting organic acids. Among these organic acids excreted by ectomycorrhizal fungi, oxalate, citrate and malate are the strongest chelators of trivalent metals. Oxalic acid is known to have the highest acid strength (Gadd 1999). This organic acid is involved in the dissolution process of common soil minerals such as apatite, biotite, phlogopite and microline (Courty et al. 2010). Many ectomycorrhizal fungi excrete oxalic acid in pure culture (Paris et al. 1996), but this organic acid excretion is also observed with ectomycorrhizas (van Schöll et al. 2006) and hyphal mats (Wallander et al. 2003). However, most of these studies have been performed in pure cultures or in pot experiments and the real contribution of ectomycorrhizal fungi in mineral weathering remains difficult to determine in natural conditions (Landeweert et al. 2001; Courty et al. 2010).

5.3 Impact of the Controlled Ectomycorrhization on Soil Microbial Functions and Phosphorus Mobilization

In forest formations, extend of extraradical mycelium of ectomycorrhizas interconnect roots belonging to the same or different ECM tree species. This common mycorrhizal network (CMN) allows a transfer of C and nutrients between host

plants (Simard and Durall 2004). Hence, the ability of ectomycorrhizal fungi to mobilize phosphorus and to transfer Pi to the host plants through this common mycorrhizal network could be supplemented by their positive selective pressure on soil microbial functions (i.e. phosphatase activity). This CMN effect is of particular importance in Mediterranean and Tropical areas where it has been clearly demonstrated that land degradation is associated with reductions in the below ground microbial diversity and/or activity (Kennedy and Smith 1995; Garcia et al. 1997).

The functioning of soil microbial community is central to understand ecosystem-level processes such as decomposition and nutrient cycling. Various standardized methodologies have been developed to determine the microbial functional characteristics (i.e. enzymatic activities, Biolog™ method, etc.). Degens and Harris (1997) proposed a method that avoids the problem of the culturability of soil microbial populations under artificial conditions. This method is based on the measurement of the patterns of in situ catabolic potential (ISCP) of microbial communities by adding individual organic substrates directly to the soil and measuring the resulting respiration response. Patterns of ISCP provide a real time measure of microbial functional diversity and this kind of measurement has shown that microbial functional diversity responded to changed land use (Degens and Vojvodic-Vukovic 1999), cropping intensity (Sparling et al. 2000), soil organic status (Degens et al. 2000), successional sequences (Schipper et al. 2001) and stress or disturbance to the soil (Degens et al. 2001). The ISCP methodology has also been used to compare functional diversity among the different soil compatments influenced or not by ectomycorrhizal symbiosis (bulk soil, rhizosphere, mycorrhizosphere and hyphosphere). In the present review, differences between microbial functionalities of these soil compartments will be reported from controlled mycorrhization experiments performed in glasshouse and in field conditions with regards to the biological processes involved in P mobilization and plant nutrition.

5.3.1 Patterns of ISCP Profiles of Microbial Communities Influenced by Ectomycorrhizal Symbiosis in Controlled Conditions

A glasshouse experiment was performed with seedlings of *Uapaca bojeri*, an endemic Euphorbiaceae of Madagascar, planted in pots filled with a natural soil collected in a forest ecosystem. Other results have shown that this tree species was highly dependent to the ectomycorrhizal symbiosis that stimulated plant growth and P nutrition (Ramanankierana et al. 2007). The aim of this study was to characterize the functional diversity in each of the soil compartment influenced by ectomycorrhizal fungi. The results showed that the patterns of ISCP of microbial communities from each of the four compartments were very significantly different (Fig. 5.2). They also showed that microorganisms able to catabolize organic acids

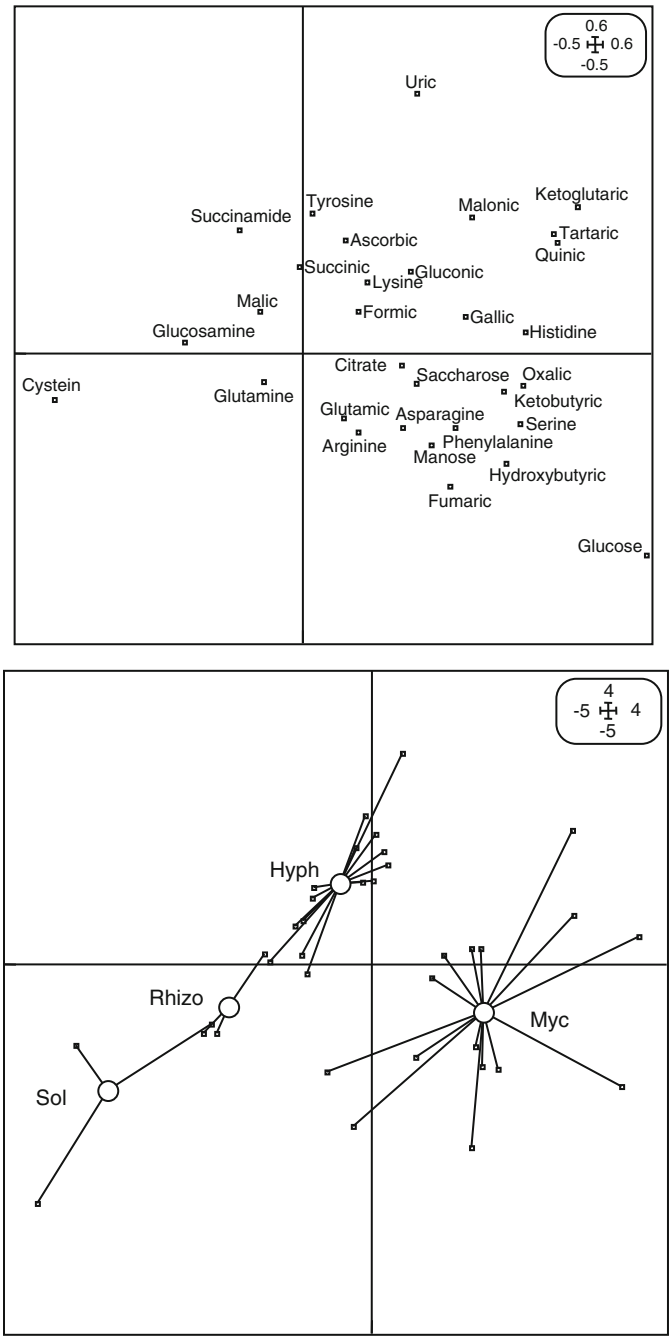

Fig. 5.2 Between-group analysis of the substrate induced respiration (SIR) responses of the bulk soil, rhizosphere, hyphosphere and mycorrhizosphere soil compartments. From Ramanankierana et al. (2006)

Table 5.1 Effect of ectomycorrhizal inoculation of *Pinus halepensis* with *Pisolithus* sp. on plant growth and needle nitrogen and phosphorus concentrations after 12-months culture in a non-disinfected soil (from Ouahmane et al. 2009)

	Treatments	
	Control (not inoculated)	*Pisolithus* sp.
Shoot biomass (mg dry weight)	312.2 (24.7) [(1)] a [(2)]	432.7 (21.5) b
Root biomass (mg dry weight)	159.7 (21.6) a	254.6 (21.8) b
Total biomass (mg dry weight)	480.9 (22.5) a	687.3 (21.6) b
Needle N content (%)	1.2 (0.1) a	1.5 (0.2) b
Needle P content (g kg^{-1})	4.1 (0.11) a	7.3 (0.13) b
Mycorrhizal colonization (%)	23.2 (1.5) a	52.5 (1.2) b

[(1)]Standard error of the mean. [(2)]Data in the same line followed by the same letter are not significantly different according to the Newman & Keuls test ($p < 0.05$)

were more abundant in the zone influenced by ectomycorrhizal fungi since it recorded a higher organic acid induced respiration in the mycorrhizosphere and hyphosphere. The authors conclude that ectomycorrhizal fungi through its exudates and more particularly through their organic acid productions induced a selective pressure on soil microbial communities. This kind of experiment has been replicated with *Pinus halepensis* and a strain of *Pisolithus* sp., selected for its high ability to mobilize P from inorganic form of phosphate (Ouahmane et al. 2009). The objective of this study was to assess how inoculation with an ectomycorrhizal fungus, *Pisolithus* sp., affects (1) the early growth of Aleppo pine seedlings and (2) the determination of functional diversity of soil microflora. After 12 months of culturing, ectomycorrhizal inoculation significantly improved the plant growth and nutrient uptake (N and P) (Table 5.1). *Pisolithus* inoculation induced strong modifications in soil microbial catabolic functions. In fact, Ectomycorrhizal inoculation led to higher average SIR responses with fumaric acid and citric acids (Fig. 5.3). This result suggested that large amounts of carboxylic acids excreted by the ectomycorrhizal fungus could exert a selective influence on soil microbial communities through a multiplication of carboxylic acids-, fumaric acid- and citric acid-catabolizing microorganisms inducing a higher SIR.

5.3.2 *Patterns of ISCP Profiles of Microbial Communities Influenced by Ectomycorrhizal Symbiosis in Field Conditions*

From our knowledge, the measurement of the patterns of in situ catabolic potential (ISCP) of microbial communities in a controlled mycorrhization experiment has been rarely realized with Mediterranean and Tropical tree species. Numerous studies have previously demonstrated that controlled mycorrhization could significantly improve the development of Australian acacias in glasshouse conditions (Cornet and Diem 1982; Duponnois et al. 2000; Duponnois and Plenchette 2003).

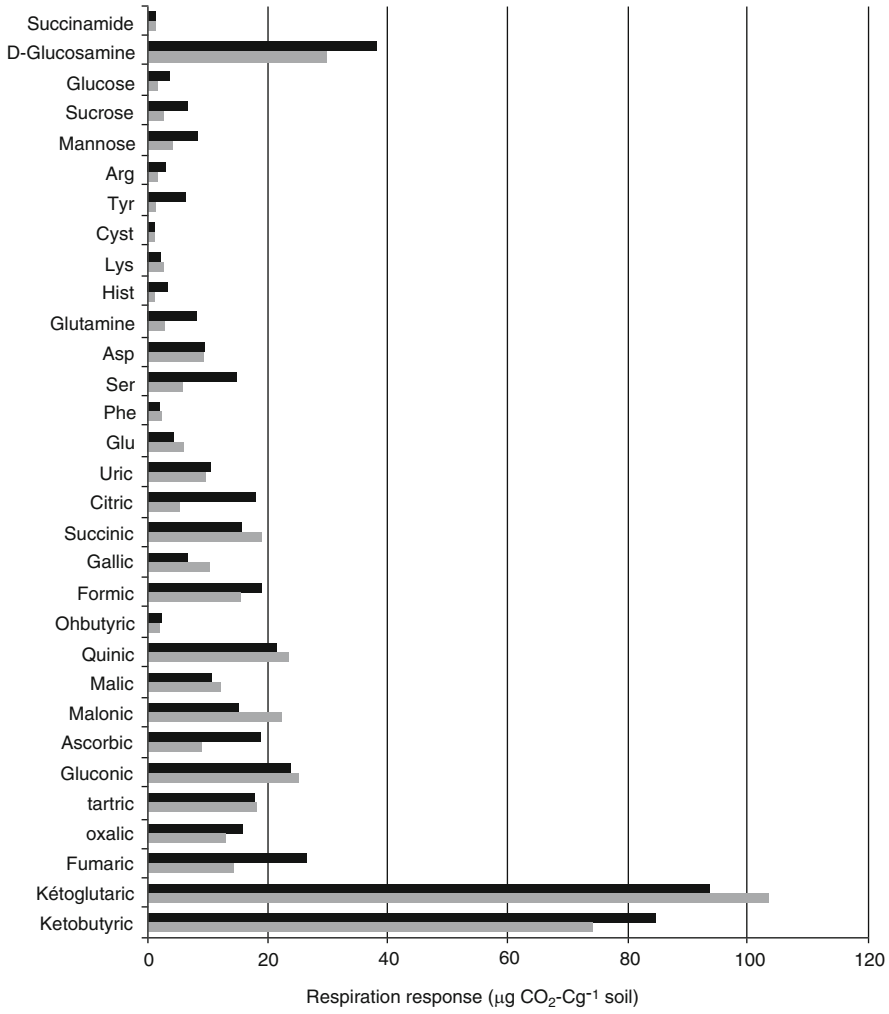

Fig. 5.3 Effect of ectomycorrhizal inoculation on soil catabolic responses. From Ouahmane et al. (2009)

However, data on the effect of mycorrhizal inoculation on host plant growth and biological soil properties in the field are very scarce. In Senegal, a field experiment was carried out to determine the effectiveness of the ectomycorrhizal inoculation with an isolate of *Pisolithus albus* (*P. albus* IR100) on the early development of an Australian *Acacia* species, *A. holosericea*, and on biological soil properties (Duponnois et al. 2005, 2007). After 2 years of plantation, ectomycorrhizal fungal inoculation significantly improved the diameter and the wood biomass of the *A. holosericea* trees as well as the N and P mineral contents per tree (Fig. 5.4).

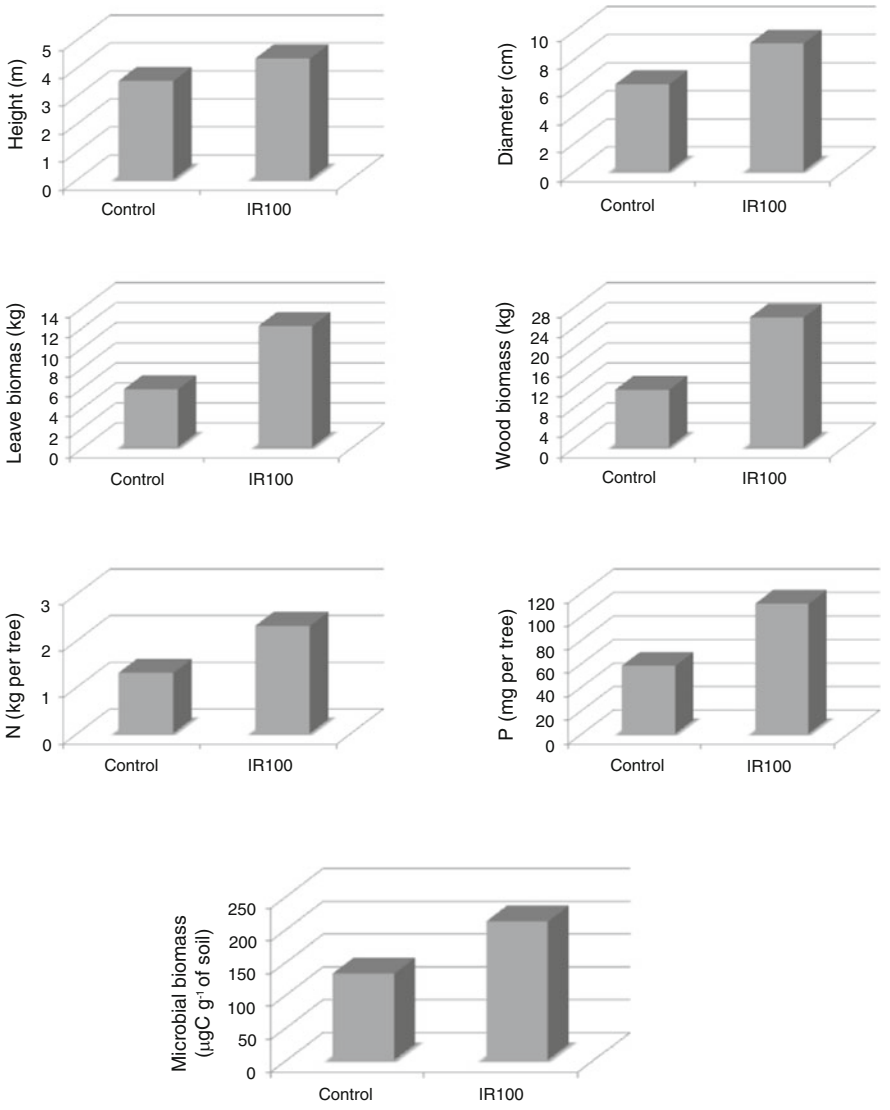

Fig. 5.4 Effect of fungal inoculation on tree growth, leaf mineral content and soil microbial biomass after 2 years of plantation in the field. From Duponnois et al. (2005)

Patterns of ISCP were determined in the soil collected out of the *A. holosericea* plantation (Crop soil), under uninoculated *A. holosericea* trees and under *Pisolithus* inoculated trees (Remigi et al. 2008). The results showed that ectomycorrhizal inoculation induced significant changes in the functions of soil microbial communities (Fig. 5.5).

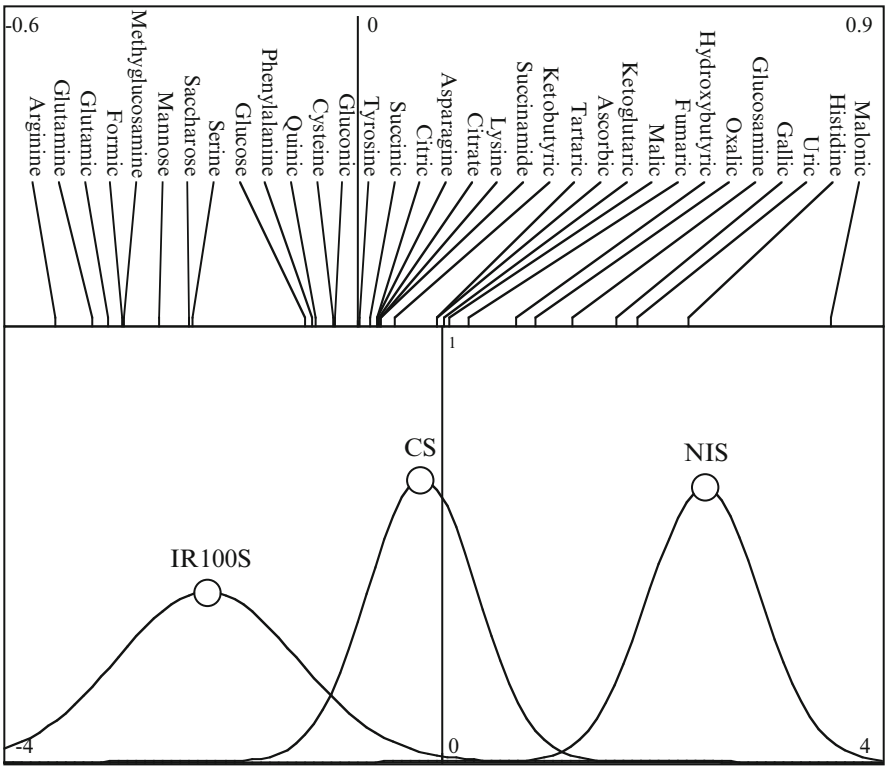

Fig. 5.5 Graphical display of the second BGA axis showing the SIRs with respect to the soil treatments. Only the second axis is used here, as the first axis merely separated the crop soil samples. The *upper part* of the figure shows the scores of the 33 substrates on the second BGA axis. In the *lower part*, the three Gauss curves represent the mean and variance of the scores of the nine soil samples (three repetitions for three treatments) on the second BGA axis. CS, crop soil; NIS, soil of plantation with uninoculated trees; IR100S, soil of plantation with *P. albus* IR100-inoculated trees. Substrates represented by *lines* curved in the same direction as corresponding Gauss curves tended to be used more in the corresponding soil samples. From Remigi et al. (2008)

5.4 Interactions Between Ectomycorrhizal Fungi and Selected PGPR

It has been already shown that some phosphate-solubilizing bacterial strains could interact with mycorrhizal fungi and enhanced plant P uptake (Kim et al. 1997; Toro et al. 1997; Muthukumar et al. 2001). These studies reported from the interactions between arbuscular mycorrhizal fungi and soil microflora. With regard to ectomycorrhizas, Frey-Klett et al. (2005) showed that phosphate-solubilizing bacteria were more abundant in the mycosphere of Douglas fir—*Laccaria bicolor* under symbiotic association and suggested that this enrichment could contribute to improve the nutrition of Douglas fir seedlings in the nursery soil. The positive

effect of the ectomycorrhizal symbiosis on this functional bacterial group was also recorded by Ramanankierana et al. (2006) who reported that the number of fluorescent pseudomonads was recorded in the mycosphere compartment similar to that in the mycorrhizosphere of hybrid larch, Sitka spruce and sycamore (Grayston et al. 1994), in the Douglas fir—*L. bicolor* mycorrhizosphere (Frey et al. 1997) and in the *A. holosericea—P. albus* mycorrhizosphere (Founoune et al. 2002a). In addition to this quantitative effect on fluorescent pseudomonads populations, ectomycorrhizal symbiosis has also modified the functional activities of fluorescent pseudomonads and, more particularly, in the mycosphere soil compartment. Thus, most of the P-solubilizing fluorescent pseudomonad strains were isolated from the mycorrhizosphere, suggested that the selective effect of the extramatrical mycelium can improve the bio-available phosphorus around the hyphae and, consequently enhanced the phosphorus uptake by the host plant directly from the soil solution or indirectly through the hyphal transfer.

The positive effect of the ectomycorrhizal symbiosis on the plant P nutrition could also stimulate the nodulation and N_2-fixation of leguminous tree species. Such effects of arbuscular mycorrhizal fungi on nodulation have been reported in *A. holosericea* with *Glomus intraradices* (Duponnois and Plenchette 2003), *G. fasciculatum* (Senghor 1998) and *G. mosseae* (Cornet and Diem 1982). In the same way, it has been shown that ectomycorrhizal fungi could enhance the number of rhizobial nodules per plant and increase nodule weight in Australian *Acacia* species namely *A. holosericea* and *A. mangium* (Founoune et al. 2002a, b, c; Duponnois et al. 2002; Duponnois and Plenchette 2003). These results suggested that the ectomycorrhizosphere effect induced chemical and physical changes in the soil around the roots but also in the physiological characteristics of the host plant that facilitate the development of rhizobia in the mycorrhizosphere soil compartment.

5.5 Conclusion

It is concluded that the ectomycorrhizal symbiosis can enhance plant growth through two trophic ways (1) directly on plant growth (hormonal and nutritional mechanisms) and (2) indirectly through a qualitative and quantitative effect on soil microflora. All these data showed the existence of soil multitrophic microbial associations resulting from interactions between the plants, the ecomycorrhizal fungal communities and soil microflora. Both biological processes underlined the major role of ectomycorrhizal symbiosis in soil functions and more particularly on soil biogeochemical cycles that ensure the nutrient availability for the cover plants. These fungal impacts on the genetic and functional diversity of soil microflora could have important implications for seedling establishment and, by extension, forest succession, dynamics and expansion. From a practical point of view, all these microbial factors have to be taken into account in improving the performance of afforestation programmes, especially in Mediterranean and Tropical areas.

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Chapter 6
Ecology of Bacterial Endophytes in Sustainable Agriculture

Pablo Hardoim, Riitta Nissinen, and Jan Dirk van Elsas

6.1 Introduction

Plants are autotrophic organisms capable of transforming light energy into chemical (carbonaceous) compounds. These photo-assimilated compounds, when secreted from plant roots, attract a variety of microorganisms that can directly affect the growth and development of the host plant. Plant roots secrete low-molecular-weight (LMW) compounds (e.g. organic acids, amino acids and sugars) next to high-molecular-weight (HMW) ones (e.g. mucilage, proteins and sloughed-off plant cells). The release of these compounds has the putative function to eliminate waste products from internal metabolic processes and to facilitate plant growth, for instance, in external lubrication and nutrient acquisition (Bais et al. 2004). Furthermore, the compounds in root exudates may affect biological processes through the regulation of mutualistic associations with neighbouring (micro)organisms. In this, beneficial interactions may be stimulated, whereas detrimental ones are antagonized (Bais et al. 2006). Two relatively well-documented types of mutualistic interactions (i.e. those with beneficial rhizobia and tumour-inducing *Agrobacterium*) exemplify the importance of plant root exudates for the initiation of the interactions. More details of these interactions are described by Fabra et al. (2012).

Until recently, plant roots have been considered as representing merely supporting plant tissues, which have the ability to absorb water and nutrients for plants to grow. However, with the increasing appreciation of how root exudates select specific soil microorganisms to interact and improve plant health (Hartmann et al. 2009), new investigations into the mechanisms involved in plant–bacterial interactions are flowing. By the process of root exudation, a rich source of 'readily available' and recalcitrant nutrients diffuses into the rhizosphere (the soil which is

P. Hardoim (✉) • R. Nissinen • J.D. van Elsas
Department of Microbial Ecology, Centre for Ecological and Evolutionary Studies, Groningen University, Nijenborgh 7, 9747AG Groningen, The Netherlands
e-mail: phardoim@gmail.com

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directly affected by plant roots), attracting diverse heterotrophic microorganisms. The latter first colonize the rhizoplane (the surface of plant roots), and, later, a selected fraction of these may occupy the internal root tissues to become endophytic. Hence, most bacterial colonization traits that are observed in rhizobacteria are expected to be present in endophytes (Hardoim et al. 2008). Furthermore, bacteria equipped with traits for efficient substrate acquisition, versatile nutrient metabolism, stress resistance and competitiveness might be at an advantage to become endophytic. In this respect, endophytes are those bacteria that occur inside a plant ('endo', inside; 'phyte', plant). In practical terms, it is often postulated that those bacteria that can be isolated from surface-sterilized plant tissues are endophytes. For the plant, common sources of bacterial endophytes are the soil surrounding roots (i.e. the exorhizosphere), the atmosphere (i.e. exophyllosphere) and vegetatively propagated plant material (e.g. seeds, stems and cuttings). Interestingly, multivariate analyses of assigned COGs (cluster of orthologous groups of proteins) from selected metagenomes, including a rice metagenome, have revealed that bacterial endophytes indeed form a distinct community when compared to bacterial communities from soil or other environmental habitats (Fig. 6.1). The metabolic profile of the collective endophytes closely resembled that found in sludge systems and, surprisingly, differed from that of the communities sampled from soil or freshwater. This suggests that, although soil might indeed be the main source of bacterial endophytes, plants provide selective forces that favour communities that possess a distinct metabolic repertoire. Furthermore, the microbial community of sludge tanks was adapted to a wide range of organic compounds, which is explained by the affluent source of nutrients being renewed constantly. To some extent, conditions inside host plants might be similar to this, thus explaining the similarity of the metabolic profiles between both systems. Here, we describe the early events—preceding the establishment of plant–bacterium associations

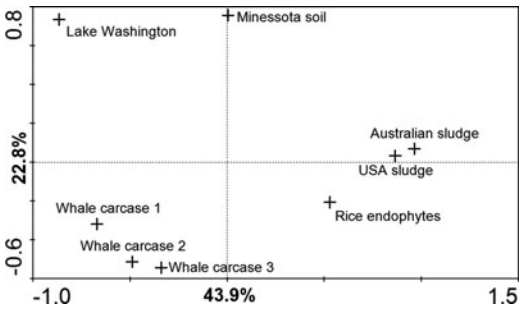

Fig. 6.1 Metabolic profile of selected metabiomes. The ordination diagram of assigned COGs (cluster of orthologous groups of proteins) entities from selected metagenome habitats is shown. The translated protein profile of the rice endophytic community was distinct from other prokaryotic communities. This analysis illustrates that endophytes might harbour metabolic pathways not found in other environments

(mainly) from soil—and analyse how bacteria adapt to and colonize niches at the root, how they are transmitted and what the bacterial properties are that improve growth of the host plant.

6.2 Recognition/Chemotaxis

The sequence of events leading to colonization of a plant by a bacterium that is to become endophytic is presumably similar, at least in the early stages, to that observed for rhizoplane or rhizosphere bacteria. Indeed, bacteria belonging to the so-called root-colonizing rhizosphere-competent bacteria—for example members of the genera *Pseudomonas* (e.g. *P. fluorescens*), *Azospirillum* (e.g. *A. brasilense*) and *Bacillus* (e.g. *B. subtilis*), all common rhizosphere inhabitants—are often found as colonizers of the internal tissue of plants (Hallmann and Berg 2006). Bacterial colonization of roots often starts with the recognition by bacteria of specific compounds that are secreted by the root tissue [Lugtenberg and Dekkers 1999; reviewed by Dardanelli et al. (2012)]. For instance, tomato roots secreting organic as well as amino acids in their exudates were found to provide chemoattractants for *P. fluorescens* (strain WCS365), but sugars had no effect on the chemotactic response (de Weert et al. 2002).

Bacteria sense, and regulate the response to, their surrounding environment via one- and two-component sensor systems (Faure et al. 2009). One-component systems are typically constituted of single proteins with input and output transmembrane domains, which lack a receiver domain and the phosphotransfer histidine kinase found in two-compound systems. Many one- and two-component systems have been identified to be involved in the recognition of root-exuded compounds, leading to active root colonization. Motility driven by chemotaxis is one of the most important and well-understood bacterial systems involved in plant–bacterium interactions. The histidine kinase CheA is responsible for the recognition of chemoattractants, while the response regulator CheY coordinates bacterial motility via flagellum-mediated chemotaxis (Szurmant and Ordal 2004). Pseudomonads as well as enteric bacteria harbour the GacS/GacA two-component regulatory system, in which GacS, the sensor kinase, recognizes still-unknown environmental signals, and GacA, the transcriptional regulator, activates the production of secondary metabolites and extracellular enzymes that enhance host colonization fitness (Heeb and Haas 2001). Recognition of legume flavonoids by the cytoplasmic membrane-associated NodD protein from rhizobia activates the transcriptional regulator LysR, leading to the production of lipochito-oligosaccharides which induce nodule formation in the host (Brencic and Winans 2005). The Nod factor is probably one of the best-known one-component systems. The one- and two-component sensor/response systems combined with other cross-regulation systems permit bacteria to perform complex information processing, allowing to coordinate appropriate responses in the dynamic rhizosphere environment.

Many biotic and abiotic factors affect root exudation. Spatial and temporal exudation patterns have been observed along the axes of the roots, creating

differential niches for diverse soil bacteria (Kuzyakov 2002). Hence, one might hypothesize that different root zones (i.e. the cork zone, root hair, elongation zone, differentiation zone and root cap) create a range of spatial niches that select specific bacterial communities, allowing to establish interactions with the plant. For instance, colonization of wheat roots by *A. brasilense* strain 245 occurs preferentially at the root hair zone and at the sites of lateral root emergence (vande Broek et al. 1993), while colonization of rice roots by *Azoarcus* sp. strain BH72 occurs preferentially in the zones of division and elongation just behind the root cap (Hurek et al. 1994) or—for rhizobial species—at those of lateral root emergence (Chi et al. 2005). Surprisingly, during growth of the root, the root cap cells are sloughed off, and while still alive (i.e. detached living cells known as border cells), they function by attracting and stimulating the growth of beneficial microorganisms, whereas repelling and inhibiting pathogenic ones (Hawes et al. 1998). Moreover, plant traits and physiological states have been shown to affect the composition and diversity of rhizobacterial communities (Hartmann et al. 2009). The effect of bacterial colonization altering root exudates was nicely demonstrated by Rudrappa et al. (2008). The introduction of the phytopathogen *Pseudomonas syringae* pv. *tomato* DC3000 onto leaves of *Arabidopsis thaliana* induced the exudation of malic acid from the roots, which promotes (in a dose-dependent manner) chemotaxis, motility and biofilm formation of *B. subtilis* FB17, thus enhancing root colonization. No biofilm formation of *B. subtilis* FB17 was observed when *Arabidopsis* was inoculated with the non-host bacterium *Pseudomonas syringae* NPS3121, suggesting that the establishment of colonization is specific to a defined bacterial infection regime.

The ability of soil bacteria to approach plant roots via chemotaxis-induced motility and effectively colonize these via attachment and microcolony formation is probably among the strongest deterministic factors for successful endophytic colonization (Compant et al. 2010). In wheat roots, the proportion of isolated bacteria with flagellar motility gradually increased from the rhizosphere to the endosphere (Czaban et al. 2007). A recent metagenomic survey of the endophytic bacterial community obtained from healthy rice root tissues revealed that all compounds of the flagellar apparatus were present in higher abundance than in other metagenomes except for the termite gut microflora metagenome (Sessitsch et al. 2012). Furthermore, the importance of chemotaxis-induced motility for root colonization was demonstrated by analysing *cheA* mutants of *P. fluorescens* WCS365, which retained motility even though they were defective in flagellum-driven chemotaxis (de Weert et al. 2002). In the competitive root colonization assay, the *cheA* mutants revealed reduced ability to compete with the wild-type strains. Besides organic and amino acids, plant secondary metabolites, especially flavonoids, have been proposed as important chemoattractants for endophytic colonization. The incorporation of flavonoids in the growth medium enhanced root colonization of rice and wheat by the endophytic bacteria *Serratia* sp. EDA2 and *Azorhizobium caulinodans* ORS571, respectively (Balachandar et al. 2006; Webster et al. 1998). Intercellular root colonization of *A. thaliana* by two diazotrophic bacteria—*A. caulinodans* ORS571 and *Herbaspirillum seropedicae*

Z67—was also stimulated by the application of the flavonoids naringenin and daidzein in low concentrations (Gough et al. 1997). These results suggest that specific classes of flavonoids might be involved in the initial signalling for beneficial plant–bacterium interactions.

6.3 Endophytic Colonization

In the vicinity of plant roots, competent bacterial endophytes need to gear their metabolisms towards a physiological state that enables optimal nutrient acquisition, niche adaptation and competition. Indeed, several studies on gene expression in rhizobacteria have shown that the genes involved in nutrient acquisition and stress adaptation, next to activation of transcriptional regulators, are among the first responders when bacteria are exposed to root exudate compounds (Somers et al. 2004). Hence, bacterial traits involved in the response to environmental stimuli (e.g. transcriptional regulators), communication (e.g. autoinducers), niche adaptation and plant colonization are important for successful interactions with the plant, in a complex process.

6.3.1 Transcriptional Regulators

Bacterial responses to environmental cues must be in perfect synchrony with their metabolic functions, and, therefore, transcriptional regulators play important roles in bacterial fitness upon interaction with the plant. The importance of transcriptional regulators in bacteria involved in root colonization was recently demonstrated by English et al. (2010). The authors inserted a transposon upstream of the *hns* gene from *Enterobacter cloacae* UW5, which increased gene expression when the strain was exposed to canola roots. Although the levels of *hns* transcripts were only up to twofold higher, the mutant strain increased its root colonization and even outcompeted the wild-type strain in a direct competition assay. The *hns* gene encodes the small histone-like protein H-NS that binds predominantly to AT-rich sequences of DNA, regions that are commonly found in promoter sequences (English et al. 2010). Adaptation to environmental stimuli occurred within minutes in *Salmonella enterica* subsp. *enterica,* where several H-NS-dependent genes were upregulated with the increase of temperature including the flagellar/chemotaxis regulon (Ono et al. 2005).

The rice endophyte microbiome comprises a high diversity and a high abundance of transcriptional regulators, which is only exceeded by the human gut metagenome (Sessitsch et al. 2012). A subset of three transcriptional regulators (i.e. belonging to the LysR-, Crp- and IclR-families) was strongly overrepresented in the rice metagenome. The physiological responses affected by these transcriptional regulators are broad, comprising the metabolism of sugars and amino acids,

transport processes, virulence, quorum sensing, pilus synthesis and motility (Korner et al. 2003; Maddocks and Oyston 2008; Molina-Henares et al. 2006). This suggests a very high degree of plasticity of responses to varying environmental stimuli, such as those represented by plant compounds.

6.3.2 Adaptation to the Niche and Adhesion

Given the fact that plant-derived compounds are the main N- and C-sources for heterotrophic soil bacteria, rhizosphere/rhizoplane bacteria (rhizobacteria), to be successful, must rapidly adapt their metabolism to the range of available nutrients. Gene expression analyses of the root-colonizing bacterium *Pseudomonas putida* KT2440 have revealed an upregulation of genes involved in metabolism and stress adaptation in the rhizosphere of corn plants (Matilla et al. 2007). Specifically, genes involved in the uptake of 'readily available' root exudate compounds (e.g. amino acids, dipeptides and polyamines) as well as aromatic compounds (e.g. phenylacetic and/or phenylalkanoic acids, plant exopolymers β-glucosidase and urease) and those encoding responses to stress (e.g. glutathione peroxidase and fatty acid *cis-trans* isomerase) and detoxification of proteins (e.g. putative efflux transporters) were upregulated. Corroborating these results, the analysis of the rice endophyte metagenome revealed a high abundance of genes involved in transport systems, mainly ATP-binding cassette (ABC) family transporters for several amino acids or polyamines as well as genes involved in the degradation of aliphatic and aromatic compounds, when compared with other selected metagenomes (Sessitsch et al. 2012). Furthermore, the rice endophyte metagenome contained an extremely high number and diversity of genes encoding enzymes potentially involved in the detoxification of reactive oxygen species (ROS), glutathione synthases and glutathione-S-transferases (GST) (Sessitsch et al. 2012).These results suggest that bacterial endophytes might be selected by harbouring a wide range of metabolic pathways, whereas by taking up the secreted metabolite waste, they might ameliorate plant stress.

Adaptation to oxic versus anoxic conditions is often required for a bacterium to survive in the vicinity of roots, especially at plants growing in flooded ecosystems. Under flooded conditions, plants like rice form heterogeneous oxic/anoxic interfaces which might create opportunities for rhizobacteria and endophytes able to perform fermentation processes (Brune et al. 2000). Under anoxic conditions, rice is known to accumulate ethanol, lactic acid and alanine at root tissues. Ethanol is one of the major carbon sources for the endophytic bacterium *Azoarcus* sp. strain BH72, whose genome harbours ten genes encoding putative alcohol dehydrogenases (Krause et al. 2006). The secretion of phytotoxic levels of ethanol may have created a niche opportunity for *Azoarcus* to colonize rice roots. This observation corroborates the data from the rice endophyte metagenome analysis, where genes involved in fermentative abilities were overrepresented (Sessitsch et al. 2012).

Adhesion to the root is mediated by cell surface structures such as polysaccharides, pili and adhesins (Hori and Matsumoto 2010). Genome analysis of *Enterobacter* sp. strain 638, a competent bacterial endophyte of poplar, revealed the presence of many genes encoding putative proteins involved in root adhesion, including hemagglutinins, curly fibres, autotransporter adhesin (YadA), type I and IV pili, cellulose biosynthesis and capsular polysaccharides (Taghavi et al. 2010). Interestingly, a number of these genes are present in genomic islands or on plasmids, suggesting their acquisition by horizontal gene transfer. The diazotrophic *A. brasilense* strain Cd has a major outer membrane protein (MOMP) involved in early host recognition (Burdman et al. 2001). MOMPs from *A. brasilense* Cd strongly adhere to root extracts of cereal plants when compared to legumes. The authors speculated that MOMPs may act as adhesins and therefore are involved in adsorption and cell aggregation on roots of selected host. Another cell surface structure, the type IV pilus, is also involved in the establishment of the endophytic bacterium *Azoarcus* sp. BH72 on the surface of rice seedling roots (Dorr et al. 1998). The mutant strains *pilA* and *pilB* (defective in pilus formation) were impaired in their proper adherence and colonization of rice roots. The role of bacterial cell surface polysaccharides [e.g. lipopolysaccharides (LPS), exopolysaccharides (EPS), capsule and peptidoglycan] in plant colonization is currently unknown. However, many of those genes were identified in the genomes of endophytic bacteria (Krause et al. 2006; Fouts et al. 2008; Bertalan et al. 2009; Taghavi et al. 2010). It is interesting that the endophytic bacterium *Azoarcus* sp. BH72 harbours many genes involved in the synthesis of cell surface compounds, but there is no gene encoding this activity in the genome of the closely related soil isolate *Azoarcus* sp. EbN1, suggesting the role of cell surface polysaccharides in the invasion/interaction of endophytes with the plant host.

Once on the root surface, bacteria might use a different type of motility, known as twitching motility, to reach their favourite entry sites (e.g. sites of lateral root emergence, root tips and/or pathogen- or predation-induced wounds). Twitching motility is mediated by type IV pili, which extend from the poles of a bacterial cell and retract, pulling forward the cell. Endophytic colonization of rice roots by the diazotrophic *Azoarcus* sp. BH72 was completely impaired in a *pilT* mutant, defective for pilus retraction, although partial colonization (50%) was observed on the root surface (Bohm et al. 2007).

6.3.3 Colonization of Internal Plant Tissues

Endorhizal Colonization—Before entering the plant internal tissues, soil bacteria colonize the rhizodermal cells. The colonization strategy varies for each bacterium–host interaction. A recent histochemical study with three bacterial species colonizing the roots of sugar beet revealed that each strain has a distinct colonization pattern (Zachow et al. 2010). For instance, *P. fluorescens* L13-6-12 and *P. trivialis* RE1-1-14 formed microcolonies (i.e. tens to hundreds bacterial

cells), respectively, on the upper parts of the roots and in compartments between root cells, as well as upon emergence of lateral roots, whereas *Serratia plymuthica* 3Re4-18 colonized—as single cells—the entire root surface as well as internal root tissues. The authors showed that each bacterial species occupied specific niches and morphologically detectable interactions were rare. These results suggest that each bacterium has its own preferred colonization sites, which may overlap in field conditions. Hence, stacking of various facilitating bacterial traits might be important for successful colonization of sugar beet roots.

Communication via quorum sensing (QS) is one of the most important bacterial traits to coordinate population behaviour (von Bodman et al. 2003). Bacterial communication by autoinducer molecules plays an essential role in endophytic colonization. QS mutant strains of *B. kururiensis* M130, impaired to produce and respond to one type of *N*-acetyl homoserine lactone (AHL), showed decreased root and aerial rice tissue colonization when compared to the wild type (Suarez-Moreno et al. 2010). Furthermore, the beneficial effects of endophytic colonization (i.e. increases in root length and branching) were reduced in QS mutant strains. Bacterial signal molecules such as lipochito-oligosaccharides and lumichrome are potentially involved in host growth stimulation (reviewed in Mehboob et al. 2009). By using the quorum-quenching approach, Boyer et al. (2008) showed that a mutant of the rice endophyte *Azospirillum lipoferum* B518 that constitutively expressed AttM lactonase (an enzyme that hydrolyzes the lactone ring of AHLs) increased the synthesis of proteins linked to transport and chemotaxis. This suggests that QS in this strain is dedicated to regulate functions involved in root colonization. In the aforementioned rice endophyte metagenome survey, genes encoding proteins for autoinducer synthesis and detection were highly abundant, with three different autoinducer systems being identified [i.e. autoinducer-2 system (AI-2), the diffusible signal factor system (DSF) and the acylhomoserine lactone system (AHL)]. This probably reflects a need for concerted gene regulation for virulence and colonization by endophytic bacteria (Sessitsch et al. 2012).

Many bacterial pathogens and symbionts might secrete or inject proteins (called effectors) to interact with plant cells. The function of effectors secreted by symbionts is still unknown, but they often differ from those from pathogens (Deakin and Broughton 2009). In the rice endophyte metagenome, all known protein secretion systems for translocation across the cytoplasmic and outer membranes were present except for compounds of the type III secretion system (T3SS; Sessitsch et al. 2012). Striking was the high abundance of genes encoding compounds of type VI secretion systems (T6SS). T6SS is involved in a broad variety of functions, from eukaryotic host infection to biofilm formation and response to stress (Bernard et al. 2010) and might be important for the endophytic lifestyle.

Soil bacteria can enter the epidermal root tissues by two processes: passively, for instance, by penetrating sites at the junction of adjacent epidermal cells (Benhamou et al. 1996) and sites at the emergence of lateral roots (Govindarajan et al. 2008) or actively, with the production of hydrolytic enzymes (e.g. exoglucanase, endoglucanase and endopolygalacturonase) involved in plant cell-wall degradation

(Reinhold-Hurek et al. 1993; Compant et al. 2005). It has been proposed that the levels of cell-wall-degrading enzymes produced by root-colonizing bacteria differentiate endophytes (low levels) from phytopathogens (deleteriously high levels) (Elbeltagy et al. 2000). Although this assumption has not been proven, it makes sense if the invader microorganisms need to avoid triggering the plant defence system. Genes encoding plant polymer-degrading enzymes were observed in high abundance and diversity in the rice endophyte metagenome (Sessitsch et al. 2012). They may contribute to endophyte entry into and spread inside the plant tissue.

Systemic Colonization—A subset of endophytic bacteria is able to colonize the aerial parts of its host plant from the root tissue (Hardoim et al. 2008; Compant et al. 2010) and even systemically colonize stem and leaf tissues. Bacterial densities in stem and leaf tissues are considerable lower than in roots, typically 10^3–10^4 cfu g^{-1} tissue. Moreover, the endobacterial diversity is also lower, indicating the need for highly specialized adaptive traits that allow thriving in the photosynthetic tissues (Hallmann 2001). Furthermore, the endobacterial populations inhabiting the aerial parts are mostly derived from the endorhiza via systemic spread through xylem vessels or intercellular spaces of parenchymatous tissue. However, as with phytopathogenic bacteria, entry from the phyllosphere via stomata or hydathodes can also occur; this has received very little attention thus far.

6.4 Vegetative Transmission

In addition to invasion of root/shoot tissue, bacteria can also be introduced into plants via propagated vegetative material (e.g. seeds, cuttings, stems, tissue culture) and thus spread to descendent generations (Hallmann et al. 1997). Although bacteria can be absent or present in very low densities in reproductive organs (10^1–10^3 cfu g^{-1} tissue; Nissinen et al., unpublished; Compant et al. 2010), seeds from many plant hosts are seen as important vectors for endophytic dissemination (Mundt and Hinkle 1976). Vertical transmission of endophytes has been observed by isolation of bacteria from cotton and rice seedlings growing aseptically on agar medium (Adams and Kloepper 2002; Hardoim et al. 2012). Furthermore, the isolation of bacteria from surface tissue and surrounding medium of rice seedlings growing aseptically on agar medium (Kaga et al. 2009) suggested that, once seeds are germinated, bacterial endophytes may move out and even colonize the surrounding plant sites. Thus, one might speculate that seed transmission of selected endophytes may be needed for plant establishment in hostile soil. This assumption was strengthened by the isolation of bacterial endophytes that were transmitted via seeds, which subsequently were found to assist the cactus seedlings to establish and grow on barren rock (Puente et al. 2009a). The dissemination of endophytic bacteria via seeds might thus be more common than previously considered and might even pose an ecological advantage for the host as plants carrying the beneficial bacteria can thus foray adverse conditions (Lopez-Lopez et al. 2010). Further studies are needed to confirm these exciting leads and assumptions.

6.5 Plant-Beneficial Properties

Plant-growth-promoting (PGP) properties of rhizosphere bacteria have been intensively studied and are well documented (see Maheshwari et al. 2012). However, the agricultural applications of PGP rhizobacteria have often led to less than optimal results. This might be due to a recurrent inability of added PGP bacteria to thrive and compete with the native soil microbiota and successfully colonize the rhizosphere (Garbeva et al. 2004). These findings, combined with the recent discovery of the high diversity and abundance of endophytic bacteria, have tremendously increased the interest in the PGP potential of endophytic bacteria. Bacterial endophytes have been shown to enhance plant growth by improving the mobilization and uptake of nutrients, by increasing stress tolerance and growth via production or (co)regulation of phytohormones and by enhancing plant disease resistance by antagonism, competition or by inducing or priming the plant's own defence systems (Compant et al. 2010).

6.5.1 Nutrient Status

Plants acts as 'miners' of Earth's crust/soils, acquiring essential nutrients for their growth mainly through root systems. Among the essential nutrients, nitrogen and phosphorus are needed in relatively high quantities; however, the availability of these elements is often limited in soil. Bacterial endophytes might help their host plants to acquire these nutrients.

6.5.1.1 Nitrogen

Nitrogen-fixing (diazotrophic) symbionts, i.e. nodule-forming rhizobia and actinobacteria, are well known and often represent highly significant N input in their respective plant hosts, in particular in nitrogen-poor soils [reviewed by Fabra et al. (2012)]. Additionally, diazotrophic bacteria have been isolated from numerous gramineous host plants, suggesting that they actively participate in biological N_2 fixation. Significant amounts of N have been shown to be incorporated into key agronomical crops like rice, sugarcane and maize by biological N_2 fixation. Although studies have shown in vivo expression in diazotrophs of the genes encoding nitrogenase and incorporation of $^{15}N_2$ gas into the host, it is still questionable whether the incorporated N is mainly due to the death and mineralization of diazotrophs or through direct and rapid transfer, as occurs in legume nodules (James 2000). Nevertheless, selected diazotrophic bacteria such as *Burkholderia* spp., *Azoarcus* sp. BH72, *Herbaspirillum seropedicae*, *Gluconacetobacter diazotrophicus* and *Azospirillum brasilense* have been reported to significantly

increase the host biomass production under controlled conditions by N_2 fixation (Bhattacharjee et al. 2008).

Studies on the diversity and community composition of associative N-fixing bacteria are common, and practically, every phylum contains species harbouring nitrogenase. Furthermore, this enzyme is conserved through evolution with ample evidence of lateral gene transfer. Thus, as only a small fraction of soil bacteria colonize gramineous plants, particular associative diazotrophic bacteria might be considered as true and successful symbionts. Endophytic diazotrophic bacteria, particularly *Gluconacetobacter diazotrophicus*, *Burkholderia* spp. and *Herbaspirillum seropedicae*, have been extensively found, for example in Brazilian sugarcane (*Saccharum* spp.) cultivars (Baldani and Baldani 2005). Similarly, *Azoarcus* sp. BH72 might be responsible for N_2 fixation in Kallar grass and rice (Hurek and Reinhold-Hurek 2003).

6.5.1.2 Phosphate

Phosphorus is one of the major plant-growth-limiting nutrients. It is likely to become more important, as the available sources of phosphorus on Earth are getting sparse. Phosphates applied to agricultural soils are rapidly immobilized and rendered inaccessible for plants. Due to this rapid immobilization, many agricultural soils have large reservoirs of phosphates, however, in an inaccessible form (Rodriguez and Fraga 1999). Many plant-growth-promoting bacteria can solubilize inorganic phosphates by secretion of organic acids, making them accessible to host plant. Phosphate solubilization is a common trait among plant–endophytic bacteria. For instance, the majority of endophytic populations from strawberry, soybean and other legumes, sunflower and cactus (59–100%) were able to solubilize mineral phosphates in plate assays (Dias et al. 2009; Kuklinsky-Sobral et al. 2004; Palaniappan et al. 2010; Forchetti et al. 2007; Puente et al. 2009b).

A survey of bacterial endophytes from sunflowers grown in irrigated or drought regime revealed that more phosphate-solubilizing endophytic bacteria were isolated from drought-exposed plants, suggesting selection for such PGPB in stress conditions (Forchetti et al. 2007). All phosphate-solubilizing bacteria also revealed other plant-beneficial properties, including the ability to grow on nitrogen-free medium and the production of several phytohormones. In addition to environmental pressure, phosphate-solubilizing endophytes might be favourable in the active growth stages of plants. Kuklinsky-Sobral et al. (2004) analysed epi- and endophytic isolates from several growth stages and cultivars of soybean. They found that 60% of the endobacterial isolates (representing dominantly Pseudomonaceae, Burkholderiaceae and Enterobacteriaceae) from the early plant growth stages were phosphate solubilizers, compared to less than 50% of the isolates from senescent plants. The majority of the phosphate-mobilizing isolates were also able to fix nitrogen and produce indole acetic acid (IAA). Likewise, Palaniappan et al. (2010) isolated endophytic bacteria from root nodules of the fabaceous plant species *Lespedeza* and found that the majority of endophytes were able to solubilize

phosphates. The authors also found that most of the endobacterial isolates harboured multiple PGP properties (i.e. phosphate solubilization, IAA and siderophore production and ACC deaminase activity).

Some controversy has surfaced about whether phosphate solubilization per se is plant beneficial, as most PGP endophytes have multiple PGP properties, as highlighted above. However, a clear correlation between phosphate mobilization and plant growth has been shown in several studies. Dias et al. (2009) analysed endobacterial isolates from strawberry, mostly representing *Bacillus subtilis* and *B. megaterium*, that were all able to solubilize calcium phosphate in plate assays. The phosphate solubilization efficiency varied markedly between isolates. The plant-growth-promotion capacity of the isolates correlated with their phosphate solubilization activity, as well as with IAA production. Puente et al. (2009b) isolated and analysed endophytic bacteria from cardon cactus, a pioneer desert plant able to establish on solid rock. The majority of endophytes were capable of solubilizing Fe/Ca-phosphates and pulverizing rock. As these bacteria were also present in cactus seeds, from where they colonized the rhizosphere of the developing seedlings, they might have a role in desert colonization and soil formation. It should be noted that many of the phosphate-solubilizing isolates were also diazotrophic, thus providing the host plant with N next to P (Puente et al. 2009a). The endophytes were tested in pot experiments, where endophyte-free cacti growing on mineral phosphate rock were amended with endophytes or nutrients or were grown under sterile conditions. The bacterized plants grew well without nutrient addition and were comparable to fertilized plants, whereas the endophyte-free cacti failed to develop. This indicated that the endophytes were able to provide the developing plantlets with phosphate as well as nitrogen (Puente et al. 2009b).

6.5.1.3 Other Nutrients

Albeit less well studied, iron chelation (via siderophores) is a common trait in endophytic bacterial communities. For instance, the rice endophyte metagenome revealed a high number of genes encoding proteins potentially involved in siderophore biosynthesis, ferric-siderophore membrane receptors, iron uptake transporters and storage proteins (Sessitsch et al. 2012). As iron is fiercely competed for in soil as well as within eukaryotic host tissues, iron-chelating bacteria can deprive putative pathogens of available iron, therefore exerting antagonistic activity.

As bacteria mobilize mineral phosphates by secretion of organic acids, they are likely also able to mobilize other mineral nutrients. *Gluconacetobacter diazotrophicus* PAl5 is a PGPB with many PGP properties, and it is able to solubilize zinc from zinc oxides and phosphates, in addition to calcium phosphates (Saravanan et al. 2007). The zinc mobilization activity is dependent on carbon availability for *G. diazotrophicus* PAl5. However, mobilization of other mineral nutrients than phosphorus by endophytes has been very little screened for.

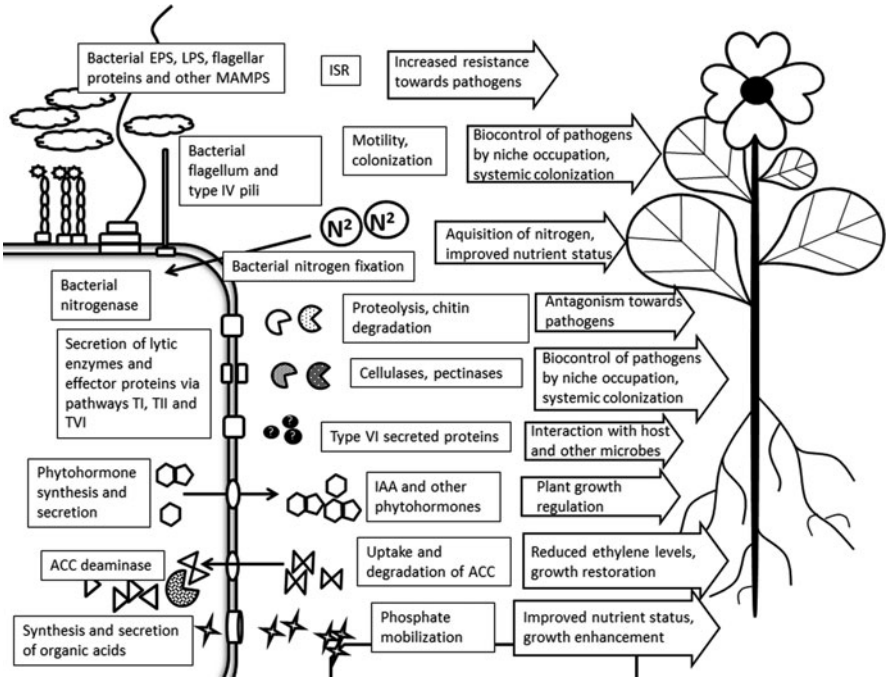

Fig. 6.2 Endophytic bacterial properties and plant-beneficial effects (*black arrows*), based on current knowledge. *EPS* extracellular polysaccharide, *LPS* lipopolysaccharides, *MAMP* microbe associated molecular pattern, *IRS* induced systemic resistance, *TI* type 1 protein secretion system, *TII* type 2 protein secretion system, *TVI* type 6 protein secretion system, *IAA* indole acetic acid, *ACC* 1-aminocyclopropane-1-carboxylate

6.5.2 Plant Growth Enhancement

In addition to improving the plant nutrient status, endophytic bacteria might stimulate plant growth by directly producing phytohormones, other growth regulators (e.g. lipochito-oligosaccharides and lumichrome; reviewed in Mehboob et al. 2009) and enhancing host anabolism (e.g. photosynthesis ability), or by regulating plant phytohormone levels (Fig. 6.2).

6.5.2.1 Production of IAA and Other Hormones

Auxins, of which IAA is most common, are phytohormones necessary for plant growth and morphological development, including cell elongation, maintenance of apical dominance, formation of vascular tissues, cell elongation and prevention of senescence. Auxins also counteract root apical dominance by cytokinins and promote the formation of lateral roots and the root system. Further, IAA prevents

the formation of ethylene in low concentrations, but stimulates ethylene synthesis in high concentrations (Woodward and Bartel 2005).

IAA production is a common trait among endophytic bacteria, and IAA-producing endophytes representing a vast range of bacterial phyla/classes have been isolated from multiple plants, including poplar, soybean, epiphytic and terrestrial orchids, cactus, potato and strawberry. IAA production by endophytic bacteria has been associated with the promotion of plant root growth, enhanced production of lateral roots and increases in root volume and biomass (Taghavi et al. 2009; Kuklinsky-Sobral et al. 2004; Tsavkelova et al. 2007; Dias et al. 2009). IAA-producing bacteria are commonly isolated from both the rhizo- as well as the endosphere. Tsavkelova et al. (2007) isolated and analysed endophytic and rhizoplane bacteria from epiphytic as well as terrestrial orchids. The endobacterial isolates, representing the genera *Erwinia*, *Bacillus*, *Pseudomonas* and *Flavobacterium*, all produced IAA. Further, on average, the endobacterial communities yielded more efficient IAA producers (as measured in cultures) than rhizoplane ones. When tested on kidney beans, supernatants from endophyte cultures significantly stimulated root formation and resulted in increases in root length as well as the number of developing roots, indicating the potential role of endobacterial auxins in root development (Tsavkelova et al. 2007).

As described in Sect. 6.5.1.2, production of IAA in PGPB is often associated with other beneficial properties. Thus, the role of IAA production has rarely been directly proven. The promotion of root growth and lateral root formation by plant-beneficial *Pseudomonas putida* GR12-2 was shown to be dependent on the presence of a functional IAA biosynthesis pathway, as plant-growth-promotion potential was lost in a *P. putida* GR12-2 IAA synthesis mutant (Patten and Glick 2002).

6.5.2.2 Enhancement of Photosynthetic Activity

Bacterial endophytes can actively alter the physiology of the host plant. Introduction of different rhizobial species, *A. caulinodans* ORS 571, *Sinorhizobium meliloti* 1021 and *Mesorhizobium huakui* 93, enhanced rice growth by stimulating photosynthetic activity and enhancing resistance to drought (Chi et al. 2005). Further studies revealed that *S. meliloti* 1021 induced the production of photosynthesis-related proteins in rice plants. Using a proteomic approach, Chi et al. (2010) showed that proteins related to Rubisco activase, pyruvate orthophosphate dikinase (catalyses the production of PEP and involved in the light and dark reactions), transport of nuclear-encoded proteins and nutrients to the chloroplast, were upregulated in the presence of the endophyte.

The promotion of photosynthetic capacity is not limited to rice/rhizobia associations. Introduction of three endophytic bacteria, i.e. *Bacillus pumilus* 2-1, *Chryseobacterium indologenes* 2-2 and *Acinetobacter johnsonii* 3-1, in sugar beet increased the plant chlorophyll content, leading to an enhanced carbohydrate synthesis when compared with uninoculated plants (Shi et al. 2010). The authors

speculated that the production of unidentified compounds by the endophytes might have led to an enhancement of electron transport and, consequently, promotion of chloroplast metabolism.

6.5.2.3 Regulation of Ethylene Levels by ACC Deaminase-Producing Bacteria

Ethylene is a highly versatile plant hormone, which is involved, for example in seed germination, fruit ripening, formation of mature xylem vessels and root hairs as well as in senescence of flowers and leaves. In plants, ethylene is synthesized from methionine via a two-step pathway. The immediate precursor is a non-protein amino acid 1-aminocyclopropane-1-carboxylic acid (ACC), which is oxidized to ethylene. Synthesis of ACC and ethylene is induced by several abiotic and biotic stressors, including flooding or drought, pathogen attack or wounding. Additionally, ethylene synthesis is induced by auxins, especially IAA and by cytokinins and inhibited by ABA. Ethylene has pleiotropic effects, and the response to ethylene is dependent on the type of plant tissue, its growth state and physiological environment. However, excessive production of ethylene associated with the stress response also inhibits root elongation and growth. Additionally, ethylene has been shown to direct auxin transport and signalling (Strader et al. 2010).

A group of plant-associated bacteria is able to degrade the ethylene precursor ACC by (bacterially encoded) ACC deaminase and utilize the end products as carbon and nitrogen sources. Hence, this forms an efficient sink for ACC. Concomitantly, these bacteria lower the ethylene levels in colonized plant tissue and restore plant growth under stressful conditions (reviewed in Glick et al. 2007). Production of ACC deaminase and associated plant growth promotion by root elongation and increase of plant biomass has been reported for numerous endophytic species, including many *Burkholderia phytofirmans* and *B. cepacia* isolates, *Methylobacterium fujisawaense* as well as for *Pseudomonas, Arthrobacter* and *Bacillus* spp. (refer to Nadeem et al. 2010 for extensive list of PGPB with ACC deaminase activity).

Abiotic Stress—Environmental stressors, such as soil salinity, extremely high or low temperature, freezing, drought, flooding or anoxia, often inhibit plant growth either directly by interfering with normal plant functioning or indirectly by the synthesis of excess stress-related ethylene and subsequent growth inhibition. Soil salinity is the major abiotic stressor in plants, being around 20% of the world's cultivated lands salt-affected. High concentrations of salts cause ion imbalances, leading to hyperosmotic stress in plants. Another stressor, low temperature (i.e. just above freezing), causes chilling stress in many tropical or sub-tropical plants. Chilling injuries, including retarded growth, leaf lesions and wilting, are often caused by loss of cell membrane functionality ensuing from changes in membrane fluidity. Salt and cold stresses are closely related to other abiotic stresses and associated with, for example elevated ethylene levels and stunted growth.

Numerous studies link the beneficial effects of inoculation with ACC deaminase-producing endophytic bacteria with increased stress tolerance and

growth in suboptimal conditions [for rhizosphere bacteria beneficial effects, see Goel and Rani (2012)]. The inoculation of tomato, cotton, groundnut, canola, maize and wheat with the ACC deaminase-producing bacteria *Achromobacter piechaudii* AVR8, *Klebsiella oxytoca* Rs-5, *Serratia proteamaculans* M35, *Enterobacter cloacae* CAL2 and *Pseudomonas* spp. increased host biomass production, lowered Na^+ and enhanced K^+ cell content compared to uninoculated plants (reviewed in Nadeem et al. 2010). *Burkholderia phytofirmans* PsJN is an intensively studied endophyte that has been associated with growth promotion and enhanced stress tolerance in several plant species, including potato, vegetables and grapevine (reviewed in Sessitsch et al. 2005). *B. phytofirmans* PsJN has ACC deaminase activity, and the plant growth enhancement under environmental stress has been postulated to be associated with ACC deaminase production by the bacterium. PsJN-inoculated grapevines showed a tenfold increase in root growth at both 26°C and 4°C. The enhanced growth was associated with an increase in plant photosynthetic capacity and starch content, as well as proline and phenolic contents in plant cells. This indicated enhanced cold tolerance of plants by PsJN inoculation (Ait Barka et al. 2006). An *acdS* mutant of *B. phytofirmans* PsJN, which was deficient in ACC deaminase activity, also lost its ability to promote root elongation in canola seedlings. Curiously, this ACC deaminase mutant also synthesized a decreased level of siderophores and increased amounts of IAA, which were suggested to result from increased levels of stationary phase sigma factor RpoS (Sun et al. 2009). This indicated co-regulation of ACC deaminase synthesis, siderophore and IAA production. Complementation with functional *acdS* restored both the ACC deaminase production and root elongation capacity, offering direct proof of the role of bacterial ACC degradation in plant growth enhancement. Curiously, complementation (in *trans*) did not reverse the IAA and siderophore phenotypes (Sun et al. 2009).

Inoculation of the alpine plant species *Chorispora bungeana* with endophytic *Clavibacter* sp. Enf12 isolated from the same plant growing under snow enhanced plant growth both at 20°C and 0°C. It also significantly attenuated the production of (ROS), oxidative damage and electrolyte leakage. Inoculation also led to elevated levels of antioxidant enzymes and proline, indicating improved control of oxidative damage and increased hardiness (Ding et al. 2011). Similarly, a cold-tolerant *Serratia marcescens* SRM isolate from summer squash significantly enhanced biomass and nutrient uptake in wheat seedlings under cold conditions. *S. marcescens* has several PGP capacities, including IAA production and phosphate solubilization, and these activities are retained at 4°C (Selvakumar et al. 2008).

Moreover, in addition to ACC deaminase and ethylene levels, other endobacterial factors are likely to play roles in plant stress tolerance and growth. Sziderics et al. (2007) studied the effect of five ACC deaminase-producing endophytes on the adaptation to abiotic stress by pepper (*Capsicum annuum*). Under moderate stress, four of the five isolates increased plant biomass. *Microbacterium* sp. EZB22, the only studied strain devoid of IAA production, failed to promote growth, despite its ACC deaminase activity, indicating that

growth enhancement is likely due to several bacterial PGPs. *Bacillus* sp. EZB8 and *Arthrobacter* sp. EZB4 were able to attenuate the induction of several stress-related genes in pepper, indicating reduced stress (Sziderics et al. 2007).

6.5.3 Resistance to Heavy Metals and Other Toxic Compounds

A new field has recently emerged, focusing on the role of the plant–endophyte partnership in the remediation of heavy metal contaminated soils. Endophytes offer several advantages to rhizobacteria: they are better maintained (less competition), they occur in a pollution gradient (plant accumulation, harvest possible), and they offer a more specific relationship with host. Therefore, heavy metal-resistant bacterial endophytes might have the ability to accumulate and/or sequester heavy metals. Furthermore, such endophytes with appropriate degradation pathways and metabolic capabilities might improve the degradation of organic contaminants and reduce phytotoxicity. Lastly, stress-ameliorating endophytes might assist their hosts to overcome contaminant-induced stress responses, and plant-growth-promoting endophytes might improve plant growth and thus contaminant extraction from soil or water (Weyens et al. 2009).

6.5.3.1 Heavy Metals

The assessment of the culturable bacterial community from the Ni hyperaccumulator *Thlaspi goesingense* revealed that the endophytic community tolerated high levels of Ni, and many endophytic strains were able to grow on ACC as sole N source when compared to those isolated from the rhizosphere (Idris et al. 2004). Sun et al. (2010) revealed that the beneficial effect of endophytic bacteria from one host plant species could be applied to another plant species. The Cu-resistant strains *Bacillus megaterium* JL35 (isolated from *Elsholtzia splendens), Sphingomonas* sp. YM22 and *Herbaspirillum* sp. YM23 (both isolated from *Commelina communis)* increased the root dry weight by 132–155% and the aboveground tissue Cu content by 63–125% when introduced onto rape (*Brassica napus*) growing in Cu-contaminated substrate. Many endophytic bacteria that are resistant to one metal show resistance to other metals as well (Kabagale et al. 2010). Hence, they might be used to improve phytoextraction in sites contaminated with multiple metals.

6.5.3.2 Organic Pollutants

Bacteria have two major assets that make them suitable to combine them with plants in cases of organic pollutant removal: (1) heterotrophic bacteria rely on organic compounds as carbon sources, and hence, they often show a great diversity

of metabolic pathways to attain their nutrition; and (2) the terminal products of their organic compound metabolism are often CO_2, H_2O and cellular biomass. On the other hand, metabolism of organic compounds by plants consists of a general transformation of more soluble forms and sequestration (Weyens et al. 2009). The inoculation of poplar with endophytic *Burkholderia cepacia* VM1468 containing the plasmid pTOM-Bu61, coding for constitutively expressed toluene degradation, revealed positive effects on plant growth in the presence of toluene and reduced the amount of toluene released via evapotranspiration when compared with poplar inoculated with the soil bacterium *B. cepacia* Bu61 (pTOM-Bu61) or uninoculated plants (Taghavi et al. 2005). Similar results were observed in lupine inoculated with *B. cepacia* L.S.2.4 (pTOM-Bu61), a natural endophyte of yellow lupine (Barac et al. 2004). These results suggest that engineering of endophytic bacteria can be a promising technique to improve phytoremediation of soils contaminated with organic pollutants. Furthermore, pea (*Pisum sativum*) plants inoculated with the endophyte *P. putida* VM1450, a bacterium possessing the metabolic pathway to degrade 2,4-dichlorophenoxyacetic acid (2,4-D), showed higher capacity for 2,4-D removal from soil than uninoculated plants (Germaine et al. 2006). It is interesting that 2,4-D is a selective systemic herbicide for the control of broad-leaved weeds and that pea inoculated with *P. putida* VM1450 showed no 2,4-D accumulation in the aerial tissues. This suggested that the bacterium might help its host by the rapid uptake and degradation of the hazardous compound.

6.5.4 Disease Resistance

Endophytic bacteria can protect their host plants from harmful microbes and pests directly by antagonism or competition for the niche (i.e. space and nutrients) or indirectly by upregulating or inducing/priming the plant defence system to respond faster and more efficiently towards invading pathogens.

6.5.4.1 Antagonism Against Fungi, Nematodes and Phytopathogenic Bacteria

Direct antagonism towards pathogens can be attained by the production of antifungal substances or fungal growth inhibitors and by antibiotics or other antibacterial metabolites. A wide variety of endophytic bacteria with antagonistic activity against fungal, bacterial and oomycete pathogens have been reported (reviewed in Lodewyckx et al. 2002). *Pseudomonas*, *Bacillus* and *Paenibacillus* spp. and strains of actinobacteria are the most commonly reported species studied as antagonistic against fungal or oomycete pathogens. Some have been successfully tested with respect to disease suppression in a wide diversity of plants, for example wheat,

potato, black pepper and ginseng (Coombs et al. 2004; Sessitsch et al. 2004; Berg et al. 2005; Aravind et al. 2009; Cho et al. 2007).

Actinobacteria are known for their production of a wide array of secondary metabolites. Coombs et al. (2004) screened 38 actinobacterial strains isolated from wheat, representing *Streptomyces*, *Microbispora*, *Micromonospora* and *Nocardioides,* for their antifungal potential against *Rhizoctonia solani*, *Pythium* sp. and *Gaeumannomyces graminis* var. *tritici* (the causal agent of take-all disease in wheat) both in vitro and by bioassays. The analyses revealed that 64% of the strains had antifungal properties in vitro assays, and 17 strains were efficient in planta (in steamed soil) against take-all disease. The active isolates were also effective under field conditions in the biocontrol against take-all as well as *Rhizoctonia* (Coombs et al. 2004).

In contrast to the high proportion of antifungal isolates in actinobacterial endophytes, Sessitsch et al. (2004) found that only 0–11% of potato endobacteria possessed activity against three fungal pathogens and the oomycete *Phytophthora cactorum*. The majority of the isolates, however, were effective antagonists against *Streptomyces scabies* and other bacterial pathogens. It is likely that potato scab affecting the potatoes in this study selected for the antagonistic endobacteria. The isolates showing antagonism against fungal as well as bacterial pathogens were from the genera *Pseudomonas*, *Paenibacillus* and *Clavibacter* (an actinobacterium).

Screening of endophytic bacteria from black pepper against *Phytophthora capsici* with three independent methods identified 14–16 antagonistic isolates based on mycelial growth inhibition on agar plate assays, lesion inhibition in cut shoot assay and foot rot suppression in microcosm assays. Three isolates with the best *Phytophthora* antagonistic capacity achieved over 70% disease suppression in greenhouse trials. *P. aeruginosa*, *P. putida* and *Bacillus megaterium* were identified as effective antagonistic endophytes for the control of *Phytophthora* foot rot in black pepper. However, although the disease suppression ability was clearly dependent on the antagonistic capacity against the causal oomycete, disease suppression rates were also dependent on the pepper cultivar (Aravind et al. 2009).

Cho et al. (2007) analysed the antifungal activity of 63 endophyte isolates from ginseng against *Rhizoctonia solani*, *Fusarium oxysporum*, *Pythium ultimum* and *P. capsici*. About 50% of the isolates were antagonistic against 2–4 pathogens, and three isolates (*Bacillus* sp., *Paenibacillus polymyxa* and *Pseudomonas poae*) had broad-spectrum antifungal activity and were antagonistic against all tested pathogens.

6.5.4.2 Induced Defences and Priming

Plants have a set of nonspecific defence mechanisms to protect them against bacterial, viral and fungal pathogens. Systemic acquired resistance (SAR) is induced by (local) exposure to pathogens. Once induced, SAR is active against a broad range of pathogens. SAR is dependent on salicylic acid (SA) as a signal molecule and is characterized by increased levels of SA and systemic induction of a

set of pathogenesis-related (PR) proteins, the best known being PR-1, PR-2 and PR-5 (Hammerschmidt 2009). SAR is effective against a broad range of biotrophic or hemibiotrophic pathogens, but is not as effective towards necrotizing pathogens. In contrast, the jasmonic acid/ethylene (JA/ET)-dependent defence pathway is effective against a broad spectrum of pathogens, including necrogenic fungi. It is associated with the systemic upregulation of PR proteins PR-3, PR-4, PDF1.2, chitinases, chitin-binding proteins and defencins (Ellis and Turner 2001). In addition to the defence reaction, ET and JA are involved in plant development.

Non-pathogenic bacteria have been long known to induce systemic resistance in plants, which is referred to as induced systemic resistance (ISR). ISR can be SA independent and dependent, and it is partially overlapping with the JA/ET pathway (e.g. van der Ent et al. 2009). ISR is effective not only against fungal but also against bacterial pathogens. Unlike the SAR- or JA/ET-dependent defence pathway, ISR activation does not lead to a massive upregulation of defence network. When Verhagen et al. (2004) screened—by microarray analysis—ISR-induced genes upon treatment of *A. thaliana* by ISR-inducing *P. fluorescens* WCS417r, they found 97 upregulated genes in roots, but no differential regulation in shoots. However, upon subsequent challenge of the plant by plant-pathogenic *P. syringae* pv *tomato*, 81 genes were found to be upregulated in shoots in plants pretreated with *P. fluorescens* WCS417r. Thus, the plants were primed to respond to pathogen attack by ISR (Verhagen et al. 2004).

ISR seems to involve both the SA and JA/ET pathways, as Niu et al. (2011) showed that *Bacillus cereus* AR156 triggered ISR in *Arabidopsis* by simultaneously activating the SA- and JA/ET-signalling pathways and associated marker genes, leading to an additive effect on the level of induced protection (for an extensive treatise of plant responses upon inoculation of rhizobacteria, see Touraine et al. 2012). Similarly, Conn et al. (2008) showed that inoculation of *A. thaliana* with endophytic actinobacteria resulted in a moderate upregulation of both defence pathways and protected the plants against subsequent challenge posed by inoculation with necrotrophic bacterial (*Erwinia carotovora*, Ecc) or fungal (*Fusarium oxysporum*) pathogens. Although endophyte treatment increased the resistance against both pathogen types, the primed defence pathways differed. Resistance towards Ecc required the JA/ET pathway, whereas resistance towards *F. oxysporum* was dependent upon SAR. Thus, endophytic bacteria were able to prime both pathways and confer resistance (Conn et al. 2008). Significantly, different *Streptomyces* strains, which (based on 16S sequence and morphology) were closely related, induced and primed different pathways as follows: *Streptomyces* sp. EN27 primarily activated the SA-dependent pathway, whereas *Streptomyces* sp. EN28 resulted in enhanced induction of the JA/ET pathway. This might be due to different secondary product profiles of these two organisms. Endophytic *Streptomyces* spp. upregulated, albeit moderately, the respective defence pathways upon ISR induction, unlike rhizobacteria. However, as one of the studied isolates, *Micromonospora* sp. EN43 did not induce PR genes, but was still able to prime *A. thaliana* upon challenge inoculation, the defence gene induction was not necessary for SR. Rather, the authors speculated that the moderate PR activation

observed was due to the *Streptomyces* spp. being detected by plants as minor pathogens. Similar observations were reported in interactions between the endophytic *Arthrobacter* sp. EZB4 and *Bacillus* sp. EZB8 and pepper, where endophyte inoculation resulted in increased proline levels indicating biotic stress. However, despite the initial (mild) stress, these endophytes increased plant biomass and protected them against abiotic stress (Sziderics et al. 2007).

At the cellular level, ISR induction has been studied by Benhamou et al. (2000), who evaluated the effects of ISR induced by endophytic *Serratia plymuthica* strain R1GC4 in cucumber (*Cucumis sativus*) seedlings against *Pythium ultimum*. Seedling treatment with *S. plymuthica* resulted in decreased disease development. Moreover, in endophyte-inoculated plants, fungal colonization was limited to the outermost root layer, and deposition of enlarged callose-enriched wall appositions was visible at sites of potential pathogen penetration. Fungal hyphae surrounded by plant-derived deposits were partially disorganized and sometimes disintegrated.

Although most PGP bacteria possess multiple PGP properties, and have simultaneous potential to enhance plant growth and incite disease resistance, the interactions seem to be bacterium/plant specific and complex. For instance, Pavlo et al. (2011) tested plant growth enhancement and defence induction towards bacterial pathogens by two potato endophytes: *Pseudomonas putida* strain IMBG294 and *Methylobacterium* sp. strain IMBG290. *P. putida* was able to protect potato against *Pectobacterium atrosepticum* and also enhanced shoot growth, but *Methylobacterium* sp. was effective in biocontrol only at an inoculum density of 10^5 cfu ml^{-1}, whereas higher inoculum levels led to ineffective disease control or even disease enhancement. In contrast, enhancement of potato shoot growth was only achieved by inoculum densities of 10^6 cfu ml^{-1}. Shi et al. (2011) evaluated *P. putida* MGY2 against papaya anthracnose, a postharvest disease caused by *Colletotrichum gloeosporioides*. They showed that MGY2-treated papaya fruits had lower disease index, lower disease incidence and lower lesion diameter. The disease suppression was associated with less softening and lowered ethylene production in papaya, making the fruit less vulnerable to infection. Additionally, MGY2-treated fruits had increased phenolics and PAL levels compared to the control, indicating activation of the defence pathway by *P. putida* MGY2 (Shi et al. 2011).

6.6 Synergistic Interactions

Recent studies have revealed that bacterial endophytes might synergistically interact with their hosts improving plant growth. Such endophytes might capture cell-secreted metabolites and other phytotoxic compounds as energy sources and thus ameliorate environmentally induced stresses. The uptake of plant carbohydrates might also trigger the production of phytohormones in endophytic bacteria. Thus, this two-sided sword-inciting double fitness might confer advantages to both partners. For example, in the case of ACC deaminase-producing bacteria

(see Sect. 6.5.2.3), intercellular ACC is sequestered and degraded by the bacterial cells to supply these with nitrogen (ammonia) and energy (α-ketobutyrate), without disturbing the nutritional balance of the plant (Glick et al. 2007). Furthermore, by removing ACC, the bacteria reduce the deleterious effect of excess ethylene, ameliorating plant stress and promoting plant growth. Thus, in this case, both the plant and the bacterium benefit from the process, awarding double fitness under adverse conditions (Hardoim et al. 2008). In the genome analysis of the endophytic bacterium *Enterobacter* sp. 638, a region was identified that encodes the uptake and metabolism of sucrose and the synthesis of VOCs (i.e. acetoin and 2,3-butanediol) (Taghavi et al. 2010). Acetoin and 2,3- butanediol are phytohormones involved in plant growth promotion and ISR (Ryu et al. 2003, 2004). In *Enterobacter* sp. 638, the production of acetoin and 2,3-butanediol was dependent on the presence of sucrose in the growth medium or poplar leaf extracts, but not on lactate as the sole carbon source. Furthermore, the transcription of genes involved in the synthesis of acetoin and 2,3-butanediol was induced by the uptake of sucrose. Therefore, the authors suggested that the uptake of sucrose, a major photosynthate in poplar trees, by *Enterobacter* sp. 638, triggers the production of the phytohormones acetoin and 2,3-butanediol promoting plant growth. These results gracefully demonstrate the synergistic interaction between some metabolites of the host plant and the activity of endophytic bacteria.

6.7 Concluding Remarks and Outlook

6.7.1 Gaps in Our Fundamental Knowledge and Future Prospects

As highlighted above, the currently emerging understanding of the mechanistic aspects of endophytic bacteria acting as beneficial partners of host plants has great potential to aid in designing strategies to substantially improve the growth and health of host plants. This is especially true when the latter have to develop under stressful conditions. Hence, associations of plants with beneficial endophytes can be seen as furnishing highly valuable additions to the 'toolbox' of sustainable agriculture. However, at the same time, the emerging research data have shown the glimpses of the extreme complexity and unicity of the interactions between any endophytic bacterium and its host plant. In particular, the co-regulation between endo- and rhizospheric bacteria and fungi, their common host plant and the environmental conditions is complex and as yet poorly understood. In the light of the current research findings, it seems that there are no simple 'key traits' that make up a successful and host-benefiting endobacterium. Rather, the beneficial effect of endobacterial presence may be the result of many properties that are compatible with, and complementary to, the host plant genotype and phenotype. Moreover, it appears that each combination of beneficial bacterium and host plant is unique, and there are no simple rules that govern its functioning.

Moreover, it appears that our current knowledge on the ecology of bacterial endophytes has strong overlaps with our understanding of prominent rhizobacteria as well as bacterial phytopathogens. Even though we did make great progress in the past two decades in describing the diversities and community compositions of bacterial endophytes across plants and even identified a range of beneficial properties that likely play roles, there are limited studies that precisely elucidate the mechanisms involved in plant–endophyte interactions. In addition, the intricacies of the 1:1 or even tripartite interactions, and the dynamics therein, are just beginning to be understood. Different approaches have been applied. For example, proteomics (Chi et al. 2010; Miche et al. 2006), transcriptomics (Rocha et al. 2007; Wang et al. 2005), metagenomics (Sessitsch et al. 2012) as well as metabolomics (Scherling et al. 2009) have been unleashed to study the dynamics of the plant–endophytic communities and their interactions.

On the positive side, several bacterial endophyte genomes have been sequenced, and this novel information will greatly help us to unravel the mechanisms of the plant–endophyte interactions. At present, in-depth studies on how bacteria become endophytic, where they reside and how they interact with the host plant are sparse, and yet they are needed to improve our knowledge and ultimately exploit it in our quest to meet the increasing food demand in a sustainable agriculture.

6.7.2 Applied Aspects of Plant-Beneficial Bacterial Endophytes

It has become clear from the aforegoing material that many factors affect the beneficial properties of endophytes. The beneficial properties are often highly bacterium and host plant specific, which suggests that rewarding effects seen on one host plant cannot be easily extrapolated to any other host. Long et al. (2008) isolated and characterized bacterial endophytes from *Solanum nigrum* and tested the plant-growth-promoting potential of the isolates on *S. nigrum* and the closely related species *N. attenuata*. The majority of the isolates that were able to promote the growth of *S. nigrum* produced either IAA or ACC deaminase. However, none of the ACC deaminase-producing strains were not able to enhance root growth in *N. attenuata*. Moreover, the introduction of another organisms, *Azoarcus* sp BH72, slightly induced the JA defence response (two proteins were upregulated) in one of two sister lineages of rice, while a sturdy JA defence response was observed on the second rice lineage (Miche et al. 2006). These results suggest an involvement of the plant genome in compatible endophyte-host interactions. Furthermore, the expression profiles induced by potentially beneficial endophytic bacteria are affected by other (microbial) residents of host plants as well (Ait Barka et al. 2000) and likely also by plant health status. Concerning the latter, Sessitsch et al. (2004) reported that the community structures of bacterial endophytes were different in potato plants performing either well or poorly in the field. Furthermore, bacterial species richness was higher in the better performing plants, either because plant performance was related to the presence and activity of beneficial endophytes or because

the conditions in the poorly performing plants were less favourable for sustaining an endophytic bacterial community.

It thus appears that, for practical purposes, we cannot easily extrapolate results obtained with one bacterium/plant system to any other system. Each such system should therefore be regarded as unique, and this goes down as far as the strain and cultivar levels. Moreover, the microbial status of the host plant, and thus the soil in which this plant is grown, plays another key role in determining the plant –endophyte status and the magnitude of the beneficial effect that is achieved. Finally, to be successful, colonization of the plant by the beneficial endophyte should follow a predictable and regular pattern, which is associated with the beneficial effect seen. In the light of the often unpredictable influences from, for example weather or climate, this issue may turn out to pose the greatest challenge to the successful use of beneficial inoculants meant to act as robust endophytes.

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Chapter 7
Strategies for the Exploration and Development of Biofertilizer

Chiu-Chung Young, Fo-Ting Shen, and Sonu Singh

7.1 Introduction

7.1.1 Plant-Growth-Promoting Rhizobacteria

Microorganisms are able to promote the growth of crops either directly stimulating plant growth or indirectly by protecting plants against pathogens. Examples of direct plant growth promotion are (1) biofertilization, i.e., the generation of nutrients to be used by the plant, such as nitrogen and phosphorus; (2) stimulation of root growth, e.g., by the hormone auxin, by bacterial volatiles, or by pyrrolquinoline quinine; (3) rhizoremediation, i.e., the degradation of soil pollutants by rhizobacteria which use nutrients secreted by the root, so-called root exudates, for their reproduction; and (4) plant stress control (Lugtenberg and Kamilova 2009). The indirect plant growth promotion or biological control by plant-growth-promoting rhizobacteria (PGPR) includes antibiosis, induction of systemic resistance (ISR), and competition for nutrients and niches. PGPR must be rhizospheric competent, able to survive and colonize in the rhizospheric soil (Cattelan et al. 1999). The bacterial surface plays an important role in the establishment of the bacteria–plant association as well as in the bacterial aggregation. Data suggesting the involvement of extracellular polysaccharides and proteins in these phenomena have been published (Burdman et al. 2000).

C.-C. Young (✉) • F.-T. Shen • S. Singh
Department of Soil and Environmental Sciences, College of Agriculture and Natural Resources, National Chung Hsing University, Taichung, Taiwan, Republic of China
e-mail: ccyoung@mail.nchu.edu.tw

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7.1.2 *Biofertilizers*

Microbial fertilizer includes active microorganisms or dormant spores. Bacteria, actinomycetes, fungi or algae, and their metabolites which provide nutrient or increase the availability of the nutrient are microbial fertilizers (Lifshitz et al. 1986; Nelson et al. 1984; Young 1990; Young et al. 1986, 1988b). The direct benefits from the microbial fertilizers are the fixation of nitrogen from the atmosphere and solubilization of mineral phosphate or potassium silicate. The increase of available nitrogen and phosphorus contributes directly to the promotion of plant growth. The symbiotic nitrogen fixation in the nodules of legumes is mainly achieved by several rhizobial members, which include *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Azorhizobium*, *Allorhizobium*, and *Burkholderia* species (Hung et al. 2005; Moreira et al. 1998; Moulin et al. 2001, Young and Chao 1989; Young and Cheng 1998). The free-living bacteria of the genus *Azospirillum* live in close association with plant roots and fix nitrogen representing one of the best-characterized PGPR (Burdman et al. 2000). Other bacteria which can serve as microbial fertilizers include members belonging to *Bacillus*, *Enterobacter*, *Frankia*, *Paenibacillus*, *Pseudomonas*, and *Serratia* (Beneduzi et al. 2010; Chen et al. 2006; Dey et al. 2004; Fabri et al. 1996; Mehnaz et al. 2001; Rekha et al. 2007).

Besides the biofertilizers originated from the microbial sources, the seaweed and earthworm can also serve as biofertilizers to provide the nutrient for the plant growth. An integrated method for the production of carrageenan and liquid fertilizer from fresh seaweeds has been patented by Eswaran et al. (2005). The earthworms have been shown to increase the rate of aerobic decomposition and composting of organic matter and also stabilize the organic residues in the sludge—removing the harmful pathogens (by devouring them and also by discharge of antibacterial coelomic fluid) and heavy metals (by bioaccumulation) (Sinha et al. 2009). The mineralization of the essential nutrients such as nitrogen, phosphorus, and potassium from the sludge enables its use as a nutritive organic fertilizer. The process for treatment of organic wastes in the presence of a geophagus earthworm *Pheretima* elongate culture was also proposed (Shankar et al. 2005). The organic wastes were converted into biofertilizers such as soil conditioning agents of fertilizer grade, culture grade, and soil grade that were provided.

7.2 Mechanisms of Plant Growth Promotion by PGPR

PGPR are free-living soil bacteria that are actually divided into three functional groups: plant-growth-promoting bacteria (PGPB), biocontrol-PGPB proposed by Bashan and Holguin (1998), and plant stress homeoregulating bacteria (PSHB) proposed by Cassán et al. (2009) that can either directly or indirectly facilitate the plant growth in optimal, biotic, or abiotic stress conditions. Indirect

plant growth promotion induced by biocontrol-PGPB includes a variety of mechanisms by which the bacteria prevent the phytopathogen deleterious effect on plant growth or development. Direct promotion induced by PGPB may include the plant provision with: fixed nitrogen; phytohormones, such as indole-3-acetic acid (IAA), gibberellic acid (GA), and cytokinin such as zeatin (Z); iron, sequestered by bacterial siderophores (Glick et al. 1999), and soluble phosphate (de Bashan and Bashan 2004). Direct stimulation induced by PSHB may include providing plants with stress-related phytohormones, like abscisic acid (Cohen et al. 2008); plant growth regulators, like cadaverine (Cassán et al. 2009); and catabolism of some molecule related with stress signaling such as bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This enzyme reduces plant ethylene level, which is increased by various unfavorable conditions and thus confers resistance to stress (Glick et al. 1999). Some of these mechanisms were briefly described and listed below:

7.2.1 Nitrogen Fixation (PGPB)

The symbiotic nitrogen fixation between Rhizobia and their legume hosts has been widely studied (Hung et al. 2005; Young et al. 1988a). Other free-living diazotrophs such as members that belong to *Azospirillum*, *Acetobacter*, *Herbaspirillum*, *Azoarcus*, and *Azotobacter* are repeatedly detected in association with plant roots (Steenhoudt and Vanderleyden 2000; Young et al. 2008b; Lin et al. 2009).

7.2.2 Phosphate Solubilization (PGPB)

Generally, soils are poor in the available P, and a large quantity of soluble forms of P fertilizer is applied to enhance plant productivity (Park et al. 2009). The easy precipitation of applied P fertilizers into insoluble forms such as $CaHPO_4$, $Ca_3(PO_4)_2$, $FeSO_4$, and $AlPO_4$ leads to an excess application of P fertilizer to crop land (Omar 1998). Applying the P-solubilizing microorganisms to soil as biofertilizer may reduce the above-mentioned problems by solubilizing the precipitated P (Chen et al. 2006; Lin et al. 2006; Nahas 1996).

7.2.3 Potassium Silicate Solubilization (PGPB)

The silicate mineral-solubilizing bacteria namely *Bacillus globisporus* were isolated from the surface of weathered feldspar and showed growth when biotite was used as the potassium source (Sheng et al. 2008). The solubilization of potassium and silicon from the silicate minerals may also contribute to the available nutrient for plant growth.

7.2.4 Phytohormone Production (PGPB)

IAA have been reported to be involved in the phytostimulatory action exerted by the *Bacillus amyloliquefaciens*, and the production of IAA through tryptophan-dependent pathway affects level of plant growth promotion (Idris et al. 2007). The fluorescent pseudomonads are also proved to be the best candidates in the production of IAA (Dey et al. 2004).

7.2.5 Insecticidal Protein Production (Biocontrol-PGPB)

Bacillus thuringiensis, a spore-forming bacterium that produces insecticidal proteins during sporulation known as crystalline inclusions, has been the subject of intensive study because of its insecticidal activity (Zhang et al. 2010).

7.2.6 Antagonism Toward Plant Pathogens (Biocontrol-PGPB)

Pseudomonas fluorescens showed ability to inhibited *Aspergillus niger* and *A. flavus* in vitro (Dey et al. 2004). Bacteria which exhibit antagonism toward *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Pyrenochaeta lycopersici*, *Pythium ultimum*, and *Rhizoctonia solani* have been described (de Brito et al. 1995).

7.2.7 Siderophore Production (Biocontrol-PGPB)

The role of siderophore in biocontrol of several fungal phytopathogens has been reported (Scher and Baker 1982). Siderophore is also known to induce systemic acquired resistance (Leeman et al. 1996). de Brito et al. (1995) have shown that the antagonistic effects observed were associated with marked increases in the percentage of siderophore producers.

7.2.8 Salicylic and Jasmonic Acid Production (Biocontrol-PGPB)

The salicylic acid biosynthetic genes were expressed in *P. fluorescens* strain P3, and the improvement of induction of systemic resistance in tobacco against tobacco necrosis virus has been approved (Maurhofer et al. 1998). *Arabidopsis thaliana* ecotype Columbia plants treated with PGPR *Serratia marcescens* strain 90-166 had significantly reduced symptom severity by cucumber mosaic virus (CMV). Strain 90-166 systemically protects *A. thaliana* against CMV by a salicylic acid and NPR1-independent and jasmonic acid-dependent signaling pathway (Ryu et al. 2004).

7.2.9 *Rhizoremediation (PGPB)*

PGPR elicit biomass increases, particularly in roots, by minimizing plant stress in highly contaminated soils (Gurska et al. 2009). Extensive development of the root system enhances degradation of contaminants by the plants and supports an active rhizosphere that effectively promotes TPH degradation by a board microbial consortium.

7.2.10 *ACC Deaminase Production (PSHB)*

One way that bacteria stimulate plant growth is through the activity of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which causes a lowering of plant ethylene levels resulting in longer roots (Shah et al. 1998). The cloned ACC deaminase gene can be expressed in *Escherichia coli* enabling this bacterium to grow on ACC as a sole source of nitrogen and confers upon both *E. coli* and *Pseudomonas* spp. strains that are transformed with this gene the ability to promote the elongation of the roots of canola seedlings (Shah et al. 1998).

7.3 Screening and Isolation of Multifunctional Bacteria

Conventional cultivation of microorganisms by using selective medium is commonly used to screen and isolate bacteria possessing plant-growth-promoting traits. These PGP traits are mainly nitrogen fixation, phosphate solubilization, IAA production, siderophore production, and ACC deaminase production. Besides, the catabolic activities of bacteria such as cellulase, amylase, xylanase, pectinase, chitinase, protease, and lipase production are also taken as the in vitro test for biofertilizer considerations. Due to various environmental factors that may affect the bacterial growth, the good results obtained in vitro cannot always be dependably reproduced under field conditions (Chanway and Holl 1993). Therefore, it is necessary to develop efficient PGPR strains in field conditions (Ahmad et al. 2008). Taking symbiotic nitrogen fixers as examples, the characterization of the nitrogen-fixing activities by acetylene reduction activity assay, their specific hosts, acid production, and tolerance to acidity, alkali, and salt will be important when suitable bioinoculants are considered.

7.4 Identification of Microbial Agents and Their Biosafety Considerations

Before application of the biofertilizers, the microorganisms present in the microbial agents should be defined clearly in order to assess the biosafety of the biological agents in the natural system. A polyphasic approach including phenotypic, chemotaxonomic,

and genotypic characterizations can be used to classify the microorganisms, which can be used to propose novel bacteria (Young et al. 2008a, 2009, 2010). The scientific name or genotype of bacterium can be determined by using molecular techniques such as 16S rDNA sequencing (genus verification) and DNA–DNA hybridization analysis (species verification) (Shen and Young 2005; Young et al. 2005), followed by the safety evaluation framework provided by the American Biological Safety Association (ABSA). The risk assessment process can be made based on the risk group level of the microorganisms referenced in the classification database for infectious agents (http://www.absa.org/riskgroups/). In many countries including the United States, infectious agents are categorized in risk groups based on their relative risk. Depending on the country and/or organization, this classification system might taken several factors into considerations, which includes pathogenicity of the organism, mode of transmission and host range, availability of effective preventive measures (e.g., vaccines), and availability of effective treatment (e.g., antibiotics). In order to assess the biosafety of the microbial agents, the toxicity of the microorganisms can be determined by testing of the acute toxicity or their environmental fate. The acute toxicity testing includes oral toxicity/pathogenicity, pulmonary toxicity/pathogenicity, intravenous toxicity/pathogenicity, dermal toxicity/pathogenicity, eye irritation/infectivity, and report of hypersensitivity incidents. The environmental fate studies include aquatic toxicity, quail toxicity/pathogenicity, and earthworm toxicity.

7.5 Bioassay

Although PGPR have been reported to influence plant growth, yield, and nutrient uptake by an array of mechanisms, the specific traits were limited to the expression of one or more of the traits at a given environment of plant–microbe interaction (Dey et al. 2004). The isolates might be screened for any of the PGPR traits and selected on the basis of germinating seed bioassay in which the root length of the seedling was enhanced significantly over the untreated control (Dey et al. 2004). A gnotobiotic system can be used to study the rhizosphere colonization by PGPR (Simons et al. 1996). Plant assays which include seed germination, seedling growth, and yield of field-grown crop (leaf and shoot dry weight, leaf surface area, plant height, seed weight, number of seed per ear and leaf area, ear dry weight of crop) (Nezarat and Gholami 2009) might be determined. For example, the seed inoculation with *Pseudomonas* spp. significantly enhanced seed germination and seedling vigor of maize. Leaf and shoot dry weight and leaf surface area were significantly increased by bacterial inoculation in both sterile and nonsterile soil. The performances of these selected strains were repeatedly evaluated for 3 years in pot and field trials (Dey et al. 2004). Seed inoculation of these isolates resulted in a significantly higher pod yield than the control, in pots, during rainy and postrainy seasons. The contents of nitrogen and phosphorous in soil, shoot, and kernel also enhanced significantly in treatments inoculated with these rhizobacterial isolates in pots during both the seasons. In the field trials, however, there was wide variation in

the performance of the PGPR isolates in enhancing the growth and yield of peanut in different years (Dey et al. 2004). PGPR significantly enhanced pod yield, haulm yield, and nodule dry weight over the control in 3 years. Seed bacterization with PGPR suppressed the soil-borne fungal diseases like collar rot of peanut caused by *A. niger* and stem rot caused by *Sclerotium rolfsii*.

7.6 Application of Biofertilizers in Combination with Chemical or Organic Fertilizers

Benefits from the application of PGPR in agriculture largely depend on the complex interactions between several factors including the nature of fertilizers selected. The biofertilizer can be used in combination with organic fertilizer to enhance availability of nutrients and hence crop productivity. The studies have been carried out to determine the fine tuning between the inoculated bacteria and different fertilizers and their effect on the growth of lettuce plants (*Lactuca sativa* L.) (Lai et al. 2008). The greenhouse experiments demonstrated that dry matter yield and mineral nutrient levels of lettuce plants were affected by different fertilizers and inoculation. The plant yield of the inoculated treatments was significantly higher than their respective noninoculated treatments. Interaction between chemical fertilizer, organic fertilizer, and inoculation also showed significant effects on yield and nutrient contents of plants. Plant growth in soil amended with pig manure fertilizer and *A. rugosum* IMMIB AFH-6 was significantly lower than in soil treated with the chemical fertilizer, but inoculation combined with half dose of chemical fertilizer significantly elevated the plant biomass. *A. rugosum* IMMIB AFH-6 facilitated the accumulation of trace minerals in higher concentrations when pig manure fertilizer was combined with full dose of chemical fertilizer. Understanding the interactions of different fertilizers in the rhizosphere helps to resolve the inconsistency in plant growth and nutrient accumulation in response to the inoculated PGPR. In order to examine the benefits of inoculation by *A. rugosum* IMMIB AFH-6, a new type of data analysis which considers both biomass and nutrient content of plants has also been proposed (Lai et al. 2008). This new method of data analysis for evaluating the efficiency of the PGPR highlighted the benefits of inoculation in terms of plant yield and nutrient accumulation and will certainly be useful in further studies.

7.7 Production and Inoculation of Biofertilizers

One factor limiting commercial interest in biofertilizers is the high cost of production for most microbial agents (Fravel et al. 1999). This may be due to high cost of substrate, low biomass productivity, or limited economies of scale. Factors that are often important in the production include carbon source, osmotic potential,

temperature, and pH (Fravel 2005). The biotechnology companies are tasked with the production of biofertilizers, as liquid, solid, or encapsulation forms through the advanced fermentation techniques. The liquid agents may be produced by fermentation with suitable and cost-effectively medium components. Solid microbial agents may be produced by solid-phase fermentation, lyophilization (freeze drying), spray drying, or encapsulation technology (Young et al. 2006). The biological agents will be used directly and contact with seed, on the surface of the seedlings or into the rhizosphere. Bacterial culture can be used along, coating with carrier or in combination with organic matter. The calcium alginate, agarose, and k-carrageenan have most often been employed as the polymeric materials of choice. The advantages of these carriers are that they are usually nontoxic and have the ability to add compounds that either enhance cellular physiology or aid in assimilating the compounds to be degraded. Recently, a study was performed to investigate the efficiency of microbial inoculants after encapsulating in alginate supplemented with humic acid on plant growth (Rekha et al. 2007). The humic acid-enriched alginate bead loaded with *Bacillus subtilis* CC-pg104 can be observed clearly by using scanning electron microscope. The results demonstrated that inoculation of the encapsulated *B. subtilis* CC-pg104 promoted plant growth similar to their respective free cells and could be a novel and feasible technique for application in agricultural industry. It is expected that new products will appear on the market soon, providing a broad activity spectrum and applicability to many other crops (Rosas-García 2009).

During the processing of the microbial agents, the quality assurance (number of bacteria) and quality control (type of bacteria) are important. The viable and culturable bacterial or propagule number in the biological agents can be calculated by plate counting method during the manufacturing process, and the bacterial type can be determined simply by observing the colony morphology or through different genotyping methods. The storage and transportation of microbial agents may also be concerned, and the spore-forming microorganisms or microorganisms which show tolerance to a wide range of pH values, temperature, salinity, or drought will be favorable.

7.8 Tracking for the Inoculants

By recognizing the physiological condition of the inoculants, it is possible to track the bacterial cells or population in the rhizosphere (Van Veen et al. 1997). Besides, the in vivo test for the PGP activity is used through the reporter gene system, in evaluating in situ gene expression by root-associated bacteria. By fusing the reporter gene in bacterial cells, it is possible to report the transcriptional activity of a promoter. The criteria such as detectability, sensitivity, reliable quantification, specific activity, and responsiveness to changes in transcriptional activity of a reporter gene system are conducted. Due to the ease and sensitivity of its detection and the large number of plasmid vectors and transposons available for making transcriptional and translational fusions, *lacZ* probably is the most common reporter

gene used (Slauch and Silhavy 1991). Measurements of gene expression of cells harboring *lacZ* gene fusions are normally made by estimating the β-galactosidase activity of a large population of cell ($>10^8$ cells/sample) and thus provide an estimate of the average level of gene expression (Loper and Lindow 2002). The abundance of β-galactosidase in individual cells can be quantified from the immunofluorescence of cells probed with a fluorescently labeled antibody specific to β-galactosidase (Kang et al. 1999). *gusA* which encodes β-glucuronidase catalyzing the hydrolysis of a wide range of glucuronides has been useful in assessing gene expression by bacterial pathogens and symbionts of plants and especially in resolving spatial patterns of in situ transcriptional activity of bacterial cells (Loper and Lindow 2002).

The green fluorescent protein (GFP) from the jellyfish was the first of several fluorescent proteins to be used as a reporter (Chalfie et al. 1994). The abundance of GFP in an individual cell can be quantified by fluorescence spectroscopy or confocal laser microscopy. Thus, the *gfp* reporter, like *lux*, can be used to assess gene transcription in individual cells, which represents an advantage over other reporter genes that measure the average transcription in a population of cells (Loper and Lindow 2002). Cellular metabolism is not required for fluorescence of GFP, so the *gfp* reporter should be useful in studies evaluating in situ gene expression by cells that are not actively growing, as may be the case in many natural habitats. Therefore, the *gfp* reporter offers an attractive alternative to the use of *lux* fusions to visualize the transcription of genes in individual cells in soil or on plant surface (Loper and Lindow 2002). By using the green fluorescent protein-expressing transconjugant strains, Chen et al. (2005) have confirmed strongly that *Burkholderia* strains can form effective symbioses with legumes.

7.9 Future Perspectives

Suggestions and criteria are given here to explore superior biofertilizers:

1. Rapid screening and selection of effectively and competitively multifunctional microbial agents, which can be used in a variety of crops or their pest hosts
2. Proper quality control system for the production of inoculants and their application in the field, in order to ensure and maximize the benefits of plant–microorganism symbiosis
3. Lasting microbial persistence of microbial agents in soil environments under stress conditions
4. Evaluation of microbial agents from agronomic, soil, and economic aspects under diverse agricultural production systems
5. Optimization of the media formula and fermentation conditions for the large-scale production of microbial agents
6. Establishment of biofertilizer management act and strict regulation for quality control

7.10 Conclusion

The sustainable management in agriculture relies on an integrated farming system, and the microbial agents may play important roles in the crop production. Microbial fertilizer serves as an alternative material in the promotion of plant growth while maintains soil fertility and productivity. Through the functional analyses of the plant-growth-promoting traits and bioassay, it is possible to select the PGPR candidates. The integrated approaches are proposed here to increase the efficiency of the biofertilizers in the rhizosphere: inoculation from a single bacterium to bacterial consortium; from sole microbial agents to a combination of microbial agents, organic fertilizer, or chemical fertilizer (biotech-fertilizer); from fast nutrient-releasing liquid fertilizer and slowly nutrient-releasing solid fertilizer to control releasing biofertilizer (biowise-fertilizer); and from microbial agents with fertilization activity to multifunctional microbial agents with both fertilization and biocontrol activities. It is also essential to take account of the protection of intellectual property (IP) of bioresources and their derivatives accompanied with newly developed biotechnique used. The biofertilizers will be brought into the markets and show huge benefits to the farmers and environments once the limitations in the research, production, and commercialization of these microbial agents have been overcome in the near future.

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Chapter 8
Endophytic Bacteria and Their Role in Legumes Growth Promotion

Tania Taurian, Fernando Ibáñez, Jorge Angelini, María Laura Tonelli, and Adriana Fabra

8.1 Introduction

Plants live in intimate association with microorganisms. Bacteria may exist as free-living organisms in soils, attached to the surface of roots or establishing symbiotic relations with plants, which encompass styles ranging from mutualistic to commensal and parasitic. The rhizosphere (the zone that surrounds the roots of plants) and roots are heavily colonized by microbes since sources of carbon and minerals are very abundant in this zone (Walker et al. 2003). Plants exude high levels of nutrients from their roots such as sugars, amino acids, organic acids, polysaccharides, and proteins (Marschner 1995). In addition to providing a carbon-rich environment, plant roots initiate cross talks with soil microbes by producing molecules that are recognized by microorganisms, which in turn produce signals that initiate colonization (Bais et al. 2006). Consequently, the rhizosphere supports large and active microbial populations capable of exerting beneficial, neutral, or detrimental effects on plant growth.

This chapter will be focused primarily on the positive interactions among legumes and endophytic (*endon* Gr., within; *phyton*, plant) bacteria (defined as microorganisms that inhabit the interior of plant tissues and organs, including nodules). These bacteria can positively influence plant growth through a variety of mechanisms, including fixation of atmospheric nitrogen (Burris and Roberts 1993), increased biotic and abiotic stress tolerance, and other direct and indirect advantages (Kloepper et al. 2004; Timmusk and Wagner 1999). They can also positively interact with plants by producing biofilms or antibiotics that protect them against potential pathogens (Ude et al. 2006) or by degrading plant-produced compounds in soils that would otherwise be allelopathic (Turner and Rice 1975).

T. Taurian • F. Ibáñez • J. Angelini • M.L. Tonelli • A. Fabra (✉)
Dpto. Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto (UNRC), Río Cuarto (Córdoba), Argentina
e-mail: afabra@exa.unrc.edu.ar

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The ability to convert atmospheric dinitrogen to ammonia is observed not only in free-living but also in symbiotic diazotrophs, including bacterial species collectively referred to as rhizobia. The interaction between these endophytes and legumes involves widely known molecular mechanisms.

The prospect of manipulating crop rhizosphere bacterial populations by inoculation of those that increase plant growth has shown considerable promise in laboratory and greenhouse studies, but under field conditions, responses have been variable. Progress in our understanding on soil biology and on the ecology and evolution of beneficial microorganisms should increase the environmental benefits of sustainable management practices to achieve better yields and to maintain soil fertility.

8.2 Plant-Growth-Promoting Bacteria

In order to enhance soil fertility and crop productivity, modern agriculture has become heavily dependent on the application of chemical inputs, including fertilizers and agrochemicals (Kiely et al. 2006). Nevertheless, the use of these products has often negatively affected the complex system of biogeochemical cycles (Steinsham et al. 2004). Moreover, it has been demonstrated that the continuous use of these chemical compounds generates environmental problems. Another fact that worsens the negative environmental effects is the low efficiency in the uptake of fertilizers by plants (Barlog and Grzebisz 2004). Therefore, the challenge is to promote more environmental-friendly agricultural practices. In this sense, a wide number of studies have been and still are focused in plant-growth-promoting bacteria as potential supplements of fertilizers, herbicides, fungicides, etc. (Adesemoye and Kloepper 2009). Microbial inoculants are promising components for integral solutions to agro-environmental problems because they promote plant growth by enhancing nutrient availability and uptake, or by holding up the health of the plants (Kloepper et al. 2004; Weller 2007; Adesemoye et al. 2008). The rhizosphere and the phylloplane of plants are habitats for a large number of microorganisms. In particular, the rhizosphere is a main spot of microbial interactions as exudates released by plant roots are an important carbon source for rhizospheric microorganisms. Many members of this microbial community have a neutral effect on the plants, while others have deleterious impact, causing diseases that result in plant death or a major reduction of its fitness and yielding. In contrast, some microorganisms can benefit the plant by promoting its growth, directly or indirectly. Plant-growth-promoting bacteria, term initially defined for rhizobacteria but that later also included bacteria isolated from different plant tissues (aerial and underground), encompasses microorganisms which, under certain conditions, promote plant growth. The use of microorganisms to benefit plant growth and to control plant pests continues being an area of rapid-expanding research. The most studied group of plant-promoting bacteria (PGPB) is the rhizobacteria (PGPR) that colonize the root surface and the portion of soil nearest

to the root. Some PGPR can reach interior tissues and generate endophytic populations not only in the roots but also in leaves and stems (Compant et al. 2005). While these rhizobacteria utilize the nutrients that are released from the host for their growth, they also secrete metabolites into the rhizosphere. Over the years, several mechanisms involved in plant growth promotion have been documented. For PGPB to exert beneficial effects on plant growth, they need to be in an intimate relationship with the host plant. The degree of intimacy can vary depending on where and in what extent the beneficial bacteria colonize the host plant (Vessey 2003). The study of plant-associated bacteria is important not only for understanding their role in the interaction with plants but also for biotechnological application in areas as the plant growth promotion (Kuklinsky-Sobral et al. 2004).

It has been suggested that plants establish a communication with PGPB to specifically attract microorganisms for their own ecological and evolutionary benefit (Hardoim et al. 2008). Owing to the complexity of plant–microbe interactions in soil, it is extremely difficult to understand the detailed mechanisms involved in these putative selection processes. However, knowledge of well-studied models as the rhizobia–plant interaction, which indicates the existence of highly evolved species-specific communication systems, could be used as reference when studying novel plant–microbe interactions (Hardoim et al. 2008).

8.2.1 Bacterial Endophytes

Plants constitute an extremely diverse niche for microorganisms. Plant-associated bacteria isolated from rhizoplane and phylloplane are known as epiphytes (Andrews and Harris 2000; Kuklinsky-Sobral et al. 2004). Those isolated and detected inside the tissues by microscopic methods that maintain their ability to infect plants are called endophytes (Azevedo et al. 2000; Kuklinsky-Sobral et al. 2004; Rosenblueth and Martinez Romero 2004; Reinhold-Hurek and Hurek 1998a). There are also some bacterial populations with lifestyles fluctuating between endophytic and epiphytic colonization (Hallman et al. 1997; Kuklinsky-Sobral et al. 2004). By colonizing internal plant tissues, endophytic microorganisms become protected from external biotic and abiotic stresses. Within endophytes, beneficial and pathogenic bacteria can be found. Among the formers, rhizobia are the most studied group which is able to fix atmospheric nitrogen inside nodules (Hardoim et al. 2008). According to their life strategy, endophytic bacteria can be classified as "obligate" or "facultative". Obligate are strictly dependent on the host plant for their growth and survival**,** and transmission to other plants occurs vertically or via vectors. On the other hand, the lifecycle of facultative endophytes can be characterized as biphasic, alternating between plants and the environment.

Colonization is an important trait of bacterial endophytes to be ecologically successful. For bacterial colonization, root cracks constitute the main portal of entry. Nevertheless, other ways of internal infection exist, such as wounds caused

by microbial or nematode phytopathogens and stomata found in leaf tissue (Hardoim et al. 2008). The sequence of events for the endophytic colonization is similar, at least in the initial phases, to that of the root surface (Hallman et al. 1997). Environmental and genetic factors are presumed to have a role in enabling a specific bacterium to become endophytic (Reinhold-Hurek and Hurek 1998b). Hardoim et al. (2008) proposed the term competent endophytes to describe bacteria having the key machinery required to colonize and persist in the endosphere. On the other hand, opportunistic endophytes are considered as competent rhizosphere colonizers that become endophytic by coincidentally entering root tissue, but lack genes that are essential to their ecological success inside the plant. Additionally, a third group named passenger endophytes has been proposed. It includes bacteria that enter plants purely as a result of chance events since they lack the machinery to either colonize surface or internal tissues (Hardoim et al. 2008). Even when all categories colonize cortical root cells, only competent endophytes are able to systemically spread throughout the entire plant (Dong et al. 2003; Zakria et al. 2007). Capacity of bacteria to colonize plant tissues both externally and internally is a desirable characteristic for seeds inoculation because such bacteria have a greater chance of influencing host development (Kuklinsky-Sobral et al. 2004). It has been described that the roots are the preferential site for epiphytic and endophytic bacteria suggesting that endophytic bacteria may travel upward from the roots into the stem during plant development (Kuklinsky-Sobral et al. 2004).

Phylogenetic diversity of epiphytic and endophytic communities has been studied, and results have shown that both are related, suggesting that endophytes are an evolved state of a previous epiphytic or rhizosphere population (Hallman et al. 1997; Sturz et al. 2000). In many endophytic bacteria–plant interactions, where no specialized structures such as root nodules are formed, the way of infection of the PGPB and their location are not as clearly understood as in legume–rhizobia symbiosis.

8.2.2 Plant Growth Promotion Mechanisms

Plant-growth-promoting bacteria have been widely studied, and several mechanisms of growth promotion have been described. Considering the mode of action, PGPB have been divided into two groups: biocontrol bacteria that indirectly benefit the plant growth and PGPB that directly affect plant growth, seed emergence, or improve crop yields (Bashan and Holguin 1998; Glick et al. 1999). Indirect plant growth promotion occurs when bacteria are able to protect the plant against soilborne diseases by reducing harm caused by pathogens (Lugtenberg and Kamilova 2009). On the other hand, the direct growth promotion occurs when bacteria stimulate plant growth by providing limited nutrients in soil or by promoting enhancement of root biomass conferring a major volume to incorporate soil nutrients. While these rhizobacteria utilize the nutrients that are released from the host for their growth, they also secrete metabolites into the rhizosphere. Several of

these metabolites can have a role as signaling compounds that are perceived by neighboring cells within the same microcolony, by other bacteria in the rhizosphere, or by root cells of the host plant (Van Loon 2007; Van Loon and Bakker 2003; Bais et al. 2004; Kiely et al. 2006). The best studied example of signal exchange is rhizobia–legume symbiosis that will be discussed later in this chapter, in which the plant releases flavonoids compounds that induce the bacterium to secrete Nod factors. This symbiosis is a prime example of an intimate relationship between a soil bacterium and its host plant and illustrates the concept behind the term "plant-growth-promoting bacteria" since, in nitrogen-poor environments, the rhizobia promotes legume plant growth by providing a limited nutrient (Van Loon 2007).

8.2.2.1 Indirect Plant Growth Promotion

There are four main groups of plant soilborne pathogens: fungi, nematodes, bacteria, and viruses. In most agricultural ecosystems, soilborne plant pathogens can be a major limitation to reach sustainable yields. The application of microbes to control diseases (biocontrol) is an environmental-friendly approach and is used as complement or alternative of agrochemicals. The term biocontrol is used not only to describe control diseases in living plants but also those occurring during the storage of fruits (also called postharvest control). Microbes able to control pathogen activity may produce secondary metabolites which are released on or near the plant surface. In contrast, the majority of agrochemicals do not reach the plant at all. Moreover, the molecules of biological origin are biodegradable compared with many agrochemicals that are designed to resist microbial degradation (Lugtenberg and Kamilova 2009). Although biocontrol studies usually focus on pathogenic microorganisms, some bacteria are also active against weeds (Flores-Fargas and O'Hara 2006) and insects (Péchy-Tarr et al. 2008; Siddiqui et al. 2005). The control of soilborne diseases by bacteria may result from competition for nutrients, antibiosis, predation, parasitism, and signal interference (Sturz et al. 2000; Van Loon 2007). Such activities are particularly important in the rhizosphere where pathogenic organisms are attracted to plant roots. However, rhizobacteria can reduce the activity of phytopathogens not only through microbial antagonism but also by activating the plant to better defend itself, a phenomenon termed "induced systemic resistance" (ISR) (Van Peer et al. 1991; Van Loon 2007).

Antibiosis

The antibiotics most commonly produced by different biocontrol bacteria include 2,4-diacetylphloroglucinol (DAPG), oomycin A, phenazine-1-carboxylic acid, ammonia, pyrrolnitrin, etc. (Whipps 2001; Verma et al. 2010). A lesser described antibiotic that showed antifungal activity produced by biocontrol bacteria is 2-hydroxymethyl-chroman-4-one, isolated from cultures of an endophytic *Burkholderia* strain

(Kang et al. 2004). An interesting point of this bacterial biocontrol trait is that production of antibiotics is highly influenced by the quantity and quality of available nutrients and is also subjected to quorum sensing (Haas and Keel 2003).

Predation and Parasitism

This biocontrol strategy has been mainly studied for the control of fungal pathogens. Parasitism of pathogenic fungi by biocontrol microorganisms occurs through the production of hydrolytic enzymes that degrade the fungal cell walls. Among them, chitinases and glucanases have been widely studied (Podile and Kishore 2006; Arora et al. 2007).

Competition for Nutrients and Niches

Biocontrol may result not only from direct interaction between the pathogen and the biological control agent but also from their competition for nutrients point of view. Then, competition between bacteria and pathogens for nutrients and niches in the rhizosphere constitutes a biocontrol mechanism. For instance, iron uptake is essential, and under starvation, several microorganisms secrete siderophores to mobilize this metal (Höfte et al. 1993). These molecules are low-molecular-weight compounds of high affinity to iron secreted by microorganisms under iron-limiting conditions (Höfte et al. 1993) that allow its incorporation from the environment. By producing siderophores, PGPB may compete with the pathogen for this nutrient (Duijff et al. 1999; Lugtenberg and Kamilova 2009) or induce systemic resistance in the plant (Leeman et al. 1996).

Induced Systemic Resistance

By this process, treatment with PGPB elicits plant defense as indicated by reduction in the severity or incidence of diseases caused by pathogens that are spatially separated from the inducing agent (Kloepper et al. 2004). This is the consequence of the plant response to compounds released by the PGPB (volatile and no volatile) and implicates a sequence of defense reactions. Many bacterial compounds induce ISR, such as LPS, flagellin, salicylic acid, and siderophores. More recently, cyclic lipopeptides, the antifungal factor Phl, the signal molecule acyl homoserine lactone (AHL), and organic volatile compounds have also been implicated (Lugtenberg and Kamilova 2009). A wide spectrum of ISR activities have been identified to be induced by rhizosphere bacteria. Among them, the activation of defense mechanisms that are also induced by pathogenic microorganisms is activated. Such mechanisms can include production of antimicrobial phytoalexins, synthesis of pathogenesis-related proteins (PRs) (Hammond-Kosack and Jones 1996),

enhanced capacity to express these defense responses upon challenge inoculation with a pathogen, phenomenon called "priming" (Conrath et al. 2006; Van Loon 2007). Other ISR responses can involve signal translation, protection against oxidative stress, and generation of structural defenses, such as wall thickening, callose deposition, and accumulation of phenolic compounds (Reymond and Farmer 1998). Plant molecules such as jasmonic acid, ethylene, and salicylic acid play a major role in this defense mechanism. Since its discovery, rhizobacteria-mediated ISR has been documented in at least 15 plant species. Once ISR is induced, plants may remain protected for a considerable part of its lifetime, indicating that this state is rather stable (Van Loon and Bakker 2006; Van Loon et al. 1998; Van Loon 2007).

Other biocontrol mechanisms are interference with activity, survival, germination, and sporulation of pathogen (Lugtenberg and Kamilova 2009). Another less studied indirect growth-promoting activity exerted by PGPB, but not less important, is the promotion or synergism of other beneficial interactions such as legume–rhizobia or plant–fungi symbioses (Vessey 2003).

8.2.2.2 Direct Plant Growth Promotion

Phytohormone production and enhancing plant nutrition are the two main mechanisms by which PGPB directly contribute to plant growth. Enhance of plant nutrition is mainly through increase of the root growth, mineral uptake, and biological nitrogen fixation (BNF).

Phytohormone-Like Molecule Production

Several studies have demonstrated production of compounds chemically and functionally similar to phytohormones. Even when production of these compounds by PGPB has been demonstrated, this growth promotion effect cannot be unequivocally attributed to them (Glick 1995; Vessey 2003; Patten and Glick 2002; Podile and Kishore 2006; Verma et al. 2010). Phytohormone-like molecules found to be produced by PGPB are auxins, cytokinins, gibberellins, abscisic acid, and ethylene (Vessey 2003; Verma et al. 2010). They stimulate density and length of root hairs causing an increase in root surface area and therefore improving the plant potential for water and mineral nutrients uptake from a larger volume of soil (Volkmar and Bremer 1998; Podile and Kishore 2006). Among these growth regulators, auxins are the most studied. These compounds affect plant growth by inducing cell enlargement and division, root development, apical dominance, increase growth rate, photo, and geotropism (Frankerberger and Arshad 1995; Verma et al. 2010). Although cytokinins are produced by several genera of PGPB, few studies have demonstrated their beneficial effects. Similarly, but investigated in a lesser extent, is the case of gibberellins (Gaudin et al. 1994; Gutierrez-Manero et al. 2001; Podile and Kishore 2006). Ethylene is usually considered an inhibitor of plant growth, but at low levels,

it can actually promote plant growth in several plant species (Van Loon 2007; Pierik et al. 2006). At moderate levels, it inhibits both root and shoots elongation, and at high levels, it enhances senescence and organ abscission (Abeles et al. 1992; Van Loon 2007). So, the interest is focused in the modulation of this plant growth modulator more than in its production by PGPB. The direct precursor of ethylene in the plant biosynthetic pathway, 1-aminocyclopropane-1-carboxylic acid (ACC), is exuded from plant roots together with other amino acids. PGPB that express the enzyme ACC deaminase, which cleaves ACC into ammonia and α-ketobutyrate, utilize these products as nitrogen and carbon sources, respectively. Under such conditions, re-uptake of ACC and its level in the roots is reduced. As a consequence, ethylene production by the roots is lowered, relieving inhibition of root growth (Glick 2005; Van Loon 2007). A second bacterial mechanism proposed to modulate plant ethylene levels is by inhibiting the enzymes of the ethylene biosynthesis pathway, ACC synthase and/or β-cystathionase (Sugawara et al. 2006; Hardoim et al. 2008). In both mechanisms, the bacteria are more efficient at modulating ethylene levels when they are closer to the plant cells in which ethylene biosynthesis takes place. Bacterial ACC deaminase is not currently known to be excreted from the bacterial cytoplasm (Glick et al. 2007; Hardoim et al. 2008). Hence, the decrease of plant ethylene levels relies on the ability of ACC deaminase expressing bacteria to take up ACC before it is oxidized by the plant's ACC oxidase (Glick et al. 1998; Hardoim et al. 2008). In this context, bacterial endophytes with high locally induced ACC deaminase activity might be excellent plant growth promoters because they ameliorate plant stress by efficiently blocking ethylene production (Cheng et al. 2007; Hardoim et al. 2008).

Volatile Compounds and Other Phytostimulators

Some rhizobacteria, belonging to phylogenetically unrelated genera such as *Bacillus* and *Enterobacter,* promote plant growth by releasing volatile compounds (Ryu et al. 2003; Lugtenberg and Kamilova 2009). Other bacterial cell components or secreted compounds have been proposed to be plant growth stimulators. Within these molecules, the protein pyrroloquinoline quinone (PQQ) has been described as a plant growth promoter in tomato and cucumber plants probably related with its antioxidant activity in plants (Choi et al. 2008). Nevertheless, its role in plant promotion has to be further elucidated since it also has antifungal activity and is able to induce systemic resistance (Lugtenberg and Kamilova 2009).

Increase of Nutrient Availability

Main mineral nutrients required for plant growth are nitrogen, phosphorus, and iron. Numerous PGPB able to increase their availability have been studied, and mechanisms involved in these effects have been determined. Among them,

and since nitrogen is the first important nutrient required for plant growth, BNF is the most studied, hence discussed further in this chapter.

Phosphate solubilization and mineralization: Even in phosphorus-rich soils, most of this element is in insoluble form, and only a small proportion (~0.1%) is available to plants (Stevenson and Cole 1999). Additionally, a large percentage of the phosphate fertilizers applied to soils precipitate into insoluble forms thus increasing the phosphorus requirement of the crop (Podile and Kishore 2006). The solubilization of insoluble phosphates in the rhizosphere is one of the most common modes of action of PGPB that enhance nutrient availability to plants (Rodriguez et al. 2006). Phosphate-mineralizing and phosphate-solubilizing bacteria (PMB/PSB) secrete phosphatases and organic acids to convert insoluble phosphates (organic and inorganic) into soluble monobasic and dibasic ions (Rodriguez et al. 2006).

Increased uptake of iron to plants by siderophore-producing bacteria: Given the importance of iron for plants, the ability to produce siderophores is a desirable PGPB trait. Microbial siderophores may stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots (Kloepper et al. 1980; Verma et al. 2010). Plants, including legumes, demonstrated their ability to use microbial siderophores as a sole source of iron (Jurkevitch et al. 1986; Verma et al. 2010).

Biological nitrogen fixation: The ability to fix atmospheric nitrogen is present in various bacterial species that are either free-living or endophytically associated with plants roots (Dobbelaere et al. 2003). BNF is the most and long time studied plant-growth-promoting effect of soil microorganisms in legumes (Cholaky et al. 1983; Sen and Weaver 1984; Vargas and Ramirez 1989; van Rossum et al. 1993; Castro et al. 1999; Taurian et al. 2002), and mechanisms involved in this symbiotic interaction will be described in the Sect. 8.3. Besides nitrogen-fixing rhizobia, several authors observed that other associated beneficial bacteria exert over this group of plants multiple plant-promoting activities such as phosphate-solubilizing activity, IAA production, and biocontrol properties (siderophore production, antibiosis, etc.) (Pal et al. 2000; Deshwal et al. 2003; Dey et al. 2004; Kishore et al. 2005; Taurian et al. 2008, 2010; Ibañez et al. 2009; Tonelli et al. 2010).

To be efficient in plant growth promotion, the PGPB should remain active under a large range of conditions, such as fluctuating pH, temperature, and concentration of different ions. These requirements are not easy to be fulfilled, which explains why several commercial inoculant products are not successful. In addition, to express beneficial traits, inoculated strains should also be able to compete successfully with other organisms for nutrients from the root and for niches on the root as well as to escape in sufficient numbers from predators (Jousset et al. 2006; Lugtenberg and Kamilova 2009). The increase of our understanding about the mechanisms of plant growth promotion and on the selection procedures of beneficial bacteria will improve the development of PGPB-based inoculants (Lugtenberg and Kamilova 2009).

8.3 The Rhizobia–Legumes Symbiotic Association

Most of the nutrients that plants require for growth are readily available, but a few, as the macronutrient nitrogen, is often limited in soils. Even when molecular nitrogen is the major component of the Earth's atmosphere, it cannot be used directly by biological systems until it is combined with the element hydrogen. This process of reduction of molecular nitrogen is commonly referred to as "nitrogen fixation" and may be accomplished biologically. Biological systems which are able of fixing nitrogen (BNF) are classified as non-symbiotic or symbiotic, depending on the requirement of one or more than one organism, respectively, involved in the process (Burris and Roberts 1993).

Diversity in the metabolic types of free-living microorganisms which are capable of BNF is very wide, including many genera of non-photosynthetic aerobic (*Azotobacter*, *Beijerinckia*) and anaerobic (*Clostridium*) bacteria or photosynthetic cyanobacteria such as *Nostoc* and *Anabaena*. However, the most important contribution to BNF comes from the nitrogen-fixing plant symbiotic association (Bishop and Premakumar 1992).

Nitrogen-fixing plant symbionts belonging to various genera of the order Rhizobiales (collectively called rhizobia) are able to invade legume roots in nitrogen-limiting environments, leading to the formation of a highly specialized organ, the nodule, where bacteria, through the induction of the nitrogenase complex, are able to convert atmospheric dinitrogen into ammonia, which is used by the plant as a nitrogen source.

Nodule formation is a complex process that requires an adequate signal exchange between the plant and the bacteria. Plants secrete flavonoids from the actively growing region of the root. Interaction of these plant signals with rhizobial Nod transcription factors activates the expression of nodulation genes in compatible rhizobial species. Nod gene products synthesize Nod factor, bacterial lipochitooligosaccharide signaling molecules. Plant perception of Nod factors potentiates immediate subcellular changes in the root epidermis and later changes in the root cortex. In the epidermis, Nod signal activates many of the early events involved in the bacterial infection process. The bacteria enter the plant via the root epidermis and induce the reprogramming of root cortical cell division and the formation of a nodule (D'Haeze and Holsters 2002).

In the best studied rhizobia–legume symbiosis, infection occurs through root hairs. The first observable event in this infection process is the curling of the root hair where bacteria become enclosed, the plant cell wall is degraded, the cell membrane is invaginated, and an intracellular structure named infection thread is formed. It is within this structure that bacteria enter the root hair cell and eventually ramify into the root cortex. Simultaneously, the root cortical cells are induced to divide to form the nodule primordium. When the infection thread reaches the cells of the primordium, the bacteria are released into cells via endocytosis, enclosed in vacuole-like structures (symbiosomes) in which they differentiate into bacteroids.

It is within these symbiosomes that the bacteria convert dinitrogen to ammonium (Oldroyd and Downie 2008).

Another mode of rhizobial infection in legumes occurs via natural wounds caused by the splitting of the epidermis and the emergence of young lateral or adventitious roots. It is known as crack entry and has been described in (sub) tropical legumes. In *Sesbania rostrata* (Dreyfus and Dommergues 1981) and *Neptunia* (Subba-Rao et al. 1995), the infection leads to the formation of intercellular infection pockets, which give rise to intracellular infection threads. However, in *Arachis hypogaea, Stylosanthes,* and *Aeschynomene,* structures resembling infection threads have never been observed, and the later penetration of bacteria to the periphery of the nodule primordia occurs intercellularly (Chandler 1978; Fabra et al. 2010).

An intriguing but still not fully understood property of the symbiosis is its host specificity, which is believed to be determined by the recognition of Nod factor structure. However, it has been recently reported that soybean host proteins related with pathogenesis (R proteins) are involved in host specificity. The involvement of R proteins in the control of genotype-specific infection and nodulation reveals a common recognition mechanism implicated in symbiotic and pathogenic plant–bacteria interactions and suggests that establishment of a root nodule symbiosis requires the evasion of plant immune responses triggered by rhizobial elicitors (Yang et al. 2010).

8.3.1 Perception of Nod Factors and Trigger of a Signaling Cascade

In legumes like *Pisum sativum*, *Medicago truncatula*, and *Lotus japonicus* where the rhizobial infection process starts in epidermal root hair cells (Brewin 2004), more than 40 host genes or loci essential for microbial endosymbiosis have been identified so far (Kouchi et al. 2010). NFR1 and NFR5 have been identified as putative Nod factor receptors from *L. japonicus* (Madsen et al. 2003; Radutoiu et al. 2003) and from *Glycine max* (Indrasumunar et al. 2009), as LYK3 and NFP from *M. truncatula* (Limpens et al. 2003; Arrighi et al. 2006), and SYM37 and SYM10 from *P. sativum* (Zhukov et al. 2008). All of them are termed LysM receptor-like kinases (LysM-RLKs) since they have a common structure composed of a single-pass transmembrane domain anchoring to an extracellular lysin motif (LysM) receptor domain and an intracellular kinase domain. At present, however, no structural study has been made on the interactions of LysM domains with specific Nod factor structures.

Another RLK involved in Nod factor signaling, located on the plasma membrane and on the infection thread membrane, has been reported (Limpens et al. 2005). It has leucine-rich repeat (LRR) and serine–threonine kinase domains and is encoded

by *M. sativa NORK*/*PsSYM19*/*LjSYMRK*/*MtDMI2*/*GmNORK* (Endre et al. 2002; Stracke et al. 2002; Mitra et al. 2004; Capoen et al. 2005; Indrasumunar 2007). Activation of the LysM-RLKs seems to be a prerequisite for the activation of this LRR-RLK, and based on downstream responses, the LysM-RLKs may have a specific role in the Nod factor signaling cascade, whereas the LRR-RLK may function more in initiating bacterial infection events (Limpens et al. 2005). In fact, it is predicted that LysM-RLK functions in both Nod factor perception and downstream signal transduction since it is required for the earliest detectable root hair responses, such as Ca^{2+} fluxes, membrane depolarization, and oscillation in cytosolic Ca^{2+} concentrations, known as Ca^{2+} spiking (Endre et al. 2002; Stracke et al. 2002). This signal transduction cascade involves potassium ion channel proteins localized in the nuclear membrane encoded by *MtDMI1, LjCASTOR,* and *LjPOLLUX* (Anè et al. 2004; Imaizumi-Anraku et al. 2005; Riely et al. 2007); nucleoporins encoded by *LjNup133* and *LjNup85* (Kanamori et al. 2006; Saito et al. 2007); and a calcium–calmodulin-dependent protein kinase (CCaMK) encoded by *MtDMI3*/*PsSYM9* (Levy et al. 2004; Mitra et al. 2004). This later protein acts downstream of Ca^{2+} spiking, while the LRR-RLK, the ion channels, and the nucleoporins seem to act upstream of oscillation in cytosolic Ca^{2+} concentrations. Many transcription factors are activated downstream CCaMK, such as nodulation signaling pathway 1 (NSP1) (Smit et al. 2005), NSP2 (Kalo et al. 2005), Ets2 repressor factor (ERF) required for nodulation (ERN) (Middleton et al. 2007), and nodule inception (NIN) (Schauser et al. 1999; Borisov et al. 2003). It has been suggested that all of them work in combination to regulate the expression of early nodulins in the epidermis (Hirsch et al. 2009). Nodulins are proteins that are coded by plant genes and are necessary for the development of symbiosis in the legume root nodules. According to their time of expression, they can be divided into early and late nodulins.

Simultaneously with this signaling cascade, bacterial infection events are triggered by the activation of the LRR Nod factor receptor. In *M. truncatula,* it has been identified 3-hydroxy-3-methylglutaryl CoA reductase (MtHMGR) as a component of this signaling via, which may be involved in the biosynthesis of cytokinins and brassinosteroids (Kevei et al. 2007). After MtHMGR activation following Nod factor perception in the epidermis, rapid responses are detected in the inner root such as rearrangements in pericycle cells (Timmers et al. 1999), the expression of the nodulin ENOD40 in cortical cells (Asad et al. 1994), and nodule development. For these responses in the inner root after exposing the outer root to Nod factors, a signaling communication seems to be necessary. In root nodule symbiosis, several hormones are reported to be important. Among them, cytokinin has been shown genetically to be essential for nodule organogenesis. *LHK1* in *L. japonicus* and *CRE1 in M. truncatula* (Gonzalez-Rizzo et al. 2006; Tirichine et al. 2007) encode a cytokinin receptor kinase, which functions in the root cortex and is involved only in nodule organogenesis, but not in the infection thread formation. Different studies have shown that downregulation, or loss of function, of this cytokinin receptor results in a decrease in nodule numbers due to the inability of plants to form nodule

primordia (Gonzalez-Rizzo et al. 2006; Murray et al. 2007), even when rhizobia infections still take place. This suggests that two temporally and spatially distinct morphogenetic programs are induced after Nod factor/rhizobia perception, one that is activated in epidermis and is related with the bacterial infection, and other initiated at the cortical cells level that is involved in the nodule organogenesis (Ferguson et al. 2010). Even when these processes occur in legumes that are infected through root hairs, in *Aeschynomeme sensitiva*, a legume infected by crack entry, it has been reported that rhizobial infection and nodule organogenesis processes are developed in absence of Nod factors (Giraud et al. 2007). In *A. hypogaea,* the synthesis of Nod factors has been studied (Taurian et al. 2008), and it is also known that they are required for cortical cells division (Ibañez and Fabra 2011) and that the Ca^{2+}/calmodulin-dependent protein kinase is expressed in roots and nodules (Sinharoy and DasGupta 2009).

In summary, on the basis of the resources established for the genome research in model legumes such as *L. japonicus* and *M. truncatula*, which are infected by infection threads formation, a number of host legume genes involved in Nod factors perception and subsequent symbiotic signal transduction have been identified in the past decade. However, this knowledge is still scarce in legumes infected by crack entry.

8.4 Diversity of Bacteria-Nodulating Legumes

Beijerinck in Holland isolated and cultivated by the first time a microorganism from inside nodules of legumes in 1888, which was named *Bacillus radicicola*. Frank (1889) firstly named bacteria isolated inside nodule as *Rhizobium leguminosarum*, and since this date, all bacteria able to nodulate legumes are called rhizobia. However, the taxonomy, and nomenclature of the root nodule bacteria, has been in constant review ever since.

The classification of the first rhizobial species was mainly based on their growth rates on a defined substrate (fast and slow growers) as well as on their legume host specificities (Baldwin and Fred 1929). Nowadays, DNA and protein sequences are widely used to infer phylogenies of rhizobia. However, it is widely accepted that genes easily transferred among species are not useful in taxonomy. In this sense, in 1970s decade, it was reported that symbiotic genes are harbored in plasmid (pSym) in fast and in some intermediate-growing species of rhizobia, whereas they are integrated in the chromosome of intermediate and slow-growing rhizobia, harbored in symbiotic islands (Sullivan et al. 2002; Crossman et al. 2008). Horizontal gene transfer (HGT) of both, pSym and symbiotic islands, has been well documented (Lozano et al. 2010; Ibañez et al. 2010; Sullivan and Ronson 1998). Then, care should be taken when using only symbiotic gene sequences for phylogenetic studies.

In the second edition of Bergey's Manual of Systematic Bacteriology published in 2005, after the analysis of 16S rRNA genes, rhizobia were included in several families within the new order Rhizobiales in the class alpha Proteobacteria (Kuykendall 2005). Taxonomy based on 16S rRNA gene sequence presupposes that genes are inherited in hierarchical manner and that each genome harbors a single copy of this gene or that multiple alleles within a single genome have identical sequences. However, exceptions to this hypothesis have now been described in various taxa (Dreyden and Kaplan 1990; Rainey et al. 1996; Condon et al. 1999; Amann et al. 2000), and therefore, discordance in 16S rRNA phylogeny may also result from HGT and recombination (Ochman et al. 2005).

Van Berkum and coworkers (2003) have reexamined the phylogenetic relationships among rhizobia by comparative analysis of 16S rRNA, 23S rRNA genes, and ITS region within the *rrn* operon sequences. Tree topologies generated with 16S rRNA gene sequences were significantly different to those corresponding to the 23S rRNA and ITS region sequences. For instance, based on 23S rRNA sequences, *Bradyrhizobium elkanii* and *B. japonicum* were placed in a single group, whereas when considering 16S rRNA sequences, they were separated into *Blastobacter denitrificans*, *Rhodopseudomonas palustris*, and *Afipia felis*.

With the current knowledge about the diversity of bacteria able to induce nodule formation on legumes, it became apparent that a common error in the rhizobial taxonomy was to consider the nodulation of legumes as an exclusive ability of rhizobia, and thus, the strains isolated from nodules that do not present the typical colonies on YMA plates were discarded. This situation dramatically changed when scientists started to use 16S rRNA gene sequencing to the identification of nodule isolates. Thus, in the past 9 years, several non-classical rhizobia but also capable of forming nodules and fixing nitrogen in legume roots have been documented and grouped within alpha and beta Proteobacteria, such as *Methylobacterium nodulans*, *Burkholderia* sp., *Blastobacter denitrificans*, *Devosia neptunia*, *Ochrobactrum lupini* and *O. cytisi*, *Phyllobacterium trifolii*, *Ralstonia taiwanensis* (renamed as *Cupriavidus taiwanensis*), *Burkholderia tuberum, B. phymatum*, *B. cepacia*, *B. mimosarum*, *B. nodosa*, and *B. sabiae* (Rivas et al. 2009).

Currently, rhizobial group is constituted by 76 species into 13 genera: *Rhizobium*, *Mesorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Bradyrhizobium*, *Azorhizobium*, *Methylobacterium*, *Burkholderia*, *Cupriavidus*, *Devosia*, *Herbaspirillum*, *Ochrobactrum*, *Phyllobacterium,* and *Shinella*. However, recent research has shown that there are many other rhizobial species in addition to these. In some cases, these new species have arisen through horizontal gene transfer of symbiotic genes (Weir 2010).

Considering the information generated in the last years, it became clear that the legume symbioses are still poorly understood and that further studies are required especially on symbionts from legumes growing in ecosystems that until now remained unexplored.

8.5 Non-symbiotic Nodule Endophytic Bacteria

Over the years, the term "root nodule bacteria" has been exclusively applied to rhizobia. However, nonsymbiotic endophytic bacteria from several genera have been isolated from legume nodules, and this will be discussed in this section.

8.5.1 Generalities

Traditionally, the term "endophytes" has been restricted to mutualistic or commensal microorganisms found exclusively in regular tissues of the host plant and excluding specific organs such as nodules and galls (Rai et al. 2007). However, in the last years, there was an increase in the number of articles dealing with bacterial endophytes obtained from nodules (specific legume organs). To avoid confusion in the following, the term "nodule endophyte" will be used to refer to nonsymbiotic bacteria that reside inside nodules of legumes but cannot induce nodule formation.

From an ecological perspective, nonsymbiotic colonization of nodules can be understood as a survival and persistence bacterial strategy. The ability to find a new ecological niche within legume nodules could allow bacteria to survive and persist in a challenging environment such as soil. In this sense, the ability of a PGPB to persist and reproduce within nodules is an advantageous and attractive trait, even when the growth promotion effect could not necessarily be performed inside the tissues of the plant. For instance, bacterial phosphate solubilization is a major direct plant-growth-promoting effect that is carried out in the rhizosphere. However, the release of phosphate solubilizing bacteria from senescent nodules ensures the presence of a stable population of this nonsymbiotic PGPB in soils. From other perspective, serious concerns have been raised since nodule can harbor bacteria reported as human pathogens. Bacteria phylogenetically related to *Klebsiella oxytoca*, *Enterobacter cloacae,* and *Pantoea sp*. were obtained from the interior of peanut nodules in Argentina (Ibañez et al. 2009; Taurian et al. 2010); *Salmonella, Erwinia, Klebsiella, Citrobacter, Pantoea, and Enterobacter* were obtained from trunk nodules of *Conzattia multiform* in Mexico (Wang et al. 2006); and *Enterobacter cloacae, Enterobacter kobei, Escherichia vulneris, Pantoea agglomerans,* and *Leclercia adecarboxilata* were isolated from nodules of wild legumes in Algeria (Benhizia et al. 2004). Furthermore, Muresu et al. (2010) indicated that the later collection of nodule endophytes possess virulence determinants such as cytotoxicity, vital stain exclusion and adhesion to epithelia, and displayed complex patterns of antibiotic resistance. Therefore, it becomes evident that the lifecycles of some endophytes are not limited to plant and soil environments and can include stages within animals and humans hosts (Muresu et al. 2010). The existence of this secondary niche for human pathogens is important from a clinical and epidemiological perspective and should be studied carefully.

The presence of bacterial isolates inside nodules belonging to genera not known to include any legume-nodulating member raised questions about their origin. One of the first hypotheses was that nodule endophytes were genuine symbionts that acquired symbiotic genes from conventional symbionts (alpha or beta Proteobacteria) through horizontal gene transfer. According to this presumption, nonsymbiotic nodule endophytes represent potential receptors of symbiotic genes and may be raw material of novel symbiotic bacteria. However, some studies led to the rejection of this hypothesis since the absence of *nod* genes has been demonstrated in peanut nodule endophytes (Ibañez et al. 2009) and spontaneous legumes from Tunisia (Zakhia et al. 2006). Alternatively, and taking into account that the legume *Aeschynomene sensitiva* and *A. indica* are nodulated without Nod factor signaling (Giraud et al. 2007), it could be proposed that nodule endophytes are novel symbiotic bacteria that use an unconventional molecular dialogue to induce nodule formation. Nonetheless, there are evidences against this hypothesis. First, nodule endophytes by themselves are not capable to induce nodulation in the original host legume or in a wide host range legume such as *Macroptilium atropurpureum* (Ibañez et al. 2009; Lei et al. 2008; Zakhia et al. 2006). Second, nodule endophytes are able to colonize nodules previously formed by the compatible rhizobial strain (Ibañez et al. 2009). Third, until now, nodulation without Nod factors is restricted to a few species. In *Arachis hypogaea* L. (peanut), a legume taxonomically related to *Aeschynomene* that is also invaded by crack entry, the requirement of Nod factor for nodule primordia formation has been reported (Ibañez and Fabra 2011). Finally, results from the culture-independent analysis of nodule occupants from native noninoculated legumes revealed that these structures are always co-occupied by a compatible rhizobial strain in viable but not culturable (VBNC) state (Muresu et al. 2008).

8.5.2 Genetic Diversity of Nodule Endophytic Bacteria

Genetic analyses indicated that nodule endophytes exhibit great diversity and represent different bacterial lineages (Table 8.1).

Considering the definition of the term "nodule endophyte," rhizobia that reside inside nodules of a legume but cannot induce their formation can also be considered within this group.

The traditional strategy used to investigate nodule-associated bacteria involves their isolation and cultivation from internal tissues of surface-sterilized nodules. In the past, isolation procedures focused primarily on cultivable microorganisms. Therefore, culturability of bacteria was a main issue. However, increasing interest in nonculturable endophytic microorganisms has recently led to the application of molecular methods for their identification (Hallmann et al. 2006). The application of a culture-independent approach led to a change in the analysis of bacterial diversity in several ecosystems and could result in a revolution of the concept of nodule endophytes in particular.

Table 8.1 Bacterial taxa described as nodule endophytes

Bacterial taxa	Host legume	Type of nodule	Reference
Agrobacterium	Diverse legumes, including *Phaseolus, Crotalaria, Mimosa*, *Onobrachis,* etc.	Determinate and indeterminate	de Lajudie et al. (1999), Gao et al. (2001), Liu et al. (2005), Mhamdi et al. (2002)
Actinobacteria	Pea	Indeterminate	Tokala et al. (2002)
Bacillus	Soybean *Cajanus cajan*	Determinate	Bai et al. (2002), Rajendran et al. (2008)
Pantoea agglomerans, Enterobacter kobei, Enterobacter cloacae, Leclercia adecarboxylata, Escherichia vulneris, Pseudomonas sp.	*Hedysarum carnosum, Hedysarum spinosissimum* subsp. *capitatum, Hedysarum pallidum*	Indeterminate	Benhizia et al. (2004)
Salmonella, Erwinia, Klebsiella, Citrobacter, Pantoea and Enterobacter	*Conzattia multiflora*	Trunk nodules	Wang et al. (2006)
Phyllobacterium, Sphingomonas, Rhodopseudomonas, Pseudomonas, Microbacterium, Mycobacterium, Bacillus, Paenibacillus	Spontaneous legumes	Determinate and indeterminate	Zakhia et al. (2006)
Agrobacterium and Enterobacteriaceae	Herbaceous legumes		Kan et al. (2007)
Agrobacterium, Phyllobacterium, Ensifer, Shinella, R. tropici, *R. leguminosarum*	*Vicia*	Indeterminate	Lei et al. (2008)
Pantoea, Serratia, Acinetobacter, Bacillus, Agrobacterium, and *Burkholderia*	Soybean	Determinate	Li et al. (2008)
Enterobacter, Klebsiella and *Pseudomonas*	*Arachis hypogaea* L.	Determinate	Ibañez et al. (2009)
Bacillus megaterium, Brevibacillus chosinensis, Microbacterium trichothecenolyticum	*Medicago sativa*	Indeterminate	Stajković et al. (2009)
Arthrobacter, Bacillus, Dyella, Microbacterium, Staphylococcus	*Lespedeza* sp.	Determinate	Palaniappan et al. (2010)

(continued)

Table 8.1 (continued)

Bacterial taxa	Host legume	Type of nodule	Reference
Micromonospora	*Arachis, Cicer, Glycine, Medicago, Lupinus, Pisum, Trifolium, Lens, Ononis, Ornithopus, Vicia, Mucuna*	Determinate and indeterminate	Cerda Castillo (2008), Trujillo et al. (2010)
Enterobacter, Pseudomonas, Achromobacter, Stenotrophomonas and *Sphingobacter*	*Clitoria ternatea* L.	Determinate	Aeron and Maheshwari (2011)
Acidovorax	*Cajanus cajan* L.	Determinate	Arya and Maheshwari (2009)

8.5.3 *Beneficial Effects of Nodule Endophytes*

The relationship between endophytes and the host plant could be neutral or beneficial through several mechanisms. Among these, nodule endophytes were mainly analyzed for phosphate solubilization, *nifH* presence and production of organic acids, siderophore, and IAA (Cerda Castillo 2008; Ibañez et al. 2009; Li et al. 2008; Rajendran et al. 2008; Trujillo et al. 2010; Zakhia et al. 2006). The results obtained are variable but indicate that many strains possess PGP activities. However, the expression of these activities inside nodules and their effects on the host legume are still unclear. However, promising effects were observed after co-inoculation of some legumes with genuine symbionts and nodule endophytes. In peanut, co-inoculation of specific symbiont (*Bradyrhizobium* sp.) and nodule endophytes of the genera *Enterobacter* led to a significantly increase in the number of nodules produced. Since it was determined that these *Enterobacter* strains produce IAA (a phytohormone that promotes the formation of lateral roots) and considering that rhizobia invade this legume at the sites of lateral root emergence, it can be speculated that the plants inoculated with these bacteria might have more sites for rhizobial infection (Ibañez et al. 2009). Another interesting cooperative effect between nodule endophytes and rhizobia has been reported (Liu et al. 2010). Co-inoculation with a mixture of *Agrobacterium* sp. II CCBAU21244 and *Sinorhizobium meliloti* induced the formation of nodules in *Wisteria sinensis* and two other woody legumes, which do not establish symbiosis with *S. meliloti* alone. Beneficial effects of co-inoculation with nodule endophytes were also observed in *Medicago* growing under sterile conditions (Stajković et al. 2009), *Cajanus* (Pandey and Maheshwari 2007; Rajendran et al. 2008) and *Lupinus,* and *Phaseolus* (Cerda Castillo 2008). Nevertheless, the mechanisms involved are still unknown. Beyond these results, experiments demonstrate that nodule endophytes constitute a population of bacteria with interesting plant-growth-promoting properties.

Considering that nodule endophytic bacteria constitute a population of microorganisms that directly or indirectly interact with rhizobial symbionts, it is possible to speculate that they could affect the development of an effective nitrogen-fixing symbiosis. However, the fact that bacteria other than rhizobia are present inside nodules has been minimized, and it represents an overlooked phenomena. Further studies are required in order to assess the effects of nonsymbiotic endophytic nodule bacteria.

8.5.4 Perspectives

Regarding nodule endophytic bacteria, there are more questions than certainties. Knowledge of the biological diversity of interactions between legumes and bacteria is still very limited. Extending the study to a greater number of legumes will conduct to a description of new nodule endophytes, possibly with novel PGP traits. In addition, the application of nonculturable approaches will significantly modify the current knowledge of nodule endophytic bacterial diversity.

Another interesting question is whether nodule endophytes only establish association with specific host plants, as occurs in rhizobia–legume symbiosis. Available information is consistent with a scenario in which plant growth promotion by native endophytic bacteria is highly species specific, regardless of whether or not they express general PGP traits (Long et al. 2008). Particular endophytes could often have important, if not essential, roles for plant growth and development. Therefore, it seems likely that plants could select for specific groups of plant-beneficial endophytes. However, the molecular dialogue regulating host specificity is still unknown.

Nodule nonsymbiotic endophytic colonization constitutes a poorly studied phenomenon, and there is still a lot to learn from bacterial ecology and population dynamics. In addition, further study is required to understand the interaction between legume-symbiotic bacteria-nodule endophyte and how it affects plant growth, nodulation, and nitrogen metabolism.

8.6 Conclusions

Information currently available clearly indicates that bacteria other than rhizobia are colonizing inside legume nodules. Many studies have shown that the coexistence of rhizobial and nonrhizobial bacteria in these organs can increase growth of different legumes. It seems to be evident that the ability of endophytic bacteria to reproduce within nodules is an advantageous strategy in their lifecycles. However, little is known about this particular host–endophyte interaction, and many questions need to be answered. In this sense, the requirement of a molecular dialogue between

legume and nodule endophytes as the established with rhizobia is unknown. Whatever the mechanisms involved, nodule legume constitute a still unexplored ecological niche for colonization by endophytes other than rhizobia.

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Chapter 9
Role of PGPR Under Different Agroclimatic Conditions

Anju Rani and Reeta Goel

9.1 Introduction

- Despite an unprecedented increase in agricultural productivity during the twentieth century, the world faces uncertainty over global food security. The most pressing issue is the predicted increase in global population. Currently, the global population could be fed by the present level of agricultural output, and the global production of food is 145% greater today than it was in 1960 (Pretty 2008). However, it is unlikely that this growth in agricultural productivity can continue to keep pace with the rising population. In addition, increases in productivity over the last 50 years mask significant variations within developing regions that reflect political, economic, and social challenges for the 1.2 billion people who currently live in poverty (Hazell and Wood 2008). Moreover, most developing countries have environmental constraints that will impede the development of agricultural systems able to meet these challenges. These include lack of water, desertification, and insufficient cultivable land. Potentially, such problems could be further exacerbated by climate change. This in turn will place an increased pressure on the available agricultural land and its management (Cummings 2009). During last few decades, agricultural production has increased due to the use of high yielding varieties and enhanced consumption of chemicals, which are used both as fertilizers to provide nutrition and as protection agents to control the damage caused by phytopathogens. Although the use of chemicals has several advantages, such as ease of handling and yielding predictable results, yet several problems related to the continuous export of fertility of the soil, yielding great amount of ecological disastrous soil damage, health problems, and high irrigation demand, etc., came into existence (Harmen 1992). Excessive use

A. Rani • R. Goel (✉)
Department of Microbiology, G.B. Pant University of Agriculture and Technology, Pantnagar, Uttaranchal 263145, India
e-mail: rg55@rediffmail.com

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of chemicals and change in traditional cultivation practices have resulted in the deterioration of physical, chemical, and biological health of the cultivable soil (Paroda 1997). Therefore, the productivity including production of a wide range of agricultural commodities under conditions of shrinking land resources and diminution of both biological potential of soil and biological wealth need to be increased. The objective of agriculture in coming decades is to optimize soil productivity (inclusive of stressed soils) while preserving its capacity to function as a healthy system. In this context, there is a strong case for using microorganisms for improved plant performance in integrated plant management systems. The use of soil microorganisms, which can stimulate plant growth, will be environmentally benign approach for nutrient management and ecosystem functions. This may ensure that nature is not exploited in the production process but is, instead, harmonized so that the entropy of environment decreases and sustainability in agricultural production is promoted (Khan et al. 2007). The management of agricultural soil is fundamental to ensuring a sustainable agricultural system. Consequently, there is increasing interest in developing and implementing the potential contribution of PGPR that are indigenous or inoculated into soils.

9.2 Plant Growth-Promoting Rhizobacteria

The use of microorganisms with the aim of improving nutrients availability for plants is an important practice and necessary for agriculture (Freitas et al. 2007). During the past couple of decades, the use of plant growth-promoting rhizobacteria (PGPR) for sustainable agriculture has increased tremendously in various parts of the world. Significant increases in growth and yield of agronomically important crops in response to inoculation with PGPR have been reported (Kloepper et al. 1980; Silva et al. 2006; Figueiredo et al. 2008; Arau´jo 2008). Studies have also shown that the growth-promoting ability of some bacteria may be highly specific to certain plant species, cultivar, and genotype (Bashan and Holguin 1998; Lucy et al. 2004).

PGPR can affect plant growth by different direct and indirect mechanisms (Glick 1995; Gupta et al. 2000). Some examples of these mechanisms, which can probably be active simultaneously or sequentially at different stages of plant growth, are (1) increased mineral nutrient solubilization and nitrogen fixation, making nutrients available for the plant; (2) repression of soilborne pathogens (by the production of hydrogen cyanide, siderophores, antibiotics, and/or competition for nutrients); (3) improving plant stress tolerance to drought, salinity, and metal toxicity; and (4) production of phytohormones such as indole-3-acetic acid (IAA) (Gupta et al. 2000). Moreover, some PGPR have the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyses ACC, the immediate precursor of ethylene in plants (Glick 1995). By lowering ethylene concentration in seedlings and thus its inhibitory effect, these PGPR stimulate seedlings root length (Glick et al. 1999). The bacteria presenting one or more of these characteristics are known as PGPR.

9.3 Rhizosphere Colonization

Plant growth-promoting rhizobacteria (PGPR) colonize the roots of plants following inoculation onto seed and that enhance plant growth. The following are implicit in the colonization process: ability to survive inoculation onto seed, to multiply in the spermosphere (region surrounding the seed) in response to seed exudates, to attach to the root surface, and to colonize the developing root system (Kloepper 1993). The ineffectiveness of PGPR in the field has often been attributed to their inability to colonize plant roots (Bloemberg and Lugtenberg 2001). A variety of bacterial traits and specific genes contribute to this process, but only a few have been identified (Benizri et al. 2001; Lugtenberg et al. 2001). These include motility, chemotaxis to seed and root exudates, production of pili or fimbriae, production of specific cell surface components, ability to use specific components of root exudates, protein secretion, and quorum sensing (Lugtenberg et al. 2001). Using molecular markers such as green fluorescent protein or fluorescent antibodies, it is possible to monitor the location of individual rhizobacteria on the root using confocal laser scanning microscopy (Sorensen et al. 2001). This approach has also been combined with an rRNA-targeting probe to monitor the metabolic activity of a rhizobacterial strain in the rhizosphere and showed that bacteria located at the root tip were most active (Lubeck et al. 2000; Sorensen et al. 2001).

An important aspect of colonization is the ability to compete with indigenous microorganisms already present in the soil and rhizosphere of the developing plant. The factors involved in these interactions has been hindered by inability to culture and characterize diverse members of the rhizosphere community and to determine how that community varies with plant species, plant age, location on the root, and soil properties. Phenotypic and genotypic approaches are now available to characterize rhizobacterial community structure. Phenotypic methods that rely on the ability to culture microorganisms include standard plating methods on selective media, community level physiological profiles (CLPP) using the BIOLOG system (Garland 1996), phospholipid fatty acid (PLFA) (Tunlid and White 1992), and fatty acid methyl ester (FAME) profiling (Germida et al. 1998). Culture-independent molecular techniques are based on direct extraction of DNA from soil and 16S-rRNA gene sequence analysis, bacterial artificial chromosome, or expression cloning systems (Rondon et al. 1999). These are providing new insight into the diversity of rhizosphere microbial communities, the heterogeneity of the root environment, and the importance of environmental and biological factors in determining community structure (Smalla et al. 2001). These approaches can also be used to determine the impact of inoculation of plant growth-promoting rhizobacteria on the rhizosphere community (Ciccillo et al. 2002; Steddom et al. 2002).

9.4 Mechanisms of Action

PGPR enhance plant growth by direct and indirect means (Glick 1995). Direct mechanisms of plant growth promotion by PGPR can be demonstrated in the absence of plant pathogens or other rhizosphere microorganisms, while indirect mechanisms involve the ability of PGPR to reduce the deleterious effects of plant pathogens on crop yield. PGPR have been reported to directly enhance plant growth by a variety of mechanisms: fixation of atmospheric nitrogen that is transferred to the plant, production of siderophores that chelate iron and make it available to the plant root, solubilization of minerals such as phosphorus, and synthesis of phytohormones (Glick 1995). Direct enhancement of mineral uptake due to increases in specific ion fluxes at the root surface in the presence of PGPR has also been reported (Bashan and Holguin 1998; Bertrand et al. 2000). PGPR strains may use one or more of these mechanisms in the rhizosphere. Molecular approaches using microbial and plant mutants altered in their ability to synthesize or respond to specific phytohormones have increased understanding of the role of phytohormone synthesis as a direct mechanism of plant growth enhancement by PGPR (Glick 1995; Persello-Cartieaux et al. 2003). PGPR that synthesize auxins and cytokinins or that interfere with plant ethylene synthesis have been identified (Garcia de Salamone et al. 2001).

PGPR that indirectly enhance plant growth via suppression of phytopathogens do so by a variety of mechanisms. These include the ability to produce siderophores that chelate iron, making it unavailable to pathogens; the ability to synthesize antifungal metabolites such as antibiotics, fungal cell wall-lysing enzymes, or hydrogen cyanide, which suppress the growth of fungal pathogens; the ability to successfully compete with pathogens for nutrients or specific niches on the root; and the ability to induce systemic resistance (Bloemberg et al. 2000; Glick 1995). Furthermore, biochemical and molecular approaches are providing new insight into the genetic basis of these traits, the biosynthetic pathways involved, their regulation, and importance for biological control in laboratory and field studies (Bloemberg and Lugtenberg 2001; Bowen and Rovira 1999; Persello-Cartieaux et al. 2003).

9.4.1 Enhancing Phosphorus Availability for Plant Growth by Rhizobacteria

Phosphorus (P) is an essential plant nutrient with low availability in many agricultural soils. Today many agricultural soils have a high total P content due to the application of P fertilizers over long periods of time. On the other hand, much of this P is in mineral forms and is only slowly available to plants (Rodriguez et al. 2006; Richardson et al. 2009). Most of the insoluble P forms are present as aluminum and iron phosphates in acid soils (Mullen 2005) and calcium phosphates

in alkaline soils (Goldstein and Krishnaraj 2007). The ability of rhizosphere bacteria to solubilize insoluble P minerals has been attributed to their capacity to reduce pH by the excretion of organic acids (e.g., gluconate, citrate, lactate and succinate) and protons (during the assimilation of $NH4^+$) (Gyaneshwar et al. 1999; Mullen 2005). These bacteria have been characterized as members of the *Bacillus, Burkholderia, Enterobacter, Klebsiella, Kluyvera, Streptomyces, Pantoea,* and *Pseudomonas* genera (Chung et al. 2005; Hariprasad and Niranjana 2009; Oliveira et al. 2009). These microorganisms grow in media with tricalcium phosphate or similar insoluble materials as the only phosphate source and not only assimilate the element but also solubilize quantities in excess of their nutritional demands, thereby making it available for plants (Martínez-Viveros et al. 2010).

Microorganisms with phosphate-solubilizing potential increase the availability of soluble phosphate and enhance the plant growth (Kucey et al. 1989; Ponmurugan and Gopi 2006). *Pseudomonas* spp. NBRI 4014 enhanced the root and shoot elongation in soybean crop at a significant level in the presence of heavy metals (Gupta et al. 2002). Similarly, phosphate-solubilizing bacteria enhanced the seedling length of *Cicer arietinum* (Sharma et al. 2007), while co-inoculation of PSM and PGPR reduced P application by 50% without affecting corn yield (Yazdani et al. 2009). In another study by Kaur (2008), it has been found that inoculation of spinach with two psychrotolerant stain *Pseudomonas putida* 710 A and *Commamonas aquatica* 710 B resulted an increase P content (Fig. 9.1) in soil as well as in plants (Table 9.1).

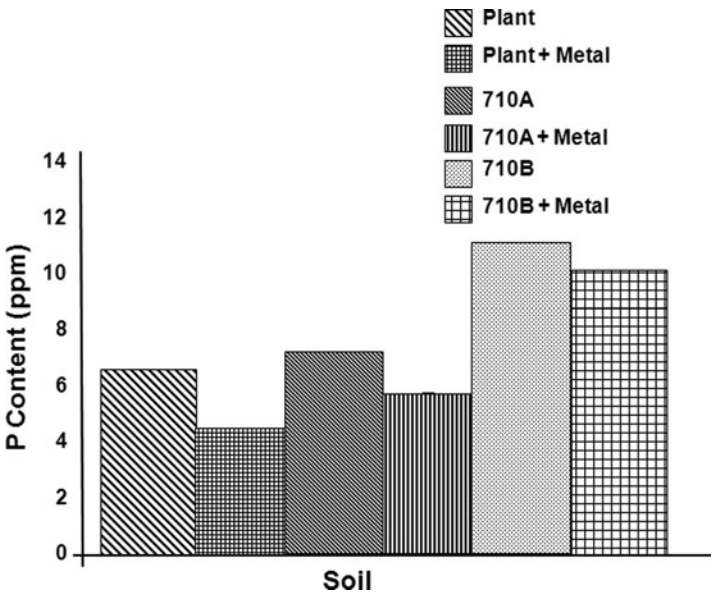

Fig. 9.1 Comparative "P" content in soil in presence of bioinoculants 710A and 710B

Table 9.1 Comparative "P" content in plants and soil in presence of bioinoculants 710A and 710B with respect to control

Strain	P solubilization (in vitro)	Treatments	P content in soil (ppm)	P content in plants (ppm)
–	–	Plants (control)	6.11 ± 0.32	6.62 ± 0.18
Pseudomonas putida 710A	(1.76 μg/ml)	Plants + *P. putida* 710A	6.41 ± 0.12 (4.9%) ↑	15.35 ± 0.20 (131.8%) ↑
Pseudomonas putida 710B	(240 μg/ml)	Plants + *C. aquatica* 710B	11.1 ± 0.52 (81.6%) ↑	18.79 ± 0.44 (183.8%) ↑

↑ % increase with respect to control
± values are SEM

The major limitation today for use of these organisms is the lack of consistent effects in mobilizing P under field conditions. This is likely due to competition with the native microflora and environmental factors that limit either the population size or activity of the PGPR. However, it is now clear that evaluation and ranking of P-solubilizing bacteria under laboratory conditions do not necessarily correspond to the efficacy of the PGPR for enhancing plant P uptake under field conditions (Richardson 2001).

9.4.2 *Facilitated Absorption of Iron by Production of Siderophores*

Iron is an essential nutrient of plants, but it is relatively insoluble in soil solutions. Plant roots prefer to absorb iron as the more reduced ferrous (Fe^{2+}) ion, but the ferric (Fe^{3+}) ion is more common in well aerated soil although it is easily precipitated in iron-oxide forms (Verma et al. 2010).

Siderophores are low-molecular-weight iron-binding molecules that are synthesized by many microorganisms (Neilands 1981). These compounds are produced by various types of bacteria in response to iron deficiency which normally occurs in neutral to alkaline pH soils, due to low iron solubility at elevated pH (Sharma and Johri 2003). Iron is essential for cellular growth and metabolism, such that Fe acquisition through siderophore production plays an essential role in determining the competitive fitness of bacteria to colonize plant roots and to compete for iron with other microorganisms in the rhizosphere (Crowley 2006). Siderophore-producing PGPR can prevent the proliferation of pathogenic microorganisms by sequestering Fe^{3+} in the area around the root (Siddiqui 2006).

Marschner and Römheld (1994) reported that plants may also utilize siderophores synthesized by microorganisms colonizing the rhizosphere; this would be a source of soluble iron for the host plant. Growth of cucumber in the presence of microbial siderophores resulted in increased plant biomass and chlorophyll content (Ismande 1998). Similarly, the growth of mungbean and pigeon pea enhanced in terms of increased root length, shoot length, and chlorophyll content in the presence of siderophore-producing *Pseudomonas putida*

KNP9 and *Proteus vulgaris* KNP3 strain, respectively (Tripathi et al. 2005; Rani et al. 2008). Uptake of microbial siderophores by plants has been attributed to microorganisms living.

9.4.3 Production of Phytohormones

The production of phytohormones by PGPR is now considered to be one of the most important mechanisms by which many rhizobacteria promote plant growth (Spaepen et al. 2007). Phytohormones are signal molecules acting as chemical messengers and play a fundamental role as growth and development regulators in the plants. Phytohormones are organic compounds that in extremely low concentrations influence biochemical, physiological, and morphological processes in plants, and their synthesis is finely regulated (Fuentes-Ramírez and Caballero-Mellado 2006). Numerous fungal and bacterial species can produce phytohormones (Tsavkelova et al. 2006). The phytohormone-producing ability is widely distributed among bacteria associated with soil and plants. Studies have demonstrated that the PGPR can stimulate plant growth through the production of auxins (indole acetic acid) (Spaepen et al. 2008), gibberellins (Bottini et al. 2004), and cytokinins (Timmusk et al. 1999) or by regulating the high levels of endogenous ethylene in the plant (Glick et al. 1998).

9.5 Survival of PGPR Under Different Agroclimatic Conditions

The rhizosphere is a complex habitat: there, the action of a growing root responding to its environment combines with that of the biotic (mostly the resident microorganisms) and abiotic soil components, which also respond to their environments. The introduction of a large amount of exogenous bacteria as an inoculant has the potential to affect these resident microorganisms, and similarly, an inoculant may be affected by them. Such interferences may result in increased, decreased, or no effect on PGPR effectiveness. Other stresses like desiccation, salinity, metals (Fig. 9.2), and temperature have direct effect on microbial population (Rani et al. 2009).

The salt pH and temperature-tolerant phosphate-solubilizing bacteria have been reported to be maximum in the rhizoplane followed by the rhizosphere and root-free soil in alkaline soils. The PSM strains with these stressed properties should therefore serve as an excellent model for studying the physiological, biochemical, and molecular mechanism of phosphate solubilization under stressed ecosystems (Khan et al. 2007).

In a study, screening and selection of cold-tolerant mutants of *Pseudomonas fluorescens* strains GRS_1, PRS_9, and ATCC13525 based on P-solubilization ability and subsequent effect on plant growth promotion under in vitro and in situ

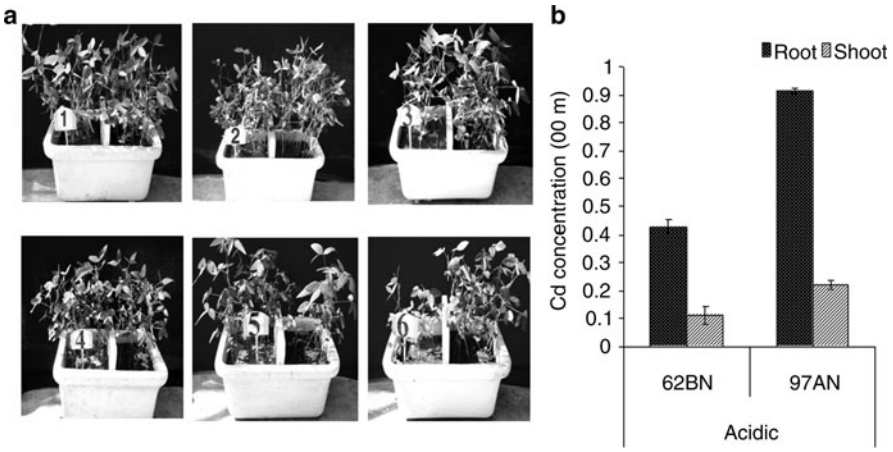

Fig. 9.2 (**a**) Effect of cadmium-resistant *P. putida* 62BN and *P. monteilli* 97AN bioinoculants on soybean growth in acidic soil, wherein (1) plants, (2) plant + cadmium, (3) *P. putida* 62BN, (4) *P. putida* 62BN + cadmium, (5) *P. monteilli* 97AN, and (6) *P. monteilli* 97AN + cadmium, respectively. (**b**) Comparative cadmium accumulation in soybean in the presence of *P. putida* 62BN and *P. monteilli* in acidic soil, respectively ($n = 3$, mean ± SEM)

condition was conducted. It has been found that there was 21-fold increase in $CRPF_2$ (GRS_1 mutant), and subsequent greenhouse trials revealed that $CRPF_2$ was a good rhizosphere colonizer as marked by a significant increase in root and shoot length of mungbean (Katiyar and Goel 2003).

In a screening of 4,800 bacterial isolates from the root-free soil, rhizosphere and rhizoplane of *P. juliflora* growing in alkaline soils, 857 morphotypes solubilized phosphate in agar. Phosphate-solubilizing ability of strain NBRI4 was higher than the control in the presence of salts (NaCl, CaCl2, and KCl) at 30°C, and it further increased at 37°C (Gaur et al. 2004). Strain NBRI2601 (Nautiyal et al. 2000) isolated from the rhizosphere of chickpea and alkaline soils could solubilize phosphorus in presence of 10% salt, pH 12, at 45°C suggesting that extensive diversity searches in appropriate habitats may lead to recovery of effective bacteria. The mechanism of osmotic stress adaptation in *P. aeruginosa* PAO1 was investigated by D'Souza-Ault et al. (1993). By using natural abundance 13C nuclear magnetic resonance spectroscopy, osmotically stressed cultures were found to accumulate glutamate, trehalose, and *N*-acetylglutaminylglutamine amide, an unusual dipeptide previously reported only in osmotically stressed *Rhizobium meliloti* and *P. fluorescens*. The intracellular levels of these osmolytes were dependent on the chemical composition and the osmolality of the growth medium. It was also demonstrated that glycine betaine, a powerful osmotic stress protectant, participated in osmoregulation in this organism (Tilak et al. 2005).

Another problem which is recently increasing is the contamination of soils with heavy metals through a variety of anthropogenic sources such as mining, the combustion of fossil fuels, metal-working factories, and the application of agrochemicals. Heavy metals tend to accumulate in the surface soil layer and can

Table 9.2 Metal-resistant bioinoculants with diverse physiological profile

Strain	Physiological profile	Metal tolerance level	Growth promotory property	Crop used	Reference
NBRI4014	Alkalophile (30°C)	Cd (0.18 mM)	P solubilization (277 μg/ml), siderophore production (143.87 μg/ml)	Soybean	Gupta et al. (2002)
KNP3	Mesophile (30°C)	Cd (1 mM) Pb (1.3 mM) Cu (1.3 mM)	Siderophore production (126.3 μg/ml)	Pigeon pea	Rani et al. (2008)
KNP9	Mesophile (30°C)	Cd (0.5 mM) Pb (1.5 mM)	Siderophore production (96.6 μg/ml)	Mungbean	Tripathi et al. (2005)
710A	Psychrotolerant (10°C)	Cd (1 mM)	P solubilization (1.76 μg/ml)	Spinach	Kaur (2008)
710B	Psychrotolerant (10°C)	Cd (0.5 mM)	P solubilization (240 μg/ml)	Spinach	Kaur (2008)

reach concentrations that are toxic for plants and living organisms (Gupta et al. 2002; Tripathi et al. 2005; Rani et al. 2008). One way to relieve heavy metal toxicity to plants might involve the use of growth-promoting bacteria. Considering our expertise in heavy metal and cold resistance, it was found that many bacterial strains which have shown ability for rescuing plant from metal toxicity and enhanced the plant growth in heavy metal-contaminated microcosm system (Table 9.2).

9.6 Challenges in Selection and Characterization of PGPR

One of the challenges in developing PGPR for commercial application is ensuring that an effective selection and screening procedure is in place, so that the most promising organisms are identified and explored. In the agricultural chemical industry, thousands of prospective compounds are screened annually in efficient high-throughput assays to select the best one or two compounds for further development. Similar approaches are not yet in place for PGPR. Effective strategies for initial selection and screening of rhizobacterial isolates are required. It may be important to consider host plant specificity or adaptation to a particular soil, climatic conditions, or pathogen in selecting the isolation conditions and screening assays (Bowen and Rovira 1999; Chanway et al. 1989). One approach for selection of organisms with the potential to control soilborne phytopathogens is to isolate from soils that are suppressive to that pathogen (Weller et al. 2002). Other approaches involve selection based on traits known to be associated with PGPR such as root colonization (Silva et al. 2003), 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Glick 1995), antibiotic (Giacomodonato et al. 2001),

and siderophore production. Further, the development of high-throughput assay systems and effective bioassays will facilitate selection of superior strains (Mathre et al. 1999).

9.7 Practical Consideration in the Use of PGPR

The concept of PGPR is now well established for both growth promotion as well as biocontrol; still the technology is not commercially successful, mainly because of the lack of reproducibility between trials conducted under controlled conditions (laboratory or glasshouse or greenhouse) and the fields. This happens so as in most cases the microbial inoculants are usually taken from one environment and introduced in another.

A common problem in much research on PGPR has been the failure to monitor the cell density of the introduced bacteria over time to confirm that inoculation was effective. In such cases, it is not possible to determine whether PGPR are responsible for the observed effects or to explain variations in efficacy of the inoculants that may be caused by management or environmental factors (Martínez-Viveros et al. 2010).

Mathematical modeling of the behavior of PGPR soil inoculants has been used to predict how various environmental factors affect the survival and activity of PGPR soil inoculants (Strigul and Kravchenko 2006). Supporting much experimental work, the model by Strigul and Kravchenko illustrates that survival and growth of newly introduced bacteria are strongly limited by competition for organic substrates with the resident microflora. PGPR are predicted to be the most effective in soils with low organic matter or stressed soils where growth of the indigenous population is restricted.

9.8 Rhizoengineering

Rhizoengineering includes strategies for manipulating plants and their root-associated microorganisms to improve plant health and productivity. Some strategies directly target plant processes that impact on growth, while others are based on our knowledge of interactions among the components of the rhizosphere (roots, microorganisms, and soil). For instance, plants can be engineered to modify the rhizosphere pH or to release compounds that improve nutrient availability, protect against biotic and abiotic stresses, or encourage the proliferation of beneficial microorganisms. Rhizobacteria that promote plant growth have been engineered to interfere with the synthesis of stress-induced hormones such as ethylene, which retards root growth, and to produce antibiotics and lytic enzymes active against soilborne root pathogens. Rhizosphere engineering also can involve the selection by plants of beneficial microbial populations. For example, some crop species or cultivars select for and support populations of antibiotic-producing

strains that play a major role in soils naturally suppressive to soilborne fungal pathogens. The fitness of root-associated bacterial communities also can be enhanced by soil amendment, a process that has allowed the selection of bacterial consortia that can interfere with bacterial pathogens. Plants also can be engineered specifically to influence their associated bacteria, as exemplified by quorum-quenching strategies that suppress the virulence of pathogens. New molecular tools and powerful biotechnological advances will continue to provide a more complete knowledge of the complex chemical and biological interactions that occur in the rhizosphere, ensuring that strategies to engineer the rhizosphere are safe, beneficial to productivity, and substantially improve the sustainability of agricultural systems (Ryan et al. 2009).

9.9 Future Prospects

As our understanding of the complex environment of the rhizosphere, of the mechanisms of action of PGPR, and of the practical aspects of inoculant formulation and delivery increases, we can expect to see new PGPR products becoming available. The success of these products will depend on our ability to manage the rhizosphere to enhance survival and competitiveness of these beneficial microorganisms. Rhizosphere management will require consideration of soil and crop cultural practices as well as inoculant formulation and delivery. Genetic enhancement of PGPR strains to enhance colonization and effectiveness may involve addition of one or more traits associated with plant growth promotion. Genetic manipulation of host crops for root-associated traits to enhance establishment and proliferation of beneficial microorganisms is being pursued.

The use of PGPR inoculants in agriculture is already proceeding and offers many opportunities to improve plant nutrition, crop yields, and disease management, while improving sustainability by reducing the need for chemical inputs. Nevertheless, as our understanding of the ecology of these bacteria improves, it should be possible to obtain a more informed explanation of the mechanisms that are involved in plant growth promotion and identify situations in which bioaugmentation with soil inoculants may be useful for increasing crop yields.

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Chapter 10
Consortium of Plant-Growth-Promoting Bacteria: Future Perspective in Agriculture

Piyush Pandey, Sandeep Bisht, Anchal Sood, Abhinav Aeron, G.D. Sharma, and D.K. Maheshwari

10.1 Dynamics of Bacterial Diversity in Rhizosphere

Soil is a dynamic, living matrix that is an essential part of the terrestrial ecosystem. It is a critical resource not only for agricultural production and food security but also toward maintenance of most life processes, and it is considered as a storehouse of microbial activity. In 1904, Hiltner coined the term "rhizosphere" referring area around the close vicinity of plant root in which bacteria are abundantly present, most often organized in microcolonies. To exploit the positive effects in rhizosphere, beneficial microorganisms are isolated from soil, cultured, and inoculated into soil (Glick 1995). These rhizobacteria utilize nutrients secreted by the plant root, and in return they influence plant growth in direct or indirect ways including increasing nitrogen uptake, synthesis of phytohormones (auxin, cytokinin), solubilization of minerals, and iron chelation (Bowen and Rovira 1999). These organisms also may suppress soilborne pathogens by producing siderophores, antimicrobial metabolites, or by competing for nutrients and/or niches (Nelson 2004). All of these

P. Pandey
Department of Microbiology, Assam University, Silchar, Assam 788011, India

S. Bisht • A. Sood
Department of Microbiology, S.B.S.P.G. Institute of Biomedical Sciences and Research, Balawala, Dehradun, Uttarakhand 248161, India

A. Aeron
Department of Microbiology, Faculty of Life Sciences, Kurukshetra University, Kurukshetra, Haryana 136 119, India

G.D. Sharma,
Department of Life sciences and Bioinformatics, Assam University, Silchar, Assam 788011, India

D.K. Maheshwari (✉)
Department of Botany and Microbiology, Faculty of Life Sciences, Gurukul Kangri University, Haridwar, Uttarakhand 249404, India
e-mail: maheshwaridk@gmail.com

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activities result in stimulation of plant growth, henceforth yield. Therefore such rhizobacteria are commonly referred as "plant-growth-promoting rhizobacteria" (PGPR) (Kloepper and Schroth 1978). In fact, use of PGPR in modern agriculture is considered as excellent eco-friendly biotechnological approach to replace harmful chemicals.

In intensive cropping system, supplementing soil nutrients by the use of chemical fertilizer is considered inevitable for obtaining optimum yield of crops. However, their utilization efficiency remains low, due to loss by volatilization, denitrification, leaching, and conversion into unavailable forms. Now it is well established that continuous use of chemical fertilizers subverts the soil ecology, disrupts environment, degrades soil fertility, and consequently shows harmful effects on human health (Ayala and Rao 2002) and also contaminates ground water (Joshi et al. 2006). Therefore, large-scale application of PGPR to crops as inoculants would be attractive as it would substantially reduce the use of chemical fertilizers and pesticides, which often pollute the environment. In addition, the application of PGPR would increase crop yield, thereby helping to feed the growing world population to ensure food security to all. A growing number of PGPR are being marketed (Bashan 1998; Pinton et al. 2001).

Recently, there has been a shift in the approach of workers, as, instead of using a single strain of plant-growth-promoting rhizobacterium as inoculants, nowadays co-inoculation of two or multiple PGPR is experimented to achieve prominent multifarious effect on productivity for improving sustainable agriculture system. Seneviratne (2003) recognized that co-inoculation and co-culture of microbes perform the tasks better than the individual microbes. However, in recent years, many studies have shown that co-inoculation of rhizobia and some plant-growth-promoting bacteria (PGPB) increases nodulation and growth in a wide variety of legumes (Bullied et al. 2002; Shaharoona et al. 2006; Tilak et al. 2006). Earlier, 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 each other through physical and biochemical activities that may enhance some beneficial aspects of their physiology (Bashan 1998). When the two different strains are made into an inoculum consortium, each of the individual strains of the consortium not only outcompetes with the others for rhizospheric establishments but also complements functionally for plant growth promotion (Shenoy and Kalagudi 2003). Combined use of plant-growth-promoting rhizobacteria is based on the principles of natural ecosystems, sustained by their constituents. In other terms, the quality and quantity of inhabitants and specific ecological parameters, *i.e.*, the greater the diversity and number of inhabitants, the higher the order of their interaction and more stable the ecosystem. This concept of combined use of plant-growth-promoting rhizobacteria is an effort to shift microbiological equilibrium in favor of increased plant growth production, nutrient uptake, and protection (Higa 1991; Parr et al. 1994). The various strategies that may be used for consortium formulation are summarized in Fig. 10.1.

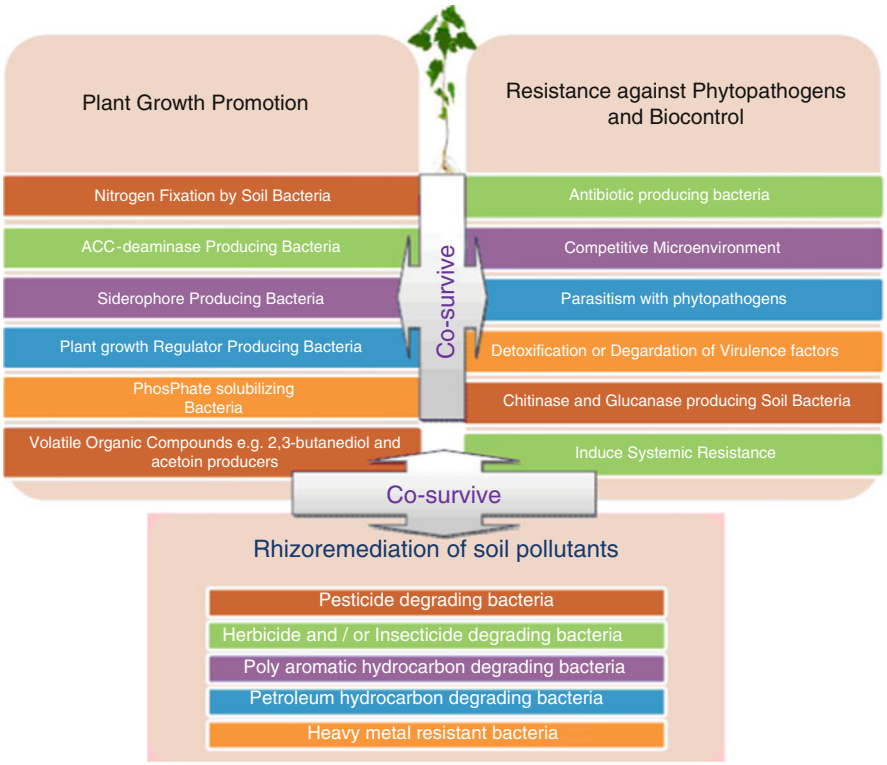

Fig. 10.1 The strategy of rhizobacteria consortium formulation may be designed to enhance the desired benefits. On one hand, diversity attributed for plant growth promotion may be accumulated in finished products of consortium, and/or properties for resistance against soilborne pathogen can be included. Additional benefits including bioremediation can also be achieved. The properties of rhizospheric competence and cosurvival of participating strains are prerequisite for effective formulation

10.2 Consortia of Rhizobia and PGPR

Legume root is colonized by numerous rhizospheric microorganisms, and these organisms have definite influence on the survival and nodulation ability of seed-inoculated rhizobia (Dashti et al. 1998; Davison 1988). There have been several reports where association of bacterial genera with wild legume or other plants improved plant yield, plant health, and nodulation (Bai et al. 2002a, 2003; Zakhia et al. 2006; Rajendran et al. 2008). Some PGPR strains enhance legume growth, nodulation, and nitrogen fixation when coinoculated with rhizobia. Examples of these are *Azospirillum* (Groppa et al. 1998), *Azotobacter* (Burns et al. 1981), *Bacillus* (Srinivasan et al. 1996), *Pseudomonas* (Grimes and Mount 1984), *Serratia* (Chanway et al. 1989; Zhang et al. 1996), and *Streptomyces* (Li and Alexander 1988). *Azotobacter* sp. is known to promote nodulation when used as coinoculum with many different

P-solubilizing organisms including *Bacillus* species (Sahin et al. 2004; Cakmakci et al. 2001). Co-inoculation of P-solubilizing bacteria and *Rhizobium* stimulated plant growth more profoundly than their separate inoculations (Perveen et al. 2002) while there is positive interaction of *Rhizobium* with P-solubilizing sp. of *Bacillus* has translated into significant yield increases of legumes (Zaidi et al. 2003). Increase in nodulation and yield components of legume crops following inoculation with N_2-fixing and P-solubilizing microbes has also been reported by other researchers (Garcia et al. 2004; Gupta 2004). Toro et al. (1998) reported that inoculation of phosphate-solubilizing bacteria (PSB) enhanced nodulation and N_2 fixation by alfalfa plants, in parallel with an increase in the P content of plant tissues, and concluded that an improvement in P nutrition of the plant resulting from the presence of PSB was responsible for increased nodulation and N_2 fixation, as it is well known that these processes are P dependent (Barea et al. 2005).

Dashti et al. (1998) and Dubey (1996) observed that nodule number and nodule weight increase as a result of co-inoculation with *Bradyrhizobium japonicum* and PGPR, for two cultivars of soybean, as compared to inoculation of the *B. japonicum* alone. In co-inoculation studies with PGPR and *Rhizobium*/*Bradyrhizobium* spp., an increase in the root and shoot weight, plant vigor, nitrogen fixation, and grain yield has been shown in various other legumes such as common bean (Grimes and Mount 1984) and green gram (Sindhu et al. 1999). Sindhu et al. (1999) reported increase in nodule number, nodule fresh weight, plant dry weight, and total plant N uptake when *Bradyrhizobium* sp. (*Vigna*) was coinoculated with *Pseudomonas* isolates. Combined inoculation of *Rhizobium* sp. with *Pseudomonas striata* or *Bacillus polymyxa* and *Bacillus megaterium* has shown significant increase in dry weight, grain yield, and phosphorus uptake over the uninoculated control in legumes (Elkoca et al. 2008). Yadegari et al. (2008) also showed that co-inoculation of PGPR with *Rhizobium* sp. and *Bradyrhizobium* sp. increases the root and shoot weight, plant vigor, and grain yield in various legumes. Additionally, Marisa and coworker also demonstrated the co-inoculation with *S. meliloti* strain 3DOh13 and *P. aurantiaca* SR1 on alfalfa plant which resulted in increase in the fresh and dry shoots and root weight of plant. Sindhu et al. (2002) showed that the effect of *Pseudomonas* strain MRS13 isolated from the rhizosphere of green gram on coinoculation with *Mesorhizobium* sp. *cicer* strain Ca181 in legumes, particularly chickpea, indicated the increased in dry weight ratios, i.e., 1.92, 1.84, and 1.98, of plant, as compared to uninoculated control. Similar results were obtained by Sindhu et al. (2002) with co-inoculation of *Pseudomonas* strain and *Mesorhizobium* which stimulated nodule fresh weight and plant dry weight. Co-inoculation studies with PGPR and *Bradyrhizobium japonicum* have also demonstrated increase in root and shoot weight, seed yield, plant vigor, nodulation, and nitrogen fixation in soybean plants (Li and Alexander 1988). An increase in grain yield, nodule dry matter, and nitrogenase activity was also obtained in chickpea inoculated with a mixture of *Azospirillum brasilense* and *Rhizobium* strains (Rai 1983).

Grimes and Mount (1984) found that a *Pseudomonas putida* strain (M17), which had been selected as a potential biological control agent, markedly increased *Rhizobium* nodulation of bean in field soils. Polonenko et al. (1987) found similar

effects of certain rhizobacteria (primarily fluorescent pseudomonads) on nodulation of soybean roots by *B. japonicum*. Numerous studies have therefore indicated that co-inoculation of *Bradyrhizobium* and certain PGPR can positively affect symbiotic nitrogen fixation by enhancing both root nodule number or mass (Polonenko et al. 1987) and increasing nitrogenase activity (Alagawadi and Gaur 1988). Zhang et al. (1996) demonstrated that co-inoculation of *B. japonicum* with *S. proteamaculans* 1-102 reduced the decrease in nitrogen concentration of plant shoots at 15°C root zone temperatures, and further, there was no difference for plant shoot nitrogen content between 15°C and 17°C ± 5°C root zone temperatures. Bai et al. (2002b) showed that co-inoculation of *Serratia proteamaculans* 1-102 and *S. liquefaciens* 2-68 with *Bradyrhizobium japonicum* on soybean [*Glycine max* (L.) Merr.] resulted in significant increased in growth, nodulation, and nitrogen fixation under controlled root zone temperatures (RZTs; 25°C, 20°C, and 15°C) in soilless media.

Actinomycetes have also been reported to improve rhizobial symbiosis in legumes. Solans et al. (2009) observed that the symbiotic effect of saprophytic actinomycetes and *Sinorhizobium meliloti* results in promotion of nodulation in *Medicago sativa* in the presence of high nitrogen. Solans et al. (2011) assayed the effect of co-inoculation of saprophytic rhizoactinomycetes *Streptomyces* MM40, *Actinoplanes* ME3, and *Micromonospora* MM18 isolated from the root nodule surface of the nitrogen-fixing actinorhizal plant *Discaria trinervis* with *Sinorhizobium meliloti* 2011 on *Medicago sativa* in fertilized soil with a low level of N (0.07 mM). The inoculation of the actinomycetes alone did not show any effect on plant growth. Meanwhile, when actinomycetes were coinoculated with *S. meliloti*, nodulation and plant growth were significantly stimulated compared to plants inoculated with only *S. meliloti*. The analysis of nodulation kinetics of simultaneous or delayed co-inoculations suggests that the effect of the actinomycetes operates in early infection and nodule development, counteracting the autoregulation of nodulation by the plant, and the reason for this stimulation is because the actinomycete was found in the symbiotic nitrogen-fixing state of the plant.

Fuhrmann and Wollum (1989) reported that co-inoculation of siderophore-producing pseudomonads with mixtures of the competing bradyrhizobia typically enhanced nodulation by *B. japonicum* strain USDA 110. Srinivasan et al. (1996) found that IAA-producing *Bacillus* isolates promoted root growth and/or nodulation when coinoculated with *Rhizobium elti* TAL 182 on *Phaseolus vulgaris* and also recorded increased nodule number, nodule fresh weight, nitrogenase activity, leghemoglobin content, and total soluble protein content in the root nodules of *P. vulgaris*.

In some instances, some endophytic genera are known to improve also the symbiosis *B. subtilis* NEB4 and NEB5 and *B. thuringiensis* NEB17 as endophytes of nodules of soybean were found to enhance growth and nodulation in greenhouse and field when coinoculated with *B. japonicum*, by providing consistent increases in nodule number, nodule weight, shoot weight, root weight, total biomass, total nitrogen, and grain yield (Bai et al. 2003). Recently, Tilak and Reddy (2006) reported increase in the yield of pigeon pea due to bacterization with endophytic *B. cereus* and *B. circulans* isolated from maize rhizosphere. However, the yield was relatively low, as compared to the treatment of these isolates in maize and wheat, possibly due to the differential response of PGPR with *Rhizobium* population in soil.

Chebotar et al. (2001) demonstrated that some plant growth regulators of *Pseudomonas* strains, but not all, increased nodule number and acetylene reduction in soybean plants inoculated with *B. japonicum*. Recently, Mañero et al. (2003) observed effect of culture filtrates of PGPR on growth, germination, and biological nitrogen fixation by lupin seedling. Role of metabolites other than phytohormones, such as siderophores, phytoalexins, and flavonoids, in enhancement of nodule formation has also been proposed (Lucas-Garcia et al. 2004), but this hypothesis has not been verified.

10.3 Consortium Comprising Free-Living PGPR

Plant growth promotion 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; Roy et al. 2009). Veen et al. (1997) critically reviewed the reasons for 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, use of multiple microbial consortia for multiple benefits can also thrive together in unique ecological niches in ideal proportions. In fact, Pratibha et al. (2011) reported several PGPR in tea rhizosphere including *Rhizobium*, *Burkholderia*, *Azotobacter*, etc., from tea garden soil of south Assam, India. Significant increase in seedling growth because of mixed culture of *Pseudomonas* and *Bacillus* on wheat under field experiments is well documented (van Elsas 1986). Inoculation with *Azospirillum halopraeferens*, a mixture of two *Azospirillum brasilense* strains and a mixture of *Bacillus licheniformis* and *Phyllobacterium* sp., has significantly increased plant height and dry weight of oilseed (*Salicornia bigelovii*) (Bashan et al. 2000). Recently, Mahmood et al. (2010) reported the influence of various rhizobacteria sp. and *Agrobacteria* sp. inoculation, singly and combined on biochemical and physiological changes of the important banana plantlets in Malaysia, Berangan cultivar (AAA). Amutha et al. (2009) studied coaggregation of *Azospirillum brasilense* with other PGPR cells using different cations and to evaluate bioinoculation effect of *Azospirillum* coaggregates on the plant height, grain yield, number of panicles, productive tillers (%), plant dry weight, and nitrogen content of rice. Dual inoculation *Azospirillum* sp. and *Azotobacter* sp. resulted in increase in total "N" content of rice and significant stimulation of their populations in rhizosphere and also increased the plant growth; concentrations of indoleacetic acid (IAA), P, Mg, and N; and total soluble sugars in wheat seedlings and shoots (Elshanshoury 1995). Similarly, it was reported that co-inoculation of two PGPR, i.e., *Enterobacter* sp. and *Pseudomonas* sp., resulted in better survival of these strains as compared to individual (Neyra et al. 1995). In an interesting report, three unrelated bacteria—methylotrophic *Methylobacterium oryzae* along with *Azospirillum brasilense* and *Burkholderia pyrrocinia*—were reported to have positive effect on nutrient uptake and therefore, the growth of tomato, red pepper, and rice plants (Aronen et al. 2002; Madhaiyan et al. 2010). Similarly, presence of *S. meliloti*

PP3, *R. leguminasorum* Pcc, and *Bacillus* sp. B1 did not have any detrimental effect on viability of PGPR strain—*Burkholderia* sp. MSSP, in wheat bran-based multispecies consortium (Pandey and Maheshwari 2007).

10.4 Consortium of Rhizobacteria in Bioremediation

Soil microbial communities are also used for biological treatment of environmental pollutants which involves the breakdown of contamination into nontoxic forms using microbiological processes (Lee et al. 1998). The advantages of employing mixed cultures as opposed to pure cultures in bioremediation have been widely demonstrated because of the synergistic interactions among members of the association. The mechanism by which isolates with bioremediation potential get benefit from synergistic interactions is considered to be complex. Yet it is possible that one species removes the toxic metabolites (that otherwise may hinder microbial activities) of the species preceding it while it is also possible that the second species are able to degrade compounds that the first are able to only partially (Alexander 1999). Rambeloarisoa et al. (1984) reported that a consortium of eight strains (comprising members of six genera) is able to effectively degrading crude oil than individual strain. Interestingly, only five of these strains were able to grow in pure cultures using hydrocarbons as sole source of C. However, when the other three strains were removed from the consortium, the effectiveness of the mixed culture was remarkably reduced. These further support the theory that each member in a microbial community has a significant role and may need to depend on the presence of other species or strains to be able to survive. Nikolopoulou et al. (2007) reported that the maximum degradation of n-alkanes (C_8–C_{11}) is achieved in treatments where bacterial consortium of *Acinetobacter* sp. T4 was applied along with *Pseudomonas putida* PB4.

10.5 AM Fungi and PGPR: Mycorrhizosphere Interaction

Arbuscular mycorrhiza fungi (AMF) are known to affect plant growth and health by improving mineral nutrition (Clark and Zeto 2000) and by increasing resistance to, or tolerance of, biotic (Cordier et al. 1996; Trotta et al. 1996) and abiotic stress (Ordookhani et al. 2010). So, the co-inoculation of AM fungi with PGPR strain provides a significant stimulation of microbial density and activity in soil. Synergistic interactions between AMF and asymbiotic N_2-fixing bacteria such as *Azotobacter chroococcum*, *Azospirillum* spp., and *Acetobacter diazotrophicus* have been reported by many researchers (Suresh and Bagyaraj 2002). Similarly, in another study, Muthukumar and Udaiyan (2006) reported the application of AM fungi and plant-growth-promoting rhizobacteria co-inoculation on the growth of bamboo plant in tropical soil with and without fertilizer.

Both AMF and PGPR complement each other in their role in N fixation, phytohormone production, P solubilization, and increasing surface absorption. Multifaceted interactions of AM fungi with various microorganisms and microfauna in the mycorrhizosphere may be positive or negative. Inoculation of tomato roots with PGPR (*Pseudomonas putida* strain, *Azotobacter chroococcum,* and *Azosprillum lipoferum*) and AMF (*Glomus intaradics* + *Glomus mossea* + *Glomus etunicatum*) has been reported to improve the quality of tomato fruit (Ordookhani et al. 2010). The positive synergistic interactions between mycorrhizosphere AM fungi and various N-fixing and P-solubilizing bacteria are the basis of application of these microbes as biofertilizer and bioprotectant agents (Bansal et al. 2002). These microbes are regulated by AMF for their own benefit, which in turn benefit the host plant. Meyer and Linderman (1986) reported enhanced mycorrhization of clover in the presence of PGPR rhizobacterium *Pseudomonas putida*. Similar observations were made later by several other researchers (Suresh and Bagyaraj 2002). All these studies suggest that colonization of plant roots by AM fungi significantly influences the mycorrhizosphere microorganisms, including PGPR. Requena et al. (1997) observed the selective and specific functional compatibility relationships in plant response between arbuscular mycorrhizal (AM) fungi, *Glomus coronatum*, native, and *Glomus intraradices,* exotic, two *Rhizobium* bacteria (NR4 and NR9, both native), and two PGPR (A2, native, and E, exotic) were screened for effectiveness by a single-inoculation trial in soil microcosms in *Anthyllis cytisoides* L., a mycotrophic pioneer legume, dominant in the target mediterranean ecosystem. A further screening for the appropriate double and triple combinations of microbial inoculants was then performed, and the parameters evaluated were biomass accumulation and allocation, N and P uptake, N_2 fixation (^{15}N), or root system quality. Overall, *G. coronatum*, native in the field site, was more effective than the exotic *G. intraradices* in co-inoculation treatments. In general, their results support the importance of physiological and genetic adaptation of microbes to the whole environment.

10.6 Enhanced Biocontrol Activity by Application of Consortium

Most of the research up till now is focused on biocontrol agents that are applied singly. Nevertheless, a single biocontrol agent is less likely to be active in all kinds of soil environment and agricultural ecosystems (Raupach and Kloepper 1998; de Boer et al. 2003) and also may result in inadequate colonization, limited tolerance to change in environment conditions, and fluctuation in production of antifungal metabolites as suggested (Weller and Thomashao 1994; Dowling and O'Gara 1994; Fukui et al. 1999). Prudent application of binary or multiple mixtures of PGPR inoculants can expand the spectrum of biocontrol activity (Felici et al. 2008). The production of hydrogen cyanide (HCN) and 2, 4-diacetylphloroglucinol (DAPG) is

a major factor in the control of soilborne diseases by *Pseudomonas fluorescens* CHA0. Co-inoculation of strain CHA0 with DAPG-producing *P. fluorescens* biocontrol strains Pf-68 and Pf-100 did result in neither a substantial alteration of hcnA nor phlA expression in CHA0 on bean roots (Jamali et al. 2009).

Consortium of PGPR is also known to improve induced systemic resistance (ISR) in host plants. Consortium and coaggregate application of *P. fluorescens* (PF-3) and *Paenibacillus polymyxa* (B-19), together with challenge inoculation of *Pyricularia oryzae* on the enhancement of ISR in rice-*Pyricularia oryzae* pathosystem, was studied under pot culture condition with rice cv.ASD-19. The application of PGPR cells, as coaggregates, was found to augment the total phenol content and defense enzyme activities such as PO and PPO content of rice plant to a higher level (Umashankari and Sekar 2011).

Though use of consortia or mixtures of two or more microbial strains to enhance the level of antagonistic substances and consistency in disease control is considered as good approach (Raupach and Kloepper 1998; Fukui et al. 1999; de Boer et al. 2003), however, proper strategy for effective screening and selection of desired strains for consortium formulation is still desirable (Walsh et al. 2001). It was found that many of the potential strains strongly inhibited others and vice versa in the in vitro assay. Similarly, some bacterial isolates showing promising attributes for plant growth promotion, like *Bacillus* sp. B7, *Pseudomonas* sp. L2, and *Rhizobium* sp. Pb, failed to survive in the presence of other potential PGPR. Earlier, inhibitory activity of pseudomonads on the other rhizobacteria has been reported by Pierson and Weller (1994).

10.7 Effect on Growth Physiology of PGPR in Mixed Inoculations

The growth physiology of various strains incorporated in mixed species consortium is an important aspect, which is sometimes ignored. The growth rate may affect the stability of artificial microbial ecosystem, in process of establishment by the application of consortium. Large difference in growth rate may result in a condition where slow growing strain gets outnumbered by fast growing partner. This imbalance may affect the colonizing abilities, affecting the plant growth.

In one study, it was found that growth of *Burkholderia* sp. MSSP was similar in monospecies and mixed species cultures with *S. meliloti* PP3. However, 25% increase in mean growth rate was recorded for *S. meliloti* PP3 when grown in mixed species of two species culture with respect to monoculture. The authors hypothesized that association with *Burkholderia* sp. favors *S. meliloti* as an adaptation of high rate of reproduction—a well-known evolved strategy that enable organisms to successfully survive and maintain themselves in communities as also explained by Andrews (1991). Derylo and Skorupska (1993) observed synergistic effect of *Pseudomonas* sp. 267 on growth of *R. leguminosarum* bv. *trifoli* 24 significantly. Shanmungam et al. (2002) cocultured *P. fluorescens* and *Rhizobium* sp. in vitro and reported positive

interaction between them. However, growth profile was measured by viable count, depending solely on their morphological characteristics.

Coimmobilization of the freshwater microalga *Chlorella vulgaris* and the plant-growth-promoting bacterium *Azospirillum brasilense* in small alginate beads resulted in a significantly increased growth of the microalga. Dry and fresh weight, total number of cells, size of the microalgal clusters (colonies) within the bead, number of microalgal cells per cluster, and the levels of microalgal pigments significantly increased (Gonzalez and Bashan 2000).

10.8 Concept and Potential of Bacterial Consortium in Future

Though the challenge of formulating a multifunctional microbial inoculum by adding appropriate microbial combinations for biotechnological approach to improve plant growth requires matching efforts, yet, with this multipurpose consortium, it can be tailored to help plants to establish, grow well, and survive in nutrient-deficient, stressful conditions.

Numerous recent studies showed a promising trend in the field of inoculation technology. Mixed inoculants (combinations of microorganisms) that interact synergistically are currently being devised. An example of this is *Azospirillum*, one of the most studied bacteria that associate with plants (Bashan and Holguin 1997a). It may associate with sugar- or polysaccharide-degrading bacteria (PDB), establishing a metabolic association where the sugar-degrading bacteria produce degradation and fermentation products used by *Azospirillum* as a carbon source, which in turn provides polysaccharide-degrading bacteria (PDB) with nitrogen. Other examples are the association between *Azospirillum* and *Bacillus* that degrades pectin, *Azospirillum* and *Cellulomonas* that degrades cellulose, and *Azospirillum* and *Emerobacter cloacae* that ferments glucose (Halsall 1993; Kaiser 1995).

Plant studies have also shown that the beneficial effects of *Azospirillum* on plants can be enhanced by co-inoculation with other microorganisms which frequently increased growth and yield of plant, compared to single inoculation; provided the plants with more balanced nutrition; and improved absorption of nitrogen, phosphorus, and mineral nutrients (Bashan and Holguin 1997a,b). Thus, plant growth can be increased by dual inoculation with *Azospirillum* and phosphate-solubilizing bacteria (Belimov et al. 1995). *Azospirillum* is also considered to be a *Rhizobium* "helper" by its stimulating nodulation, nodule activity, and plant metabolism and resistance to unfavorable conditions (Fabbri and Del Gallo 1995; Itzigsohn et al. 1993). Other successful combinations include *Azospirillum* or *Azotobacter* mixed with *Streptomyces* (Elshanshoury 1995) and *Azospirillum* with the fungal biocontrol agent *Phialophora radicola* (Flouri et al. 1995). Mixed inoculation with diazotrophic bacteria and arbuscular mycorrhizal fungi creates synergistic interactions that may result in a significant increase in growth, in the phosphorus content in plants, enhanced mycorrhizal infection, and an enhancement in the uptake of mineral nutrients such as phosphorus, nitrogen, zinc, copper, and iron

(Barea 1997; Chanway and Holl 1991; Garbaye 1994; Gori and Favilli 1995; Isopi et al. 1995; Linderman 1992; Linderman and Paulitz 1990; Rozycki et al. 1994; Singh et al. 1990).

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Chapter 11
Signals in the Rhizosphere and Their Effects on the Interactions Between Microorganisms and Plants

N.S. Paulucci, J.C. Vicario, A.B. Cesari, M.B. García, M.S. Dardanelli, and W.F. Giordano

11.1 Introduction

Molecular and genetic research in the field of the interactions between microorganisms and plants will allow great development in the areas of chemistry, biology, and agronomy. Plants depend on the ability of roots to communicate with microbes, but many bacteria and fungi are also dependent on the associations with plants, which are often regulated by roots. Although most soils are low in nutrients, roots release different types of compounds of either low or high molecular weight. With the aim to potentiate agricultural practices, it is important not only to know the diversity of molecules released by the roots but also to determine which type of communications exists between plants and soil microorganisms.

11.2 The Microbial Ecology of the Rhizosphere

The term "rhizosphere" was coined by Lorenz Hiltner in 1904 (Hiltner 1904), who realized that root exudates of different plants support the development of different microbial communities and that plant nutrition is affected by the microbial composition of the rhizosphere.

The rhizosphere is a highly dynamic open system with temporal and spatial changes of biotic factors, such as those resulting from physiological and morphological

N.S. Paulucci • J.C. Vicario • A.B. Cesari • M.B. García • M.S. Dardanelli (✉) • W.F. Giordano
Departamento de Biología Molecular, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta Nacional N36, Km. 601, CP X5804BYA Río Cuarto, Córdoba, Argentina
e-mail: mdardanelli@exa.unrc.edu.ar

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changes of the growing root system, and of abiotic factors, such as rain, irrigation, and drought. Consequently, the microbial adaptations to each particular situation are difficult to understand (Spaepen et al. 2009). The rhizosphere is generally seen as a cylindrical volume of soil surrounding roots.

However, although its inner limit, the root surface, is easily recognizable, its outer boundary is much more complex to identify (Belnap et al. 2003). The main drivers of the formation of the rhizosphere are the development of water and solute gradients around roots, which can alter the physical, chemical, and biological properties of the soil (Raynaud 2010). These gradients are created because the root either adds or extracts water and solutes from the root surface (the rhizoplane), which move through the soil or are altered in response to root modifications of the physical and chemical properties of the soil (Raynaud 2010). The microbial ecology of the rhizosphere relates to the study of interactions of microorganisms both with each other and with the environment surrounding the plant root. The analysis of nucleic acids directly extracted from rhizosphere soils provided an opportunity to study a much broader spectrum of microorganisms residing in the rhizosphere (Berg and Smalla 2009). Some bacterial species living in the rhizosphere and inside the roots can affect growth either positively or negatively. Bacteria that favorably affect plant growth and yield of commercially important crops are denominated plant-growth-promoting rhizobacteria (PGPR) (Burdman et al. 2000; Dobbelaere and Okon 2007; Spaepen et al. 2009; Medeot et al. 2010). PGPR activity has been reported for strains belonging to a group that includes different diazotrophic bacterial species and strains belonging to genera such as *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Gluconacetobacter*, *Herbaspirillum*, *Klebsiella*, *Paenibacillus*, *Pseudomonas*, and *Serratia* (Glick 1995; Spaepen et al. 2009). Rhizobia can also be considered as soil bacteria with PGPR activity, where root colonization and growth promotion of rice, cereals, and other nonlegumes have been reported (Chabot et al. 1996).

Plant-growth-promoting capacity has been related to different physiological activities: (1) synthesis of phytohormones, such as cytokinins, gibberellins, and auxins; (2) enhancement of factors affecting mineral nutrition, such as phosphorus solubilization; (3) protection of plants against phytopathogens; (4) increases in root surface area; (5) enhancement of beneficial symbioses of the host; and (6) combination of different modes of action (Persello-Cartieaux et al. 2003; Somers et al. 2004). More recently, however, attention has been focused on the plant-growth-promoting capacity of endophytes (bacteria that reside within living plant tissue without causing substantial harm to their host) because they can improve plant growth and development both directly and indirectly (Badri et al. 2009).

On the other hand, roots anchor the plant in the soil, but most importantly, the whole nutrient transfer from the soil to the aboveground plant parts is channeled through the roots, and roots are important storage organs in perennial plants. This makes roots attractive to herbivores. However, plant roots are no passive victims of attacking herbivores and microorganisms (Bonkowski et al. 2009). They have direct and indirect defense compounds involving communication and interaction with other organisms (Bonkowski et al. 2009).

11.3 The Roots: Contribution to the Rhizosphere and Effect of Plant Roots on the Activity of Microbial Communities

The several compounds released by the roots to the soil can be chemically classified as organic or inorganic. Each has varied functions within the rhizosphere.

Most root products, including specific compounds typical of the secondary metabolism of each plant species, are available to colonizing microbes. Some authors estimate that plants release between 20% and 50% of their photosynthates through their roots (Barriuso et al. 2008).

Organic acid materials, carbohydrates, and amino acids serve as carbon and energy sources for microorganisms. In addition to such molecules, there is an entire range of insoluble products occurring in the root (e.g., cellulose, lignin, protein), which can be lost from it by cell exfoliation and root pruning (Babalola 2010). Other compounds, such as flavonoids, are involved in signal exchange between the legume and rhizobia or in the defense system of plants (Cooper 2007). Moreover, some components of rhizodeposition have been shown to interfere with quorum sensing-dependent bacterial communication systems (QS) (Teplitski et al. 2000).

There are different organisms that are able to produce and secrete compounds that mimic the QS signals of bacteria and thus affect the behavior of associated bacteria (Bauer and Teplitski 2001; Bauer and Robinson 2002). Different plants, such as pea, crown vetch, alfalfa, rice, soybean, and tomato, have also been shown to produce substances that appear to mimic the activities of acylhomoserine lactones (AHLs) and have specific effects on QS-regulated behaviors in bacteria (Teplitski et al. 2000; Daniels et al. 2002; Gao et al. 2003). On the basis of these observations, it has been suggested that plants possess a great potential for different soil and may select the most suitable bacteria (Simms and Taylor 2002).

Plants use great part of their energy on the regulation of the production of new cells, especially those of roots. For example, border cells can rapidly attract and stimulate growth in some microorganisms and repel and inhibit the growth in others. Such specificity may provide a way to control the dynamics of adjacent microbial populations in the soil to foster beneficial associations and inhibit pathogenic invasion (Hawes et al. 1998).

Emerging lateral roots promote the release of even more nutrients via plant cell lysis. Because of the outward diffusion of nutrients and the inward movement of salts and minerals during transpiration, chemical gradients form around the root and create a range of distinct microbial habitats (Beattie 2006). Furthermore, mature roots produce less mucilage and fewer cell lysates, due to the absence of border cells and emerging lateral roots, and leak less water due to the deposition of a water-impermeable suberin layer around epidermal cells. Consequently, the developing roots generally support fast-growing microorganisms like bacteria, whereas mature roots support slower-growing microorganisms like fungi and actinomycetous bacteria (Beattie 2006).

On the other hand, rhizosphere microorganisms can enhance root exudation of carbon and energy sources or flavonoids (Dardanelli et al. 2008b). A major component of root secretions is mucilage, which contains hydrated polysaccharides,

organic acids, vitamins, and amino acids, and is an excellent substrate for microbial growth. Mucilage binds water and thus helps to form a well-hydrated environment for the roots and rhizosphere microorganisms (Beattie 2006).

Changes in some of the components of the rhizosphere can affect either the entire system or part of it. The composition of the rhizodepositions secreted by germinating seeds and roots is dependent on the plant species, the plant age, the root zone, the plant mineral nutrition, stress effects (Maloney et al. 1997; Baudoin et al. 2002, Dardanelli et al. 2008a, 2010), and other environmental factors such as the presence of microbes. The production of molecules by the roots may not be constant, thus altering the production pattern of, for example, flavonoids (Dardanelli et al. 2008a, 2010), and it is probable that such changes can affect the microbial communities along time.

Rhizosphere interactions are based on complex exchanges that evolve around plant roots. Root-based interactions between plants and organisms in the rhizosphere are highly influenced by edaphic factors; however, the belowground biological interactions that are driven by root exudates are more complex than those occurring above the soil surface (Bais et al. 2004). A number of multifaceted processes consisting of physical, chemical, and biological modifications occurring at the root–soil interface result in the formation and development of the rhizosphere. Microbe–microbe interactions are crucial to understand the dynamic processes characteristic of rhizosphere establishment and maintenance affecting plant growth and health (Barea et al. 2005). Therefore, the rhizosphere is the ideal system to study when seeking to understand the critical functional interdependence and metabolic capabilities of complex microbial communities containing different organisms. The various mycorrhizal associations allow plants to colonize and grow efficiently in suboptimal environments, and, in most terrestrial ecosystems, mycorrhizae, not roots, are the chief organs of nutrient uptake by land plants (Barea et al. 2005).

11.4 Signals Exchanged Between Legumes and Rhizobia

Before colonization, it is assumed that there is a continuous dialog of signals between microorganisms and plants. During germination and growth, plants release many compounds or signals into their rhizosphere, including amino acids, organic acids, sugars, aromatics, and various other secondary metabolites (Somers et al. 2004). These signals include phenolic compounds, particularly flavonoids, which are known as key signaling components (Steinkellner et al. 2007). Scervino et al. (2006) reported that flavonoids exhibit a genus-specific and species-specific effect on arbuscular endomycorrhizae (AM). In addition, strigolactone exudates by different roots are known to induce hyphal branching in AM fungi, a condition for successful root colonization (Akiyama et al. 2005).

The enriched environment around plant roots allows the establishment of interactions between soil bacteria and the roots. These relationships can be beneficial, pathogenic, parasitic, or saprophytic and exert important effects on plant

development and productivity. Microorganisms colonize mineral soil particles as well as plant roots. They may cause either plant diseases or a wide range of beneficial effects, including legume plant growth promotion through biological nitrogen fixation (BNF). When environmental nitrogen is limited, soil bacteria known as rhizobia interact with roots of leguminous plants to produce symbiotic nodules, inside which atmospheric nitrogen is reduced to ammonium for use by the plant, while the bacteria receive carbohydrates from the plant in a protected environment. Establishment of this symbiosis relies on an exchange of signals between the legume and the rhizobia, where the microsymbiont nodulates a particular group of related legume species.

The plant initiates the "molecular dialog" 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. Flavonoids also affect human and animal health because of their role in the diet, which is ascribed to their antioxidant properties and estrogenic action and to a wide range of antimicrobial and pharmacological activities (de Rijke et al. 2006).

Bacteria 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. Figure 11.1 shows how plant roots can communicate with rhizobacteria and establish active rhizospheric interactions.

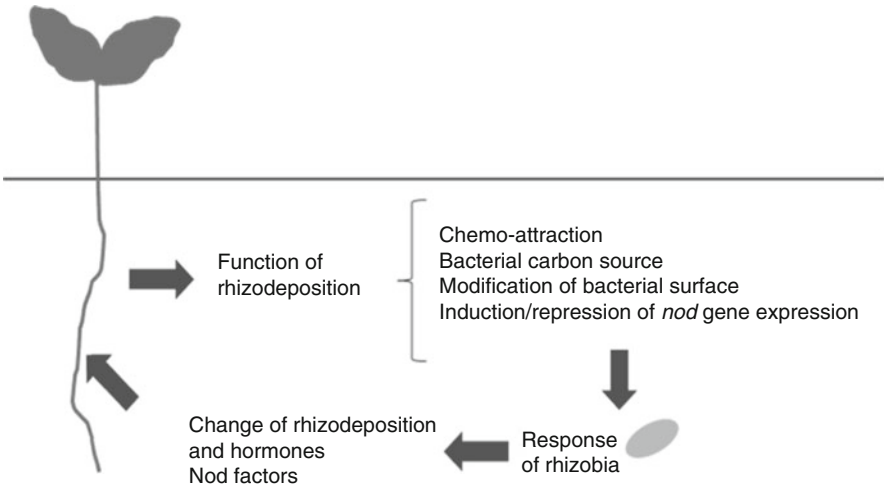

Fig. 11.1 Schematic summary of the involvement of rhizodepositions in the establishment of rhizobia–legume symbiosis

The early events of the symbiotic process, collectively designated as preinfection, take place while the bacterial cells are still outside the root tissues. These events include a rhizobial chemotactic approach to the root, root colonization, attachment to root surfaces particularly to emerging root hairs, hair deformation and curling, and induction at a distance of a meristem and cortex proliferation at special locations in the root (Long 1989). Some of these events require the participation of legume root exudates, where a range of substances of root origin, amino acids, sugars, organic acids, flavonoid compounds, polysaccharides, peptides, enzymes, lectins, and other glycoproteins may influence the behavior of rhizobia. Many of these exudate components are rhizobial chemoattractants (Bergman et al. 1988). Particular attention has been paid to exuded flavonoid compounds because they may play an important role in chemotaxis (Caetano-Anolles et al. 1988) and regulate the expression of the *nod* genes in the rhizosphere (Maxwell et al. 1989). Macromolecular components of the root exudate, including lectins, also influence the process of infection and nodulation by rhizobia in legume systems (Wall and Favelukes 1991) (Table 11.1).

Flavonoids are studied mainly for their antioxidant properties and, as mentioned above, for their role in the establishment of rhizobia–legume symbiosis. However, it has been recently demonstrated that flavonoids can also affect nutrient availability through soil chemical changes. Despite the well-investigated role of flavonoids

Table 11.1 Summary of main molecules involved in the legume–rhizobia symbiosis

Stages	Molecule	Function
Initial symbiotic process	Plant	
	Flavonoids (flavones, isoflavones, flavanones, flavonols, anthocyanins) Phenolic acids	Attraction of rhizobia by the plant Inductor of plant–microbe symbioses
	LysM receptor kinases	Recognition of bacteria by the host
	Enzymes and proteins (amylase, hydrolase, invertase, peroxidase, phenolase, lectin phosphatase, polygalacturonase, proteases,) Fatty acids	Plant defense; nod factor degradation Biological activities
	Rhizobia	
	Lipo-chitin oligosaccharides	Activation of LysM receptor kinases
Early steps of plant infection	Plant	
	Lectins	Attachment of bacteria to plant root
	Rhizobia	
	Cellulose	Enhancement of contact of bacteria to the root surface
	Polysaccharides	Protection against stress, attachment
Later stages of nodulation	Plant	
	Hormones (cytokinin)	Nodule organogenesis
	Rhizobia	
	Nitrogenase	Reduction of nitrogen to ammonium
	Hydrogenases	Hydrogen recycling

Source: Somers et al. (2004), Brechenmacher et al. (2010), Mandal et al. (2010)

in BNF, surprisingly little is known about the mechanisms of release from plant roots. The highly specialized release patterns in space and time suggest the presence of tightly controlled mechanisms for the regulation of these processes (Cesco et al. 2010). The production and release of root-derived compounds are commonly constitutive but may be induced by biotic or abiotic stress, as described previously in this chapter. The mechanism by which plant roots secrete compounds is thought to be mainly a passive process mediated through three separate pathways: diffusion, ion channels, and vesicle transport (Neumann and Romheld 2000; Bertin et al. 2003). ABC transporters, importers, and exporters of compounds from the cell and multidrug and toxic compound extrusion transporters have been implicated in transport of flavonoids to the vacuole (Yazaki 2005).

Lectins released from plant roots affect bacterial attachment and biofilm formation. Plant lectins are proteins that reversibly and nonenzymatically bind specific carbohydrates (De Hoff et al. 2009). They play important roles during the early stages of the interaction between the host plant and the symbiotic bacteria, particularly in the initial attachment of rhizobia to root epidermal cells. Soybean lectin causes a dose-dependent increase of attachment and biofilm formation on polystyrene surface by *Bradyrhizobium japonicum* wild-type USDA 110 cultures (Pérez-Giménez et al. 2009). Preincubation of rhizobia with soybean lectin increases bradyrhizobial adhesion to soybean roots (Lodeiro et al. 2000). Exopolysaccharides seem to be involved in *B. japonicum* biofilm formation on both inert and biotic surfaces (Pérez-Giménez et al. 2009). Biofilm formation on plants appears to be associated with symbiotic and pathogenic responses, but it is unclear how plants regulate the association. However, it is clear that biofilms function as structures that are resistant against stress factors such as desiccation, UV radiation, predation, and antibiosis, which help create protective niches for rhizobia (Rinaudi and Giordano 2010).

As the initial stages of symbiosis formation occur in the root zone and on the root surface, scientists' attention has been focused on the role of root exudates and their components in symbiosis formation, and little is known about seed exudates. However, the first interaction steps between the symbionts occur during legume seed germination when biologically active substances secreted by the seeds can modulate the symbiotic properties of nodule bacteria and affect the formation of efficient symbiosis (Mel'nikova and Omel'chuk 2009). In this context, L-canavanine, an arginine analog found exclusively in the seeds of legumes, has been reported to be as abundant as up to 5% (dry weight) of some leguminous seeds (Weaks 1977). In addition to serving as a nitrogen source for the germinating seedlings, L-canavanine is also known to serve as an allelopathic substance by inhibiting the growth of certain bacteria and phytophagous insects (Rosenthal 2001). Handelsman et al. (1990) showed that canavanine exuded from alfalfa seeds has the potential to affect the population biology of *Bacillus cereus* (Emmert et al. 1998). L-Canavanine is incorporated in place of L-arginine into nascent protein chains during synthesis, resulting in altered protein structure and function and eventually leading to death of the targeted cell (Rosenthal 2001).

11.5 Concluding Remarks

The region around the root, known as the rhizosphere, is relatively rich in nutrients, due to the loss of plant photosynthates from the roots, named rhizodepositions. Consequently, the rhizosphere supports large and active microbial populations capable of exerting beneficial, neutral, or detrimental effects on plant growth. The importance of rhizosphere microbial populations in the maintenance of root health, nutrient uptake, and tolerance of environmental stress is now recognized. A multiplicity of signals controls the responses of plants and their associated organisms in the rhizosphere. Progress has been recently made in characterizing the process of rhizosphere colonization, identification, and cloning of bacterial genes involved in nitrogen fixation, phosphorus solubilization, production of plant growth regulators, and suppression of plant diseases. The interactions/relationships of rhizosphere microorganisms with their hosts must be analyzed in legume production under sustainable agriculture systems.

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Chapter 12
Role of Plant: Microbe Interactions in the Sustainable Development of Muga Sericulture

Bala Gopalan Unni, Basabrani Devi, Yelena Kakoty, Sawlang Borsingh Wann, Archana Borah, and Pallavi Dowarah

12.1 Introduction

Root colonizing bacteria (rhizobacteria) that exert beneficial effects on plant development via direct or indirect mechanisms have been defined as plant-growth-promoting rhizobacteria (PGPR). PGPR were first defined by Kloepper and Schroth (1978) to describe soil bacteria that colonize the roots of plants following inoculation onto seed and that enhance plant growth. There has been much research interest in PGPR, and there is now an increasing number of PGPR being commercialized for crops improvement. The addition of compost and compost teas promotes existing PGPR and may introduce additional helpful bacteria to the field. The absence of pesticides and the more complex organic rotations likely promote existing populations of these beneficial bacteria. However, it is also possible to inoculate seeds with bacteria that increase the availability of nutrients, including solubilizing phosphate, potassium, oxidizing sulfur, fixing nitrogen, chelating iron, and copper. Phosphorus (P) frequently limits crop growth in organic production. Nitrogen-fixing bacteria are miniature of urea factories, turning N_2 gas from the atmosphere into plant available amines and ammonium via a specific and unique enzyme they possess called nitrogenase. Although there are many bacteria in the soil that "cycle" nitrogen from organic material, it is only this small group of specialized nitrogen-fixing bacteria that can "fix" atmospheric nitrogen in the soil. Special groups of microorganisms can make sulfur more available and do occur naturally in most soils. One of the most common ways that PGPR improve nutrient uptake for plants is by altering plant hormone levels. This changes root growth and shape by increasing root branching, root mass, root length, and/or the amount of

B.G. Unni (✉) • B. Devi • Y. Kakoty • S.B. Wann • A. Borah • P. Dowarah
Biotechnology Division, North-East Institute of Science & Technology (CSIR), Jorhat 785 006, Assam, India
e-mail: bgunni@rrljorhat.res.in; bgunni@yahoo.com

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root hairs. This leads to greater root surface area, which in turn, helps it to absorb more nutrients.

PGPR have attracted much attention in their role in reducing plant diseases. Some PGPR, especially if they are inoculated on the seed before planting, are able to establish themselves on the crop roots. They use scarce resources and thereby prevent or limit the growth of pathogenic microorganisms. Even if nutrients are not limiting, the establishment of benign or beneficial organisms on the roots limits the chance that a pathogenic organism that arrives later will find space to become established. PGPR have also been reported to enhance the nutrient accumulation and growth of oil palm seedlings (Amir et al. 2005) and production of high-yielding and good-quality banana (Mia et al. 2005).

Insects are used as a model system for studying humoral immunity because of their ability to invade diverse range of ecological niches. All insects defend themselves against bacteria and parasites, using cellular and humoral systems that are rapidly activated in infected animals. Antibacterial peptides are obtained from the insects after injection of live bacteria or any substance which induces its production of these peptides in response to infection. These peptides show antimicrobial activities against various pathogens. Because of this, it has a wide application in developing novel antibiotics. Bacterial strains isolated from diseased insects are used to study the antibacterial protein production in insects along with other bacteria.

12.1.1 PGPR in Research

Over the years, the PGPR (plant-growth-promoting rhizobacteria) have gained worldwide importance and acceptance for agricultural benefits. These microorganisms are the potential tools for sustainable agriculture and the trend for the future. Scientific researchers involve multidisciplinary approaches to understand adaptation of PGPR to the rhizosphere, mechanisms of root colonization, effects of plant physiology and growth, biofertilization, induced systemic resistance, biocontrol of plant pathogens, production of determinants, etc. Biodiversity of PGPR and mechanisms of action for the different groups like diazotrophs, bacilli, pseudomonads, Trichoderma, AMF, rhizobia, phosphate-solubilizing bacteria and fungi, and lignin-degrading, chitin-degrading, cellulose-degrading bacteria and fungi have been studied. Different bacteria that have been reported as PGPR belong to the following genera: *Pseudomonas, Bacillus*, *Azospirillum, Agrobacterium, Azotobacter, Arthrobacter, Alcaligenes, Serratia, Rhizobium, Enterobacter, Burkholderia, Beijerinckia, Klebsiella, Clostridium, Vario-vovax, Xanthomonas,* and *Phyllobacterium* (Bullied et al. 2002; De Freitas and Germida 1990; De Silva et al. 2000; Lucy et al. 2004; Lugtenberg et al. 2002; Quadt-Hallmann et al. 1997; Saubidet et al. 2002). Among these, *Pseudomonas* and *Bacillus* are the most widely reported PGPR (Adesemoye et al. 2008).

12.1.2 PGPR in Sericulture

The application of PGPR that colonizes the rhizosphere results in modification of plant health and development (Kloepper et al. 1980). The properties of PGPR offer great promise for agronomic application. Recent progress in understanding their diversity, colonizing ability, mode of action, formulation, and application has facilitated their development as reliable components in the management of sustainable agricultural system. Although significant control of plant pathogens or direct enhancement of plant development has been demonstrated by PGPR in the laboratory and greenhouse, results in the field have been less consistent (Bowen and Rovira 1999). Growth, development, and economic characters of silkworms are influenced to a great extent by nutritional content of their food plants (Pant and Unni 1980). So the association between Som plants (*Persea bombycina*) and rhizobacteria was studied to determine if well-characterized PGPR strains which demonstrate growth promotion in other plants also enhance plant growth in Som, *P. bombycina*, one of the main primary host plants of Muga silkworm (*Antheraea assamensis,* Helfer), and has significant effects on their health and survival (Unni et al. 2008). In our study based upon the growth-promoting activity in Som plants using three combinations of strains, it can be concluded that PGPR (*Bacillus* spp., *Streptomyces* spp., *Pseudomonas* spp., and *Chromobacterium* spp.) caused maximum increase in growth of Som plants, and fiber quantity and quality in Muga silkworms. The investigation has proved useful to the sericulture farmers. The genus *Pseudomonas* has been studied showing usefulness in plant-growth promotion and biological control. Many fluorescent Pseudomonas strains (e.g., *Pseudomonas aeruginosa*) which colonize the rhizosphere exert a protective effect on the roots through the production of in situ antibiotic compounds that promote growth and prevent microbial infections (Jenni et al. 1989) (Fig. 12.1).

12.1.2.1 Antibacterial Proteins from Insects

An essential feature of success of insects has been their ability to invade and to exploit diverse ecological niches. Insects possess both cellular and humoral immune systems. In cellular immunity, mechanisms such as phagocytosis and encapsulation are operative (Bowmen and Hultmark 1987; Dularay and Lackie 1985; Ratcliffe et al. 1985; Rizki and Rizki 1984). Antibacterial peptides were initially isolated in 1980 from insect hemolymph after challenging cecropin pupae with the live bacteria (Hultmark et al. 1980). More than 150 different antibacterial peptides and proteins have now been purified from different insect species. Antibacterial molecules that have been extensively studied are lysozyme, defensins, and cecropin and attacin families of antimicrobial peptides (Boman 1998; Barra et al. 1998; Hultmark et al. 1983; Cociancich et al. 1994; Lamberty et al. 1999; Matsuura et al. 2007). It is thought that these molecules play a critical role in early immune defense against bacterial infection; failure of its induction may cause

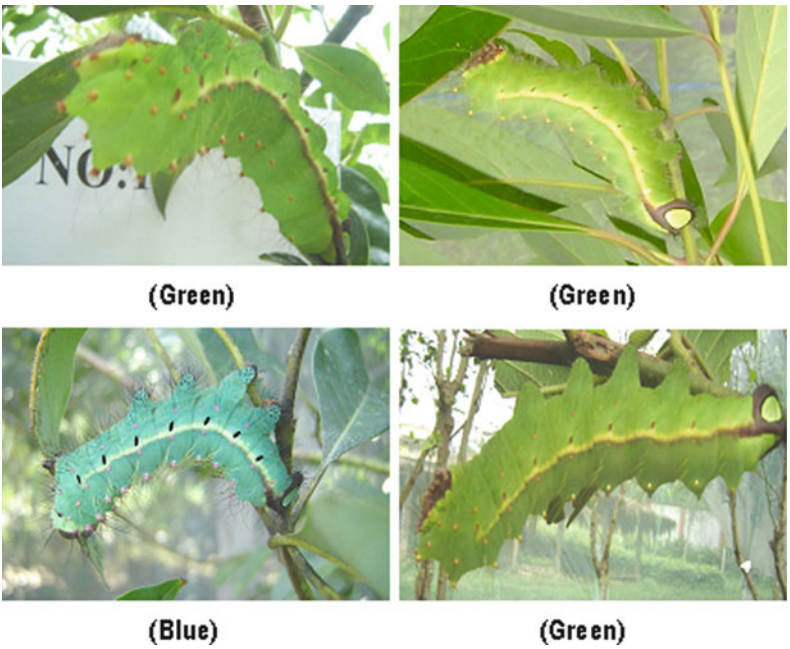

Fig. 12.1 Variation in larval color (*green and blue*) of Muga silkworm *Antheraea assama* accessions

a serious infection in insects. Upon detection of bacteria, a complex genetic cascade is activated, which ultimately results in the synthesis of a battery of antibacterial peptides and their release into the hemolymph (Otvos 2000). Thus, the insects represent the most extensive and successful defensive strategies against infection.

12.1.2.2 Antibacterial Proteins from Different Insect System

The larvae of *Galleria mellonella* (wax moth) and pupa of *Hylophora cecropia* are the two model systems which were used for studying the cell free immunity in insects. According to Bowmen and Hultmark (1987), in *Galleria*, *Cecropia*, and other insects, a primary injection with either live nonpathogenic bacteria or heat-killed pathogens induced the immune state in them. Pupae of *Cecropia* spend 6–9 months in diapauses, their winter hibernation. During this period, the metabolism of the insects is reduced to a few percent of a normal level. Such a dormant insect can be immunized without an immediate break of the diapauses, and therefore, it is possible to obtain a fairly selective labeling of RNA and proteins made as a response to an infection. The antibacterial activity in *Cecropia* pupae rose with the synthesis of 12–15 proteins, which were separated by SDS-PAGE. Lysozyme was the first immune protein from the larva of *Galleria mellonella* and *Bombyx mori* (De verno et al. 1984). It was claimed that lysozyme was the main bactericidal

protein in insect hemolymph. But there was a poor correlation between the lysozyme concentration and the ability of an insect to withstand a secondary infection. Moreover, lysozyme does not kill gram-negative bacteria Boman and Steiner (1981) isolated P9 protein named cecropin. In earlier studies, Morishima et al. (1988, 1990) have purified cecropin-like peptide from the silkworm larvae immunized with *E. coli*. Although cecropin have been isolated from several species of lepidopteran and dipteran insects, they have not been found in other orders of insects. They are small-molecular-weight proteins, which are polarized having basic N-terminal end and hydrophobic C-terminal part. They show antibacterial activity against many kinds of gram-negative and gram-positive bacteria (Hara and Yamakawa 1995). A cecropin-like peptide called GM cecropin was isolated from the hemolymph of larvae of wax moth, *Galleria mellonella* immunized against *E. coli* and its antibacterial activity was examined in radial diffusion assay. It was a low-molecular-weight protein similar to cecropins (Stephens and Marshall 1962; Chadwick 1970; Hoffmann et al. 1981; Mak et al. 2001; Chong et al. 2004). Antibacterial proteins were isolated and purified from the larvae and pupae of *Manduca sexta* (Hurlbert et al. 1985; Spies et al. 1986). These were also bacteria-induced, low-molecular-weight cecropin D-like basic proteins effective against gram-positive and gram-negative bacteria (Dickinson et al. 1988). Brook (1971) studied the inflammatory response of *Manduca sexta* against the microsporidium, *Nosema sphingidis*. It proved that insects do have a strong inbuilt mechanism to fight against minor infections and remove them from circulation. Similar kind of antibacterial protein has been isolated, purified, and cloned from *Phorima ferranovae*, a Dipteran. It was called Diptericin, and its cDNA was cloned for transcriptional profile study (Reichhart et al. 1989). Sun and Fallon (2002) isolated, purified, and characterized the genomic DNA encoding cecropin from *Aedes albopictus* mosquito cell line. Teshima et al. (1986) isolated and purified similar kind of inducible antibacterial protein from silkworm. Antibacterial protein from Chinese oak silkmoth, *Antheraea pernyi*, was compared with that of cecropia moth (Qu et al. 1982; Qi et al. 1984). Here, antibacterial protein was induced as in cecropia pupa by injecting viable *E. coli* or *Enterobacter cloacae* in diapausing pupae, which in turn produced a set of immune protein, and the major antibacterial protein was found to be cecropin D. The general structure of cecropin having a charged N-terminal region (residue 1–10) followed by a long hydrophobic stretch (residue 22–32) was well conserved in cecropin isolated from *A. pernyi*. The homology between the cecropin from the two insects suggests that they originated from a single ancestral gene. In both the insect system, it was found that expression of these proteins require de novo synthesis of RNA and proteins. It was also shown that the antibacterial activities continue to rise for about a week and then it gradually declines. According to Qu et al. (1982), a comparison of the sequences of cecropin B and D from *Hylophora* and *A. pernyi* shows that there are only a few minor differences between the two species. Insect attacin was identified in *Hyalophora cecropia* (Hultmark et al. 1983; Kockum et al. 1984; Sun et al. 1991). It consists of four subunits of equal size (Pye and Boman 1977). Hultmark et al. (1983) have shown that attacin is in fact composed of at least six distinct

proteins, termed as attacin A-F with a molecular weight of approximately 22,000 Da. They have narrow spectrum antibacterial activity against gram-negative bacteria (Kockum et al. 1984). Attacin is divided into different groups depending upon their amino acid composition and N-terminal sequences. Attacin A-D constitutes basic group, and attacin E-F constitutes acidic group. The basic groups have positively charged N-terminal and hydrophobic C-terminal. The two acidic forms had slightly different sequence. Several lines of evidence indicate that the attacins are made in preprofrom deletion of amino acid at both C- and N-terminals, making them active proteins (Lee et al. 1983; Kockum et al. 1984). It mainly acts on the outer membrane of the gram-negative bacteria (Engstrom et al. 1984a). This protein has been isolated from *A. pernyi*, *M. sexta*, *S. peregrina*, and recently in *A. assama*. Insect defensins have been isolated from several orders of insects such as Dipteran, Hymenopteran, Coleopteran, Trichopteran, Hemipteran, and Odonata (Hoffmann and Hetru 1992; Cociancich et al. 1993). However, defensins have not yet been observed in Lepidopteran insects. All types of antibacterial proteins have been reported in Lepidopteran insects except for the insect defensins. It was found highly effective against gram-positive bacteria including human pathogenic bacteria such as *Staphylococcus aureus*, whereas they do not exhibit strong activity against gram-negative bacteria (Boman et al. 1991; Hara and Yamakawa 1995). The study of insect immunity makes us understand the basic mechanism they use to protect themselves from antigens. Many experiments were carried out to activate their immune response by injection with bacteria. Choudhury et al. (2004), for the first time, reported the induction of immunity in *Antheraea assama* larvae by injection of heat-killed cells of *Pseudomonas aeruginosa* strain AC-3. It was observed that the immune response in fifth instar larvae of *Antheraea assama* reaches a maximum level after 3 days of injection. The antibacterial activity of the immune plasma rose against *Pseudomonas aeruginosa* AC-3 was effective against *P. aeruginosa, E. coli*, and *B. subtilis*. Jyotsana et al. (2005) isolated and purified an antibacterial protein similar to attacin from hemolymph of Muga silkworm (*Antheraea assama*). It was found to be 23 kDa, basic protein effective against *Pseudomonas aeruginosa* AC-3, bacteria causing "flacherie" in these silkworms. Hara and Yamakawa (1995) isolated a novel type of antibacterial peptide, "moricin," from *Bombyx mori* that showed antibacterial activity against *Staphylococcus aureus*. It was a basic peptide and consisted of 42 amino acids and also effective against several gram-negative and gram-positive bacteria. Abraham et al. (1995) purified an antibacterial protein from the larvae of silkworm, *Bombyx mori*. The purified protein was a single polypeptide chain of molecular weight 16 kDa, whose 20 N-terminal amino acid sequence showed homology with the N-terminal amino acid sequence of lysozymes of other species. This protein was found to have antibacterial activity against *E. coli* and *Micrococcus luteus*. It was found to be active against bacteria as well as fungi. Both showed no obvious similarities in amino acid sequence with other antibacterial peptides (Lamberty et al. 2001a, b). From the larvae of flesh fly, antibacterial proteins sarcotoxin I and II and lectins were purified and characterized (Komano et al. 1980; Okada and Natori 1983; Takahashi et al. 1985; Ando et al. 1987). Kaaya et al. (1987) induced synthesis of cecropin- and attacin-like antibacterial factors in the hemolymph of *Glossina*

morisitans. Majority of antibacterial proteins isolated from Lepidopteran and Dipterans are made up of 20–40 amino acid residues which have either α-helical or β-pleated sheet structure and are positively charged (Bulet et al. 1999). Chung and Ourth (2000) isolated a novel antibacterial peptide together with lysozyme by gel filtration. Molecular mass of this antibacterial protein was estimated to be 12 kDa. The N-terminal amino acid sequence of 12-kDa protein was different from those of antibacterial molecules found in other insects and has not been identified before. It was named as viresin; it showed antibacterial activity against several gram-negative bacteria including *E. cloacae* but not against gram-positive bacteria.

12.1.2.3 Site of Antibacterial Protein Synthesis

Faye and Wyatt (1980) have reported the synthesis of several proteins by cultured fat body from immunized, diapausing pupae of *H. cecropia*. De verno et al. (1984) have reported that a mixture of fat body and cell free hemolymph from non-immunized larvae of *G. mellonella* showed antibacterial activity when incubated with bacteria in vitro. It has been shown that the first stage after injection of bacteria into *H. cecropia* is the phagocytosis of these microbes by granular hemocytes (Abu and Faye 1981). The actual mechanism of triggering protein synthesis is unknown. But on the basis of above result, Boman and Steiner (1981) suggested that it originates from the hemocytes. They had also suggested that humeral immunity, in particular its induction, is a nonspecific process. The initial hemolymph response of insects to foreign particles is mediated by circulating hemocytes. Insect's hemocytes are extremely efficient at removing foreign particles such as bacteria, fungi, nematodes, etc., by phagocytosis, nodule formation, or encapsulation (Salt 1970).

12.1.2.4 Elicitors of Antibacterial Protein Synthesis

In *G. mellonella,* there was 50-fold increase in lysozyme activity in 24 h after injection of living or heat-killed cells of *Micrococcus luteus*, *Bacillus cereus*, and *B. subtilis* or heat-killed cells of *Pseudomonas aeruginosa* (Mohring and Messner 1968). A similar response was observed after injection of sterile distilled water, Ringer's solution, and methylene blue. Chadwick (1970) reported that lysozyme activity increased two- to sevenfold after injection of bacteria as well as water and saline. Kanost (1983) reported that equal number of cells of living and formalin-killed bacteria of the same species induced equivalent levels of lysozymes. An increase in enzyme activity was observed following injection of *M. lysodeikticus*, lipopolysaccharide from *E. coli* and *S. marcescens*, latex beads, saline, etc. (Anderson and Cook 1979).

12.1.2.5 Mode of Action of Antibacterial Proteins

A general feature of antimicrobial peptides is their action in selectively disturbing microbial membrane integrity either by disruption or pore formation in lipid bilayers. The resulting openings lead to diffusion of intracellular ions and small molecules, which is followed by the collapse of the transmembrane electrochemical gradient and then microbial cell death (Bechinger 1999). Engstrom et al. (1984b) studied the action of attacin on *E. coli* and found that attacin causes an alteration in the permeability properties of the outer membrane, which results in an improved access of lysozyme and of cecropin to their targets in the cell wall and the cytoplasmic membrane, respectively. Later in a study with the pupae of the giant silk moth, *Hyalophora cecropia*, in response to a bacterial infection. Carlsson et al. (1991) found that attacin specifically inhibits the synthesis of several outer membrane proteins including OmpA, OmpC, OmpF, and LamB (approximate molecular weights, 30,000, 38,000, 37,000, and 47,000, respectively) and that the inhibition occurs at pretranslational level. Cociancich et al. (1993) showed that cecropin disrupts the permeability barrier of the cytoplasmic membrane of *M. luteus*, resulting in a loss of cytoplasmic potassium, a partial depolarization of the inner membrane, a decrease in cytoplasmic ATP, and an inhibition of respiration. These permeability changes reflect the formation of channels in the cytoplasmic membrane by defensin oligomers. In a study, Gazit et al. (1994) found that Cecropin B isolated from hemolymph of *Bombyx mori* binds phospholipid membranes preferentially as monomers lying on the surface, rather than cooperatively as bundles that form transmembranal pores via a "barrel stave" mechanism. Boman et al. (1993) isolated two types of cecropin, P1 and PR-39, from pig intestine and studied their mechanism of action on *E. coli* K-12 and found that cecropin P1 kills bacteria by lysis. From the isotope incorporation experiments, it was found that PR-39 kills bacteria by a mechanism that stops protein and DNA synthesis and results in degradation of these components.

12.1.2.6 Gene Expression of Antibacterial Protein

The gene encoding the antibacterial peptide in insects has largely been cloned. Dimarcq et al. (1994) cloned a Drosophila gene encoding a preprodefensin. The gene was intronless and present in a single copy/haploid genome, mapped at position 46CD on the right arm of the second chromosome. Its upstream region revealed the presence of multiple putative cis-regulatory sequences. A novel gene named enbocin had been cloned from immunized *Bombyx mori* cDNA library (Kim et al. 1998). Its genomic sequence revealed that the transcription unit was about 1.2 Kb, and the coding sequence was interrupted by an intron of 660 bases. Recombinant enbocin expressed under the control of the baculovirus polyhedron promoter that showed the broad range of antibacterial activities against gram-positive and gram-negative bacteria.

12.1.2.7 Application of Antibacterial Proteins

In our study we used Muga silkworm, *Antheraea assama*, as a model system for studying humoral immunity because of its economic importance and due to its uniqueness of producing golden yellow silk found only in North East India. Choudhury et al. (2004) induced the immunity in *Antheraea assama* by injection of heat-killed strain of *Pseudomonas aeruginosa* in larvae of silkworm. The immune plasma was found to be effective against *Pseudomonas, E. coli*, and *B. subtilis* (Jyotsana et al. 2005) by the isolated antibacterial protein from the hemolymph of Muga silkworm pupae after injection with *Pseudomonas* DAS-01. The amino acid composition showed mainly the presence of basic amino acid which gave the protein a negatively charged and hydrophilic N-terminus. This protein showed antibacterial activity against *Pseudomonas aeruginosa*, causative organism of "flacherie" disease in Muga silkworm. The molecular weight was determined by LC-MS and was found to be positively charged with molecular weight 23 kDa (Unni et al. 2009).

The antimicrobial peptides synthesized in vivo have wide application. To develop novel antibiotic peptides, which substitution and hydrophobicity by leucine substitution. These analogues showed enhanced are useful as therapeutic drugs, analogues were designed to increase net positive charge by lysine antibacterial and antitumor activity with hemolysis (Park et al. 2003). Sarmasik and Chen (2003) reported the coning of bacterial diseases in fishes by the production of transgenic fish expressing endogenous cecropin. They succeeded in expressing the cecropin gene in fish under control of cecropin B signal peptide. Similar expression studies have been done in insect, plants, and bacteria. The short persistence of cecropin B peptide in plants, due to posttranslational degradation, is a serious impediment in its effective utilization for developing bacterial resistance transgenic plants. Two DNA constructs encoding the full-length precursor of cecropin B peptide and the mature sequence of cecropin B peptide preceded by a signal peptide derived from rice chitinase gene were transformed in rice. The development of lesions resulting from infection by *Xanthomonas oryzae* was significantly controlled in the infected leaflet of transgenic lines, when compared with the control plants (Sharma et al. 2000).

Nowadays, cecropin is being seen as substitute to chemicals used by farmers for protecting their plants from bacteria and fungi (Ekengren and Hultmark 1999; Andra et al. 2001; Kirill et al. 2001).

Research into the application of nonsymbiotic rhizosphere microorganism like PGPR has opened a new door for improving the biocontrol efficacy of the agent. Identifying different mechanisms of action facilitate the combination of strains, bacteria, or bacteria with fungi to hit pathogens with a broader spectrum of microbial weapons (Compant et al. 2005). Along with this biotechnology can be applied for the production of vaccine using insect as a model system.

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Chapter 13
Arabidopsis as a Model System to Decipher the Diversity and Complexity of Plant Responses to Plant-Growth-Promoting Rhizobacteria

Guilhem Desbrosses, Fabrice Varoquaux, and Bruno Touraine

13.1 Introduction

Plant-growth-promoting rhizobacteria (PGPR) are naturally occurring soil microorganisms that colonize the rhizosphere of many plant species and confer beneficial effects, including plant growth stimulation and reduced susceptibility to pathogens (Van Loon et al. 1998; Bloemberg and Lugtenberg 2001; Dobbelaere et al. 2003; Bashan et al. 2004; van Loon 2007; Babalola 2010). PGPR have been applied to a wide range of crops and agricultural conditions for the purpose of enhancing plant growth and health, hence improving crop yields (Kloepper et al. 1989, 1991, 2004; Zhuang et al. 2007). While the mechanisms involved in biocontrol, especially the induced systemic resistance (ISR) elicited by PGPR, have been investigated in details (Van Loon et al. 1998; Kloepper et al. 2004; van Loon 2007), the mechanisms involved in plant growth promotion are still elusive. The main reason for this lack of knowledge is that the mechanisms involved in plant nutritional and developmental responses underlying plant growth promotion have received little or no attention, in comparison with the ISR mechanism that has been investigated in details. Indeed, until recently, all the studies on the elicitation of plant growth promotion by PGPR focused on the bacterial partner without consideration of plant's physiology.

One difficulty in studying plant–PGPR interactions is the manifold species of PGPR that can elicit growth promotion and the manifold plant species that respond positively to PGPR. The second difficulty lies in the weak host specificity of PGPR, though these bacteria exhibit differences in their metabolism, their localization in roots and rhizosphere, and their potential modes of action, whereas third difficulty

G. Desbrosses • F. Varoquaux • B. Touraine (✉)
Laboratoire des Symbioses Tropicales et Méditerranéennes (UMR UM2/IRD/Cirad/SupAgro/INRA), Université Montpellier 2, CC 002, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France
e-mail: touraine@univ-montp2.fr

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is that growth promotion is an integrative phenotype that does not correspond to a specific function but is the reflection of many developmental and nutritional processes, all of which being potential targets for PGPR action. Until recently, studies on plant growth promotion by beneficial rhizobacteria used the plant as a broad phenotype to screen bacterial strains in their ability to stimulate plant growth and to identify bacterial compounds or genes. However, to understand how plants respond to PGPR requires also focusing on the plant partner to identify the plant's targets and determine how signaling pathways are modified by PGPR interaction. Obviously, a plant approach of plant–PGPR interaction needs to use a model plant species, so that to avoid diluting research efforts and to provide genetic and genomic tools. In addition, to determine how PGPR modulate plant nutritional and developmental processes, it is necessary to use a model plant in which there is a deep knowledge of the molecular and cellular bases of these processes. *Arabidopsis thaliana* is unequivocally the best plant model, and in the last decade, a few groups have begun to use it to investigate the signaling pathways involved in the plant growth promotion response to rhizospheric bacteria. These investigations, although not numerous and very recent, already revealed unexpected effects of PGPR and propose new paradigms to explain plant responses. This chapter presents new insights of plant responses to PGPR that are responsible for the stimulation of plant growth, obtained in *Arabidopsis*, excluding other aspects of the plant–PGPR interaction, such as recruitment of beneficial soil bacteria by root exudates, rhizosphere colonization, indirect effects on plant nutrition like solubilization of nutrients in the soil, and biocontrol processes including ISR.

13.2 PGPR Emit Volatile Organic Chemicals that Elicit Plant Developmental Responses

Plants are powerful producers of low-molecular-weight volatile organic chemicals (VOCs) of diverse nature in response to either internal clues (e.g., developmental stages) or external stimuli. Historically, the first gaseous plant signal discovered was the hormone ethylene, which is involved in both plant development and defense (Abeles et al. 1992; Bleecker and Kende 2000). A blend of chemically diverse compounds, including fatty acid derivatives, terpenes, indole, and molecules from other chemical families, have been shown to have important roles in the interaction between the plant and its immediate environment, neighboring plants, and attackers, including pathogenic microorganisms and herbivores (Paré and Tumlinson 1999; Farmer 2001; Piechulla and Pott 2003). Although it was known that microorganisms release VOCs (Stotzky and Schenck 1976) and the fact that similar compounds have been identified as signal molecules for plants, the role of volatiles emitted by bacteria in plant development was barely discerned until recently. In their pioneering work on the effects of bacterial VOCs on plant growth promotion, Ryu et al. (2003) showed that some PGPR strains, including *Bacillus subtilis* GB03 and

Bacillus amyloliquefaciens IN937a, stimulate the growth of *A. thaliana* seedlings cultivated in one part of divided Petri dishes when these bacteria were cultivated in the other part of the plates. The partition of Petri dishes forming a tight seal between the bacterial and the plant media, only airborne signals can be transmitted from one side to the other side as evidenced by plant growth promotion. Since growth promotion was not obtained with the *Escherichia coli* DH5α used as a nongrowth-promoting control strain, this simple experiment elegantly demonstrates that some PGPR release VOCs that support plant growth. Furthermore, only three of the seven PGPR strains tested by Ryu et al. (2003) led to increased growth rate of *Arabidopsis* in the divided Petri dishes system indicating that the synthesis of bioactive VOCs is a strain-specific phenomenon. Collecting and analyzing VOCs emitted by the two PGPR strains *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a and by the non-growth-promoting strains *E. coli* DH5α and *Pseudomonas fluorescens* 89B61, the authors identified 2,3-butanediol and acetoin as volatile components released by the two growth-promoting strains but not from the nongrowth-promoting ones; pharmacological application of 2,3-butanediol and inoculation with bacterial mutants deficient in 2,3-butanediol and acetoin synthesis indicated that these VOCs were responsible for the plant-growth-promoting effect.

To investigate the effects of VOC emission from different rhizobacterial strains, Gutiérrez-Luna et al. (2010) cocultivated *Arabidopsis* with 12 bacterial strains isolated from the rhizosphere of lemon plants (*Citrus aurantifolia*) in partitioned Petri dishes and analyzed plant fresh weight and root system architecture. Differences in plant growth promotion were related to differential modulation of root system architecture. Emitted VOC analysis identified both common and specific compounds, and comparison with the phenotypic data suggests that differential VOC emission can modulate plant growth promotion and root system architecture in response to the PGPR strains.

The growth of *Arabidopsis* seedlings was drastically inhibited by cocultivation with *Serratia odorifera* 4Rx13 in partitioned Petri dishes in one set of experiment (Vespermann et al. 2007) while it was promoted in a second set (Kai and Piechulla 2009). This strong difference in *Arabidopsis* pattern responses to 4Rx13 was attributed to sealed or nonsealed Petri dishes (Kai and Piechulla 2009): plant growth promotion was obtained when Petri dishes were sealed with parafilm, while seedlings did not develop in the nonsealed setup (the seedlings stopped growing at very early stage after germination and they were albino). Using tripartite Petri dishes to trap CO_2 with barium hydroxide, Kai and Piechulla (2009) showed that there was a significant rise of CO_2 levels in sealed Petri dishes due to bacterial growth and that elevated CO_2 level was responsible for the plant growth promotion by 4Rx13. The deleterious effect of bacterial cells in nonsealed Petri dishes would be due to VOCs emitted at ambient CO_2 concentration. The 4Rx13 bacteria release more than 100 volatile compounds, among which dimethyl disulfide (DMDS) and ammonia have growth-inhibiting effect on *Arabidopsis* (Kai et al. 2010). The other volatile substances extracted had no effect on *Arabidopsis* growth. However, the bioassay used to test the effects of these VOCs could have been insufficient, and the extraction method may have failed to isolate all bioactive molecules; it cannot

be excluded that some 4Rx13 emitted VOCs have antagonistic or synergistic effects with DMDS and NH_3 on plant growth.

The possibility that bacterial CO_2 production affects the plant growth in plant–PGPR interaction studies has to be considered. However, the application of purified VOCs or genetic approaches like in the study reported by Ryu et al. (2003) demonstrated that PGPR-emitted VOCs have beneficial effects on plant growth besides having the role of possible increase in CO_2 level. In addition, investigating the targets of these VOCs in plants by using *Arabidopsis* mutants further identified specific effects that cannot be attributed to the provision of supra optimal level of CO_2. Using *Arabidopsis* mutant lines defective in hormonal pathways, Ryu et al. (2003) showed that the ethylene, gibberellin, and brassinosteroid-signaling pathways were not involved in the promotion of growth by *Bacillus* spp. GB03 and IN937a strains-emitted VOCs. Although an *eir1*/*pin2* mutant deficient in one IAA efflux transporter retained the growth promotion response to both PGPR strains, the authors could not exclude the possibility that the auxin signaling pathway be implicated in the response of *Arabidopsis* seedlings to VOCs. Indeed, because of the great number of auxin efflux transporters acting in the various tissues and organs, the IAA transport pattern required for the GB03 response could still operate normally in the mutant. Moreover, because no mutant in the IAA transduction pathway has been included in this study, the implication of the auxin signaling pathway itself was not directly tested. A cytokinin receptor *cre1* mutant did not exhibit growth promotion when exposed to GB03, suggesting a role for the cytokinin signaling pathway in plant response to VOC emission by this strain. By contrast, the other *Bacillus* spp. strain tested in this study, IN937a, still promoted the growth of *cre1* mutant seedlings, which suggests that (1) several plant signaling pathways are targets of PGPR-originating VOCs responsible for developmental changes and growth stimulation, and (2) volatile blends released by GB03 and IN937a differ in their composition.

Farag et al. (2006) further characterized 38 volatile metabolites from the GB03 and IN937a strains, most of these compounds being branched-chain alcohols not identified in the previous study by Ryu et al. (2003). Comparison of the GB03 and IN397a VOCs profiles showed apparent differences, with IN397a producing higher amounts of 3-methyl-1-butanol, 2-methyl-1-butanol, and butane-1-methoxy-3-methyl. It can be speculated that the release of these alcohols only in IN937a volatile blend is responsible for plant growth promotion through a cytokinin-independent pathway. In any case, the fact that both *Bacillus* spp. strains similarly promote the *Arabidopsis* growth using VOCs as elicitors of plant signaling pathways, but differ in the composition of their VOCs bouquets and in the regulatory pathways targeted, is extremely interesting to understand the diversity and specificity of mechanisms responsible for plant growth promotion by rhizobacteria. Because of the availability of a large number of mutant lines altered in the different steps of hormones synthesis, transport, sensing, and transduction, the model plant *Arabidopsis* provides the tools to investigate further the specific targets of VOCs emitted by the two *Bacillus* spp. strains.

13.3 PGPR Elicit Plant Developmental Responses by Modulation of Plant Hormonal Pathways

The plant hormones have roles in both plant physiology and development, thus determining plant growth. Most of the PGPR genera secrete auxins, gibberellins, etc., and it has been considered that plant hormones produced by PGPR strains are essential in plant growth promotion. However, the results of recent investigations using Arabidopsis have questioned this hypothesis, showing that PGPR can elicit plant hormonal pathways without a hormone of bacterial origin be involved, as detailed below.

13.3.1 The Auxin Signaling Pathway Is Elicited by IAA-Nonproducing PGPR

Probably, the most widely accepted mechanism for plant growth promotion by PGPR remains the synthesis of auxin by bacterial cells and its release in the rhizosphere (Loper and Schroth 1986; Dobbelaere et al. 1999; Spaepen et al. 2007). This hypothesis has been supported by studies that used auxin-deficient bacterial mutants especially with the PGPR strains *Azospirillum brasilense* sp245 (Barbieri and Galli 1993) and *Pseudomonas putida* GR12-2 (Patten and Glick 2002), two high auxin producers. Furthermore, some studies reported a correlation between the growth parameters (root and shoot elongation, root and shoot dry weight) of inoculated seedlings and the in vitro auxin production by several PGPR strains (e.g., Khalid et al. 2004). Other studies, however, failed to find such a correlation (e.g., Kishore et al. 2005). This discrepancy indicates that bacterial production and release of indole-3-acetic acid (IAA) is likely to be one mechanism that can affect positively plant growth by some rhizobacteria strains but that this mechanism cannot be generalized among all the PGPR. In addition, until very recently, no investigation has been made in the plant partner to confirm the implication of auxin in plant responses and to characterize the role of the plant auxin signaling pathway in specific plant developmental or metabolic responses.

The only studies that actually investigated the implication of auxin signaling pathway in plant responses to PGPR took advantage of the model plant *Arabidopsis* to perform genetic or reverse genetic approach. In the earliest of these very few studies (Persello-Cartieaux et al. 2001), a screen for *Arabidopsis* mutants insensitive to the inoculation with a *Pseudomonas thivervalensis* strain was developed based on the reduction of primary root length upon inoculation. The screen resulted in the isolation of two *aux1* mutants impaired in the major transporter for the influx component of auxin polar transport. This finding thus reinforces the conclusion of rhizobacterial studies that auxin is involved in plant responses to PGPR. More precisely, genetic evidence obtained by Persello-Cartieaux et al. (2001) demonstrates that IAA influx is required for the rhizobacteria-induced root

morphology change, but there is no direct evidence that the auxin signal transduction pathway is implicated in this response.

More recently, two studies, one with a VOC producing PGPR (Zhang et al. 2007) and the other with a VOC nonproducing PGPR (Contesto et al. 2010) showed the implication of the plant auxin pathway in growth promotion response without elicitation by bacterial auxin. The modifications of *Arabidopsis* transcriptome by *B. subtilis* GB03-emitted VOCs (plants and rhizobacteria positioned on separate sides of partitioned Petri dishes, see Sect. 13.2) identified several auxin biosynthesis genes as upregulated by GB03 VOCs (Zhang et al. 2007). The authors further showed that these genes were specifically upregulated in the shoots of GB03-exposed plants. Using a transgenic *DR5::GUS* auxin marker line (Ulmasov et al. 1997) revealed that auxin accumulation decreased in leaves while increased in roots with GB03 exposure. These opposite changes in organ auxin contents suggest that GB03 VOCs activate basipetal auxin transport (Zhang et al. 2007). Application of the auxin transport inhibitor 1-naphthylphthalamic acid both prevented GB03-mediated decrease in shoot auxin level and thwarted GB03-mediated growth promotion. All together, the discovery by Zhang et al. (2007) stated that bacterial VOCs devoid of auxin or other known plant hormones regulate auxin homeostasis and cell expansion (histochemical analysis of leaves section and induction of a group of genes involved in cell-wall loosening) provides a new paradigm as to how PGPR promote plant growth.

We showed that this behavior is not restricted to VOCs-emitting rhizobacteria and gave further indications on the implication of the auxin signaling pathway by investigating root architecture modifications in *Arabidopsis* seedlings inoculated with the PGPR strain *Phyllobacterium brassicacearum* STM196 (Contesto et al. 2010). This strain (former isolate 29-15) was isolated from the roots of canola plants grown on a canola field soil of the Burgundy region (France) (Bertrand et al. 2001; Mantelin et al. 2006b). This particular strain was selected among the canola-associated rhizobacteria isolated in this study for its higher efficiency to promote canola growth in a bioassay. A detailed analysis of the effect of STM196 on canola seedlings grown in vertical Petri dishes showed that plant growth promotion is accompanied by an increase in the individual lateral root growth rate (Larcher et al. 2003). Similar growth promotion and root architecture changes are recorded in the model plant *A. thaliana* (Mantelin et al. 2006a), making this PGPR a good model to decipher the signaling pathways involved in plant responses to beneficial rhizobacteria. Using a mutant severely altered in IAA transduction (*axr1*, Estelle and Somerville 1987; del Pozo et al. 2002), we demonstrated for the first time that not only the IAA molecule is involved in the beneficial effect of some PGPR but also that the IAA transduction pathway per se is actually implicated in this response (Fig. 13.1). The free IAA level was significantly lower in the roots of STM196-inoculated wild-type plants than in the roots of noninoculated ones, which would be difficult to reconcile with IAA release by the PGPR. Consistent with the lack of a significant provision of bacterial auxin to the plant roots, STM196 appears to be a very low-IAA producer: its capacity to synthesize and release IAA is dramatically

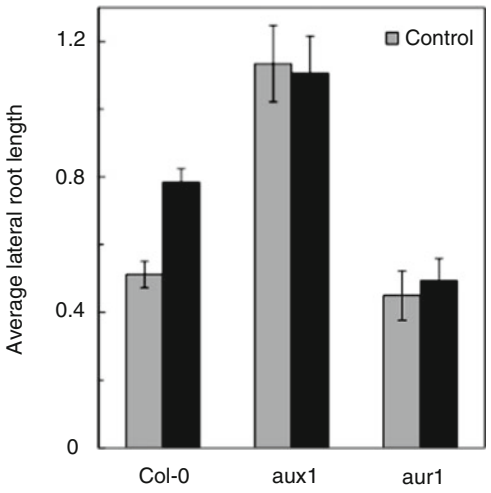

Fig. 13.1 The stimulation of lateral root growth by the PGPR strain *Phyllobacterium brassicacearum* STM196 involves the auxin signaling pathway. This rhizobacterium increases the average lateral root length of *Arabidopsis thaliana* wild-type plants (Col-0 ecotype) grown on a solid medium in Petri dishes. Both mutations in the major IAA influx transporter (aux1) and in the IAA receptor complex (axr1) block this response. Data drawn from Contesto et al. (2010)

lower than that of the well-characterized *A. brasilense* sp245 strain often used in studies that support a role for bacterial auxin in plant growth promotion (Barbieri and Galli 1993; Spaepen et al. 2008), and equal to that of its IAA-low-producing *ipdc* mutant (Costacurta et al. 1994). In support to the hypothesis that bacterial IAA is not involved in root development changes of STM196-inoculated *Arabidopsis* seedlings, the root system phenotype of these plants—longer lateral roots—differs markedly from the root phenotype of sp245-inoculated plants—increased number of lateral roots, strong shortening of primary root, shorter lateral roots—whereas this latter phenotype resembles the root phenotype obtained by addition of IAA to the medium supplied to noninoculated seedlings (Contesto et al. 2010). Transgenic *DR5::GUS* plants indicated higher IAA accumulation levels in the tissues where it normally accumulates (within primary and lateral root meristems) and an extension in size of these regions toward older tissues along the central cylinder. This change in the distribution of IAA within the root system explains the apparent contradiction between the decreased total root IAA content in STM196-inoculated plants and the implication of IAA transduction pathway in lateral root response. Therefore, STM196 must specifically affect the IAA transport without either providing supra-auxin to the plant or increasing total IAA production by the plant.

This new paradigm of a modification of IAA distribution within plant organs and tissues upon inoculation with PGPR raises several questions. Firstly, the strains *B. subtilis* GB03 and *P. brassicacearum* STM196 used in the studies that led to this discovery are very unlikely to elicit IAA redistribution in plant via the same bacterial molecules: while in the experiments by Zhang et al. (2007) the elicitors were necessarily VOCs, in fact STM196 fails to elicit the root development changes reported by Contesto et al. (2010) when the roots are separated from

the inoculums (unpublished data). Identifying these different bacterial molecules is certainly an important aspect for further research issue. Secondly, with regard to the plant response, this shows that besides its key role in plant organs specialization, organization, and development and in plant adaptation to abiotic constraints (e.g., unidirectional light or gravity force, nutrient availability at the root surface), the polar auxin transport is also a major integrator of plant responses to biotic interactions. The polarity of auxin transport at the tissue and organ levels is determined by the subcellular localization of auxin carriers, especially PIN efflux transporters, which is regulated by specific kinases, phosphatases, and GTPases (Benjamins and Scheres 2008). The details of this polarity loop are far from being completely deciphered yet, but it is likely that some of its elements are, directly or indirectly, the targets of PGPR components. Identifying these targets is another important perspective of future research. The PGPR–*Arabidopsis* interaction is a good model to help understanding how the polarity loop can integrate possibly antagonistic or synergistic effects from internal cues, abiotic constraints, and biotic interactions.

By contrast with STM196 (Contesto et al. 2010), *Bacillus megaterium* UMCV1 caused the same effects on *Arabidopsis* mutants altered in the auxin and ethylene pathways than in the wild-type plants, suggesting that this PGPR promotes growth and alters root system architecture through an auxin- and ethylene-independent mechanism (Lopez-Bucio et al. 2007). With regard to the auxin signaling pathway, the mutants used were *aux1* and *axr4*, thus blocking the same step (the AXR4 protein is specifically involved in AUX1 trafficking, Dharmasiri et al. 2006). Because the polar auxin transport depends much more on efflux transporters than on influx transporter, the fact that *aux1* and *axr4* mutations did not thwart the UMCV1 effect cannot be considered as a definite evidence for the nonimplication of the auxin signaling pathway in the growth-promoting effect of this PGPR strain. To demonstrate that UMCV1 alters root system architecture and promotes plant growth through an auxin-independent pathway would require using a mutant altered in the auxin transduction pathway. Lopez-Bucio et al. (2007) also reported concomitant GUS staining decrease in primary root tips and increase in lateral root primordia of transgenic *DR5::GUS* plants inoculated with UMCV1. These observations indicate that UMCV1 modifies auxin transport within roots, though with a different profile than GB03 or STM196 do.

Since published studies that investigated the implication of auxin signaling pathway in plant responses to PGPR used IAA-low-producer strains, their conclusions do not dismiss the possibility that high-IAA-producing PGPR can promote plant growth by releasing auxin in the rhizosphere as proposed earlier. To investigate this process would require characterizing the cellular and molecular responses of *Arabidopsis* to high-IAA-producing bacterial strains. Unfortunately, the literature provides no report that describes in detail the effect of such PGPR on *Arabidopsis* development parameters and/or investigates the signaling pathways elicited in the plant.

13.3.2 The Relationship Between PGPR and the Ethylene Signaling Pathway Is Complex

One hypothesis for the effect of PGPR on root system architecture is based on the reduction of plant ethylene production by bacterial ACC deaminase activity (Glick 2005). Indeed, the enzyme ACC deaminase (AcdS) that catalyzes the cleavage of the plant ethylene precursor, 1-amino cyclopropane-1-carboxylic acid (ACC), in α-ketobutyrate and ammonia is found in many plant-beneficial as well as pathogenic bacteria (Blaha et al. 2006). Rhizobacterial AcdS activity could divert ACC from ethylene biosynthesis and thereby lower the level of ethylene in plant root (Glick et al. 1998). Bacterial AcdS enzyme can effectively compete with plant ACC oxidase (ACO) despite a higher affinity of ACO than AcdS for ACC, provided that the AcdS level is 100- to 1,000-fold greater than the ACO level. Glick et al. (1998) argue that such a situation is likely to occur because ACO is an enzyme normally present at very low levels in plant cells, so that PGPR AcdS activity could actually lower ethylene levels in plant roots except when ACO is induced by environmental conditions or developmental stage. The best evidence in favor of Glick's hypothesis is that AcdS-deficient mutants of the *P. putida* GR12-2 and UW4 PGPR strains were found unable to promote root elongation of canola seedlings as the wild-type strains did (Glick et al. 1994; Li et al. 2000). However, the lack of studies on the plant partner did not permit to investigate the real impact of bacterial AcdS activity and the possible involvement of the ethylene signaling pathway in plant response to PGPR until studies have recently been conducted with the model plant *Arabidopsis*.

Seedlings from *Arabidopsis*-ethylene-insensitive mutant lines *etr1* and *ein2* displayed enhanced total leaf area upon exposure to GB03 VOCs, indicating that ethylene sensing and signaling is not involved in growth promotion by this PGPR (Ryu et al. 2003). Similarly, *etr1* and *ein2* mutants are not impaired in growth promotion responses to *B. megaterium* UMCV1 (Lopez-Bucio et al. 2007). Consistent with these two reports, we found no difference between the root system architecture of *Arabidopsis* seedlings inoculated with $AcdS^-$ mutants of *P. brassicacearum* STM196, *P. putida* UW4, *Rhizobium leguminosarum* bv. *viciae* 128C53K, and *Mesorhizobium loti* MAFF303099, and the root system architecture of seedlings inoculated with their respective wild-type counterparts (Contesto et al. 2008; Desbrosses et al. 2009). All together, these studies negate an essential role of bacterial AcdS activity and plant ethylene signaling in the activation of root development and plant growth by PGPR. One possible explanation for the discrepancy with Glick's reports is that the bacterial AcdS activity would be insufficient to compete with the plant ACO activity in the growth conditions used in these three studies. The capacity of PGPR to lower ethylene concentration in plant's roots and thereby affect plant development and growth, therefore, cannot be dismissed. In the reports of differential plant response patterns to $AcdS^-$ and

wild-type PGPR cells, however, the implication of the ethylene signaling pathway in plant responses is yet to be demonstrated.

Besides root system architecture, another useful phenotypic response to investigate the role of ethylene in plant–PGPR interaction is root hair elongation (Contesto et al. 2008; Desbrosses et al. 2009). Indeed, inoculation of *Arabidopsis* seedlings with PGPR strains led to a dramatic stimulation of root hair elongation: their length was increased by two- to threefold upon inoculation with the STM196, UW4, 128C53K, and MAFF303099 strains (Contesto et al. 2008). The AcdS$^-$ mutant cells of all four strains led to a slightly but significantly stronger stimulation of root hair elongation. Genetic studies in *Arabidopsis* have identified the ethylene and auxin signaling pathways as the main regulators of root hair elongation (Pitts et al. 1998), suggesting that these two hormonal pathways are the main regulators of root hair development. The differential root hair pattern response to AcdS$^-$ and wild-type STM196, therefore, is consistent with a lowering of plant ethylene level by AcdS activity of wild-type bacterial cells. On the other hand, AcdS activity does not explain the root hair elongating effect of PGPR, as it actually antagonizes this effect, indicating that PGPR elicit an ethylene-independent pathway to elongate root hairs. Consistent with this conclusion, we found that STM196 is able to stimulate root hair elongation in all the *Arabidopsis* mutant lines altered in the ethylene signaling pathway we tested (Desbrosses et al. 2009). Remarkably, mutants altered in auxin signaling also maintain full capacity to elongate root hairs when inoculated with STM196. These findings are much unexpected since, as mentioned above, auxin and ethylene are considered as the main regulating factors of root hair elongation. Nevertheless, this shows that PGPR must elicit some ethylene- and auxin-independent pathway(s) in the plant, not excluding demonstrated implication of the auxin signaling pathway and possible implication of the ethylene signaling pathway in some plant responses. Among other, this is strong evidence that multiple signaling pathways are elicited by PGPR and that the multiple plant responses are the result of a complex combination of these various regulations (so-called additive hypothesis, see Bashan et al. 2004). In addition, the fact that PGPR can induce a strong root hair elongation independently to auxin and ethylene pathways raises the question of what other plant factor can overcome these regulations.

In summary, the results published for PGPR–*Arabidopsis* interactions suggest that the impact of bacterial AcdS activity is rather modest and probably affects specifically local processes such as root hair elongation (Desbrosses et al. 2009). By contrast, more integrated processes that depend upon systemic regulation by shoot-derived compounds, such as root development and root system architecture, are unlikely be affected by AcdS activity of PGPR. Another difficulty with the Glick's model is the existence of a negative feedback loop affecting ethylene biosynthesis (Guzman and Ecker 1990), so that stimulated ethylene emission can be induced indirectly by PGPR while typical ethylene responses are not displayed.

13.4 PGPR Thwart the N-Dependent Regulation of Lateral Root Development

From the observation that the inoculation with *P. brassicacearum* STM196 induced increased lateral root growth in *Arabidopsis* whereas increasing NO_3^- has the opposite effect (Zhang et al. 1999; Tranbarger et al. 2003), we investigated how these two processes interfere with each other (Mantelin et al. 2006a). Remarkably, STM196 countervails the inhibitory effect of high NO_3^- on lateral root development: the negative correlation observed between NO_3^- concentration and lateral root length was not observed in STM196-inoculated *Arabidopsis* (Fig. 13.2). Considering that regulatory pathways in plants are highly interconnected as illustrated by hormonal signaling cross talks, it is not so surprising that plant development integrates the antagonistic effects of a biotic agent and an abiotic constraint via some process. However, to our knowledge, the alteration of nitrate-dependent control of root architecture by STM196 (Fig. 13.2) is the first example of such interference between metabolic-dependent and PGPR-induced developmental controls. Inhibition of lateral root development by high NO_3^- is a systemic mechanism by which the high N status in leaves control the root system architecture, and it is usually considered that the leaf NO_3^- is the sensing pool (Scheible et al. 1997; Forde and Lorenzo 2001). Because no systematic correlation was found between leaf NO_3^- and lateral root length in STM196-inoculated seedlings, we concluded that the inoculation with STM196 alleviates the N-dependent regulation of root development downstream the sensing of N status. The mechanism by which NO_3^- accumulation in leaf regulates lateral root development is not known yet, but some pieces of evidence suggest that auxin

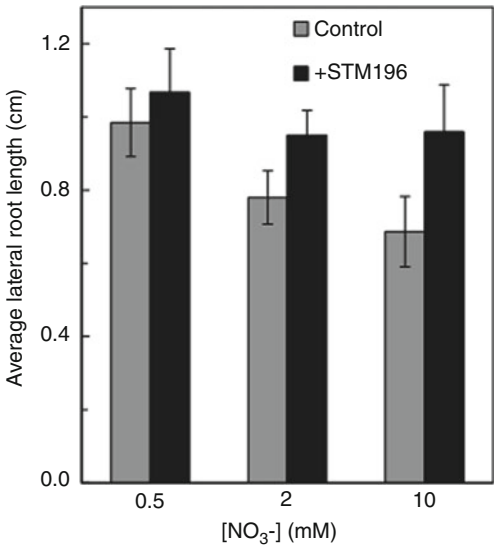

Fig. 13.2 Nitrate-dependent regulation of lateral root development and PGPR-elicited lateral root growth are antagonistic processes. High external NO_3^- concentrations reduce root development, but the *Phyllobacterium brassicacearum* STM196 PGPR strain thwarts this effect. Indeed, STM196-inoculated *Arabidopsis* seedling displayed a NO_3^--independent lateral root development. Data drawn from Mantelin et al. (2006a)

be involved in the shoot-to-root signaling. For instance, transferring *Arabidopsis* seedlings from 50 to 1 mM NO_3^- led to concomitant decrease in shoot IAA concentration and increase in root IAA concentration within 24 h, followed by a restoration of lateral root development (Walch-Liu et al. 2006). Since changes in polar IAA transport are implicated in the lateral root development response to STM196 (Contesto et al. 2010), it is likely that integration of bacterial-originating signals and N-status-sensing to control root system architecture involves regulation of IAA homeostasis.

13.5 PGPR Modify Nutrient Uptake Both Indirectly and Directly

A common hypothesis to explain plant growth stimulation by beneficial rhizobacteria considers that PGPR primarily increase root surface area due to lateral root proliferation induced by bacterial auxin, and increased plant growth is a consequence of increased nutrient and water acquisition. However, this model suffers from several problems. First, because hormones have systemic effects in plant and their impact is never restricted to a unique organ, it would be very unlikely that PGPR affect root development through plant hormonal pathway without altering shoot development. Second, root ion transporters are known to be regulated by internal cues related to the nutritional demand (e.g., see Imsande and Touraine 1994; Lappartient and Touraine 1996; Lappartient et al. 1999; Nazoa et al. 2003), so that the regulations of root development and ion transporter activities are antagonistically coordinated to maintain acquisition rate (Touraine 2004). If PGPR specifically exerted an effect on root development, therefore, nutritional demand controls should downregulate the ion uptake systems, so that the whole acquisition rate of nutrient by the plant would not change and shoot growth would not increase if drawn solely by higher acquisition rate as hypothesized. In other words, to elicit both increased nutrient acquisition rate and plant growth promotion, PGPR must interfere with the development–nutrition normal coordination. To achieve this, they needs to promote shoot like root growth, so that the whole plant growth rate is higher and the increased nutritional demand draws nutrient uptake to sustain this supra biomass production. In addition, as discussed below, PGPR can also more directly stimulate the ion transport systems in root, thus modifying the coordination of developmental and nutritional processes. Thirdly, a model that would consider local effects as the main plant responses to PGPR is not consistent with transcriptome studies of PGPR-inoculated *Arabidopsis* plants, which show larger modifications of the gene expression profiles in shoots than roots (Cartieaux et al. 2003). Finally, the *B. subtilis* GB03 strain has been shown to augment photosynthetic efficiency through the modulation of endogenous sugar/ABA signaling (Zhang et al. 2008b, see Sect. 13.6). Such a regulatory role in plant acquisition of energy provides the PGPR with a

supplementary mechanism to affect leaf metabolism and development not only as a consequence of the enhanced nutrient acquisition that would be drawn by increased root surface area.

The impact of PGPR on nutrient uptake systems has been much less studied than their effects on root development. In canola, both NO_3^- and K^+ net influx rates per unit root surface area increased upon inoculation with *Achromobacter* sp. strain U80417 (Bertrand et al. 2000). The net H^+ efflux was also enhanced, so that increased NO_3^- and K^+ uptake rates may be part of a general increase in ions uptake rate as a consequence of root cells plasma membrane energization by enhanced proton pump activity. Supra acidification of the rhizosphere by plant roots has also been observed with *Arabidopsis* seedlings exposed to GB03 (Zhang et al. 2009), suggesting that the stimulation of root H^+ ATPase may well be a general response to PGPR. Alternatively, the increased nutrient demand associated to stimulated growth rate in inoculated plants could be responsible for increased NO_3^- and K^+ uptakes. Indeed, nutrient uptake is controlled by plant demand, so that it does not depend only on nutrient availability and local regulation exerted in roots but also systemic regulations that link ions transport activity in roots to the whole plant nutritional status (Imsande and Touraine 1994). Therefore, it is difficult to determine whether ions uptake increase is a consequence or a cause of the growth stimulation by PGPR.

The difficulty in determining whether PGPR primarily stimulate plant growth or enhance nutrient uptake lies in the fact that, as discussed by Mantelin and Touraine (2004) for nitrogen uptake, the consequences are about the same, since mineral nutrient are assimilated and diluted in the biomass produced in both cases. Such a chicken and egg question cannot be resolved by a combination of microbiology and whole plant physiology approaches. To decipher the links between PGPR, plant development and growth control, and uptake and nutrition processes, it is necessary to identify the plant signaling pathway elicited. Using the model plant *Arabidopsis* provided some pieces of evidence, but these indications are still very fragmented and do not give a clear picture of PGPR effects on nutrient uptake.

One indication in favor of "developmental" rather than "nutritional" primary effects is that PGPR do exert a direct effect on developmental processes such as root hair elongation and lateral root development independently to any change in nutrient uptake and assimilation (see above). The fact that STM196 thwarts the N-dependent regulation of lateral root development independently to an effect on NO_3^- uptake or distribution is also a strong support for the "developmental" hypothesis. However, it must be kept in mind that PGPR elicit a large array of responses in plants via multiple signaling pathways ("additive hypothesis," see above), and it is all the more feasible that the PGPR induce both developmental processes and nutrient transport and metabolism. Hereafter are summarized evidence recently published that demonstrate the capacity of PGPR to induce modifications of nitrate, sodium, and iron ion transports in *Arabidopsis*.

13.5.1 *The Effect of* Phyllobacterium brassicacearum *STM196 on Nitrate Uptake Can Be Direct or Indirect*

In *Arabidopsis*, measurement of NO_3^- uptake led to contradictory results: NO_3^- influx was increased in seedlings 24 h after transfer on STM196-inoculated medium while it was reduced 7 days later (Mantelin et al. 2006a). Moreover, it is difficult to draw conclusion from the results since the efflux component has not been measured, so that the pattern response of net NO_3^- uptake rate is not known. The accumulation of nitrate and ammonium transporters transcript was very slightly or not significantly changed upon STM196 inoculation, except for the *NRT2.5* and *NRT2.6* genes (Mantelin et al. 2006a). These two genes are likely to be involved in plant response to STM196 but, their function being unknown, their role is still elusive. In any case, they are mostly expressed in shoots (Mantelin et al. 2006a) and their mutations do not induce significant changes in NO_3^- uptake rate (unpublished data), and STM196 is unlikely to exert a transcriptional regulation on NO_3^- uptake. Nevertheless, STM196 must increase NO_3^- uptake rate per unit root surface area in *Arabidopsis* because total N content increased in STM196-inoculated plants (Mantelin et al. 2006a), and taking into account the relative root and shoot growth rate increases, N acquisition rate per unit root weight is higher in STM196-inoculated plants than in noninoculated plants. Although contributions of N_2 fixation by associated bacteria to the plant N budget have been reported for several plants, with higher levels in sugar cane, the impact of N_2 fixation by PGPR is still debated, and it is rarely credited for the stimulation of plant growth (for review see Dobbelaere et al. 2003; Vessey 2003). Specifically, STM196 is unlikely to fix N_2 nitrogen (Mantelin et al. 2006a) and to supply an alternative source of nitrogen to *Arabidopsis* since it does not restore growth to nitrate-reductase-deficient mutant grown in a NO_3^--free medium (unpublished data). Therefore, the increased N acquisition in STM196-inoculated *Arabidopsis* requires net NO_3^- uptake rate through the root cells plasma membrane be increased upon STM196 inoculation. As discussed above, the PGPR can stimulate NO_3^- uptake either as a consequence of increased N demand in PGPR-inoculated plants or due to a more direct effect on NO_3^- transporter activity concomitantly to its effect on plant growth (also see Mantelin and Touraine 2004). Also, increased H^+ ATPase activity recorded in canola (Bertrand et al. 2000) and *Arabidopsis* (Zhang et al. 2009) with other PGPR can be part of the explanation for increased NO_3^- uptake in STM196-inoculated *Arabidopsis*.

13.5.2 Bacillus subtilis *GB03 Induces Salt Tolerance by Manipulation of a Sodium Transporter Expression and Accumulation of Osmoprotectants*

Salt stress can damage plants by several mechanisms, including water deficit, ion toxicity, nutrient imbalance, and oxidative stress. Plant response mechanisms to salt

stress include, among others, movement of Na^+ and K^+ ions and the production of osmoprotectants such as proline, glycine betaine, and sugar polyols (Wang et al. 2003). These two mechanisms are induced by GB03 VOCs, as demonstrated by experiments performed on GB03-exposed *Arabidopsis* seedlings treated with high NaCl exogenous concentration (Zhang et al. 2008a) or exogenous mannitol (Zhang et al. 2010).

Under salt stress, exposure of *Arabidopsis* seedlings to GB03 VOCs concurrently down- and upregulates *HKT1* expression in roots and shoots, respectively (Zhang et al. 2008a). In *Arabidopsis*, the HKT1 transporter functions in the shoots' phloem tissues to retrieve Na^+ from the xylem, thus facilitating shoot-to-root Na^+ recirculation (Berthomieu et al. 2003). By removing large amounts of Na^+ from the leaves, and consequently maintaining a high K^+/Na^+ ratio in leaf tissues, this recirculation would play a crucial role in plant tolerance to salt. In addition to its role in Na^+ recirculation, however, HKT1 is also involved in Na^+ uptake by roots so that its activities in roots and shoots may have opposite effects on Na^+ accumulation in plants (Rus et al. 2001). Consistent with the dual role of HKT1 in shoots and roots, the differential regulation of *HKT1* expression in these two organs of plants exposed to GB03 resulted in reduced accumulation of Na^+ and increased accumulation of K^+ in both organs of salt-stressed seedlings (Zhang et al. 2008a). Consistent with the effect of GB03 on HKT1 and the role of this transporter in salt tolerance, GB03 increased shoot growth of salt-stressed *Arabidopsis* wild-type seedlings, but it failed to rescue salt-stressed *hkt1* mutant seedlings from elevated Na^+ accumulation and stunted foliar growth. These results demonstrate that a PGPR strain can regulate the expression of specific plant transporter and consequently control ion homeostasis in plant organs.

In addition to its effect on Na^+ transports, GB03 enhances the biosynthesis and accumulation of the osmoprotectants choline and glycine betaine in plants under mannitol- and drought-induced dehydration stress (Zhang et al. 2010). Upon 100 mM exogenous mannitol stress, *Arabidopsis* plants exposed to GB03 VOCs exhibited increased phosphoethanolamine *N*-methyltransferase gene (*PEAMT*) transcript level compared with stressed plants that were not exposed to the bacteria. The enzyme PEAMT catalyzes the three methylation steps to produce choline, the precursor of glycine betaine, from phosphoethanolamine. Consistent with *PEAMT* transcriptional regulation, endogenous choline and glycine betaine metabolite pools were strongly increased by GB03 treatment. The *xipotl* (*peamt*) mutant line failed to display GB03-induced tolerance to exogenous mannitol, which confirms a role for *PEAMT* in GB03-induced osmotic stress tolerance. Zhang et al. (2010) found similar levels of abscisic acid (ABA) in the shoots and roots of osmotic-stressed plants with or without GB03 exposure, suggesting that GB03-induced osmoprotection is ABA independent. The regulatory pathways responsible for GB03-dependent regulation of *HKT1* and *PEAMT* expression, lower Na^+ accumulation and higher K^+ accumulation, and increased accumulation of choline and glycine betaine remain to be identified.

13.5.3 Bacillus subtilis *GB03 Stimulates Iron Reduction and Uptake by Roots*

In their investigation on the effects of the GB03 strain on *A. thaliana*, the Paré's group has also demonstrated that GB03-emitted VOCs activate the plant's iron acquisition machinery leading to increased iron assimilation (Zhang et al. 2009). In dicots and nongraminaceous monocots, iron acquisition is performed through the strategy 1 process: under iron-deficient conditions, the plant acidifies the soil through activation of a plasma membrane H^+-ATPase of the root epidermal cells, leading to increased iron solubility, and Fe^{3+} chelates are reduced by a specific root reductase prior to transport of released Fe^{2+} ions across the root plasma membrane via Fe^{2+} transporters (Curie and Briat 2003). Exposure of *Arabidopsis* seedlings to GB03 led to a stimulation of all these activities (Zhang et al. 2009). Firstly, GB03 acidifies the rhizosphere, both directly due to chemical effects of some unidentified VOCs and indirectly through increased root proton efflux. Secondly, GB03 upregulates the expression levels of *FRO2* and *IRT1* genes, coding respectively for a Fe^{3+} chelate reductase and a Fe^{2+} transporter. As a result, GB03-exposed *Arabidopsis* has enhanced ferric chelate reductase activity and increased iron content. Microbial siderophores have been observed to facilitate iron uptake by plants (Vansuyt et al. 2007), but the partition that separates *Arabidopsis* from the bacteria and the fact that none of the VOCs characterized so far have known siderophore activity strongly suggest that bacterial siderophores are not implicated in the stimulation of iron uptake by GB03. Some volatiles compounds can be classified as organic acids which could participate at the rhizosphere acidification (Farag et al. 2006), but the main effect of GB03 is via the elicitation of plant activities, namely H^+-ATPase, ferric chelate reductase, and Fe^{2+} transporter.

In plants cultivated without PGPR, induction of *FRO2* and *IRT1* is observed under iron starvation conditions, and these transcriptional regulations have been shown to involve the Fe-deficiency-induced transcription factor FIT1 (Colangelo and Guerinot 2004). Consistent with a role for FIT1 in the GB03 induced increase in *FRO2* and *IRT1* expression, GB03 induced *FIT1* expression in wild-type *Arabidopsis* and it failed to increase root ferric reductase activity and plant iron content in *Arabidopsis fit1* mutants (Zhang et al. 2009). All together, the study by Zhang et al. (2009) again shows that a PGPR strain can modify plant activities by interfering with plant regulatory processes, thus activating plant transduction cascades. However, similar to the other activities elicited by PGPR described before, neither the bacterial chemicals nor the plant sensors and factors that interfere with downstream plant regulatory processes have been identified yet: how GB03 induces *FIT1* expression is not known. Reciprocally, characterization of plant factors involved in FIT1 induction by GB03 may reveal insights into regulatory steps in plant iron uptake and homeostasis.

13.6 Augmentation of Photosynthetic Activity by *Bacillus subtilis* GB03 Involves Modulation of ABA and Sugar Signaling

Again by exposure to the GB03 strain in partitioned Petri dishes, Zhang et al. (2008b) showed that strain augments the photosynthetic capacity by increasing photosynthetic efficiency and chlorophyll content in *Arabidopsis*. GB03 suppressed classic glucose signaling responses, including hypocotyl elongation and seed germination inhibitions by high exogenous glucose. Concurrently, GB03 led to higher hexose accumulation in shoots. GB03, therefore, attenuates glucose inhibitory effects through the repression of sugar signaling rather than by lowering sugar accumulation. Furthermore, GB03 failed to enhance the photosynthetic activity of two *Arabidopsis* mutants defective in hexokinase-dependent sugar signaling, indicating that it augments photosynthesis through repressing hexokinase-dependent, rather than hexokinase-independent, sugar signaling (Zhang et al. 2008b). In addition, GB03-exposed plants exhibited a reduction in ABA biosynthesis transcript levels and shoot ABA levels. Since sugar signaling is known to overlap with the ABA transduction pathway (Rolland et al. 2006), the reduction of ABA levels could explain the repressed glucose signaling in GB03-exposed plants. Consistent with this hypothesis, exogenous ABA thwarts GB03-induced increases in photosynthetic efficiency and chlorophyll content. Overall, this study demonstrates that some PGPR can affect photosynthesis through the modulation of endogenous ABA/sugar signaling regulatory pathways. Considering that PGPR modulate many plant hormonal pathways, as illustrated by the studies performed with *Arabidopsis* summarized herein, and that these pathways interfere with sugar signaling, directly like the ABA pathway or indirectly, this result is not so surprising. On the contrary, the modulation of sugar signaling is likely to be a general feature of PGPR pattern responses, though it remains very elusive.

13.7 Concluding Remarks and Future Research Perspectives

Until very recently, the studies on plant growth promotion by PGPR exclusively focused on the bacterial partner without consideration of plant's physiology. This approach did not succeed in unraveling the mechanisms elicited by the rhizobacteria that operate to promote plant growth. Using *A. thaliana* to investigate how a PGPR can stimulate the growth of plants has demonstrated to being very efficient when considering the limited number of researchers involved in *Arabidopsis*–PGPR studies and the complexity of the biological system. This success lies in the availability of genetic and genomic tools; the deep molecular, cellular, and physiological knowledge; and the experimental convenience of this model species (Fig. 13.3). Indeed, several lessons already arise from this short story (less than a decade).

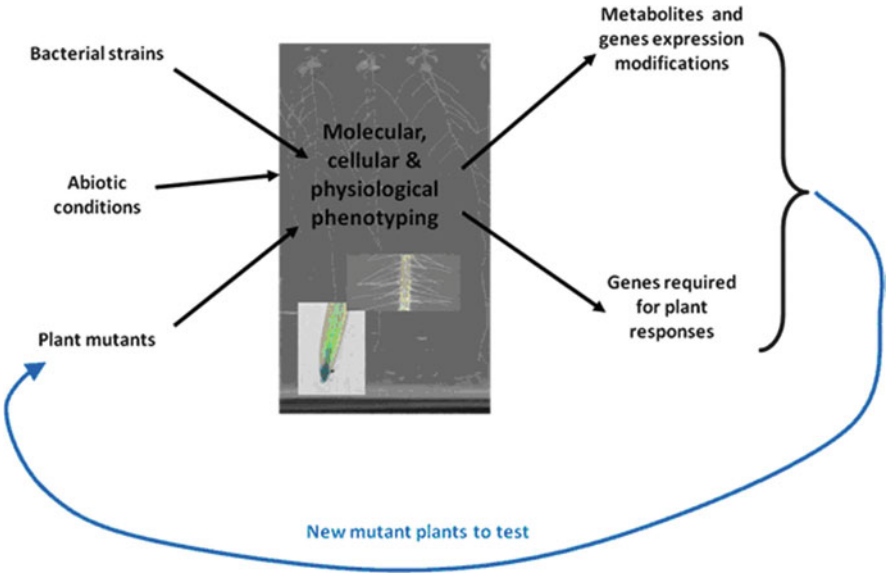

Fig. 13.3 The model plant *Arabidopsis thaliana* provides unique tools to investigate the common and strain-specific signaling pathways involved in plant responses to PGPR because of the largest availability in plant genetic tools, the deepest knowledge in plant biology, and its experimental convenience. Molecular, cellular, and physiological phenotypic responses to inoculation with a PGPR strain (illustrated by IAA accumulation pattern visualized in a DR5::GUS transgenic plant, root hairs, and root system architecture) allow identifying genes and metabolites that are affected by the rhizobacterium and, using mutant plants, the genes that are required for some of these responses. These data lead to question the role of other genes in plant response to PGPR, hence providing new mutants for testing molecular, cellular, and/or physiological response pattern to inoculation. This reverse genetic approach already revealed the occurrence of numerous, and often unexpected, plant response to PGPR, and it will unravel the underlying mechanisms of the complex regulatory network elicited in plants by beneficial rhizobacteria

The first lesson to be drawn from *Arabidopsis*–PGPR studies is that there is an enormous diversity of plant responses to PGPR: a single strain can elicit several hormonal pathways, modulate the activity of several transporters, modify photosynthetic activity and other physiological processes, etc. Furthermore, the list of plant responses is certainly far from being completed. In addition, these various responses are interconnected by plant regulatory pathways, making difficult the identification of PGPR targets.

The second lesson is that to classify a specific PGPR strain as a "phytostimulator" or a "biofertilizer" would not be very meaningful for two reasons: (1) the strains that have been the most extensively studied with *Arabidopsis*, GB03 and STM196, have been proved to affect both plant developmental and nutritional processes, and (2) regulations of nutrition and development are so tightly interconnected in plants that signaling pathways are not entirely distinguishable.

The third lesson is that PGPR not only can modulate plant hormonal pathway, as postulated before, by providing hormones or hormone-like molecules to plant roots, but they also modulate plant hormonal pathways per se. This ability to subtly modify plant endogenous regulatory pathways establishes a new paradigm that showed the complexity of plant responses to PGPR.

The fourth lesson relates to the plant biology knowledge: among the modifications of developmental or physiological traits induced by PGPR, some appeared to involve yet unidentified regulatory pathways. For instance, GB03 induces FIT1 via unknown mechanism (Zhang et al. 2009), so that characterizing the plant factors involved in FIT1 induction by GB03 may reveal to be a useful way to get insights into regulatory steps in plant iron uptake and homeostasis. Another example is the regulation of root hair elongation: while this process has been considered to be mainly dependent upon auxin and ethylene signaling pathways up to now, STM196 is able to dramatically elongate root hairs independently to both hormonal pathways (Contesto et al. 2008; Desbrosses et al. 2009), indicating that another key regulator of root hair development remains to be discovered. These two examples, and other that could be drawn from the studies summarized herein, show that PGPR–*Arabidopsis* interaction studies may be a good model to decipher new regulatory pathways, or new interactions between known pathways, in plants.

In conclusion, the PGPR–*Arabidopsis* interaction appears to be a very powerful model to decipher plant responses to PGPR, but also plant regulatory mechanisms and how a plant integrate endogenous signals with environmental, both biotic and abiotic, signals. Further research using this model will need to combine approaches from the molecular to the ecophysiological level to make a clear picture emerging of such a complex network.

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Chapter 14
Interactions of Plant-Parasitic Nematodes and Plant-Pathogenic Bacteria

Zaki A. Siddiqui, Rukshima Nesha, Neelu Singh, and Subha Alam

14.1 Introduction

Plant-parasitic nematodes are cosmopolitan parasites, exploit all parts of the host plant, and affect virtually every crop. Plant-parasitic nematodes are devastating parasites of crop plants, reducing the overall yield or lowering the market value of crops (Sasser and Freckman 1987; Barker et al. 1994). It has been estimated that overall yield loss averages 12.3% annually; this figure approaches 20% for some crops (Sasser and Freckman 1987; Koenning et al. 1999). Plant-parasitic nematodes range from 250 μm to 12 mm in length, averaging 1 mm, to about 15–35 μm in width. There are two main types of plant-parasitic nematodes: ectoparasitic and endoparasitic. The ectoparasitic type lives outside the plant, feeding on roots with the ability to move about 3 ft to find a host, depending on the soil and species. Endoparasitic types penetrate the root, then enter and live inside it. Each type goes through development stages: starting from an egg, then four juvenile stages (molting after each one), and an adult stage. In addition to the more well-known root-knot nematode, there are many others, most of them named for physical characteristics. They include ring, dagger, sheath, stubby-root, spiral, pin, lesion, stem and bulb, and foliar nematodes. In fact, nematodes occupy all parts of vascular plants including leaves (*Aphelenchoides* spp.), stems (*Bursaphelenchus xylophilus*), tubers (*Globodera rostochiensis*), corms (*Radopholus similis*), and roots (*Heterodera* and *Meloidogyne*). To date, most attention has been focused on the root-parasitic species, and various classification schemes based on the site of feeding within the root have been developed (Dropkin 1969; Hussey and Grundler 1998; Wyss 1997).

Z.A. Siddiqui (✉) • R. Nesha • N. Singh • S. Alam
Department of Botany, Aligarh Muslim University, Aligarh 202002, Uttar Pradesh, India
e-mail: zaki_63@yahoo.co.in

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Nematodes deploy a broad spectrum of feeding strategies, ranging from simple grazing to establishment of complex cellular structures including galls in host tissues (Bird and Koltai 2000). Various models of feeding site formation have been proposed, and a role for phytohormones has long been speculated, although whether they perform a primary or secondary function is unclear (Bird and Koltai 2000). Sedentary endoparasitic nematodes are root parasites that interact with their hosts in a remarkable way. These obligate biotrophic pathogens establish an intimate relationship with their host plants, inducing the redifferentiation of root cells into specialized feeding cells. The successful establishment of feeding cells is essential for nematode development. Root-knot nematodes, of the genus *Meloidogyne*, have evolved strategies enabling them to induce feeding cell formation in thousands of plant species, probably by manipulating fundamental elements of plant cell development (Caillaud et al. 2008).

Many of the bacteria that are associated with plants are actually saprotrophic and do no harm to the plant itself. However, a small number, around 100 species, are able to cause diseases (Jackson 2009). Bacteria pathogenic for plants are responsible for devastating losses in agriculture and are a major problem worldwide for agriculture. There are 21 phyla within the domain Bacteria. Plant-pathogenic bacteria are found in three phyla: the Firmicutes, the Actinobacteria, and the Proteobacteria. The important genera include *Clavibacter, Curtobacterium, Rathayibacter*, *Leifsonia*, *Nocardia*, *Rhodococcus*, *Streptomyces*, *Bacillus, Clostridium*, *Spiroplasma*, *Agrobacterium, Sphingomonas, Acidovorax, Burkholderia, Ralstonia, Xylophilus, Erwinia, Pseudomonas, Xanthomonas*, and *Xylella*. List of plant-pathogenic bacteria is maintained by the International Society for Plant Pathology Committee on the Taxonomy of Plant Pathogenic Bacteria (Bull et al. 2008; ISPP-CTPPB; http://www.isppweb.org/about_tppb.asp).

14.2 Interactions of Plant-Parasitic Nematodes with Bacteria

Since the first report of an interaction of *Meloidogyne* sp. with *Fusarium oxysporum* on cotton (Atkinson 1892), numerous interactions of plant-parasitic nematodes with the plant-pathogenic fungi, viruses, bacteria, and nematodes have been described. Hunger (1901) first reported the possible association between plant-parasitic nematodes and plant-pathogenic bacteria. He noted that tomato plants cultivated in nematode-infested soil were severely attacked by *Pseudomonas solanacearum*, in comparison to those cultivated in nematode-free soil remained healthy. Carne (1926) established that *Anguina tritici* is a carrier of *Corynebacterium tritici*, the causal agent of yellow slime bacteriosis of wheat.

Plant-parasitic nematodes alone can sap the vitality of a plant, but they can also facilitate infection of additional pathogens. Plant-parasitic nematodes as primary pathogens favor establishment of secondary pathogens which alone cannot infect plant under normal condition. Primary pathogens induce changes in the host whereas secondary pathogens after infection by primary pathogen participate

actively and alter the process of pathogenesis. Secondary pathogens generally colonize dead cells induced by primary pathogens (Mayol and Bergeson 1969). Nematodes are of tremendous important as a component of disease complexes because when plant is infected by one pathogen, its response to additional invaders is altered. These alterations exert significant influence upon disease development, etiology of pathogens involved, and ultimately on disease control. It is therefore important to consider the role of primary pathogen and its relationship with secondary pathogen and their ultimate effect on host plant. Reviews and book chapters on the interactions of plant-parasitic nematodes with bacteria (Pitcher 1963, 1965; Sitaramaiah and Pathak 1993), other plant pathogens (Riedel 1988; Taylor 1990), and root-nodule bacteria (Siddiqui and Mahmood 1995) have appeared in last few decades. Ways in which nematodes participate in disease complexes include serving as vectors or agents of pathogen transmission, providing portals of entry, inducing necrotic infection courts, modifying the physiology of host, breaking of host resistance to other pathogens, etc. Disease development in complex diseases may also be controlled by changes in rhizosphere microflora mediated by the nutritional quality and quantity of exudates from nematode-parasitized roots which enhance or suppress growth of other organisms. By limiting host root development, nematodes may induce drought stress in the host, a factor thought to influence development of some plant diseases. Interactions between plant-parasitic nematodes and bacteria on different plants have been summarized in Table 14.1. Plant disease complexes involving nematodes and bacteria have two types of relationships:

(a) The expression of disease symptoms occurs only when both nematodes and bacteria are present together; neither pathogen inoculated separately reproduced the disease.
(b) Each pathogen acts independently and not directly influenced by others; generally, nematodes enhance the incidence of disease.

14.3 The Role of Nematodes in Interactions with Bacterial Pathogens

Interactions of plant-parasitic nematodes with host plants exhibit most elaborate feeding sites and evolutionary most advance form of parasitism (Bird and Koltai 2000). All parasitic nematodes should be considered to be equally evolved, and differences between parasitic strategies reflect adaptations to exploit different ecological niches within the host. Root parasites (*Meloidogyne* and *Heterodera* spp.) hatch in soil as L2 larva which penetrates and migrates within a host root to establish permanent feeding sites that are characterized by extensive modifications to host cells. The nematode undergoes dramatic developmental and morphological changes and adopts a sedentary life style. Eggs are either released in masses on the surface of the root gall or encased in the body of the female forming cyst.

Table 14.1 Plant-parasitic nematodes and plant-pathogenic bacterial disease interactions

Bacterium	Nematode	Host	Role of nematode in bacterial disease	Reference
Agrobacterium				
A. rhizogenes	*Pratylenchus vulnus*	Rose	The bacteria may penetrate roots through injuries caused by *P. vulnus*	Munnecke et al. (1963)
A. tumefaciens	*Meloidogyne javanica*	Peach	Nematodes increase crown gall incidence	Nigh (1966)
A. tumefaciens	*M. hapla*	Raspberry	Crown gall infections occurred only in presence of *M. hapla* in two of the three cultivars evaluated	Griffin et al. (1968)
A. tumefaciens	*M. javanica*	Almond	Increased crown gall incidence in the presence of nematodes	Orion and Zutra (1971)
A. tumefaciens	*Pratylenchus penetrans*	Raspberry	Galled root supported high nematode population	McElroy (1977)
A. tumefaciens	*Rotylenchulus reniformis*	Grapevine	Speculated that nematode facilitate field infection by bacterium	Lele et al. (1978)
A. radiobacter var. *tumefaciens*	*M. incognita*	Cotton	Disease severity increased by presence of nematodes	Zutra and Orion (1982)
A. tumefaciens	*P. penetrans*	Raspberry	Number of galls in both susceptible and resistant cultivar increased with the increase in nematode inoculum	Vrain and Copeman (1987)
A. tumefaciens	*M. incognita*	Tomato	Bacterium stimulated development and reproduction of *M. incognita* when applied to the opposite split root	El-Sherif and Elwakil (1991)
A. vitis	*M. hapla*	Grapevine	Combined inoculation of roots with *M. hapla* and *A. vitis* resulted in an increased level of root infestation	Sule and Lehoczky (1993)
A. tumefaciens	*Meloidogyne* spp.	*Prunus* spp.	Root-knot nematode and *A. tumefaciens* galls were numerous and homogenous under high inoculum pressure	Rubio-Cabetas et al. (2001)
Clavibacter (*Corynebacterium*)				
C. michiganense subsp. *insidiosum*	*Ditylenchus dipsaci*	Alfalfa	Bacterium was transmitted to alfalfa by nematodes	Hawn (1963, 1965)
C. insidiosum	*M. hapla*	Alfalfa	Observed relationship between incidence of bacterial wilt and *M. hapla*	Hunt et al. (1971)

C. tritici	*Anguina tritici*	Wheat	Nematode is essential as vector of bacterium for yellow rot disease	Gupta and Swarup (1972)
C. michiganense	*M. incognita*	Tomato	*M. incognita* increased bacterial canker on tomato	De Moura et al. (1975)
C. rathayi	*Anguina* sp.	Ryegrass	Toxin produced in the plant tissues in response to presence of bacterium	Stynes et al. (1979)
C. fascians	*Aphelenchoides fragariae* or *A. fragariae*	Strawberry	Demonstrated that nematode and *C. fascians* are necessary to produce "cauliflower" disease of strawberry	Crosse and Pitcher (1952), Pitcher and Crosse (1958)
Clavibacter sp.	*Anguina funesta* and *A. tritici*	Wheat	*Clavibacter* sp. adhered to both *Anguina funesta* and *A. tritici*, but differences in the nature of adhesion were noted	McClure and Spiegel (1991)
Pseudomonas				
P. solanacearum	*M. incognita acrita*	Tobacco	Wounding of roots by nematodes larvae facilitate infection of bacterium	Lucas et al. (1955)
P. caryophylli	*Helicotylenchus nannus* *Meloidogyne* spp.	Carnation	Wounding the roots by nematodes facilitate entry of *Pseudomonas* into the roots	Stewart and Schindler (1956)
P. solanacearum	*H. nannus*	Tomato	Simulated by substituting mechanical injury for nematode feeding	Libman et al. (1964)
P. solanacearum	*M. hapla*			
P. marginata	*M. javanica*	Gladiolus	Nematodes increased severity of gladiolus scab	El-Goorani et al. (1974)
P. syringae	*Criconemoides xenoplax*	Plum	More extensive canker developed on trees infected with nematodes	Mojtahedi et al. (1975)
P. solanacearum	*M. incognita acrita*	Potato	Nematodes kill roots and promote emergence of pathogenic bacteria	Jatala and Martin (1977a, b)
P. solanacearum	*M. incognita*	Eggplant	More number of plants wilted (40%) when the nematode and bacterium were inoculated simultaneously	Reddy et al. (1979)
P. solanacearum	*M. incognita*	Tomato	Caused synergistic effect on wilt symptoms	Napiere and Quinio (1980)
P. marginalis *P. viridiflava* *P. corrugata*	*Helicotylenchus dihystera* *M. hapla* *P. penetrans* *D. dipsaci*	Alfalfa	Nematodes interacted with three pseudomonads to produce greater growth reductions than were obtained with single pathogen	Bookbinder et al. (1982)

(continued)

Table 14.1 (continued)

Bacterium	Nematode	Host	Role of nematode in bacterial disease	Reference
P. fluorescens	*Ditylenchus dipsaci*	Garlic	Mixed inoculation showed that *P. fluorescens* penetrated the plant tissues better when *D. dipsaci* was present	Caubel and Smason (1984)
P. solanacearum biotype 3	*M. javanica*	Eggplant	The combined effects of bacterium and nematode on eggplant were greater than independent effects of either	Sitaramaiah and Sinha (1984a, b)
Ralstonia solanacearum	*M. incognita*	Eggplant	Combined effects of two pathogens provided synergistic effect on the development of wilt symptoms	Swain et al. (1987)
P. solanacearum	*M. incognita* race 1	Tomato	Nematode infection increased the severity of the vascular wilt disease	Chindo et al. (1991)
P. mendocina	*M. incognita*	Tomato	Bacterium inhibited nematode multiplication	Siddiqui and Husain (1991)
R. solanacearum	*M. incognita* *R. reniformis*	Tomato	At high temperature, *M. incognita* greatly increased wilt severity in susceptible and resistant cultivars, but the *R. reniformis* had no such effect	Deberdt et al. (1999)
P. solanacearum	*M. incognita*	Banana	Presence of nematode population 10 days prior to bacterium accelerated the wilt disease development to the maximum	Pathak et al. (1999)
R. solanacearum	Nematodes	Potato	Disease severity increases if bacterium is found in association with root nematodes	Stansbury et al. (2001)
R. solanacearum	*Meloidogyne* spp.	Potato	Controlling *Meloidogyne* spp. did not decrease bacterial infection incidence	Charchar et al. (2007)
R. solanacearum	*M. hapla*	Alfalfa	Bacterium and nematode together result in increased incidence and disease severity	Partridge (2008)
R. solanacearum	*M. incognita*	*Coleus* and *Withania*	Simultaneous inoculation of both pathogens with *Fusarium* exhibited more early disease symptoms	Mallesh et al. (2009)
R. solanacearum	*M. incognita*	Eggplant	In the presence of bacterium, nematode activities including population build up in soil and in roots were reduced	Hussain and Bora (2009)

Depending on the particular nematode and host as well as environmental conditions, there are between one to four generations per year (Bird and Koltai 2000). Nematodes participate in disease complexes in following ways.

14.3.1 Nematodes as Vector

Phytoparasitic nematodes transmit certain bacteria which can incite diseases. Nematodes mainly carry pathogens from soil to plant or from plant organs to meristematic tissues. Kalinenko (1936) has proved that various nematodes such as *Pratylenchus pratensis, Helicotylenchus multicinctus*, and *Aphelenchus avenae* are vectors of bacteria. Nematodes extracted from the roots of *Scorzonera tausaghyz* were washed in distilled water and transferred to an agar culture; the resultant bacterial growth was identified. The same species of bacteria were found in the culture medium as were found in the roots of the plant, namely, *Erwinia carotovora*, *Xanthomonas phaseoli*, *X. necrosis*, *Pseudomonas fluorescens*, and *Bacillus mesentericus*. Fungus *Dilophospora alopecuri* is introduced into apical meristem of wheat by *Anguina tritici*. Attempts to produce the disease in the absence of the nematode have been unsuccessful (Atanasoff 1925; Leukel 1948). Lordello and Joly (1961) investigated simultaneous attack of artichoke by a nematode *Protorhabditis oxyuris* and four bacterial species and concluded that nematodes are not primary pathogens but are carriers of bacteria. Similarly, *Ditylenchus dipsaci* sometimes transmits the causal agent of bacterial wilt of alfalfa, *Corynebacterium insidiosum*, and feeding by nematode results in greater wilt severity than when the bacterium occurs alone (Hawn 1971). In general, nematodes parasitizing the roots, stems, leaves, and seeds of plants facilitate the penetration and transmission of bacteria. More often, the bacteria are first transmitted from the soil to plant tissues, where they spread throughout the infested plant; they are less often transmitted from plant to plant.

14.3.2 As Wounding Agent

Nematodes feeding cause physical damage to host plant and provide direct passage for pathogenic bacteria especially when the pathogen is not strong enough to break mechanical barriers of the host. Stewart and Schindler (1956) concluded that endoparasitic and ectoparasitic nematodes aggravated bacterial wilt by wounding the roots and allow bacteria to enter the plant. Wilt inducing bacteria depends mainly on wounds for penetration and establishment of an infection court (Goodman et al. 1967). The stylet opening of plant-parasitic tylenchid nematodes ranges from 0.2 to 1 μm in diameter and restricts the passage of pathogenic bacteria into intact plant cell. Number of bacterial pathogens normally inhabit soil and may become pathogenic on roots. At low nematode population levels, crown gall

symptoms were no more severe than those occurring in plants inoculated with bacterium alone after wounding (Nigh 1966). Similarly, initial damage by *Rotylenchulus reniformis* facilitates entry and establishment of *A. tumefaciens* and disease development. Bookbinder et al. (1982) reported that *M. hapla*, *Pratylenchus penetrans*, and *Helicotylenchus dihystera* produced wounds in alfalfa roots which were invaded by *Pseudomonas* spp. Numbers of studies explain that mechanical root injury or root wounding of plant cell by nematodes is important factor for introduction of bacterial pathogen in host (Libman et al. 1964; Johnson 1966; Johnson and Powell 1969; Jatala and Martin 1977a, b; Sitaramaiah and Sinha 1984a, b). Pitcher (1965) noted that wounds created by nematodes apparently favor bacteria more than fungi because bacteria are less adapted for penetrating the host's epidermis. Disease symptoms similar to those which occur in nematode-bacterium wilt interactions were simulated by substituting mechanical injury for nematode feeding (Libman et al. 1964; Lucas et al. 1955). Predisposing effect of nematodes has been attributed to the creation of wounds which leak nutrients and allow soil bacteria to multiply both in the lesions and in the rhizosphere (Kurppa and Vrain 1985). Lucas et al. (1955) demonstrated that wounding of roots by penetration of *M. incognita* larvae facilitates infection of tobacco roots by *Pseudomonas solanacearum*. There are strong indications that nematodes, especially root-knot nematodes, may induce physiological and/or biochemical changes in their hosts which enhance the development of pathogenic bacteria and/or predispose their host to bacterial pathogens. Griffin et al. (1968) demonstrated that *M. hapla* was necessary for establishment of *A. tumefaciens* in raspberry tissue. However, these authors referred to other works that wounding of roots by other agents permits the infection of the bacteria. Nematodes improve bacterial growth (Weischer 1968) but mostly measured by plant symptoms and not by qualitative analyses of bacteria.

14.3.3 Nematode Infection Causes Necrosis

Wounding of a host, by some species of nematodes, results in decay of root tissues, which may favor ingress of certain additional pathogens (Baldwin 1977). These pathogens are often unspecialized and may be facultative parasites, i.e., they generally survive on dead plant tissues, but are also capable of invading living tissue. Generally, lesion nematodes produce characteristic necrotic lesions (darkened areas of dead tissue) on the surface and throughout the cortex of infected roots. The lesions turn from reddish-brown to black and are initially spotty along the root surface. As the nematodes continue to migrate and feed within the roots, the lesions can coalesce to become large necrotic areas of tissue that may eventually girdle the root. Tissue distal to the lesion is frequently sloughed off. Severe damage from high populations of lesion nematodes can result in a stunted and necrotic plant root system. The extent of lesion formation can be accelerated during concomitant root invasion by other soil-borne plant pathogens, and sometimes, these interactions can develop into synergistic disease complexes. The wounds inflicted on plant roots and

other belowground plant parts by lesion nematodes can serve as infection courts for pathogenic soil microbes (Davis and MacGuidwin 2000). This appears to be particularly true in disease complexes that involve lesion nematodes and wilt inducing bacteria.

14.3.4 Nematodes Act as Modifier of Substrate

All parasitic nematodes have extensive stylet that is connected to a well-developed pharynx containing three or five gland cells. Marked changes in the shape and volume of the pharyngeal glands were observed that appeared to correlate with key events in establishment of the parasitic interaction. In root-knot and cyst nematodes, the subventral glands seem to be more active before host penetration, with the reduction of secretary activity coordinated with onset of parasitism (Endo 1987; Endo and Wegin 1988) at which time activity of the dorsal gland increases (Bird 1983). Similarly, phytohormones play a role in feeding site formation and, indeed, may be the key factors in modulating the host-parasite interaction. Direct biochemical methods have shown that root-knot nematode-induced galls have elevated levels of auxin and its precursors (Balasubrama and Rangaswami 1962; Viglierchio and Yu 1968). In addition, cytokinin levels were found to be increased in nematode-infected roots (Bird and Loveys 1980). Root-knot nematodes have been shown to produce biologically active cytokinin (Bird and Loveys 1980).

Powell and Nusbaum (1960) first demonstrated the modification in the substrate due to nematode infestation provide an advantage to pathogen. Creation of an infection court is one way in which nematodes modify a host to enhance infection by additional pathogens. However, there is increasing evidence that nematodes modify host substrates in more subtle ways. Changes in biochemistry of the host are probably the most important factors favoring disease complexes involving nematodes (Slack 1963). Nematodes may induce production of host metabolites which are favorable to other pathogens, or they may destroy host metabolites that provide resistance to potential pathogens (Pitcher 1965). Johnson and Powell (1969) reported that root-knot nematodes act as modifiers of infested tissues so that infected tissue and surrounding cells become more suitable for bacterial colonization. The plants inoculated with the nematodes 3 to 4 weeks prior to bacterial inoculation develop bacterial wilt symptoms to a greater extent than plants inoculated with nematodes and bacteria simultaneously. *Meloidogyne* sp. induces gross physiological changes in a host. Thus, infection with root-knot nematodes prior to inoculation with bacterial pathogen is more likely to result in a synergistic disease complex, than when inoculations are simultaneous. The nematodes substantially alter host physiology so that a subsequently introduced pathogen is favored (Powell 1971; Yang et al. 1976).

14.3.5 Nematodes as Breakers of Disease Resistance

It is observed that a resistant cultivar to bacterial pathogen becomes susceptible in the presence of plant-parasitic nematodes as nematodes bring about physiological changes favoring the bacterial pathogen. Using tobacco variety Dixie Bright 101 which is resistant to bacterial wilt, Lucas et al. (1954) obtained similar results in experiments on infestation by gall nematodes *M. incognita acrita* and infection with bacteria *P. solanacearum*. Three variants of these causative agents were added to experimental pots of cultivated tobacco plants: a suspension of bacteria, soil infested with gall nematodes, and lastly both components. Within 21 days, 10%, 0%, and 100% of the tobacco plants were infested with bacterial wilt, respectively. Alfalfa cultivars with high resistance to wilt by *Corynebacterium insidiosum* may be diseased by this bacterium when *Ditylenchus dipsaci* is present (Hawn and Hanna 1967). Field resistance in potato to *P. solanacearum* was broken down when plants were infected with *M. incognita acrita* (Jatala and Martins 1977a, b). Similarly, Reddy et al. (1979) observed that when eggplant cultivar "Pusa purple cluster" highly resistant to *P. solanacearum* was inoculated together with *M. incognita* a greater number of plants wilted. Nematodes may alter hosts to such an extent that such plants may become susceptible to organisms to which they are otherwise resistant.

14.3.6 Nematode Infection Changes Rhizosphere Microflora

Nematodes seem to favor all stages of bacterial infection and development by modifying the composition of the root leachates. They can promote the growth of microorganisms in the rhizosphere. Moreover, these modifications of the rhizospheric environment may limit the development of organisms antagonistic to the pathogenic bacteria. Their feeding sites and the cells they modify, especially the giant cells induced by root-knot nematodes, may serve as a favorable substrate which helps the bacteria to establish within the plant and promote their development. Nematode-induced or nematode-produced factors appear to be translocated from the nematode feeding sites to other parts of their host, especially in the above ground parts. These factors seem to modify the resistance of the host to the bacteria and/or directly stimulate bacterial growth. The balance between the rhizosphere microflora and plant pathogens and soil microflora and plant pathogens is important in host-pathogenic relationship. The biochemical qualities of root exudates and the presence of antagonistic microorganisms play an important role in the proliferation and survival of root infecting pathogens in soil either through soil fungistasis, inhibition, or antibiosis of pathogens in the rhizosphere. Disease development in complex diseases may be controlled by changes in rhizosphere flora mediated by the nutritional quality and quantity of exudates from nematode-parasitized roots which enhance or suppress growth of organisms antagonistic to plant pathogens

(Riedel 1988). Such exudates may also overcome fungistasis. By these mechanisms, nematodes also exert influence on their own reproduction and cohabitation in host plants.

14.4 Effect of Bacterial Pathogens on Plant-Parasitic Nematode

Relatively few interactions involving nematodes and bacteria have been investigated as because bacterial pathogens are less in number as compared to fungi and viruses. *Agrobacterium*, *Clavibacter* (*Corynebacterium*), *Ralstonia*, *Pseudomonas*, and *Xanthomonas* are the most common genera of bacteria commonly associated with nematodes in disease complexes. Effect of bacteria on disease complexes may be of following types.

14.4.1 Toxin Production by Bacteria

Limited information is available on the production of toxins by bacteria. The association between *Anguina funesta* (*Anguina agrostis*) and *Clavibacter* sp. (*Corynebacterium rathayi*) infesting *Lolium rigidum* produces toxin. Galls produced by *A. funesta* in annual ryegrass become toxic to nematodes when colonized by the bacterium (Stynes et al. 1979). Pitcher (1963) in his studies on the interaction of *Aphelenchoides fragariae* and *Corynebacterium fascians* found that bacteria at first increase but then decrease the rate of population growth of nematodes. The mechanism of this interaction is unknown, and the possible production by toxins by bacteria had adverse effect on nematodes. Infection of tobacco roots by *P. solanacearum* caused decrease of *M. incognita* in roots (Lucas et al. 1955; Johnson and Powell 1969). The contents of giant cells degenerated following bacterial invasion, leaving virtually empty cells resulting into the death of root-knot nematodes. The strong antagonistic effect of *A. tumefaciens* on the reproduction of *P. penetrans* was observed on raspberry (Vrain and Copeman 1987). Similar result has been observed in another interaction study (Pitcher and Crosse 1958). Bird et al. (1980) concluded that toxin production is associated with an interaction between nematode-infected plant cells and the bacterium.

14.4.2 Inhibits Nematode Development

Pitcher (1963) suggested that the bacteria modify host tissues which do not favor nematode multiplication. Lucas et al. (1955) reported that infection of tobacco roots by the *P. solanacearum* caused a decrease of *M. incognita* in roots. The adverse effects on nematode are expected as these pathogens share and compete for same

host substrate. The unfavorable effect of bacteria pathogen on nematode may also be due to the destruction of feeding sites, impaired nutrition, and harmful byproducts produced by bacterial colonization. Similarly, Swain et al. (1987) reported inhibitory effect of *R. solanacearum* on *M. incognita*. Inoculation of *M. incognita* alone produces more galls and egg masses compared to its association with *R. solanacearum* (Hussain and Bora 2009). It may be due to the reason that establishment of the bacteria induces certain changes in root system which are not favorable for nematodes. Bhagawati et al. (1996) and Hazarika (2003) reported significant poor galls and egg masses in jute when *M. incognita* was associated with *R. solanacearum*.

14.4.3 Nematodes and Bacteria Together May Result in a Different Disease

Symptoms of disease of the host plant usually appear much faster and are more pronounced when two pathogens are present, than when just one infested the host. The host reaction may or may not be synergistic. This is largely influenced by environmental factors; the effect of these factors on nematode injury to plants was reviewed by Smart (1964). Sometimes, the presence of both the nematode and the other pathogen is necessary for production of certain types of symptoms, as it has been shown by Pitcher and Crosse (1958) and Blinov (1969) in their work on the association of *Aphelenchoides fragariae* and *Corynebacterium fascians* in "cauliflower" disease of strawberries. The expression of "cauliflower" symptoms depends also upon the cultivar studied. Interaction of *P. penetrans* and *A. tumefaciens* on raspberry might be causing the sudden decline, which is not a symptom characteristic of either pathogen alone (McElroy 1977). The yellow ear rot or tundu disease requires both the nematodes *A. tritici* and *Clavibacter tritici* for the expression of complex disease. Surface-sterilized nematode larvae alone caused only ear-cockle disease; the bacterium alone was not capable of causing disease (Gupta and Swarup 1972).

14.5 No Effect of Nematodes on Disease Complexes

Despite rapid advances on certain aspects of plant-pathogenic bacteria, many economically important pathosystems are largely unexplored, and biologically relevant life stages of even familiar systems remain poorly understood. We know remarkably little about interactions between microbes in a plant, and the effects of quantitative virulence factors. Not all species of nematodes assist in the development of bacterial wilt; in few cases, no effect of nematodes in diseases complexes was observed. Experiments conducted by Lucas and Krusberg (1956) stated the

ectoparasitic root nematode *Tylenchorhynchus claytoni* exerted no influence on the appearance of bacterial wilt in tobacco variety Dixie Bright 101. Neither did the ectoparasitic nematode *Xiphinema diversicaudatum* on the severity of bacterial wilt in carnation caused by *Pseudomonas caryophylli* (Stewart and Schindler 1956). Generally, plant age, cultivar (resistant or susceptible), nematode inoculums levels, type of nematode parasitism (ecto or endo), environmental conditions, and their interaction with the type of microorganism have significant effect in determining the role of nematode in disease complexes.

14.6 Conclusion

In nature, plants are rarely exposed to the influence of only a single pathogen, particularly in soil environment. Roots are constantly exposed to a wide range of microorganisms which are likely to influence one another because they occupy the same habitat. It is reasonable to expect the infection by one pathogen may alter the host response to subsequent infection by another. It is apparent that plant-parasitic nematodes are involved in disease complexes and play a major role in synergistic interactions. Disease complexes are major economic hazards posed by nematodes, and interaction studies involving nematodes and bacteria should receive more attention of plant pathologists. The understanding of nematode-induced physiological and biochemical changes induce in their hosts that are responsible for the predisposition of the host plants to bacterial pathogens could be the necessary bases to develop control strategies against these parasites. More multidisciplinary research between biochemists, geneticists, and pathologists is necessary to understand the interrelationships between nematodes, bacteria, and plants. The improved understanding of relationship among host plant, nematodes, and bacteria will enhance our ability to control these plant diseases and the damage caused by them.

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Chapter 15
PGPR-Mediated Systemic Resistance for Sustainable Agriculture

B. Meena

15.1 Introduction

Plant-growth-promoting rhizobacteria (PGPR) are known to control a wide range of phytopathogens like fungi, bacteria, viruses, etc., and they are known to control these pathogens by biocontrol mechanism which may be by competition or antagonism; however, the most studied phenomenon is the induction of systemic resistance by these bacteria in the host plant, thereby containing the invading pathogens (Van Loon et al. 1998). Several studies have indicated that PGPR may stimulate the production of biochemical compounds associated with host defense; massive accumulation of phytoalexins and phenolic compounds; increase in the activities of PR proteins, defense enzymes, and transcripts; and enhanced lignification.

The induction of SAR using various ISR inducers has been of recent interest with quite reasonable success. Induced resistance against Fusarium wilt of carnation caused by *F. oxysporum* f.sp. *dianthi* was found by prior application of *Pseudomonas* spp. strain WCS417r (Van Peer et al. 1991). Zhou and Paulitz (1994) found systemic induced resistance against the root pathogen (*Pythium aphanidermatum*) in cucumber by application of *Pseudomonas* spp. to a root spatially separated from the pathogen-inoculated root. Colonization of tobacco roots by *P. fluorescens* strain CHAO resulted in reduction of tobacco leaf necrosis virus disease, showing its ability to induce systemic resistance (Maurhofer et al. 1994a). Fusarium wilt of radish (*F. oxysporum* f. sp. *radicis-lycopersici*) was controlled by *P. fluorescens* strain WCS374 by inducing systemic resistance (Raaijmakers et al. 1995). Raupach et al. (1996) opined that treatment of cucumber seeds with PGPR strain 89B-72 (*P. fluorescens*) resulted in induction of systemic resistance against fungal and bacterial diseases.

B. Meena (✉)
Sugarcane Research Station, Tamil Nadu Agricultural University, Sirugamani, Trichy 639115, Tamil Nadu, India
e-mail: meepath@rediffmail.com

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Induced resistance against Fusarium wilt of carnation caused by *Fusarium oxysporum* f. sp. *dianthi* was found by prior application of *Pseudomonas* spp. strain WCS417r (Van Peer et al. 1991). Verhagen et al. (2009) studied the ability of *P. fluorescens* CHAO to induce resistance in grapevine against *Botrytis cinerea* and highlighted the importance of salicylic acid, pyochelin, and pyoverdin in priming phytoalexin responses and induced resistance. Vleesschauwer et al. (2008) demonstrated the ability of *P. fluorescens* WCS374r to trigger ISR in rice (*Oryza sativa*) against the leaf blast pathogen, *Magnaporthe oryzae*, and found that WCS374r-induced resistance is regulated by an SA-independent but jasmonic acid/ethylene-modulated signal transduction pathway.

15.2 PGPR as Plant Growth Promoters

The bacteria that provide some benefit to plants are of two general types: those form a symbiotic relationship with them and those that are free-living in the soil but are often found near, on, or even within the roots of plants. Free-living soil bacteria are usually referred to as plant growth-promoting rhizobacteria or PGPR. The beneficial effects of these bacteria have been variously attributed to their ability to produce various compounds including phytohormones, organic acids, and siderophores to fix atmospheric nitrogen, to solubilize soil phosphate, to produce antibiotics that suppress deleterious rhizobacteria, or to show some other unidentified mechanisms.

Several strains of *P. fluorescens* increase the plant growth of rice and cotton by 27% and 40%, respectively, when the bacterium was applied to seeds (Lin et al. 1992). Schippers et al. (1987) documented an increase in fresh weight of root and shoot of tomato, cucumber, lettuce, and potato as a result of bacterization with *Pseudomonas* strains. The growth promotion of winter wheat by treating seeds with several strains of *Pseudomonas* spp. under greenhouse and field condition was reported by De Freitas and Germida (1992). Dubeikovsky et al. (1993) suggested that indole acetic acid (IAA) production by *P. fluorescens* might influence the development of black currant cuttings. The strongest effect was observed as changes of root system weight and morphology. The stimulating IAA-mediated effect of bacterial inoculation on the development of the roots of the cuttings was observed.

Significant plant growth promotion with increased runner length and increased leaf number per plant in cucumber by seed and soil application of PGPR was reported (Wei et al. 1996). Williams and Asher (1996) achieved improvement in seedling emergence in proportion of healthy seedling in sugar beet by *Pseudomonas* sp. when compared to seedlings from untreated seeds. Seed coating with pseudomonad isolates like BHU1, A19, and C185 resulted in significantly greater root length, root and shoot biomass, pod yield, and nodule number of groundnut compared with the control (Pal et al. 1999). This may be attributed to various factors such as ACC (1-aminocyclopropane-1-carboxylate, sigma) deaminase

activity, siderophore production, and increase in root length. These bacteria might have enhanced the uptake of nutrients resulting in healthier and better root system and resulting in improved plant growth.

15.3 PGPR as Biocontrol Agents

Fluorescent pseudomonads commonly isolated from rhizosphere have been shown to protect plants from fungal infection (Kloepper et al. 1986). *P. fluorescens* and *P. putida* suppress several major plant pathogens. The suppression depends on the ability of the bacteria to colonize the roots and production of an antibiotic phenazine-1-carboxylic acid (PCA), a siderophore called pyoverdine and an antifungal factor (AFF). Antagonistic activity of *P. fluorescens* has been reported against *Xanthomonas citri* (Unnamalai and Gnanamanickam 1984) and *Sarocladium oryzae* (Sakthivel and Gnanamanickam 1986). Along with the disease control, an increase in yield in the bacterized plots was also noted.

15.3.1 Soilborne Pathogen Suppression by Fluorescent Pseudomonads

Soilborne plant pathogens cause significant damage to crop production worldwide. Disease symptoms caused by these plant pathogens include damping-off, root rots, foot rots, and wilting. For several soilborne plant pathogens including *Gaeumannomyces graminis* var. *tritici, Fusarium oxysporum*, *F. solani*, *Phytophthora cinnamomi, Rhizoctonia solani,* and *Sclerotium cepivorum*, disease-suppressive soils have been described (Cook and Baker 1983). In these soils, expression of disease is limited despite the presence of a virulent pathogen, a susceptible crop and environmental conditions favorable for disease development. In several of these suppressive soils, microbial populations that are antagonistic toward the pathogen play a key role in disease suppression. Selected strains from many genera of bacteria isolated from these suppressive soils have the potential to reduce plant diseases when applied to the plant root environment (Weller et al. 2002). In rice, seed treatment followed by root dipping and foliar spray with *P. fluorescens* showed higher induction of ISR against sheath blight pathogen, *R. solani* (Radjacommare et al. 2004).

15.3.1.1 Foliar Pathogen Suppression by Fluorescent Pseudomonads

In cucumber, PGPR strains showed a higher level of induced resistance against foliar diseases, viz., angular leaf spot caused by *P. syringae* pv. *lachrymans* and

Table 15.1 Systemic protection induced by foliar application of *P. fluorescens* Pf1 formulation on late leaf spot of groundnut

Position of groundnut leaves sprayed with *P. fluorescens*	Disease intensity (grade) in leaves inoculated with *Cercosporidium personatum*		
	Lower leaf	Middle leaf	Upper leaf
Lower leaf			
Sprayed	4.1[a]	3.1[a]	2.2[a]
Unsprayed	7.2[c]	5.6[b]	3.8[b]
Middle leaf			
Sprayed	4.8[b]	2.9[a]	2.4[a]
Unsprayed	7.0[c]	5.4[b]	3.5[b]
Upper leaf			
Sprayed	4.6[b]	2.8[a]	2.2[a]
Unsprayed	7.1[c]	5.2[b]	3.6[b]

Data represent mean of five replications
Data followed by the same letter in a column are not significantly different ($p = 0.05$) by DMRT

anthracnose caused by *Colletotrichum orbiculare* (Zehnder et al. 2000). In groundnut, seed treatment and foliar spray with *P. fluorescens* Pf1 induced systemic resistance (ISR) against late leaf spot by inducing various defense mechanisms in the host plant (Meena et al. 2000) (Table 15.1). Similarly, Viswanathan and Samiyappan (2001) reported PGPR-mediated ISR against red rot disease in sugarcane. Application of PGPR strains showed enhanced resistance to bacterial speck and spot of tomato (Kavitha and Umesha 2007).

Meena et al. (1999) observed the induction of chitinase and glucanase activities in rice leaves in response to application of *P. fluorescens*. Fusarium wilt of radish caused by *F. oxysporum* f.sp. *radicis-lycopersici* was controlled by *P. fluorescens* strain WCS374 by inducing systemic resistance (Raaijmakers et al. 1995). The influence of plant growth promotion and ISR resulted in enhancing the disease resistance in tea plants against blister disease by PGPR bioformulations (Saravanakumar et al. 2007).

Insect and Nematode Pest Control by Fluorescent Pseudomonads

Fluorescent pseudomonads are shown to be effective against certain insect and nematode pests (Ramamoorthy et al. 2001). Tian et al. (2007) reported that fluorescent pseudomonads act synergistically on nematodes through the direct suppression of nematodes, promoting plant growth and facilitating the rhizosphere colonization. Pechy-Tarr et al. (2008) found that when *P. fluorescens* CHAO or Pf5 injected into the hemocoel, even low doses killed the larvae of the tobacco hornworm *Manduca sexta*. Significant suppression of nematode multiplication by PGPR was due to its capability of altering root exudates, which could alter nematode behavior and suppress nematode population in root system (Kloepper et al. 1992).

15.4 Mechanisms of Disease Control

Beneficial fluorescent pseudomonads can promote plant growth and induce disease suppressiveness by several mechanisms. It includes (1) antibiotic production, (2) production of bacteriocins, (3) production of siderophores, (4) production of hydrolytic enzymes, (5) production of other metabolites, (6) phytoalexins production, (7) interference in quorum sensing, (8) reduction in ethylene production, and (9) induction of systemic resistance. PGPR suppress the pathogen directly by antagonism through the production of various secondary metabolites or indirectly by inducing plant-mediated defense reactions (van Loon et al. 1998). Multiple interactions occur between the bacteria and between bacteria and other microorganisms involving competition, antibiosis, parasitism, and predation. Various interactions also occur between bacteria and plant roots that can be beneficial, neutral, or harmful to the plant.

15.4.1 Siderophore Production

Soil pseudomonads generally produce fluorescent, yellow-green water-soluble siderophores with both hydroxamate and phenolate groups. These siderophores have been classified as either pyoverdins or pseuobactins (Neilands 1981). The production of these siderophores has been linked to their disease suppression ability (Loper and Buyer 1991). Siderophores are unique compounds meant for iron uptake. Siderophores are produced by virtually all bacteria and fungi under iron-limiting conditions. They have a very high affinity for ferric iron and are secreted during growth under low iron conditions. They form a complex with available iron making it unavailable to other organisms. Geels and Schippers (1983) have reported that siderophore producing *Pseudomonas* strains are more effective in protecting potato crops from disease than some antibiotic producing strains. Production by certain fluorescent pseudomonads of extracellular, water-soluble, yellow and green pigments in KB medium that fluoresce in UV light is known for a hundred years. All such pigment (or siderophore)-producing pseudomonads *P. aeruginosa*, *P. fluorescens*, and *P. putida* belong to one intrageneric homology group. Pseudobactin, the first siderophore, was isolated, purified, and characterized by X-ray crystallography from *P. fluorescens* strain B10. Some other identified Pseudomonas siderophores are Pseudobactin A214, produced by a deleterious *Pseudomonas* strain A214, and Pseudobactin 358, produced by *P. putida* strain WCS 358.

15.4.1.1 Antibiotic Production

Antibiotics encompass a chemically heterogeneous group of organic, low-molecular-weight compounds by microorganisms at low concentrations; antibiotics are

deleterious to the growth or metabolic activities of other microorganisms (Thomashow et al. 1997). Antibiotic production by some fluorescent *Pseudomonas* spp. is now recognized as an important factor in disease suppression ability of these strains. Compounds such as phenazines (Thomashow and Weller 1988), pyoluteorin (Howell and Stipanovic 1979), tropolone (Lindenberg 1981), pyocyanin (Dahiya et al. 1988), 2,4-diacetylphloroglucinol (Keel et al. 1990), and pyrrolnitrin (Howell and Stipanovic 1979) have been isolated from soil in fluorescent Pseudomonas.

Antibiotic production by fluorescent pseudomonads is now recognized as an important feature in biological control of plant diseases, and a number of researchers are now interested in investigating the genetics of these compounds. Some pseudomonads have been recognized as antagonists of plant fungal pathogens and antibiotic producers. A single strain of *Pseudomonas* produces several different antibiotics. In particular, the pseudomonad product pyrrolnitrin serves as a lead compound for new fungicides fenpiclonil and fludioxonil. Since PGPR being a potential candidate in disease management through multiple modes of action, it becomes highly imperative to know about the role of antibiotics in the management of plant pathogens.

The compound 2,4-diacetylphloroglucinol (DAPG) is a phenolic molecule produced by certain plant-associated fluorescent pseudomonads of worldwide origin (Thomashow et al. 1997). It has antifungal, antibacterial, antihelminthic, and phytotoxic properties. DAPG is a polyketide synthesized by condensation of three molecules of acetyl coenzyme A with one molecule of malonyl coenzyme A to produce the precursor monoacetylphloroglucinol, which is subsequently transacetylated to generate DAPG by a biosynthetic route utilizing chalcone synthase (CHS)-type enzyme (Shanahan et al. 1992). The mechanisms of action of DAPG are lysis of the inner mitochondrial membrane, vacuolization of the nuclear envelop, inhibition of hyphal elongation, golgi vesicle trafficking, and localized alteration of plasma membrane. DAPG produced by several strains of *P. fluorescens* not only has activity against a wide range of plant pathogenic fungi but also has antibacterial, antihelminthic, and phytotoxic properties (Keel et al. 1992).

Phenazine comprises a large family of heterocyclic nitrogen containing brightly colored pigments with broad-spectrum antibiotic activity. Several strains of fluorescent pseudomonad produce antifungal metabolites, namely, phenazines (Thomashow et al. 1997). 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. Pyrrolnitrin (3-chloro-4-(2′-nitro-3′chlorophenyl) pyrrole) is a broad-spectrum antifungal metabolite produced by many fluorescent strains of the genus *Pseudomonas*. The biological control agent, *P. fluorescens* BL915, contains four gene clusters involved in the biosynthesis of antifungal molecule pyrrolnitrin from the precursor tryptophan (Hamill et al. 1970). Pyoluteorin is an aromatic polyketide antibiotic consisting of a resorcinol ring, which is derived through polyketide biosynthesis. It is produced by several *Pseudomonas* sp., including strains that suppress plant diseases caused by phytopathogenic fungi (Maurhofer et al. 1994b). It mainly inhibits the oomycetous fungi, including *Pythium ultimum* against which it is strongly active. When applied to

seeds, pyoluteorin-producing pseudomonads decrease the severity of *Pythium* damping-off.

HCN Production

Hydrocyanic acid (HCN) is produced by many rhizobacteria and is postulated to play a role in biological control of plant pathogens. Production of hydrogen cyanide (HCN) by certain strains of fluorescent pseudomonads has also been involved in the suppression of soilborne pathogens. Production of HCN by *P. fluorescens* strain CHAO was implicated in suppression of black root rot of tobacco (Stutz et al. 1986) and take-all of wheat (Defago et al. 1990). HCN production by *P. fluorescens* inhibited the mycelial growth of *Pythium* in vitro (Weststeijn 1990).

Haas et al. (1991) have found that HCN production by some fluorescent Pseudomonas may in fact be detrimental to plant growth since it has been implicated in the reduction of potato yields. In contrast with the detrimental effect on plant growth, Voisard et al. (1989) presented evidence that HCN is beneficial to biological control. Pseudomonad-PGPR increase potato yields, according to one theory, by reducing HCN production by deleterious rhizobacteria, through siderophore-mediated competition for Fe. (III), which is required for HCN production. There are suggestions that the biocontrol of pathogens through HCN production by certain fluorescent Pseudomonas may be due to the induction of plant resistance against certain pathogens. The role of HCN production by fluorescent Pseudomonas in the control of root pathogen is not yet clear. It is possible that HCN production in the rhizosphere has different effects on different plant types.

Competition in Soil and Root Colonization

The crucial factor in the success of biological control by fluorescent pseudomonads is their ability to colonize the rhizosphere and their persistence throughout the growing season. It is well known that different fluorescent pseudomonads have different abilities to colonize a particular root niche. Unless an organism can compete favorably with other organisms and effectively scavenge and utilize favorable nutrients, it will not constitute a significant proportion of the rhizosphere population. Paulitz (1990) has given a depth account of competition for nutrients and infection sites on root surfaces. The competitive exclusion of deleterious organisms by fluorescent pseudomonads at the root may also be a significant suppressive trait of these biocontrol strains. This mechanism has been suggested to play a role in the biocontrol by strains of fluorescent Pseudomonas against species of *Fusarium* and *Pythium* (Elad and Chet 1987). The competitive exclusion of deleterious rhizosphere organisms is directly linked to their ability to successfully colonize a root surface. All disease-suppressive mechanisms exhibited by fluorescent pseudomonads are essentially of no real value unless these bacteria can successfully establish themselves at the root environment.

Competition between pathogenic and saprophytic microorganisms for organic materials released from the roots reduces growth and/or pathogenic activity of the pathogens. The involvement of competition for nutrients in biological control by fluorescent *Pseudomonas* sp. was suggested in several studies. Nutrient competition varies in different rhizospheres, depending on the available sources of carbon, nitrogen, sulfur, phosphate, and micronutrients. It is not yet very clear whether a superior ability utilizing a particular type of nutrient or nutrients provides advantage to fluorescent pseudomonads. The competitive exclusion of deleterious organisms by fluorescent pseudomonads at the root may also be a significant suppressive trait of these biocontrol strains (Elad and Chet 1987).

With the advent of genetic engineering, many more traits not present in fluorescent pseudomonads can be introduced into desired strains to enable them to combat pathogens more effectively. Chitinase degrades chitin, which is an inherent part of many plant deleterious fungi and insects. The introduction of this property into a desirable fluorescent pseudomonad could impart a biocontrol property into the strain. A better understanding of root colonization and disease suppression mechanisms is needed before suitable strains can be directly selected or engineered by genetic means into commercially viable products. A major problem to date has been the inconsistency of the performance of strains as disease-suppressive agents. The possible reason for this is that organisms respond to different stimuli in their environment, which leads to an alteration in metabolic activities. It may be necessary to genetically modify these metabolic traits to improve the consistency of the performance of strains under different conditions. Information on these aspects is now emerging, and research along these lines will increase the impact of fluorescent pseudomonads on the biocontrol of root diseases in the commercial world.

15.5 Induction of Systemic Resistance (ISR)

Induced resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated (van Loon et al. 1998). ISR has been known for many years, but only recently, it has been correlated with the systemic activation of several plant defense responses via a signal transduction pathway initiated locally at the initial site of pathogen attack (Audenaert et al. 2002). Classical inducers of ISR include pathogens, heat-killed or attenuated pathogens, synthetic chemicals, metabolic products of hosts or infectious agents, and incompatible pathogens. Bacterial strains differ in their ability to induce resistance in different plant species, and plants show variation in the expression of ISR upon induction by specific bacterial strains (van Loon et al. 1998).

Induced resistance results from perception of rhizobacteria by plant roots which give rise to an increased level of resistance that is expressed upon subsequent infection by a pathogen. Localized induction of resistance at the site where eliciting bacteria are present on the roots is difficult to demonstrate because a challenging pathogen will also be subject to bacterial antagonism at this same location.

In contrast, no direct interaction between inducing bacteria and a challenging pathogen is possible when each organism is present at spatially separated sites and no contact between the two is established. Enhanced resistance due to ISR by PGPR is achieved by induction of defense compounds of phenylpropanoid pathway and PR proteins (pathogenesis-related proteins).

Systemic acquired resistance (SAR) is typically a response to a localized infection or an attenuated pathogen, which is manifested in subsequent resistance to a broad range of other pathogens. Some biocontrol agents include a sustained change in the plant, increasing its tolerance to infection by a pathogen, a phenomenon known as ISR. In some cases, it is clear that induced resistance by biocontrol agents involves the same suite of genes and gene products involved in the well-documented plant response known as SAR, but this is not always the case. Induced resistance brought about by prior inoculation of the host by a pathogen, avirulent or incompatible forms of a pathogen, or heat-killed pathogens has been attributed to induced physiological response of the host plant against subsequent inoculation by the virulent pathogens.

ISR in plant has been demonstrated in over 25 crops, including cereals, legumes, solanaceous plants, etc., against a wide spectrum of pathogens. Mutants of *P. fluorescens* CHAO that do not produce the siderophore pyoverdine do not induce SAR, suggesting a novel role for bacterial metabolites in disease suppression. It has been shown that the biocontrol agent *P. fluorescens* strain CHAO that does not produce the siderophore pyoverdine does not induce SAR, suggesting a novel role for bacterial metabolites in disease suppression (Maurhofer et al. 1994a).

ISR triggered in some rhizobacterial strains depends on salicylic acid (SA) signaling in the plants. Induced resistance by *P. aeruginosa* 7NSK2 was found to be iron-regulated and involved three siderophores, pyoverdine, pyochelin, and salicylic acid. Salicylic acid is also a precursor in the production of SA-containing siderophores, such as pseudomonine in *P. fluorescens* WCS374 (Audenaert et al. 2002). Additional support for induction of resistance by bacterially produced SA comes from the study in which the SA biosynthetic genes of *P. aeruginosa* PAO1 were expressed in the non-SA-producing *P. fluorescens* strain P3 and improved ISR in tobacco against tobacco necrosis virus (TNV) (Maurhofer et al. 1998). Another line of evidence for induced resistance, which may or may not involve SAR, is that some biocontrol agents suppress disease when they are applied far from the site of infection by the pathogen and they cannot be found at the infection site (Wei et al. 1991). Furthermore, in suppression of Fusarium wilt by *P. fluorescens*, preparations of lipopolysaccharides from the bacterial cell surface induce resistance as effectively as the living bacteria, demonstrating that biocontrol is not necessarily due to transport of the bacteria or an antibiotic through the plant (Leeman et al. 1995). The transcriptome of rhizobacteria-ISR in *Arabidopsis* revealed that root colonization by *P. fluorescens* WCS417r did not lead to transcriptional changes in the leaves, whereas in the roots, there is a large set of genes that are differentially transcribed (Verhagen et al. 2004). Whether or not biocontrol agents suppress disease by inducing resistance, it is essential that SAR and biocontrol strategies be compatible

because future agricultural practices are likely to require the integration of multiple pest control strategies.

15.5.1 Induction of Compounds of Phenyl Propanoid Pathway by Fluorescent Pseudomonads

Several studies have indicated that PGPR may stimulate the production of biochemical compounds associated with host defense; massive accumulation of phytoalexins and phenolic compounds; increase in the activities of PR proteins, defense enzymes, and transcripts; and enhanced lignification. Peroxidase (PO) catalyzes the last step in the biosynthesis of lignin and other oxidative phenols. PO is associated with disease resistance in plants. In groundnut, increased activity of PO was observed due to application of *P. fluorescens*, and PO isoforms were expressed at higher levels (Meena et al. 2000). Phenylalanine ammonia lyase (PAL) is the first enzyme involved in phenylpropanoid pathway and plays a key role in the biosynthesis of phenolics and phytoalexins. An increase in the level of mRNAs encoding for PAL was recorded in the early stage of interaction between bean roots and various rhizobacteria (Zdor and Anderson 1992). When cucumber roots were treated with *P. corrugata* 13 or *P. aureofaciens* 63-28, PAL activity was stimulated in root tissues in 2 days, and this activated accumulation lasted for 16 days after bacterization (Chen et al. 2000).

Induction of higher PPO activity was noticed in tomato and hot pepper pretreated with fluorescent pseudomonads strain against *Pythium* diseases (Ramamoorthy et al. 2002). Phenolics are fungitoxic in nature and increase the physical and mechanical strength of the host cell wall. Treatment of pea seeds with *P. fluorescens* strain 63-28 resulted in formation of structural barriers, viz., cell wall apposition (papillae) and deposition of newly formed callose and accumulation of phenolic compounds at the site of penetration of invading hyphae of *P. ultimum* and *F. oxysporum* f.sp. *pisi* (Benhamou et al. 1996). M'Piga et al. (1997) reported that application of *P. fluorescens* strain 63-28 brought about cell wall thickening, deposition of phenolic compounds, and formation of callose resulting in restricted growth of *F. oxysporum* f.sp. *radicis-lycopersici*. Such rapid defense reactions at the site of fungal entry delay the infection process and allow sufficient time for the host to build up other defense reactions to restrict pathogen growth.

15.5.1.1 Induction of PR Proteins

Pathogenesis-related proteins are designated as PRs and are defined as proteins coded by the host plant but induced specifically in pathological or related situations. They are not only accumulated locally in the infected leaves but also induced systemically associated with the development of systemic induced resistance against further infection by pathogens (van Loon et al. 1994). Originally, five

main classes of PRs (PR1–PR5) were characterized by both biochemical and molecular techniques in tobacco (van Loon et al. 1987).

Induced resistance by PGPR is associated with the accumulation of PR proteins (Radjacommare et al. 2004). Maurhofer et al. (1994a) reported that ISR by *P. fluorescens* strain CHAO against TNV in tobacco was associated with accumulation of PR proteins namely β-1,3-glucanases and endochitinases. They also established that ineffective strain P3 did not accumulate PR proteins indicating involvement of PR proteins in induction of resistance. Meena et al. (2000) found that application of *P. fluorescens* in groundnut leaves enhanced the PAL (phenylalanine ammonia-lyase) activity (Table 15.2) and chitinase activity (Table 15.3) and

Table 15.2 Effect of foliar application of lower leaves with *P. fluorescens* on phenylalanine ammonia-lyase (PAL) activity in upper leaves inoculated with *C. personatum*

Spraying with *P. fluorescens* on lower leaves	Inoculation with *C. personatum* on upper leaves on 2nd day	PAL activity (nmol trans-cinnamic acid min^{-1} g^{-1} fresh weight)				
		Days after inoculation with *C. personatum*				
		0	1	3	5	7
+	+	131^{a}	172^{a}	204^{a}	192^{a}	164^{a}
+	−	86^{c}	91^{b}	121^{b}	104^{cd}	96^{b}
−	+	84^{c}	96^{b}	114^{b}	101^{d}	82^{bc}
−	−	71^{d}	74^{c}	81^{c}	72^{d}	65^{c}

The superscript letters represent that the significance was tested at 5% level.
+ = Treatment with *P. fluorescens* or *C. personatum*
− = Mock inoculation
For mock inoculation with *P. fluorescens*, lower leaves were sprayed with talc powder formulation (1 kg ha^{-1}) without Pf1
For mock inoculation with *C. personatum*, upper leaves were sprayed with sterile water
Data followed by the same letter in a column are not significantly different ($p = 0.05$) by DMRT

Table 15.3 Effect of foliar application of lower leaves with *P. fluorescens* on chitinase activity of upper leaves inoculated with *C. personatum*

Spraying with *P. fluorescens* on lower leaves	Inoculation with *C. personatum* on upper leaves on 2nd day	Chitinase activity (nmol GlcNAc min^{-1} mg^{-1} fresh weight)				
		Days after inoculation with *C. personatum*				
		0	1	3	5	7
+	+	4.8^{a}	12.8^{a}	15.2^{a}	16.1^{a}	13.5^{a}
+	−	2.6^{b}	6.4^{b}	9.2^{b}	11.6^{ab}	8.5^{ab}
−	+	3.4^{ab}	7.4^{b}	9.6^{b}	8.2^{b}	6.4^{b}
−	−	1.9^{c}	2.2^{c}	2.6^{d}	2.2^{c}	1.8^{c}

The superscript letters represent that the significance was tested at 5% level.
+ = Treatment with *P. fluorescens* or *C. personatum*
− = Mock inoculation
For mock inoculation with *P. fluorescens*, lower leaves were sprayed with talc powder formulation (1 kg ha^{-1}) without Pf1
For mock inoculation with *C. personatum*, upper leaves were sprayed with sterile water
Data followed by the same letter in a column are not significantly different ($p = 0.05$) by DMRT

induced 30-kDa glucanase and 23-kDa thaumatin-like protein (TLP) when compared to untreated leaves. Pieterse et al. (1996) reported that ISR induced by *P. fluorescens* strain WCS 417r in *Arabidopsis* is not associated with PR gene activation. Inoculation of tomato plants with same strain has similarly induced the production of plant chitinases upon challenge inoculation with the wilt pathogen, *F. oxysporum* f.sp. *radicis-lycopersici* (M'Piga et al. 1997). Velazhahan et al. (1998) reported that TLPs were induced in rice plants that were infected with sheath blight fungus, *Rhizoctonia solani*.

15.6 Factors Affecting Effectiveness of PGPR as Biocontrol Agents

Colonization of the root and rhizosphere is considered important in achieving maximum effectiveness in biological control (Suslow 1982), yet little is known about soil physical, chemical, and biological factors which influence multiplication, spread, and survival of these bacteria in the rhizosphere. It has been reported that colonization of roots by pseudomonads was affected by water, temperature, and soil microflora. Park et al. (1988) suggested that bacterial size, charge properties, and presence of capsular material might be important, as are physical attributes of the soil. A number of factors can differentially affect the population size of different strains (Loper et al. 1984).

Temperature has rarely been considered as a factor affecting the performance of biocontrol agents in seed treatment. Harman (1991) observed that *Trichoderma hamatum* applied to pea seeds was not effective in controlling *Pythium* damping-off at temperature below 17°C or above 34°C. Organic matter present in soil also has an important impact on microorganism associated with plants in the rhizosphere. It enhances rhizosphere proliferation, which in turn may affect the competitive ability of the inoculant (Suslow 1982). Upadhyay et al. (1991) found that antagonistic activity of *P. cepacia* was greatly influenced by nutritional and environmental conditions and reported up to 90% inhibition when xylose and ammonium compounds were used as carbon and nitrogen sources, respectively, at slightly acidic pH and 30–37°C temperature. Nutritional and environmental activity of *P. cepacia* makes it one of the most nutritionally versatile bacteria, capable of using as a sole carbon source a large number of carbohydrates and their derivatives, amino acids, and other nitrogenous derivatives (Shanahan et al. 1992). They showed that DAPG production by *P. fluorescens* was reduced in response to glucose as carbon source in culture media and may reflect differences in medium formulation and regulation of DAPG production.

Production of metabolites in vitro by *Pseudomonas* spp. depends on cultural conditions, and in situ production is likely to be even more sensitive to the physical and chemical environments in the rhizosphere. The production of antibiotic compounds by fluorescent *Pseudomonas* spp. is influenced by different chemical

conditions. Gutterson et al. (1986) showed that glucose stimulated the production of some antibiotics by a strain of *P. fluorescens*, whereas the production of other antibiotics was inhibited by glucose. Siderophore production by *Pseudomonas* is influenced by a great variety of factors, e.g., concentration of iron, carbon, and nitrogen sources; level of phosphate; pH; light; and temperature. Therefore, Misaghi et al. (1988) suggested that siderophores might not be produced in sufficient quantities in soil to have any significant biocontrol effect.

15.7 Conclusion

Fluorescent pseudomonads showing various modes of action especially rhizosphere colonization, antibiotic production, and induction of systemic resistance would certainly potential biocontrol agents for the management of pest and diseases of crops. In addition to crop protection, improving plant growth and yield parameters makes these rhizobacteria as useful agents in sustainable agriculture. The technology of commercial use of biocontrol agents has tremendous potentials. The greatest achievement has been the elucidation of the mechanism through which the biological control operates. In recent past, PGPR have frequently been introduced into soil for promotion of plant growth and suppression of plant pathogens. Although some success in agricultural trials has been reported, a major constraint has been the poor productivity and considerable variability of results obtained. A key factor affecting the results may have been a varying degree of establishment and survival of the introduced bacterial population. In spite of this knowledge, the comprehensive understanding necessary to predict bacterial survival and population dynamics under field conditions is still lacking.

The inconsistency in performance of these PGPR strains is a major constraint to their widespread use as biocontrol agent in commercial agriculture. However, genetic manipulation of PGPR has the potential to construct significantly better strains with improved biocontrol efficacy. Further, the efficacy of biocontrol bacteria can be improved by developing the better cultural practices and delivery systems that favor their establishment in the rhizosphere. The applications of mixture of biocontrol agents may be a more ecologically sound approach because it may result in better colonization and better adaptation to the environmental changes occurring throughout the growing season. Future strategies are required to clone genes involved in the production of antibiotics, siderophores, and other metabolites and to transfer these cloned genes into the strains having the good colonization potential along with other beneficial characteristics such as nitrogen fixation. In near future, the biotechnological approaches used in manipulation of bacterial traits will lead to improve biocontrol activity leading to better plant growth.

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Chapter 16
Potential of Plant Growth-Promoting Rhizobacteria for Sustainable Agriculture

R.Z. Sayyed, M.S. Reddy, K. Vijay Kumar, S.K.R. Yellareddygari, A.M. Deshmukh, P.R. Patel, and N.S. Gangurde

16.1 Introduction

Plants require many elements for their nutrition, among which 13 elements should be supplied through soil. Nitrogen, phosphorus, and potassium are the three major plant nutrients. These elements are continuously removed from soil and transferred into plants. Thus, there is a continuous demand for replenishment of these elements which is usually fulfilled by fertilizers. The fertilizer industry greatly depends on petroleum reserves, which in next few decades will be almost exhausted. Further use of chemical fertilizers is deleterious to the environment and injurious to human and soil health. Therefore, there is an urgent need to adopt alternate sources of sustainable fertilizers like biofertilizer (Bagyaraj and Aparna 2009).

R.Z. Sayyed • P.R. Patel • N.S. Gangurde
Department of Microbiology, P.S.G.V.P Mandal's, S.I. Patil Arts, G.B. Patel Science and STSKVS Commerce College, Shahada, Maharashtra 425 409, India

M.S. Reddy (✉)
Department of Entomology and Plant Pathology, Auburn University, 209 Life Sciences Building, Auburn, AL 36849, USA
e-mail: munagrs@auburn.edu

K. Vijay Kumar
Scientist (Plant Pathology), Acharya N G Ranga Agricultural University, Institute of Frontier Technologies, Tirupati-517502
e-mail: kotamvk@gmail.com

S.K.R. Yellareddygari
Department of Entomology & Plant Pathology, 209 Life Sciences Bldg, Auburn University, Auburn, AL 36849

A.M. Deshmukh
Department of Microbiology, Dr. B.A. Ambedkar Marathwada University Sub-center, Osmanabad, Maharashtra 413 501, India

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Table 16.1 Fertilizer requirement and production in India (million tons)

Year	2000	2011	2031	2051
Requirement	19.0	20.2	27.3	31.3
Production	14.9	15.8	20.9	23.9
Gap	4.1	4.4	6.4	7.2

The current population [2008] of India is 1.16 billion and the current gross cropped area is 193 million ha. Food grain production is 212.05 million tons (mt). With the current rate of increase in population, it is projected that India will have a population of 1.5 billion by 2025 AD. To feed this population, we must produce 310 mt of food grains. Although the advances in agriculture have resulted in approximately a four times increase in crop productivity, the scope for increasing the gross cultivated area [horizontal expansion of production] is marginal, and at the most it may increase to 200 m ha, i.e., an addition of 10 m ha in the future. Our food production in next 30 years must be doubled to feed the bulging population. In short, we should have vertical expansion of production. For this purpose, supplementation of soil with farm manures, composts, green manures, and biofertilizers is indispensable (Bagyaraj and Aparna 2009).

The fertilizer (NPK) production in India is less than the required amount (Table 16.1). Organic wastes and the biofertilizers are the alternate sources to bridge the future gaps. Such an integrated approach will help to sustain soil health and productivity and thus should be advocated and popularized among the farming community. Therefore, a major emphasis is being placed on exploiting the biodiversity of nitrogen fixers such as phosphate solubilizers/mobilizers and plant growth-promoting rhizobacteria (PGPR) (Bagyaraj and Aparna 2009).

16.2 Chemical Fertilizer

Chemical fertilizers are those fertilizers which are prepared synthetically from nonrenewable sources. They are mostly inorganic in nature. The most commonly used chemical fertilizers include NPK fertilizers.

16.2.1 Demerits of Using Chemical Fertilizers

Continuous use of chemical fertilizers in agriculture possesses numerous problems (Bagyaraj and Aparna 2009).

- Production of chemical fertilizers consumes nonrenewable energy, e.g., petroleum. Petroleum prices are increasing day by day with high speed, which leads to an increase in the prices of chemical fertilizers.

- Most of the developing countries are importing chemical fertilizers from developed countries and ultimately losing their foreign currency.
- Large amounts of chemical fertilizers applied in soil are immobilized and not available to the plant. Eighty-five percent of inorganic soluble phosphate that is added in the soil chemically reacts with soil components and is converted into insoluble inorganic phosphate, which is of no use.
- There is increasing demand for chemical fertilizers. The application dose of chemical fertilizers has increased six- to sevenfold in twentieth century. As a result, developing countries are facing the problem of scarcity of chemical fertilizers.
- The cost of chemical fertilizer is unbearable to poor farmers of developing countries, and day by day it is increasing due to the rising cost of petroleum.
- The extensive, indiscriminate use of chemical fertilizer adversely affects the natural balance of the soil/crop ecosystem and microbial ecology environment, which results in widespread decline in the crop yields.
- Excessive use of chemical fertilizers creates a number of pollution problems. For example, eutrophication of lakes and increased nitrate content in water adversely affect human health. Hence, there is a need for a cheaper source of nutrients which can be fulfilled by integrated nutrient management and adoption of new technologies including biofertilizers.

16.3 Biofertilizers

Biofertilizers are latent or living cells of microorganisms which mobilize and augment the availability of plant nutrients. The term microbial inoculums may also be used for biofertilizers. Biofertilizers are preparations containing microorganisms in sufficient numbers that improve plant growth and nutrition (Verma 1993).

Biofertilizer production and marketing trends indicate that phosphobacteria (PSB) are most commonly produced and used, which indicate high marketing potentialities of PSB as compared to other biofertilizers like *Azotobacter*, *Azospirillum*, *Acetobacter*, and *Rhizobium*. Biofertilizer production in the northern states is less compared to the southern states of India (Table 16.2).

Among the leading biofertilizer states, Tamil Nadu produces and uses the highest quantity followed by Karnataka state.

16.3.1 Merits of Using Biofertilizers

Biofertilizers have many advantages over chemical fertilizers.

Table 16.2 Biofertilizer production in different states of India

Indian State	Production (MT)	Actual biofertilizer production (in MT)						
		Azotobacter	*Azospirillum*	*Acetobacter*	*Rhizobium*	P solubilizers	Others	Total
HP	75	2.48	0	0	2.39	0	0	4.87
Punjab	2	0	0	0	1.47	0	0	1.47
Haryana	75	5.82	0	0	8.33	4.58	0	18.73
Delhi	1	0.39	0.037	0	0.17	0.28	0	0.877
Rajasthan	75	17.11	0	0	13.9	13.87	0	44.85
UP & UK	225	76.22	8.34	35	45.99	101.92	0	267.46
AP	265	1.92	14.12	0.7	43.11	38.3	39.29	109.56
Karnataka	1,835	76.392	88.454	0.004	68.28	385	2,023.29	2,641.0
TN	1,870.4	16.428	794.714	5.46	180.90	664	128.99	1,819.0
Pondicherry	75	0	7.79	0	0.725	7.3	7.63	23.45
Kerala	225	3.72	43.2	0	0.05	4.23	3.6	55
Total	4,723	200.48	956.65	41.16	365.32	1,219	2,202	4,986.4

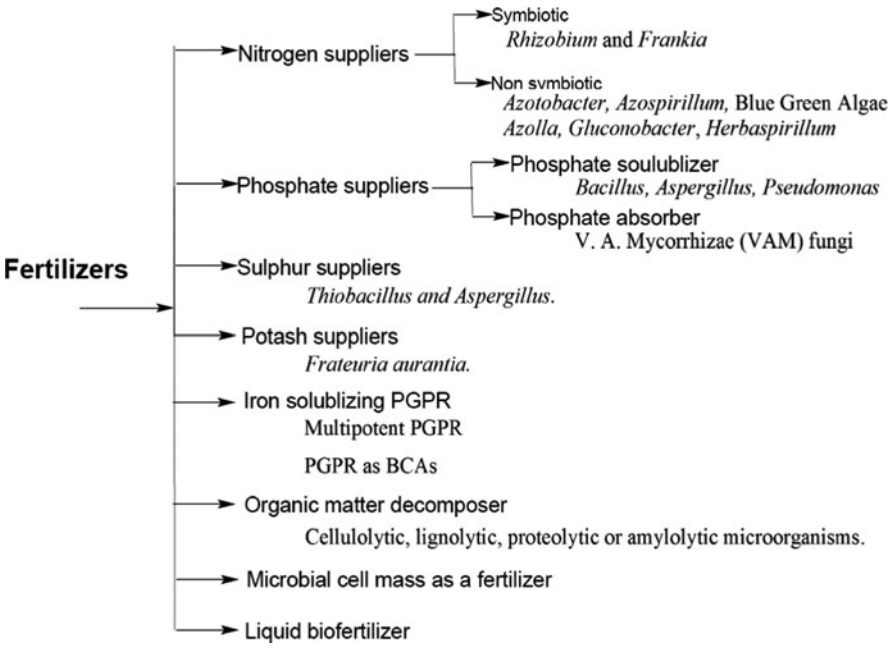
- Biofertilizers are very cheap compared to chemical fertilizers because the infrastructure and equipment required for growth of microorganism is inexpensive.
- The production of biofertilizer is very simple. They can be manufactured in any simple microbiology laboratory. It requires low investment, small space, and little labor and equipment compared to the production method of chemical fertilizers.
- Biofertilizers are natural; their self-replication circumvents repeated application. They are safe for human and animals and do not create a pollution problem.
- A few microorganisms used as biofertilizers also control plant pathogens.
- They may supply other nutrients and increase fertility of soil. The *Azotobacter* added in the soil for nitrogen fixation may have amylolytic or proteolytic activity. Thus, *Azotobacter* also helps in development of humus.
- Biofertilizers may prevent soil erosion as they produce extracellular, capsular polysaccharide, which is viscous in nature, adheres to the soil particle, and prevents soil erosion.
- They may supply vitamins and plant growth hormones like auxins, ethylene, abscisic acid, cytokinin, pantothenic acid, indole acetic acid, and gibberellin.
- Biofertilizers convert immobilized chemical fertilizers into soluble forms and can act as a renewable supplement to chemical fertilizers and organic manures.

16.3.2 Limitations of Using Biofertilizers

- *Contamination*—Biofertilizers generally get contaminated with microorganisms if strict sterile precautions are not taken.
- *Shelf life*—Shelf life of carrier-based biofertilizer at room temperature is at a maximum of 6 months. Hence, biofertilizers must be sold by industries within a time frame.
- *Efficacy*—The efficiency of biofertilizer is mainly dependent on environmental conditions; therefore, many times good results are not obtained.
- *Trained man power*—Workers preparing biofertilizers are not properly trained. They must package the biofertilizers in plastic bags, transport them from industry to market, and sell them to the consumers. Retail shopkeepers are usually not interested in selling biofertilizers.

16.3.3 Types of Biofertilizers

Biofertilizers are broadly divided into the following eight main categories



3.3.1 Nitrogen Suppliers

(a) Symbiotic—*Rhizobium* and *Frankia*
(b) Nonsymbiotic—*Azotobacter*, *Azospirillum*, Blue Green Algae, *Azolla*, *Gluconobacter*, *Herbaspirillum*

3.3.2 Phosphate Suppliers

(a) Phosphate Solubilizers—*Bacillus*, *Aspergillus*, *Pseudomonas*
(b) Phosphate Absorber—V. A. Mycorrhizae (VAM) fungi

3.3.3 Sulfur Suppliers—*Thiobacillus and Aspergillus*
3.3.4 Potash Solubilizers—*Frateuria aurantia*
3.3.5 Iron Solubilizing PGPR

(a) Multipotent PGPR
(b) PGPR as BCAs

3.3.6 Organic Matter Decomposer—cellulolytic, lignolytic, proteolytic or amylolytic microorganisms
3.3.7 Microbial Cell Mass as a Fertilizer
The whole biomass of microorganisms can be used as a good source of biofertilizers
3.3.8 Liquid Biofertilizer

Liquid biofertilizers offer many advantages over carrier-based biofertilizers. These include ease of application, proper mixing, and good growth of microbial cell in liquid medium

16.3.3.1 Nitrogen Suppliers

Nitrogen is present abundantly (78% by volume) in the atmosphere and is fixed by several bacteria living freely in soil or in symbiosis with plants. A profile of different bacteria which fix atmospheric nitrogen and use them as biofertilizers is given in Table 16.3.

Table 16.3 A profile of different N_2 fixing biofertilizers

Type of biofertilizer	Function/ contribution	Limitation	Used for crops
Rhizobium (Symbiotic)	Fixation of 50–100 kg N/ha 10–35% increase in yield Leaves residual nitrogen	Fixation only with legumes Visible effect not reflected in traditional area Needs optimum P and Mo	Pulse legumes like chickpea, red gram, pea, lentil, black gram etc.; Oil seed legumes like soybean and groundnut, forage legumes like clover and leucaena; Tree legumes like Leucaena
Azolla (Symbiotic)	Fixation of 30–100 kg N/ha Yield increase 10–25%	Survival difficult at high temperature Great demand for phosphorus	Only for flooded rice
Azoobacter Azospirillum (Nonsymbiotic)	Fixation of 20–25 kg N/ha 10–15% increase in yield Production of growth-promoting substances	Demands high organic matter	Wheat, maize, cotton sorghum, sugarcane, pearl millet, rice, vegetables and several other crops
BGA (Phototropic)	N_2 Fixation 20–30 kg N/ha 10–15% increase in yield Production of growth-promoting substances	Effective only in submerged rice Demands bright sunlight	Flooded rice

Symbiotic Nitrogen Fixers

Symbiotic association between bacteria and leguminous plants has been known since the initial role of microorganisms in agriculture. *Rhizobium* and *Frankia* sp. are the best examples of symbiotic nitrogen fixers.

1. *Rhizobium*
   Rhizobium is a symbiotic nitrogen-fixing biofertilizer used for leguminous crops. It forms nodules on the roots of leguminous plant. This is a crop-specific inoculant, able to form nodules on the roots of specific leguminous plants only. There are seven groups according to specificity (Table 16.4).
   Other genera of the *Rhizobiaceae* family, including *Azorhizobium*, *Bradyrhizobium*, and *Cyanorhizobium*, can also form root nodule on the roots of leguminous plant. The nodule contains leg hemoglobin, which gives pink color to legume nodules. Leg hemoglobin plays an important role in nitrogen fixation. The efficient strain of *Rhizobium* can fix 40–200 kg/ha N in the soil.
   Rhizobia isolated from root nodules are screened for efficiency through plant tubes, leonard jars, pot experiments, microplot experiments, and finally multilocational field trails. Efficient strains, thus selected, are mass multiplied in Congo Red Yeast Extract Mannitol Broth (CRYEMB), mixed with carrier material like lignite, packed in polythene bags and stored either in cold room or at room temperature. The shelf life of the inoculum is usually 6 months, and it should contain a viable *rhizobial* count of 10^8–10^9 cells/g carrier.
   Legume seeds are treated with an adhesive-like sucrose or jaggery solution, and then rhizobial inoculum is added and mixed thoroughly. About 450–500 g inoculum is required for treating seeds to be sown in a hectare. Seed treatment is usually done either in the early morning or in the late evening and air dried before sowing. Seeds, thus treated, contain 10^5–10^6 rhizobia per seed. Rhizobia are compatible with fungicides like Captan and Ceresan and insecticide like Malathion. For sowing in very acidic soils, lime pelleting at the rate of 9 kg lime per hectare can be done over and above rhizobial treatment. Several experiments conducted in India have shown that nearly 50% nitrogenous fertilizer can be saved through inoculation with efficient Rhizobia (Subba Rao et al. 1993), and rhizobial inoculations have brought out increased grain yield (Table 16.5).
2. *Frankia [Actinorhiza]*

Table 16.4 Crop Inoculation Group and Host Specificity of *Rhizobium* sp.

Rhizobium sp.	Host/Crop inoculation grouping
R. leguminosarum	Pea
R. phaseoli	Bean
R. trifoli	Clover
R. meliloti	Afla alfa
R. lupine	Lupini
R. japonicum	Soyabean
Rhizobium sp.	Cowpea

Table 16.5 Quantity of nitrogen fixed and percentage increase in yield of legumes due to *Rhizobium* inoculation

Crop	N fixed	Average increase in grain yield over control (%)
Chick pea	85–110	28
Cow pea	80–85	36
Ground nut	50–60	21
Lentil	90–100	26
Mung bean	50–55	49
Pigeon pea	168	67
Urad bean	50–55	29

Frankia which belongs to Frankiaceae family of Actinomycetes and is a Gram-positive, symbiotic, nitrogen-fixing bacteria. It is filamentous in nature and bears a chain of spores. It forms nodules in nonleguminous trees and shrubs like *Casuarina*, *Alnus*, *Ceonothus*, *Coriaria*, *a*nd *Myrica*. About 200 plants species from 25 genera, 8 families, and 7 orders, all from nonleguminous angiosperms, have been found to form nodule symbiosis with *Frankia*. It can fix atmospheric nitrogen up to 300 kg/N/year. *Frankia* is a very slow-growing organism on culture media, and thus no commercial inoculant preparation is available.
They are more effective in plants like *Casuarina* and *Alnus*. Root nodules formed by *Frankia* are hard, have a diameter of 5–10 mm, and a weight of 1 g in dry matter.

Non-symbiotic Nitrogen Fixers

Nonsymbiotic nitrogen fixers are free-living soil microorganisms which fix nitrogen freely in the soil. This soluble nitrogen from soil is utilized by crop plants. These nonsymbiotic nitrogen fixers include *Azotobacter* sp., *Azosirillum* sp., BGA, *Acetobacter* sp., and *Azolla* sp.

1. *Azotobacter*
   Azotobacter is a non-symbiotic, nitrogen-fixing, free-living aerobic bacteria commonly present in soil. *Azotobacter indicum* is suitable in acidic soil. Other species of *Azotobacter* include *A. chroococum*, *A. vinelandi*, *A. beijerncki*i, *A. insignis*, *A. macrocytogenes*, and *A. nitrocaptans*. *Azotobacter* forms a microcyst as a resistant structure in old culture. Hence, it is used in liquid biofertilizer with a shelf life of at least 5 years. *Azotobacter chroococum* is commonly used in neutral alkaline soil. It is characterized by the presence of insoluble black pigment. Nitrogen-free mannitol agar is used for isolation of *Azotobacter*.
   Azotobacter is grown on Ashby's, Waksmann no. 77, or Jensen's medium, mixed with the carrier material, and used as described earlier for *Rhizobium*. *Azotobacter* can be used for cereals, vegetables, mulberry, and sugar cane (Table 16.6). Nearly 25–50% of N fertilizer applications can be reduced through

Table 16.6 Yield enhancement in sugarcane due to *Azotobacter* inoculation

N_2 levels	Cane yield (t/ha)	
	Azotobacter	Control
150 kg N/ha	175.58	149.61
200 kg N/ha	195.92	171.36
250 kg N/ha	196.03	177.39

Table 16.7 Yield enhancement of cereals due to *Azospirillum* inoculation

Crop	Grain yield (t/ha)		
	Control	Inoculated	Increase (t/ha)
Rice	3.93	4.54	0.61
Wheat	4.94	5.75	0.81
Pearl millet	2.62	2.79	0.17
Sorghum	4.46	5.38	0.92
Maize	3.98	7.20	3.22

Azotobacter inoculation, and the same amount of increase in crop yield (Table 16.7) can be obtained through *Azotobacter* inoculation (Bagyaraj and Indira 1994).

2. *Azospirillum*
   Baldani et al. (1975) isolated the nitrogen-fixing, free-living, spiral-shaped bacterium, *Azospirillum*, for the first time. It is capable of colonizing the root zone and fixing 20–40 kg/ha N in the soil. The optimum temperature for the growth of *Azospirillum* is 32–35°C. It is more effective with sorghum, maize, mustard, and wheat.
   There are four major species of *Azospirillum* used in acidic soil. They include *A. lipoferum*, *A. brasinense*, *A. amazonense*, and *A. seropedicae*. While *A. halopreferans* is found in saline soil, *Azospirillum* does not show association with roots of dicotyledons.
3. *Azospirillum and Azotobacter diazotrophicus*
   These bacteria live in associative symbiosis with the host. *Azospirillum* can be multiplied on N-free Bromothymol Blue medium, mixed with the carrier material, and used as described earlier for *Azotobacter*. Recent studies in India have shown up to 11% increase in yield of cereals like rice, wheat, sorghum, maize, and pearl millet (Wani 1992) (Table 16.7). A new bacterium *Herbaspirillum*, taxonomically related to *Azospirillum*, was isolated from Brazil. It was found to be associated with grasses and to fix atmospheric nitrogen. Indira and Bagyaraj (1996) have recently isolated a bacterium, *Azotobacter diazotrophicus*, from 17 forage grasses in and around Bangalore, India. It is a saccharophillic bacterium and thus is associated with plants like sugarcane, sweet potato, and sweet sorghum. These bacteria can be multiplied in N-free malate medium, mixed with the carrier material, and used for inoculating sugarcane.
4. *Blue Green Algae* (*BGA*)
   BGA or cyanobacteria are photosynthetic, prokaryotic bacteria commonly present in moist soil. They form a bluish green mat on standing water and constitute 70%

of total algae occurring in moist soil. They are able to fix atmospheric nitrogen 20–30% kg/ha and are widely used for rice crop. Members of *Aulosira*, *Nostoc*, *Anabeana*, *Tolypothrix*, *Plectonima,* and others are able to fix atmospheric nitrogen via a thick-walled heterocyst.

Cyanobacteria (BGA), like *Nostoc*, *Anabaena*, and *Tolypothrix*, can be multiplied by farmers in plots of 20 m × 1 m × 22 cm lined with a polythene sheet, pH neutralized by adding lime starter culture, and inoculated in the plots with water. A thick layer of algal growth, which develops in 15 days, is allowed to dry. Flakes are then collected and used as inoculum. To control predators and insects, carbofuran or ekalux, at the rate of 125 g/plot, can be used. From one plot of 20 m^2 up to 10 kg algal inoculum can be obtained. This inoculum can be stored for many years.

In the main field, algal inoculum is broadcast over standing water at the rate of 10 kg/ha, 1 week after transplanting rice seedlings. Experiments conducted have shown nearly 10% increase in grain yield due to algal inoculation and an addition of 20–25 kg N/ha per season.

5. *Azolla*

*Azolla i*s a water fern harboring N-fixing alga, *Anabaena azollae*, in the leaf as a mat on a water surface. It can fix 40–60 kg N/ha. The growth rate of *Azolla* is very fast, but it is susceptible to temperatures above 40°C. It needs adequate phosphorous for growth. It consists of 94% water, 5% nitrogen, and 1% mineral. *Azolla* leaves have small cavities in the upper surface in which BGA, *Anabeana*, reside. It decomposes rapidly in the soil and multiplies more rapidly in winter than in summer. *A. nilotica*, *A. piñata,* and *A. microphylla* are common species found in nature.

Azolla is grown in 1-m^2 plots 3 weeks before transplanting rice. Inoculation is done at the rate of 100 g/m^2 (i.e., 0.1 t/ha) 15–20 days after transplanting. *Azolla* multiplies 100-fold, resulting in a yield of 10–15 t/ha. Furadon (0.02%) is added to prevent insect damage. *Azolla*, thus multiplied, is added to the main field at the rate of 10 fresh material/ha and incorporated into soil before transplanting rice. The other method includes growing *Azolla* along with a standing crop of rice as a dual crop. The incorporation method is better. About 30–50 kg/N/ha/season is added by *Azolla*. *Azolla* also adds organic matter and thus improves the physicochemical properties of soil. Field experiments have shown that nearly 50% N fertilizer can be saved through *Azolla* application (Subba Rao et al. 1993). Inoculation of rice field with *A. microphylla* alone is reported to increase the rice yield by 6.22%; however, combination of *A. microphylla* and urea is reported to increase the yield (25.59%) by manifold (Bagyaraj and Aparna 2009).

Pabby et al. (2003) carried out comparative studies on freshly separated and cultured symbionts of six *Azolla* species, e.g., *A. microphylla*, *A. filiculoides*, *A. caroliniana*, *A. rubra*, *A. mexicana,* and *A. pinnata*, with free-living strains of *Anabaena variabilis* and *Nostoc* sp. They found that the amount of chlorophyll *a*, proteins, sugars, amount of ammonia excreted, the levels of glutamine synthetase (GS) and nitrate reductase (NR) enzymes, and acetylene reducing activity

(ARA) were significantly higher in freshly separated symbionts, while their cultured counterparts exhibited higher activities of N-assimilation enzymes. Ray et al. (1979) have also reported similar findings in *Azolla* endosymbionts.

Fresh isolates had significantly higher protein accumulation, i.e., 193.4 μg/m in cultured symbiont of *A. filiculoides* vis-a-vis 112.5 μg/ml in the case of freshly separated symbiont of *A. microphylla*. Presence of high amounts of sugars was correlated with the fact that carbon is directly supplied to symbionts from its host, while in the cultured state the symbiont may have reverted back or modified its mode of nutrition to an autotrophic type.

The cultured symbiont of *A. filiculoides* also exhibited significantly high alkaline phosphatase activity. The presence of high phosphatase activity in its endosymbiont is definitely relevant for a better understanding of P metabolism in this symbiotic association. Pabby et al. (2003) claimed that freshly separated symbionts, with low GS and high ammonia accumulation, seem to be the best biofertilizers.

Heterocyst frequency of 20–25% was observed in freshly separated symbionts compared to 7–10% in its cultured counterparts. The size of heterocysts was almost twofold higher in freshly separated symbionts. The acetylene reduction activity (ARA) of symbionts cultured from *A. caroliniana*, *A. pinnata,* and *A. rubra w*as 3–30-fold higher than the corresponding freshly separated symbionts.

Arora and Singh (2003) have analyzed biomass accumulation and nitrogen-fixing potential of six different *Azolla* species, namely *A. lliculoides*, *A.* mexicana, *A. microphylla*, *A. pinnata*, *A. rubra,* and *A. caroliniana*. Among them, *Azolla microphylla* gave the highest biomass production and relative growth rate followed by *Azolla caroliniana*. Both these strains exhibited high nitrogenase activity on the 14th day of growth, which declined on further incubation. *Azolla microphylla* and *Azolla rubra* were more tolerant to salinity than others. Low biomass production, relative growth rate, and lower nitrogenase activity were reported in *Azolla pinnata*, *w*hich was unable to grow in saline medium. Gopalaswamy and Kannaiyan (1998) also reported good biomass production and doubling time of 5.8 days at 14-day incubation in *A. microphylla* during its growth in IRRI medium. Manna and Singh (1990, 1991) have reported peak nitrogenase activity in *A. caroliniana* and *A. pinnata* on the 14th day of growth under field conditions. Kannaiyan (1989) observed that different species of *Azolla* showed higher nitrogenase activity on the 14th day.

A. microphylla was found to perform better than other species in its capacity to produce biomass and N-free nitrogen. This species has been taken up for outdoor mass multiplication for further application as inoculum in paddy fields in the northern region of India.

6. *Acetobacter [Gluconobacter]*

Acetobacter diazotrophicus is found on root, stem, and leaves of sugar cane-fixing atmospheric nitrogen. It fixes 15 kg N/ha/year. It is commonly used in the

sugar field. It can tolerate high sucrose concentration and low pH and secretes plant growth hormones, i.e., Indol acetic acid.

7. *Herbaspirillum*
   Herbaspirillum is an associant symbiont that fixes atmospheric nitrogen on the roots of sugarcane. It is a spiral-shaped bacterium, which also produces growth-promoting hormones.

16.3.3.2 Phosphate Suppliers

Phosphorous is the second most important plant nutrient. It is supplied to the plant by the activity of microorganisms. There are two types of phosphate suppliers.

Phosphate (P) Solubilizers

Tropical soils are deficient in phosphorus. Furthermore, most of them fix P and thus make it unavailable for plant growth. It is estimated that in most tropical soils, 75% of superphosphate applied is fixed and only 25% is available for plant growth. There are some fungi and bacteria like *Bacillus polymyxa*, *Pseudomonas striata*, *Aspergillus awamori*, and *Penicillium digitatum* which can solubilize an unavailable form of P to an available form. India has 260 mt of rock phosphate deposits (Jaggi 1991). Cheaper sources for rock phosphate, which are available in India, are Mussorie rock phosphate, Udaipur rock phosphate, and others. These can be used along with phosphate-solubilizing microorganisms. This combination will save a considerable amount of foreign exchange as the raw material for the manufacture of superphosphate is imported.

Every year farmers are adding soluble inorganic phosphate in the form of chemical fertilizers. Eighty-five percent of this soluble inorganic phosphate chemically reacts with soil constituent and is converted into insoluble inorganic phosphate. Thus, soils are generally rich in insoluble inorganic phosphate. Many microorganisms solubilize phosphate. Some examples are *Aspergillus awamori*, *A. flavus*, *Bacillus megaterium*, and *Pseudomonas striata*.

The P-solubilizing bacteria or fungi can be mass multiplied on Pikovaskaya's broth and mixed with the carrier material used. Several studies conducted by scientists from IARI, New Delhi, found that by inoculation with phosphate-solubilizing microorganisms (PSMs), along with rock phosphate, it is possible to produce the same amount of yield as with superphosphate (Tilak 1993).

Plant roots cannot absorb insoluble inorganic phosphate that is present abundantly in soil. Many of the microorganisms are able to produce a variety of organic acids, such as formic acid, acetic acid, propionic acid, lactic acid, and butyric acid, that solubilize insoluble inorganic phosphate into soluble inorganic phosphate (Patil et al. 2001). In addition, a diverse group from autotrophs to heterotrophs and diazotrophs to phototrophs is known to secrete phosphatases that help to solubilize inorganic P from insoluble sources (Illmer and Schinner 1992).

Table 16.8 Yield enhancement of various crops due to PSM inoculation

Bioinoculant	Crop	Increase in the yield (%)
IARI Microphos	Chick pea	23
	Potato	60
B. firlmus NCIM 2636	Paddy	12
Glomus faciculatum	Banana	
B. megaterium + *G. faciculatum*	Banana	30
Phosphobacterium	Sword bean variety SBS 1	70
Ps. Striata	Soybean in sandy alluvial soil	60
Ps. Striata	Chick pea	25

Tilak (1991), Datta and Banik (1994), Sowmya and Anusaya (1998), Govindan and Arjunan (1998), Gaur (1985), and Shinde and Saraf (1994) have reported the PSMs as efficient PGPR for increasing the yield of various crops (Table 16.8).

Phosphate Absorber

There are certain fungi which form symbiotic association with the roots of plants and help in the uptake of phosphorus. They are called mycorrhizal fungi. Ectomycorrhizal fungi, like *Pisolithus*, *Laccaria*, *Amanita*, *Scleroderma*, *Russula*, *Tricholoma*, and others, form associations with tree species belonging to family Pinaceae, Betulaceae, and Fagaceae. These fungi increase the surface area of absorption of the roots and thus help in absorption of nutrients, especially those less mobile in soil solution like P. They also help in the uptake of water and protect roots from root pathogens. These fungi are culturable and hence can be mass produced and used as carrier-based inoculants for inoculating forest nurseries (Bagyaraj and Padmavathi 1993).

Arbuscular mycorrhizal (AM) fungi colonize roots of several crop plants important in agriculture, horticulture, and tropical forestry. They are zygomycetous fungi belonging to the genera *Glomus*, *Gigaspora*, *Acaulospora*, *Sclerocystis* among others. These are obligate symbionts and cannot be cultured on laboratory media. They help plant growth through improved phosphorus nutrition and protect the roots against pathogens. They are multiplied as a pot culture, using a suitable host, root pieces, and the substrate as inoculum. They are currently recommended for use in transplanted and nursery-raised crops because of the difficulty in inoculum production. Several studies have shown that they improve seedling growth and vigor of many plants important in agriculture like tobacco, finger millet, and chillies. They also aid horticulture crops like tomato, citrus, cardamom, and mango and trees like *Leucaena*, *Dalbergia*, *Acacia*, *Casuarina,* and *Tectona*. Very few field studies have been conducted with vesicular arbuscular mycorrhizae (VAM) fungi. These studies clearly determined that nearly 25–50% of phosphatic fertilizer can be saved through inoculation with efficient VAM fungi (Table 16.9). Further studies are urgently needed to mass produce AM fungi (Bagyaraj 1992).

Table 16.9 Effect of VAM inoculation on yield of tomato and capsicum

Crop	Variety	Treatment	Recommended level of P	Yield (kg/Plot)
Tomato	Rashmi	*Glomus intraradices*	50%	66.78
	Pusa Ruby	*Glomus intraradices*	50%	73.91
Capsicum	Bharath	*Glomus monosporum*	50%	14.30
	California Wonder	*Glomus monosporum*	50%	13.90

Vesicular Arbuscular Mycorrhizae

VAM is a fungus that forms a symbiotic association with root systems of higher plants. The partners in this association are members of fungi belonging to *Basidiomycetes*, *Ascomycetes*, *Zygomycetes*, and most vascular plants. The term mycorrhiza comes from the Greek myco, meaning fungus, and rhiza, meaning root, which translates literally to fungus root. VAM fungi colonize the root system and increase the growth and yield of the plant. The improved plant growth is due to increased nutrition uptake, particularly P, Zn, and other micronutrients.

VAM developed special structures called vesicles and arbuscules. The finger-like projections are arbuscules and the circular, ellipsoidal, or rectangular shapes are vesicles. VAM have an endosymbiotic association. Arbuscules help in the transfer of nutrients from soil to the root system. Vesicles are storage houses. The benefits of these to plants are that plants get a continuous supply of water, phosphorous, and other nutrients like Mo, Ca, Zn, Fe, Cu, and Mg. They also give physical protection to the plant root, preventing infection by a plant pathogen.

16.3.3.3 Sulfur Suppliers

Although sulfur is present in large quantities in soil, it is not easily available to crop plants due to its inorganic insoluble nature. It is solubilized by sulfur-oxidizing bacteria like *Thiobacillus thioxidans* and *T. novelis*, which oxidize inorganic insoluble sulfur into a soluble and available form that is absorbed by plant roots.

16.3.3.4 Potash Solubilizers

Frateuria aurantia are capable of solubilizing potassium. Certain crops require a good amount of potash. These biofertilizers are used in crops like banana. They can increase crop yield by 20–25%.

16.3.3.5 Iron-Solubilizing PGPR

PGPR or Yield-Increasing Bacteria (YIB), which are present in close vicinity to plant roots or its surface, play a crucial role in promoting plant health/growth

(Sindhu et al. 1997) through different mechanisms including iron nutrition through siderophores. In this context, siderophore-producing microbes function as efficient PGPR (Kloepper 1993, Kloepper et al. 1996, Glick 1995) with multifunctional potential for plant growth promotion (Sayyed et al. 2005a, b, 2007a, b) and biocontrol of phytopathogens (Sayyed et al. 2008, 2009; Sayyed and Chincholkar 2009).

Siderophore-Producing PGPR for Iron Nutrition of Plant

Siderophores (Sid = iron, Phores = bearers) are low-molecular-weight (<10,000 D), virtually ferric-specific ligands produced by microbes as scavenging agents in order to combat low iron stress (Kintu et al. 2001, Rane et al. 2005). The most important biotechnological use of siderophores occurs in the rhizosphere region of the plant where they provide iron nutrition to the plant, serve as first defense against root-invading parasites and help in removing toxic metals from polluted soil. Siderophores may be involved in the beneficial uptake of ions other than iron like aluminum, chromium, cobalt, copper, zinc, and molybdenum. There is sufficient evidence available regarding iron uptake by plants through microbial siderophores, which convert the insoluble form of iron into a soluble form (Chincholkar et al. 2000).

Multi-potent PGPR

PGPR are those rhizobacteria which promote plant growth directly by releasing phytohormones, fixing nitrogen in the rhizosphere, solubilizing insoluble forms of nutrients such as phosphate, promoting mycorrhizal function, and regulating ethylene production in plant roots. In addition, some rhizobacteria have the capacity to suppress major plant pathogens and play an important role in plant defense mechanisms (Bloemberg and Lugtenberg 2001; Sayyed and Chincholkar 2009).

In addition to the common *Azotobacter* sp., *Rhizobium* sp., and *Azospirillum* sp., a number of other bacteria, including various species of *Pseudomonas*, *Acetobacter*, *Alcaligenes*, *Klebsiella*, *Enterobacter*, *Xanthomonas*, and *Bacillus* sp., have been considered as PGPR (Sayyed et al. 2005a, b; Sayyed et al. 2010).

PGPR as Biocontrol Agents (BCAs)

Every year severe global economic losses to agricultural crops are encountered due to plant diseases caused by more than 60 pathogens that result in the loss of 30% crop yield of around Rs. 20,000 crores. Traditional practice of chemotherapy has increased crop production. On the other hand, it has adversely imbalanced the agro-ecosystems (Nehl et al. 1996; Yang and Crowley 2000). The increasing public awareness of these problems has stimulated interest in the research on the use of

Table 16.10 Siderophore-bearing rhizobacteria for controlling infestations in cash crops

Biocontrol agent	Target pathogen/disease	Crop
Ps. fluorescence	*Erwinia carotovora*	Potato
	Gaeumannomyces graminis Take-all	Wheat
	Fusarium glycinia	Wheat
	Sarocladium oryzae	Soybean, Rice
Ps. putida	*Fusarium* sp.Wilt	Radish
	Fusarium solani	Cucumber
	Erwinia carotovora	Beans, Potato
Ps. cepacia	*Fusarium oxysporum*	Onion
B. subtilis	*Fusarium roseum*	Corn
Bacillus sp.	*Rhizoctonia*, *Pythium*, Rot & Take all	Wheat
Rhizobium sp.	*Macrophomina phaseolina*	Soybean
Bradyrhizobium sp.	*Fusarium solani*	Sunflower
	Rhizoctonia solani	Mungbean

biocontrol methods as an important and vital component of Integrated Pest Management (IPM) or Integrated Plant Disease Management (IPDM) for crop disease management (Chincholkar et al. 2000; Sayyed et al. 2008).

Siderophore-producing PGPR, like *Pseudomonas* sp. and others, have been implicated in the biocontrol of several diseases like damping-off of cotton caused by *Pythium ultimum*, root rot of wheat caused by *Pythium* sp., potato seed piece decay caused by *Erwinia carotovora*, vascular wilts caused by *Fusarium oxysporum*, and stem rot of pea nut caused by *Rhizoctonia solani* and *Sclerotium rolfsii* (Table 16.10). Recently, a great deal of work has been conducted on the biocontrol of plant diseases, which has led to the development of commercial bioproducts. Some of the commercially available bacterial biocontrol products are listed in Table 16.11.

The mechanism responsible for this biocontrol activity includes competition for nutrients and niche, induced systemic resistance (ISR), and the production of antifungal metabolites (AFMs) (Nielsen et al. 1999). Most of the identified *Pseudomonas* biocontrol strains produce AFMs, which inhibit the growth of phytopathogenic fungi (Downing and Thomson 2000), of which phenazine, pyrrolnitrin, 2, 4-diacetylpholoroglucinol (DAPG), pyoluterin, viscosinamide (Bloemberg and Lugtenberg 2001), and tensin (Nielsen et al. 2000) are the most frequently detected classes (Thrane et al. 2000).

Phytohormone Providing PGPR

Production of phytohormones is a mechanism of action for the enhanced plant growth response (Jamsen 2000). *Pseudomonas syringae* and *P. fluorescence* were capable of producing Indole Acetic Acid (IAA), leading to increased shoot–root ratio in sugar beet and black currant, respectively. Young et al. (1992) reported that the BCAs induce root elongation on cucumber and tomato. Hoflich and

Table 16.11 A partial list of commercially available biocontrol products for controlling soil borne pathogens

Trade Name	Biocontrol organism	Target pathogen/disease	Crop under application	Formulation	Mode of application
Bio-save 10[5] and Biosave 11[5]	*Pseudomonas syringae*	*Botrytis cinerea*, *Mucor pyriformis*, *Geotrichum candidum* and *Penicillium* sp.	Citrus and pome fruit	Wettable powder	Post harvest Drench, dip and spray
BlightBan A506[6]	*Pseudomonas fluorescence*	*Erwinia amylovora*	Almond, cherry, apple, potato, etc	Wettable powder	Post harvest Drench, dip and spray
Conquer[8] = Victus[26]	*Pseudomonas fluorescence*	*Pseudomonas tolassii*	Mushroom	Aqueous biomass suspension	Spray
Epic[11]	*Bacillus subtilis*	*Rhizoctonia solani*, *Fusarium*, *Alternaria* and *Aspergillus* sp.	Cotton and legumes	Dry powder 5.5×10^{10} spores/g	Drip and potting mix in row
Galltrol-A[13]	*Agrobacterium radiobacter*	*Agrobacterium tumifaciens* Crown gall	Fruit, nut and ornamental nursery plants	Suspension 1.2×10^{11} cfu	Suspension used for seeds/ seedlings, root, soil drench
Intercept[14]	*Pseudomonas cepacia*	*Rhizoctonia solani*, *Fusarium* and *Pythium* sp.	Maize, cotton and vegetables	–	–
Kodiak[11], Kodiak HB	*Bacillus subtilis*	*Rhizoctonia solani*, *Fusarium*, *Alternaria* and *Aspergillus* sp.	Cotton and legumes	Dry Powder 5.5×10^{10} spores/g	Slurry mixture for seed treatment
Norback 84 C[17]	*Agrobacterium radiobacter*	*Agrobacterium tumifaciens* Crown gall	Fruit, nut and ornamental nursery plants	Aqueous suspension 8×10^{10} cfu/ 100 ml	Root, stem, cutting, dip or spray
System 3[22]	*Bacillus subtilis*	Seedling pathogens	Bean, barley, cotton, peanut	Dust	Seed treatment

Weise (1992) also observed that cytokinins of *Pseudomonas fluorescence* stimulated growth and yield increase in winter wheat, winter rape, oil radish, mustard, and peas in pot and field experiments.

PGPR Secrete an Array of Enzymes

Enzymes in the rhizosphere have been considered as indicators of biological activity of rhizosphere microbes. Buildup of phosphorous and nitrogen in the soil has been reported to be the result of the activity of phosphates and dehydrogenase (Kaushik 2001). Adholeya (2001) has reported the influence of fertility level and previous microbial inoculation on the activity of dehydrogenase and total microbial counts. Talukdar (2001) has analyzed rice–legume–rice and rice–rice cropping systems and reported the presence of dehydrogenase as a good indicator of soil health. Besides dehydrogenases, acid and alkaline phosphatases have also been reported to be an indicator of soil health (Johri et al. 2003).

16.3.3.6 Organic Matter Decomposers

Decaying plant leaves, or baggas, are continuously added to the soil in large amounts. Microorganisms producing extracellular digestive enzymes can be used as a biofertilizer. Microorganisms with cellulolytic activity, lignolytic activity, and proteolytic activity are organic matter decomposers. *Trichoderma*, *Cellulomonas*, and *Arthrobacter* are used as organic matter decomposers.

16.3.3.7 Microbial Cell Mass as Fertilizer

The remaining saprophytic microbial mass at the end of any fermentation process can be used as organic fertilizer. Microbial mass consists of proteins, enzymes, vitamins, organic acids, and plant growth hormones. Chitin present in fungal biomass stimulates chitinolytic bacteria, which prevent fungal pathogen infection. In many fermentation industries, it is a waste product and hence is very cheap.

16.3.3.8 Liquid Biofertilizers

Recently liquid biofertilizers are capturing the market. Microcysts of *Azotobacter* and endospores of *Bacillus* are resistant structures that can survive for a long time. They are also used in liquid biofertilizer to increase the shelf life. One can obtain a high number ($>10^9$) of CFU in liquid biofertilizer. It is also easy to maintain sterility and there are no chances of contamination. Moreover, application of biofertilizer is easy; they can be packed in plastic bottles with an attractive cover.

It is proven that the use of liquid biofertilizers increases the crop yield as compared to carrier-based biofertilizers.

16.4 Applications of Biofertilizers

Biofertilizers can be used to treat seeds before sowing by using adhesives, dipping the seedlings in biofertilizer solution before transplanting and treating the soil by mixing it with Farm Yard Manure (FYM) during plant development.

16.4.1 Seed Treatment

Fifty grams of crude sugar, or jaggery, or 20 g of gum is dissolved in 500 ml of water. The mixture is heated for 15 min, cooled properly, and then 200 g of inoculant is poured to form a slurry. This preparation can be used for surface treatment of 10 kg of seeds. Seeds are dried in the shade and used immediately for sowing.

16.4.2 Root Surface Treatment

In the root surface treatment method, the type of biofertilizer application is dissolved/mixed in water and applied to the surface of roots. In this application, 1–2 kg of biofertilizer is mixed in 50 l of water. Roots of seedling from 10–15 kg of seed are dipped in the slurry for 10–13 min followed by transplantation of seedling into soil.

16.4.3 Soil Treatment

Soil application of biofertilizers involves the mixing of microbial biomass with soil/compost/FYM. In this application, about 0.5–1 kg of biofertilizer is uniformly mixed with 10–15 kg of FYM or compost. This mixture is spread on the soil before sowing or at the time of sowing.

16.4.4 Precautions in the Use of Biofertilizers

- Specific biofertilizers should be used for specific crops.

- Biofertilizer packets should be stored in the shade to prevent direct exposure to sunlight.
- Plastic bags of biofertilizer should be handled carefully because water evaporates through the minute holes and can cause drying of biofertilizer.
- Seeds are treated first with pesticide and then with biofertilizers.
- Enough moisture and organic matter must be present in the soil for effectiveness of biofertilizers.
- pH of the soil should be favorable for microbial growth.

16.5 Biofertilizer Demand and Availability

The annual requirement and present production capacity for different biofertilizers not only show a tremendous gap but also indicate the potential for organized production (Venkataraman and Tilak 1990) (Table 16.1). About 30 m ha are under pulses, including pulse and forage legumes in our country. The requirement for *Rhizobium* cultures to cover an entire cultivation area in India will be around 15,000 tons of carrier-based material, of which the present production is only around 800 tons. Similarly, four lakh tons of BGA are required to cover the entire rice cultivation area in India. This indicates the need to augment efforts and to plan a phased strategy (Tilak and Saxena 1986).

In India, besides a large number of private and semi-government organizations, the National Biofertilizer Development Centre, sponsored by the Ministry of Agriculture, Government of India, with 6 regional and 49 subcenters, the establishment of the National Facility for *Rhizobium* Germplasm Collection at the Indian Agricultural Research Institute, New Delhi, and Mycorrhizal Germplasm Collection at Tata Energy Research Institute, New Delhi, by the Department of Biotechnology, Government of India, are important developments that reflect our concern to harness biofertilizers in our agricultural economy. Several organizations are developing that provide biofertilizer inoculant (Table 16.12), conventional

Table 16.12 Addresses of organizations in India that provide biofertilizer inoculants

Sl No.	Name of organization	Address of organization
1	Nodule Research Laboratory	Bidhan Chandra Krishi Viswabidyalaya, Mohonpur, Nadia, Gujarat
2	National Laboratory	Agricultural Department, Govt. of West Bengal. 230, AJC Bose Road, Kolkata 40, WB
3	Vivekananda Institute of Biotechnology	Nimpith Ashram, 24-Pargana (South), WB
4	Department of Forestry	Government of West Bengal. 6A, Raja Subhodh Chandra Mullik Square, Kolkata 13, WB
5	Development Research Communication and Service Centre	58A, Esplanade Road, Kosba, Kolkata 42, WB

Table 16.13 List of companies producing biofertilizer with their products

Sl No.	Biofertilizer company	Type of product
1.	Agro-Biotech Research Centre Ltd.	*Rhizobium*, *Azospirillum*, *Azotobacter*, Phosphobacteria
2.	Bio-Potash (*Frateuria aurantia*)	VAM
3.	Biotech International Limited	Bioazoto, Biophos, Biopotash, *Biospirillum*
4.	Biotech Consortium India Limited	BGA biofertilizer
5.	Jain Irrigation System Ltd, Jalgaon, MS	Vermicompost
6.	Bhubani Products Indian	Vermicompost
7.	Barod Feed Concern, Ujjain, MP	Organic, Natural, Biofertilizers, Agricultural fertilizers, Organic manure
8.	Sai International Trading Company, Kerala	Organic fertilizers, Biofertilizers
9.	Neem Products, Noida, Uttar Pradesh	Neem fertilizer
10.	Akshat Farms, Udaipur, Rajasthan	Bioorganic fertilizer, Compost, Vermin and earthworm compost, Vermiculture
11.	Total Agri Care Concern Private Limited, Kolkata, West Bengal	Micronutrient fertilizers, Organic manures, Natural fertilizers, Organic fertilizers
12.	Suriya Farms, Madurai, Tamil Nadu	Vermicomposts, Vermi Culture, FYM Press Mud, Coco Pith
13.	Srinivasa Marine Chemicals, Madurai, Tamil Nadu	Seaweed fertilizer
14.	Associated Alcohols and Beverage Ltd., Bharwaha, MP	Organic, Bioorganic, Agriculture and Micronutrient fertilizers, Organic manures
15.	Scientific Agriculture Lab., Tamil Nadu	Biofertilizer
16.	Ruchi Biochemicals, Mumbai, Maharashtra	Biomedical, Organic and natural fertilizers
17.	RBM Trade Care Pvt Ltd., Bhavnagar	Natural fertilizer
18.	Gujarat Agricultural Fertilizer	Organic fertilizer
19.	Rays Bio Energy Pvt. Ltd., Raipur, CG	Bioorganic products, Bioorganic fertilizers

Azotobacter and *Rhizobium* biofertilizers, and various other fertilizers to the market (Table 16.13).

16.5.1 Limitations of Biofertilizer Technology

There are several constraints in biofertilizer production and its commercialization. These constraints can be physical, chemical, biological, and technological.

16.5.1.1 High Temperature

The growth and multiplication of microbial cells are affected by temperature and loss of water due to excess temperature/heating, which can lead to a decrease in moisture content. During the summer months in some parts of India, temperatures are very high. Care must be taken to avoid transportation of biofertilizer packets in direct sunlight. Storage of biofertilizers should not be done in containers/sheds where the temperature becomes very high. While making packets, care must be taken to see that the carrier material has enough moisture (about 40%). If the carrier material is very dry, the population of biofertilizers will decrease rapidly.

16.5.1.2 Acidic or Saline Conditions

The growth and multiplication of microbial cells are affected by pH. Highly acidic or saline soils will adversely affect the population of an introduced biofertilizer. It is recommended that in acidic soil seeds be pelleted with lime (after treating with biofertilizer) and in saline soils with gypsum. In soils less than 10 ppm available P, application of P fertilizer is essential, without which the nitrogen-fixing biofertilizers will not function effectively.

16.5.1.3 Antagonism by Native Soil Population

There are high chances of antagonism of biofertilizers by native soil population. Soil flora and fauna can parasitize or devour biofertilizer added to soil. Bacteriophages, called rhizophages, can destroy rhizobia added to soil. Some protozoa like *Vorticella* and nematodes like *Meloidogyne* can act as predators of biofertilizers added to soil. *Azolla* is subjected to attack by pests, especially larvae of various lepidopteras and dipterous insects and aphids. Cyanobacteria can be attacked by phages, myxobacteria, and grazers like snails and mosquito larvae.

16.5.1.4 Technical Difficulties

Raw material—Peat and lignite, which are good carrier materials, are available only in specific places like Nilgiris and Neyveli, which results in high transportation costs to biofertilizer manufacturing units.

Microbial strains—Suitable strains of biofertilizers for different crops have been identified, and therefore a specific strain should be used for a specific crop.

Contamination-free technology—It is general practice to carry out biofertilizer production under nonsterile conditions. This results in considerable contamination.

Suitable technology to minimize contamination, such as gamma irradiation of the carrier material and injection of liquid culture into polythene bags containing sterile carrier material, should be developed.

Inadequate quality assurance—Substandard quality of inoculants is one of the most important factors resulting in failure in the field and a lack of farmer confidence in the product. The Bureau of Indian Standards (BIS) has prepared ISI specifications for only *Rhizobium* and *Azotobacter* inoculants. These were prepared in 1977 and need revision. ISI specification for other biofertilizers is not available.

The BIS has no network to test biofertilizers samples. They send samples to agricultural universities or research institutes in order to obtain an ISI mark. Once a manufacturer gets an ISI mark, they continue production without routine checking.

16.6 Recommendations

- All crops should be inoculated with suitable biofertilizers.
- Work on newer biofertilizers like *Azotobacter diazotrophicus* and plant growth-promoting rhizobacteria should be strengthened.
- Technology for mass production of VAM fungi should be given top priority.
- The standards developed by BIS for *Rhizobium* and *Azotobacter* have to be reviewed by considering the research data collected since 1977. The BIS standards have to be developed for new types of biofertilizers, such as *Azospirillum*, phosphorus-solubilizing microorganisms, VAM, and BGA. The quality control laboratories should be established at the state agricultural universities to determine and monitor the quality of biofertilizers.
- In order to exploit the root associations with microorganisms, collaboration with agronomists is essential.
- There is an urgent need to improve the inputs of organics and biological nitrogen fixation and to increase the production of quality inoculants and popularize their use in Indian agriculture. To achieve this, we need to exploit the enormous biodiversity of diazotrophs, PSMs, and PGPR in diverse environments and under-explored ecosystems.
- Exploit the biodiversity of nitrogen fixers, phosphate solubilizers/mobilizers, and PGPR for biofertilizer applications in diverse cropping systems.
- Improve biofertilizer technology with particular reference to quality, carriers, consortia, delivery systems, and testing methods.
- Expand biofertilizer research and application in drylands, mountainous regions, tribal areas, and other under-explored ecosystems.
- It is essential to develop more effective and competitive strains that are tolerant to high temperatures, drought, nitrate, acidity, and other abiotic stresses (Rao 2003). Newer formulations of mixed biofertilizers, improvement of inoculant quality, and development of best delivery systems are crucial for making further progress in taking the technology to farmers' fields. The problem is so acute that it is beyond any single type of nutrient source to meet the challenges of

appropriate nutrient supply. Integrated use of all the sources, such as mineral fertilizers, organic manures, and biofertilizers, is the only alternative for improving soil fertility.

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Chapter 17
Contribution of N_2 Fixation for the World Agriculture

André Luís Braghini Sá, Armando Cavalcante Franco Dias, Manoel de Araújo Teixeira, and Rosana Faria Vieira

17.1 Introduction

Nitrogen is generally considered the most limiting nutrient for plant growth since it is part of the composition of nucleic acids, proteins, and polysaccharides. Although the atmosphere is composed of 78% nitrogen gas, eukaryotic organisms such as plants and animals cannot directly use this element, due to the absence of the enzyme nitrogenase. To break it, so that its atoms can combine with other atoms, substantial amounts of energy are needed. This enzyme breaks the triple bond of N_2, which diffuses into the porous space, reducing it to NH_3, which may be used by various agencies. Only a few phylogenetic groups of prokaryotes are capable of performing the process of biological nitrogen fixation, i.e., some genera of bacteria, cyanobacteria, and the genera *Frankia*. The industrial process that converts N_2 to NH_3, on the manufacture of nitrogen fertilizers, is costly since it requires, besides hydrogen-derived gas oil and a catalyst-containing iron, high temperatures and high pressures. Besides, the high-cost nitrogen fertilizers also have low utilization efficiency by plants, usually not exceeding 50%. For Brazilian agribusiness, replacement of these fertilizers by inoculation with nitrogen-fixing bacteria could save billions of dollars. In the case of soybeans, for example, which requires

A.L.B. Sá (✉) • M. de Araújo Teixeira
Laboratory of Applied Microbiology, University of Vale do Sapucaí – UNIVAS, 37.550-000 Pouso Alegre, Minas Gerais, Brazil
e-mail: biobragh@yahoo.com.br

A.C.F. Dias
Center for Nuclear Energy in Agriculture, University of São Paulo, CENA/USP, São Paulo, Piracicaba 13.400-970, Brazil

R.F. Vieira
Laboratory of Environmental Microbiology, CNPMA - Embrapa Meio Ambiente, 13.820-000, Jaguariúna, São Paulo, Brazil

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Table 17.1 Genera and species of *Rhizobium* (Family Rhizobiaceae) that form nodules on legumes roots

Species	Legumes	Source
Rhizobium miluonense	*Lespedeza chinensis*	Gu et al. (2008)
Rhizobium fabae	*Vicia faba*	Tian et al. (2008)
Rhizobium multihospitium	*Robinia pseudoacacia*	Han et al. (2008)
Rhizobium tubonense	*Oxytropis glabra*	Zhang et al. (2011)
Rhizobium sphaerophysae	*Sphaerophysa salsula*	Lin et al. (2011)
Ensifer mexicanum	*Acacia angustissima (Mill.) Kuntze*	Lloret et al. (2007)
Sinorhizobium chiapanecum	*Acaciella angustissima*	Rincón-Rosales et al. (2008)
Mesorhizobium albiziae	*Albizia kalkora*	Wang et al. (2007)
Mesorhizobium gobiense	*Glycyrrhiza uralensis, Lotus corniculatus, Oxytropis glabra, and Robinia pseudoacacia*	Han et al. (2008)
Rhizobium lusitanum	*Phaseolus vulgaris*	Valverde et al. (2006)
Mesorhizobium tarimense	*Glycyrrhiza uralensis, Lotus corniculatus, Oxytropis glabra, and Robinia pseudoacacia*	Han et al. (2008)
Azospirillum doebereinerae	*Sesbania* spp., *entre outros*	Moreira et al. (2006)
Bradyrhizobium pachyrhizi	*Pachyrhizus erosus*	Ramires-Bahena et al. (2009)
Bradyrhizobium jicamae	*Pachyrhizus erosus*	Ramires-Bahena et al. (2009)

240 kg N ha^{-1} to produce 3,000 kg of grain/ha^{-1}, inoculation resulted in savings of 3.3 billion for Brazilian agriculture in 2006.

Soil bacteria, collectively referred to as rhizobia, are members, among others, of the bacterial order Rhizobiales of the α-Proteobacteria with the unique ability to establish a dinitrogen (N_2)-fixing symbiosis on vegetable roots and on the stems of some aquatic vegetables. Among the species responsible for fixation in legume stand out *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium*, and *Mesorhizobium* (Table 17.1).

The genus *Parasponia* (Ulmaceae) is the only nonlegume plant to form nodules of N_2 fixation in association with *Rhizobium* (Trinick 1979). Biological N_2 fixation by vegetable-rhizobia symbioses is importance to the environment and to world agriculture. Perception of vegetable root exudates triggers the production of rhizobial *Nod factor* signals, which are recognized by compatible plant receptors leading to the formation of root nodules, in which differentiated bacteria (bacteroids) fix N_2 (Oldroyd and Downie 2008). Earlier this century, species of bacterial genera were described, such as *Burkholderia*, *Methylobacterium*, *Ochrobactrum*, *Ralstonia*, and *Phyllobacterium* and their capacity for noduling vegetables (Table 17.2). These bacteria are also known as rhizobia, despite their name has become inappropriate.

Bacteria of the genus *Burkholderia* have been isolated from nodules of *Aspalathus carnosa* and *Machaerium lunatum* (Moulin et al. 2001) and also *Mimosa caesalpiniifolia* nodules (Chen et al. 2008). In the latter case, the name *Burkholderia sabiae* was proposed to the bacteria which were isolated from rot nodules.

Table 17.2 *Burkholderia* species isolated from legumes nodules

Species	Legumes	Source
Burkholderia nodosa	*Mimosa bimucronata, M. scabrella*	Chen et al. (2007)
Burkholderia sabiae	*Mimosa caesalpiniifolia*	Chen et al. (2008)
Burkholderia tuberum	Tropical legumes	Vandamme et al. (2002)
Burkholderia phymatum	Tropical legumes	Vandamme et al. (2002)
Burkholderia sp.	*Medicago sativa*	Garau et al. (2009)
Devosia yakushimensis	*Pueraria lobate (Willd.) Ohwi*	Bautista et al. (2010)

Ralstonia taiwanensis and *Ralstonia eutropha* were isolated from nodules of *Mimosa* in China (Chen et al. 2001) and India, respectively. In Spain, *Phyllobacterium trifolii* was isolated from nodules of *Trifolium pratense*. The *P. trifolii* is also capable of forming nodules on *Lupinus* sp. (Valverde et al. 2005). Also, in Spain, *Ochrobactrum cytisi* was isolated from *Cytisus scoparius* (Zurdo-Piñero et al. 2007).

17.2 Nitrogen Fixation in Legumes

The biological fixation of N_2 is a key process for agricultural sustainability, especially in tropical soils, since they generally are deficient in N. In Brazil, soya and beans are two crops of major economic and social importance, for which there are commercial inoculants. Black beans (*Phaseolus vulgaris* L.) constitutively one of the main sources of protein for the low-income population. In this culture, the sparse nodulation and the lack of response to inoculation in field experiments frequently had been frequently reported worldwide, raising questions about the benefits of inoculation (Graham 1981). This fact is attributed to intrinsic characteristics of the host plant, particularly the nodulation promiscuity (Michielis et al. 1998), as well as the great sensitivity to other nodulation-limiting factors, such as the high rate of N_2. Of the approximately total of 26 million of inoculants marketed, produced in Brazil and imported in 2003, 99% were for soybeans and only 1% for other crops, especially *Phaseolus vulgaris*. The use of new strains in this culture, however, has shown increases of 900 kg ha^{-1} compared with the control without inoculation and nitrogen fertilization (Hungria et al. 2000). Similarly higher yields of bean strains have been obtained using new, not used in commercial inoculants (Mostasso et al. 2001). Other studies showed good yields of bean when inoculated with the strains UFLA 02–100, UFLA 02–127, UFLA 02–86, in relation to the reference strain CIAT 899 (Soares et al. 2006). These results encourage more demand for isolates that can ensure an efficient nodulation of bean crops.

Soybean was introduced in Brazil around 100 years ago, but large-scale commercial cultivation did not begin until the 1960s. Brazilian soils were originally free of soybean bradyrhizobia, but inoculation has established populations of some few strains in most of the 12×10^6 ha which are today cultivated with this crop

(Vargas and Hungria 1997). Previous evaluations performed in Brazil have demonstrated that some crop rotations and the no-tillage management system favor bradyrhizobia populations, nodulation, nitrogen fixation rates, and yield (Voss and Sidiras 1985). Ferreira et al. (2000) have highlighted the potential benefits of agronomic practices, such as the no-tillage system and crop rotations with legumes, in agricultural sustainability and maintenance of rhizobia populations and diversity. Furthermore, within this diversity, it is possible to select adapted strains with high rates of N_2 fixation.

The biological nitrogen fixation may also enhance plant growth under extreme conditions of soil, even if not by increased N_2 fixation. Contamination of some elements, for example, arsenic (As) of natural resources is a global environmental problem. Arsenic contaminated ground water has been reported in over 20 countries including Bangladesh, India, China, and the USA (Rahman et al. 2006). In conclusion, the ability of the soybean *B. japonicum* symbiosis to form root nodules, and thus the ability to fix atmospheric N, is not tolerant of excess As. This appears to be due to a reduction in root hairs at high As concentrations resulting in a reduction of infection sites rather than the sensitivity of *B. japonicum* to elevated As in the growing medium. However, *B. japonicum* had a positive effect on plant biomass production that was maintained at the highest As concentration tested possibly via the production of growth-promoting hormones. The production of auxin, a plant growth hormone, by rhizobia has been widely quoted in the literature (Boeiro et al. 2007). According to Lambrecht et al. (2000), many species of rhizobia produce IAA, and many studies indicate that changes in the concentration of endogenous auxin are a prerequisite for nodule organogenesis. Besides the production of IAA, the production of gibberellic acid (GA) by strains of *Rhizobium* spp. has also been reported (Patten and Glick 1996). Boeiro et al. (2007) report for the first time the production of IAA, GA_3, zeatin, ethylene, and abscisic acid (ABA) in strains of *B. japonicum* using quantitative methods. The strains exhibited different capabilities to produce phytohormone, and this fact may have important implications for the formulation of inoculants. The potential use of rhizobial bacteria to mitigate the negative effects of excess As (and possibly other heavy metals) on biomass production of plants is currently being further investigated (Reichman 2007). Similarly, water deficiency is a major limiting factor of plant productivity and symbiotic nitrogen fixation, but some bacteria salt tolerant can help the plant growth. The bacterium *Ensifer meliloti* bv. *mediterranense* 4H41, salt tolerant, enhanced the growth and the grain yield of common bean under water deficiency, unlike strains of *Rhizobium tropici* CIAT 899 and *Rhizobium etli* (Mnasri et al. 2007).

Peat inoculants applied to the seed as a slurry (hereafter referred to as "peat slurry") is the most commonly used method to inoculate grain legumes with rhizobia (e.g., *Bradyrhizobium* spp., *Mesorhizobium* spp., *Rhizobium* spp.). A key to the effectiveness of peat inoculants is that sterilized inoculants can support high concentrations of rhizobia, generally 10^9–10^{10} cells g^{-1} peat at manufacture (Hartley et al. 2005). Granular inoculants have been widely used in North America, and detailed studies from this region highlight the potential benefits of their use

(Clayton et al. 2004; Gan et al. 2005). Granular inoculants provide a major advance in the flexibility of inoculants application, particularly for large commercial farms. Overall, these studies demonstrate that certain granule types have the potential to be used in Australia with grain legumes, particularly in circumstances when seed-applied inoculants are problematic, such as where seed fungicides or insecticides need to be applied. However, granular inoculants formulations differ substantially in their potential to produce nodules on a range of grain legumes (Denton et al. 2009).

17.3 Nitrogen Fixation in Graminous Plant

With the exception of legumes and actinorhizal plants, no other plant family, in association with bacteria, it has the ability to form nodular structures, which may be a biological nitrogen fixation. Nevertheless, with the use of different methodologies, the existence of high levels of nitrogen fixation in grasses of economic importance (Table 17.3), such as sugarcane, rice, and others has been demonstrated. It is not known what exact microorganisms are responsible for this setting although several diazotrophic endophytes have been isolated. The nitrogen fixation by *Gluconacetobacter diazotrophicus* has been rarely proved. It is impossible to say with certainty whether the effects are sometimes observed result of N_2 fixation associated with plants, or to factors such as hormonal effects, and increased uptake of soil N, due to increased root growth, induced by bacteria. However, it is argued that these microorganisms have an advantage over the associative diazotrophic root since they occupy space more closely linked to the host and therefore have greater access to carbon sources. Furthermore, they colonize niches protected from oxygen, which is necessary for the expression and activity of nitrogenase (Dobbelare et al. 2003). From the standpoint of biotechnology, bacteria that have more than one characteristic to promote growth, for example, nitrogen fixation, to produce growth hormones, among others, have greater potential for application in the field, aiming to increase agricultural production (Verma et al. 2001). However, the effect of inoculation with *Azospirillum*, although widely studied, the results are inconclusive.

Table 17.3 Species of nitrogen-fixing bacteria isolated from gramineous plants

Species	Host	Source
Azospirillum brasilense	Rice, sugarcane, wheat	Tarrand et al. (1978), Tejera et al. (2005)
Azospirillum oryzae	Rice	Xie and Yokota (2005)
Azotobacter chroococcum	Sugarcane	Tejera et al. (2005)
Azospirillum melinis	*Melinis minutiflora*	Peng et al. (2006)
Burkholderia tropica	Corn	Reis et al. (2004)
Burkholderia unamae	Corn, sugarcane	Caballero-Mellado et al. (2004)
Burkholderia silvatlantica	Corn, sugarcane	Perin et al. (2006)
Sphingomonas azotifigens	Rice	Xie and Yokota (2006)

Wide variety of diazotrophic and nondiazotrophic bacteria has been isolated from the rhizosphere of sugarcane and endorhizosphere using culture media free of N. Although *Acetobacter diazotrophicus* and rhizosphere bacteria appear to be isolated in the greatest number, it is impossible to attribute the N_2 fixation in this plant to any specific bacteria since there is no known correlation between number of diazotrophic bacteria and their ability to fix N_2 (James and Olivares 1998). The N_2 fixation by sugarcane depends on optimal concentrations of water supply and availability of P, K, and micronutrients, mainly molybdenum (Oliveira et al. 2006, Urquiaga et al. 1992).

Recently, Embrapa Agrobiology in Rio de Janeiro launched the inoculant containing diazotrophic bacteria for use in sugarcane. The inoculant is a mixture of strains of five species (*Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans*, *Azospirillum amazonense*, and *Burkholderia tropica*). The use of this technology has the main impact on the substitution of nitrogen fertilizer application in the first year of cultivation of sugarcane.

17.4 Nitrogen Fixation and Plant Growth Promotion

Numerous data show that nitrogen fixation in bacteria naturally occurring in soil is usually affected by interactions with plant and other microbes. Several microorganisms are considered to be PGPR (plant-growth-promoting Rhizobacteria) but the mechanisms of the growth promotion are diverse, for example, phosphate solubilization and antibiotic production to antagonize plant pathogens.

Although several studies demonstrate the benefits of co-inoculation of legumes with rhizobia and some endophytic bacteria, little is known about the mechanisms that promote the increase of nitrogen fixation. Bai et al. (2002) demonstrated that three bacteria isolated from the surface disinfested from soybean nodules when inoculated together with rhizobia increased nodulation and plant weight compared to those receiving just rhizobia. These bacteria were classified as *Bacillus* species (*Bacillus thuringiensis* and *Bacillus subtilis*) and were unable to form nodules or increase the growth of soybeans when inoculated alone. These bacterial isolates could be formed endospores and adaptable to the formulation of inoculants for applying manure to the field. Some rhizobial and *Azospirillum* strains are more effective and competitive when introduced to the rhizosphere together with other bacteria and fungi (Burdman et al. 1998). The inoculation with the strain *Burkholderia cepacia* SAOCV2 promotes the growth of common bean by several mechanisms that include P mobilization, antagonism toward pathogenic species of *Fusarium* and, indirectly, increase in nodulation that may lead to an increase in N_2 fixation (Peix et al. 2000).

The positive effect of combined inoculation of some endophytic bacteria with *Rhizobium* spp. can be attributed to an early nodulation, an increase in the number of nodules, or a general improvement in root development. The PGPR which

increases the efficiency of the symbiotic process in a legume not necessarily what it does in other legumes. The strain *Bacillus* sp. CECT 450 although it increased nodulation in bean (*Phaseolus vulgaris* L.), when co-inoculated with *Rhizobium tropici* CIAT 899, they have reduced nodulation in soybean when co-inoculated with *Bradyrhizobium japonicum* USDA 110. The strain of *Bacillus* sp. CECT 450 did not affect growth of bean when inoculated alone, suggesting that its action is mediated in the plant by *Rhizobium* (Camacho et al. 2001). In chickpea (*Cicer arietinum* L.), the beneficial effect of co-inoculation of seeds in the process of fixing atmospheric nitrogen was also evident (Goel et al. 2002). In this case, the author used strains MRS23 and CRP55b (*Pseudomonas* and *Mesorhizobium* sp.). One hundred days after sowing, there were 100% more nodules in co-inoculated chickpea than in pants that received rhizobia only. Beneficial effects of legumes under stressful conditions has also been found, when using bacteria capable of producing deaminase of 1-aminocyclopropane-1-carboxylic acid (ACC), which reduces the production of ethylene. Under the action of the enzyme deaminase, ACC is converted into α-ketobutyrate and ammonia, preventing the formation of ethylene. Such fact has been observed with bean co-inoculated with *Pseudomonas putida* under conditions of low phosphorus. Direct evidence of the positive effect of rhizobial strains containing ACC deaminase activity in nodules of leguminous plants have already been obtained for alfafa (Ma et al. 2006).

17.5 Conclusion

Nitrogen fixation by endophytes has been often proved. However, it is argued that these microorganisms have an advantage over the associative diazotrophic root since they occupy space more closely linked to the host and therefore have greater access to sources of carbon. Furthermore, they colonize niches protected from oxygen, which is necessary for the expression and activity of nitrogenase. From the point of view of biotechnology, bacteria that have more than one characteristic for growth promotion, for example, fix atmospheric nitrogen, produce growth hormones, among others, have greater potential for application in the field, aiming to increase agricultural production.

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Chapter 18
Plant Probiotics in Phosphorus Nutrition in Crops, with Special Reference to Rice

Md. Tofazzal Islam and Md. Motaher Hossain

18.1 Introduction

Phosphorus (P) is the second most important and most frequently limiting macronutrient for plant growth. P makes up about 0.2% of a plant's dry weight (Schachtman et al. 1998). It is a component of the key macromolecules such as nucleic acids, phospholipids, and adenosine triphosphate (ATP) in the cells. Consequently, P is one of the important players in the process of photosynthesis, nutrient transport, and energy transfer. It is also involved in key enzymatic reactions in major metabolic and signaling pathways (Theodorou and Plaxton 1993). Therefore, adequate P nutrition is essential for proper growth and yield of any crops.

In soils, P exists in major four "pools," namely, solution, organic, inorganic, and microbial biomass P (Fig. 18.1) (Busman et al. 2009; Richardson and Simpson 2011). Soil P can also be classified into only two broad groups, namely, organic and inorganic P. Organic P comes from organic sources such as plant residues, manures, and microbial tissues. On the other hand, inorganic forms of soil P consist of apatite minerals (the original source of all P), complexes of iron, aluminum and calcium phosphates, and P adsorbed on clay particles. The solubility of these P compounds, as well as organic P, is extremely low, and only very small amounts of soil P are in solution at any one time.

A large portion of inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized soon after their application and becomes unavailable to plants (Dey 1988; Yadav and Dadarwal 1997). In acidic soils, P becomes insoluble by

M.T. Islam (✉)
Department of Biotechnology, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur 1706, Bangladesh
e-mail: tofazzalislam@yahoo.com

M.M. Hossain
Department of Plant Pathology, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur 1706, Bangladesh

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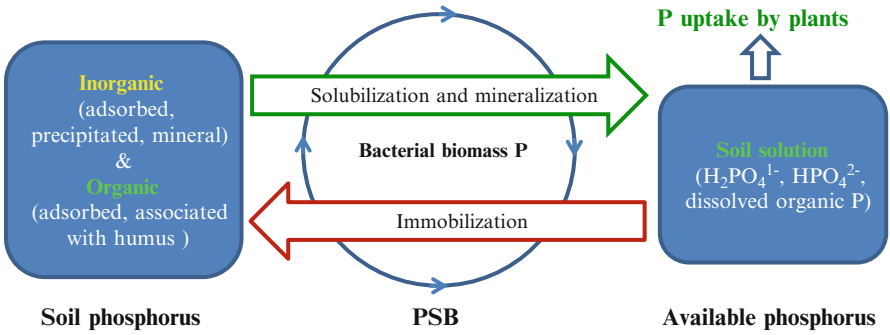

Fig. 18.1 A simplified schematic representation of the role of phosphate-solubilizing bacteria (PSB) to P availability in soil and uptake by plants (adapted from the scheme of Richardson and Simpson 2011)

reacting with iron and aluminum, whereas in alkaline condition, it reacts with calcium and becomes unavailable to the plants. Farmers are thus asked to apply P fertilizers in severalfold excess in order to overcome this problem. This adversely affects both the environment (e.g., eutrophication) and economy (Elser and Bennette 2011). Therefore, the release of insoluble and fixed forms of P is an important aspect of increasing soil P availability and production of crops.

Rice is the most important food crop in the world. In the past 40 years, the productivity of rice has been increased in more magnitude than area of rice cultivation. This tremendous increase in productivity is due to the introduction of modern varieties along with the increased use of agrochemicals and new agriculture technologies (Brink 1997). There has been a steady increase in the use of P fertilizers in rice production (Syers et al. 2008). The only source of raw materials for phosphate fertilizers is rock phosphate (Tilman 1998). Global demand for rock phosphate is currently around 148 million tons per year (Smil 2000a, b; Gunther 2005), and the demand for P is predicted to increase by 50–100% by 2050 (Steen 1998; EFMA 2000). However, mineable resources of P are limited, and hence, recycling programs of P are urgently needed (Elser and Bennette 2011). If the current rate of extraction continues, the global commercial phosphate reserves will be depleted by 50–100 years (Runge-Metzger 1995; Steen 1998; EcoSanRes 2003). A significant reduction in the use of phosphate fertilizers could be achieved if solubilization of the large reservoir of soil-insoluble P in tropical and subtropical soils is made available to the crop plants (Rodriguez and Fraga 1999; Turner and Haygarth 2001; Vessey 2003; Thakuria et al. 2004).

To solubilize P in P-deficient soils, plants have evolved diverse array of strategies to uptake adequate P under P-limiting conditions, including modifications to root morphology, carbon metabolism, membrane structure; exudation of organic acids, protons, and enzymes; and association with mycorrhizal fungi, and harboring phosphate-solubilizing microorganisms including bacteria (Marx 2004; Begum and Islam 2005). However, rice is very poor in association of mycorrhizal fungi in flooding conditions and also secretion of organic acids (Lipton et al. 1987;

Begum et al. 2005). Therefore, inoculation of growth-promoting bacteria such as phosphate-solubilizing bacteria (PSB) seems to be an alternative approach for P nutrition in rice under P-deficient tropical soils (Thakuria et al. 2004; Islam et al. 2007a).

Probiotics are living microorganisms which when administered in adequate amounts confer a health benefits of the host (Joint Food and Agriculture Organization/World Health Organization Expert Consultation 2001; Hamilton-Miller et al. 2003). This term is commonly used for bacteria such as *Lactobacillus* spp., *Bifidobacterium* spp., etc., that passage through the gastrointestinal tract of animals and human and might prevent, or even cure, diarrhea (Haas and Defago 2005). Recently, the term "plant probiotics" has been used to describe plant-associated microorganisms, which enhance the growth of the host plants when applied in adequate amounts. Plant probiotics, which is known to enhance the solubilization of fixed soil P and applied phosphate fertilizer, resulting in better P nutrition in crop plants, are known as PSB (Yahya and Al-Azawi 1989; Abd-Alla 1994; Mehta and Nautiyal 2001). The ability of some soil bacteria to convert insoluble forms of phosphorus to an accessible form is an important trait in plant-growth-promoting bacteria (PGPB) for increasing plant yields (Islam et al. 2007a; Azziz et al. 2011).

The concept of microbial enhancement of P availability to plants is not new. Gerretsen (1948) first showed that pure cultures of soil bacteria could increase the P nutrition of plants under controlled conditions through solubilization of precipitated forms of calcium phosphates. Since that study, many examples of microbially mediated P mobilization and characterization of different microorganisms have been reported (Richardson 2001; Gyaneshwar et al. 2002; Khan et al. 2007, 2010; Harvey et al. 2009; Richardson et al. 2009; Zaidi et al. 2009). There has been a large body of literature describing potential uses of plant-associated bacteria as agents of P nutrition in plants. Increased solubilization of fixed soil phosphates and applied phosphates ensuring higher crop yield has been reported on inoculation of PSB including *Pseudomonas*, *Bacillus*, *Rhizobium*, *Micrococcus*, *Flavobacterium*, *Burkholderia*, *Achromobacter*, *Erwinia*, *Pantoea, Streptomyces, Acinetobacter,* and *Agrobacterium* (Rodriguez and Fraga 1999; Islam et al. 2007a, b; Trivedi and Sa 2008). How soil bacteria dissolve fixed phosphates and make them available to plant uptake was an interesting question for research since mid-twentieth century (Gerretsen 1948; Sperber 1957). The widely recognized mechanism of phosphate solubilization mediated by plant-associated bacteria include production of low-molecular-weight organic acids such as gluconic, oxalic, 2-ketogluconic, citric, succinic, lactic, and malic and/or secretion of hydrolytic enzymes (e.g., phosphatases, phytases) (Sahu and Jana 2000; Vessey 2003; Chen et al. 2006; Hill and Richardson 2006; Rodriguez et al. 2006; Vyas and Gulati 2009; Lim et al. 2007). Members of the bacterial genera such as *Pseudomonas*, *Bacillus, Streptomyces, Pantoea,* and *Burkholderia* have been reported among the most efficient PSB as well as important bioinoculants due to their multiple functions in improving soil nutrient status, secretion of plant growth regulators, and suppression of soilborne pathogens (Rodriguez and Fraga 1999;

Botelho and Mendonça-Hagler 2006; Vassilev et al. 2006; Gulati et al. 2008; Vyas et al. 2009; Vyas and Gulati 2009; Islam 2011; Islam and Hossain 2012).

The use of chemical P fertilizers is the best means to circumvent P deficiency in crop plants, but their use is always limited due to their spiraling cost and concern over environmental pollution (Zaidi et al. 2009). Therefore, to increase the availability of P and to reduce the use of chemical fertilizers, solubilization of insoluble P by plant-probiotic bacteria has been considered as an attractive alternative to expensive chemical phosphatic fertilizers. Despite their potential as low-input practical agents of plant nutrition, application of PSB has been hampered by inconsistent performance in the field tests, which is usually attributed to their rhizosphere competence (Islam et al. 2007a, b; Richardson and Simpson 2011). Therefore, better understanding on interactions of plant roots with these probiotic bacteria associated with enhanced P acquisition by crop plants is needed for increasing the efficiency of nutrient management in soil and also to promote eco-friendly low-input sustainable agriculture (Rengel and Marschner 2005; Vyas and Gulati 2009). Although some good reviews have recently been published on plant-growth-promoting microorganisms (Lugtenberg and Kamilova 2009) or soil-microorganism-mediating phosphorus availability (Richardson and Simpson 2011), however, none of them has focused on plant probiotics in phosphorus nutrition in major crop plants like rice. This chapter comprehensively reviews advances in research on plant probiotics capable of solubilizing soil insoluble P, their mechanism of action, and their potential use for biofertilization of P in crop plants. Convenient bioassay methods to screen PSB, development of commercial formulation, and application of PSB in the field for growth and yield of crop plants are also discussed, with special reference to rice.

18.2 Isolation, Screening, and Characterization of PSB

Diverse genera of phosphate-solubilizing bacteria inhabit in soil and plant rhizosphere. To better understand the ecological significance and role of these PSB in plant nutrition, isolation and characterization of these bacteria from diverse ecological niches have become an interesting area of research in agriculture.

18.2.1 Isolation of Bacteria and Screening PSB

Microorganisms are central to soil nutrient cycling. The rhizosphere is a rich niche for diverse microorganisms including bacteria. These microorganisms share photosynthates exuded from plant roots. Among them, the rhizosphere bacteria play a significant role in mediating the transformation of complex form of essential nutrient elements into more available form for rapid acquisition by the plants. Many bacteria have the ability to solubilize P in soil and make it available to growing

plants (Antoun et al. 1998). These PSB constitute less than 1% of total bacterial populations in the soils (Kucey 1983) and occur in both fertile and P-deficient soils (Oehl et al. 2001).

Literature survey reveals that PSB have been isolated from diverse environment including rhizoplane (Islam et al. 2004a, b, 2007a, b; Alam et al. 2008a, b; Afzal and Bano 2008) and rhizosphere (Thakuria et al. 2004; Naik et al. 2008) of rice; aerobic rice field (Panhwar et al. 2011a); rice straw compost (Hameeda et al. 2008); rhizosphere of wheat (Kumar et al. 2001), banana (Naik et al. 2008), maize (Oliveira et al. 2009), and associate with pepper plants (Canbolat et al. 2006); Moroccan phosphate-mining center (Hamdali et al. 2008); high-altitude rhizospheric soils (Selvakumar et al. 2011); Salado river basin of Argentina (Castagno et al. 2011); basidiomes of *Suillus grevillei* and tomato rhizosphere (Gamalero et al. 2004); soils in India (Sharma et al. 2007); rhizosphere, roots, and nodules of chickpea (Gull et al. 2004); pasture and waste land at low pH (Pal 1998); and rhizosphere of *Hippophae rhamnoides* (Vyas and Gulati 2009), subalpine Himalayan forest site (Pandey et al. 2006), and Quebec soils (Chabot et al. 1996). Table 18.1 shows a list of PSB strains and their sources of isolation and effects on plant and soils.

Isolation of bacteria from roots, rhizosphere, soils, and other environmental samples are done by using dilution plate or streak culture on suitable agar medium (Deora et al. 2006; Islam et al. 2007a). After isolation of bacteria, the potential of isolates as phosphate solubilizer is generally assessed by screening through precipitated phosphate agar assay. The precipitated phosphate agar assay is extensively used in the initial isolation and screening for PSB (Pikovskaya 1948; Lindsay 1979; Halder et al. 1991; Abd-Alla 1994; Wenzel et al. 1994; Pal 1998). Bacteria capable of solubilizing phosphate minerals are grown on an agar medium amended with insoluble phosphates (such as dicalcium, tricalcium, aluminum, or iron phosphate) as the only P source and produce a visible clear zone around their colonies. The production of a clear or halo zone on the plate is due to the Secretion of organic acids into the surrounding medium (Pikovskaya 1948). The persistence of their phosphate-solubilizing capacity is checked by successive subcultures in the same medium. The most efficient P-solubilizing strain, with the largest solubilization halo zone, is selected for further studies (Valverde et al. 2006).

Phosphate solubilization efficacy of bacteria is influenced greatly by medium composition and its pH. Therefore, various nutrient compositions in the agar medium are used to screen bacteria. For example, of the 443 fluorescent pseudomonad strains screened, only 80 strains (18%) produced phosphate solubilization on Pikovskaya's (PVK) agar medium by inducing clear zones around the bacterial colonies (Naik et al. 2008). Similarly, PSB colonies have been isolated by serial dilution of rice roots and loosely adhering soils directly on Pikovskaya's agar-containing tricalcium phosphate (TCP) as a P source (Thakuria et al. 2004). TCP agar was also used in screening strains of *Azotobacter chroococcum* (Kumar et al. 2001) and *Acinetobacter* sp. (Ogut et al. 2010) for phosphate solubilization. Chabot et al. (1996) qualitatively screened rhizobacteria isolated from soil for P solubilization in DCP plates by the observation of a distinct halo zone around the colonies.

Table 18.1 Some phosphate-solubilizing bacteria, their source, and their effects on plant growth

PSB strain	Source	Test crop	Effect on plant and soil	Reference
Bacillus spp. PSB9 and PSB16	Aerobic rice field	Aerobic rice	Enhance P uptake, plant biomass, plant height, and root morphology	Panhwar et al. (2011a)
			Enhance P solubilization, leaf chlorophyll content, photosynthesis rate, and root development	Panhwar et al. (2011b)
			Enhance plant biomass and root growth	Panhwar et al. (2011c)
Bacillus sp. RM-2	Rhizosphere of cowpea	Cowpea	Promote plant growth and yield	Nain et al. (2012)
Pantoea agglomerans	Rhizosphere of *Oryza rufipogon*	Rice (*Oryza sativa* L.)	Promote growth and dry weight	Zeng et al. (2012)
Streptomyces anthocysnicus Psd1 *Ps. aeroginosa* Psd5 *Pseudomonas pieketti* Psd6 and *Bacillus* sp. Psd7	Rhizosphere of rice	Rice	Increase grain yield	Thakuria et al. (2004)
Acinetobacter sp. BR-12 *Klebsiella* sp. BR-15 *Acinetobacter* sp. BR-25 *Enterobacter* sp. BR-26 *Microbacterium* sp. BRS-1 and *Pseudomonas* sp. RS-2	Rhizoplane of rice cv. BR29	Rice	Solubilize P from tricalcium phosphate (TCP)	
B. megaterium A12ag	–	Rice	Enhance seed germination, growth, and yield	Sapsirisopa et al. (2009)
B. firmus NCIM-2636	–	Rice	Increase grain and straw yield	Datta et al. (1982)
Staphylococcus epidermidis TP06 *Erwinia tasmaniensis* TP08 *Ps. aeruginosa* TP13 *Ps. aeruginosa* TP14 *Ps. aeruginosa* TP16 *B. subtilis* TP27 and *Ps. aeruginosa* TP373	–	Rice and bean	Enhance seed germination, shoot and root length, and biomass	Duarah et al. (2011)

Ps. striata	–	Rice	Increase yield and nutrient uptake	Kundu and Gaur (1984)
Pseudomonas sp. RM3M *Ps. denitrificans* PsD6 *Ps. rathonis* PsR47 *B. laevolacticus* BcL28 *B. amyloliquefaciens* BcA27 *Arthrobacter simplex* ArS43 and *Rhizobium meliloti* URM1	Rhizosphere of wheat and maize	Wheat, maize, and cotton	Enhance shoot and root length of wheat and maize; P-uptake of cotton	Egamberdiyeva et al. (2003)
Streptomyces griseus BH7 and YH1, *Micromonospora aurantiaca* KH7 *St. cavourensis* BH2	Moroccan phosphate-mining centers	Wheat	Increase biomass production and higher aerial growth of plant	Hamdali et al. (2008)
Azotobacter chroococcum P1, P4, P9, P11, P12, M14, M15, M22, M26, and M37	Wheat rhizosphere	Wheat	Increase plant height, root biomass, spike length, spikelet size, grain weight, grain yield, and straw yield	Kumar et al. (2001)
Ps. lurida strain M2RH3	High-altitude rhizosphere soil	Wheat	Enhance growth and nutrient uptake	Selvakumar et al. (2011)
Pseudomonas sp. strain 54RB	Rice	Wheat	Increase root and shoot weight, plant height, spike length, grain yield, seed P content, leaf protein, and leaf sugar content	Afzal and Bano (2008)
Acinetobacter sp. WR922	Rhizosphere of wheat	Wheat	Increase wheat P content and dry matter	Ogut et al. (2010)
Pseudomonas sp. BR2	Wheat rhizosphere	Wheat	Increase seedling emergence, help mycorrhization, and increase plant growth of wheat fertilized with TPR	Babana and Antoun (2005)
			Promote plant growth when applied with AM fungi	Babana and Antoun (2006)

(continued)

Table 18.1 (continued)

PSB strain	Source	Test crop	Effect on plant and soil	Reference
Bacillus M-13 *Bacillus* RC01	M-13 from pepper plants; RC01 from wheat rhizosphere	Maize	Increase available phosphate in soil and plant biomass	Canbolat et al. (2006)
Rhizobium leguminosarum bv. *phaseoli* strains P31 and R1	Soils	Maize and lettuce	Increase dry matter of maize and lettuce	Chabot et al. (1996)
Serratia marcescens EB 67 *Pseudomonas* sp. CDB 35	Rice straw compost	Maize	Increase plant biomass and grain yield	Hameeda et al. (2008)
Enterobacter agglomerans		Maize	Promote plant growth	Laheurte and Berthelin (1988)
Bacillus sp. B17 *Burkholderia* sp. B5	Maize rhizosphere	Maize	Increase P efficiency	Oliveira et al. (2009)
Ps. putida B0	Sub-alpine Himalayan forest site	Maize	Promote plant growth	Pandey et al. (2006)
Ps. trivialis BIHB 745, BIHB 747 *Ps. poae* BIHB 808, and *Pseudomonas spp*. BIHB 756	Rhizosphere of *Hippophae rhamnoides*	Maize	Increase plant growth (shoot and root) and NPK contents in plant tissues	Vyas and Gulati (2009)
Bacillus sp. PAS-2	Pasture and waste land of pH 4.8	Finger millet, maize, amaranth, buckwheat, and French bean	Increase vegetative and grain yield	Pal (1998)
Ps. petida accessions 9 and 41	-	Barley	Increase biological and grain yield	Mehrvarz et al. (2008)
Mesorhizobium mediterraneum PECA21	-	Barley and chickpea	Increase dry matter, phosphorus, nitrogen, potassium, calcium, and magnesium content	Peix et al. (2001)
Ac. calcoaceticus IH9, OCI 1	Rhizosphere of grasses	Barley and chickpea	Promote plant growth	Peix et al. (2009)
Bacillus sp. (M-13)	Pepper plants	Barley and sugar beet	Increase grain and biomass yields of barley and leaf, root and sugar yield of sugar beet	Sahin et al. (2004)

Brevibacillus reuszeri (P-solubilizing) *R. rubi* (both N_2-fixing and P solubilizing)	Rhizosphere of tea	Broccoli	Increase yield, chlorophyll content, and uptake of macro- and micronutrients by broccoli	Yildirim et al. (2011)
PSB strains CPS-2, CPS-3, and Ca-18	Rhizosphere, roots, and nodules of chickpea	Chickpea	Increase shoot length, shoot dry weight, and nodulation	Gull et al. (2004)
Ps. fluorescens, B. megaterium	Soil	*Cicer arietinum*	Increase radicle and plumule length	Sharma et al. (2007)
Pseudomonas sp. PSB 5 with *M. ciceri* RC4 + *A. chroococuum* A10 + *Bacillus* sp. PSB9	–	Chickpea	Increase seed yield, grain protein, and P uptake	Wani et al. (2007)
Sinorhizobium meliloti strain RD64 (IAA over producer)	Engineered from *S. meliloti* strain 1021	*Medicago truncatula*	(1) Improve N_2 fixation and P mobilization (2) Increase dry matter, shoot, and root fresh weight	Bianco and Defez (2010)
B. subtilis strain SJ-101	–	Indian mustard plant	Plant growth promotion, and attenuation of soil Ni by biosorption and bioaccumulation	Zaidi et al. (2006)
Pantoea eucalypti	Salado River Basin (Argentina)	*Lotus tenuis*	Plant-growth promotion and solubilization of phosphates	Castagno et al. (2011)
Enterobacter sp., *B. subtilis*	–	Onion	Promote establishment of AM endophytes, increase biomass, N and P accumulation in plant tissues, and promote biogeochemical P cycling	Toro et al. (1997)
Ps. fluorescens 92rk and P190r	Strain 92rk from basidiomes of *Suillus*	Tomato	Increase shoot and root fresh weight and leaf P content alone	Gamalero et al. (2004)

(continued)

Table 18.1 (continued)

PSB strain	Source	Test crop	Effect on plant and soil	Reference
	grevillei and P190 from tomato rhizosphere		or in combination with AMF *G. mosseae*	
B. subtilis (TT_0), *B. circulans* (TT_8)	TT0 from Oat rhizosphere TT8 from Pigeon pea rhizosphere	Mung bean	(1) Increase P availability in alluvial soil; (2) increase root and shoot biomass, straw and grain yield, and P and N uptake	Gaind and Gaur (1991)
PSB strains T1, G2, and U1	Oxisol, Central Java in Indonesia	–	Enhance P solubilization from Ca_3PO_4 and $AlPO_4$ through secretion of organic acids	Prijambada et al. (2009)
Burkholderia tropicalis, Acidocella sp., *Acidiphilium* sp., *Acidovorax* sp., *Burkholderia* sp.	Rhizoplanes of plants tolerate to acid sulfate soils	Rice, *Melaleuca cajuputi, and Scleria sumatrensis*	Increase P solubilization from $AlPO_4$	Pengnoo et al. (2007)
Acinetobacter sp. CR 1.8	Rhizosphere of rice	–	Increase P solubilization from Ca_3PO_4 and $AlPO_4$	Chaiharn and Lumyong (2009)

However, organic P solubilization can be determined in the same basic media as DCP, but phytic acid replaced the DCP precipitate as the P source. Qualitative estimation of P solubilization by *Pseudomonas putida* B0 was conducted using Petri dish assays on Pikovskaya agar (Pandey et al. 2006). For isolating PSB from soil sample, Peix et al. (2001) inoculated serial dilutions of emulsified soil samples to Petri dishes with yeast extract agar supplemented with a 0.2% of TCP and considered the colonies surrounded by a clear zone after incubation for 7 days. To improve the clarity of the clear/halo zone, dyes such as bromophenol blue and alizarin red S are often used in the agar media (Cunningham and Kuiack 1992; Gupta et al. 1994). Valverde et al. (2006) isolated PSB by plating serial dilutions of soil samples in the medium, containing poorly soluble TCP and bromophenol blue, and subsequently by selecting colonies showing large solubilization halos.

The precipitated phosphate agar assay is an old method which has been used to assess P-solubilizing ability of PSB. It is a very fast and easy-to-use method and can be used to screen large number of isolates quickly and simultaneously. An alternate microbiological growth medium, National Botanical Research Institute's phosphate growth medium (NBRIP), which is comparable to PVK, was developed by Nautiyal (1999) for screening PSB. The NBRIP agar medium has currently been used by many researchers to qualitatively test the phosphate-solubilizing ability of the isolated bacteria. Islam et al. (2007a) isolated and purified a total of 30 bacterial strains from the rhizoplane of rice by repeated streak culture on NBA medium and initially tested for their phosphate-solubilizing activity by an agar assay using NBRIP medium supplemented with 1.5% Bacto agar (Fig. 18.2). Similarly, a total of 130 heterotrophic bacterial isolates showing different degrees of mineral

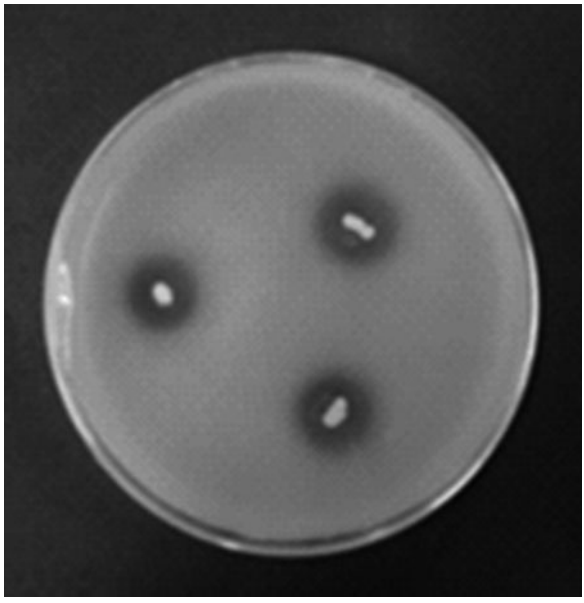

Fig. 18.2 Photograph showing solubilization of tricalcium phosphate (halo zones) by *Acinetobacter* sp. strain BR-25 isolated from the rhizoplane of rice cv. BRRI dhan 29

TCP-solubilizing activities were isolated from the thousands of bacteria through a screening program on NBRIP plates containing $Ca_3(PO_4)_2$ as a sole P source (Pérez et al. 2007). However, this method requires more time, labor, and chemicals, which is the limiting factor for a speedy qualitative screening of several thousands of bacteria. Therefore, a modification of the NBRIP medium was done by using BPB, a blue-colored dye that decolorizes owing to a drop in pH of the medium, as an indicator to quickly evaluate the phosphate solubilization based upon visual observations (Mehta and Nautiyal 2001). This method is also widely used as a reliable and qualitative indicator of the phosphate-solubilizing activity. For example, the phosphate-solubilizing activity of each of 36 strains isolated from the rhizosphere and rhizoplane of corn was evaluated in the NBRIP-BPB medium using tricalcium phosphate as the P source (Espinosa-Victoria et al. 2009). P-solubilizing ability of *Bacillus* sp. M-13 was demonstrated based on the qualitative methods on NBRIP-BPB medium (Sahin et al. 2004). Similarly, to confirm the observation on NBRIP medium, the 130 purified PSB isolates were further tested by Perez et al. (2007) on NBRIP-BPB medium.

18.2.2 Characterization and Molecular Identification of PSB

Polyphasic taxonomical studies, which include phenotypic, genetic, and phylogenetic information, have been widely used in microbial diversity studies (Vandamme et al. 1996). In order to determine the phenotypic diversity of phosphate-solubilizing bacteria, characterization was done on the basis of Gram stain, motility, fluorescence on King's B (KB) medium, presence of cytochrome oxidase, production of arginine dihydrolase, levan formation, gelatin liquefaction, nitrate reduction, growth at 4°C and 42°C, and carbon utilization profile (Naik et al. 2008). Similarly, the characterization of PSB strains such as *Pseudomonas* sp. RM3M, *Ps. denitrificans* PsD6, *Ps. rathonis* PsR47, *B. laevolacticus* BcL28, *B. amyloliquefaciens* BcA27, *Arthrobacter simplex* ArS43, and *Rhizobium meliloti* relied on Gram stain, morphology, spore formation, motility, nitrate reduction, and gas production from glucose (Egamberdiyeva et al. 2003). Sharma et al. (2007) characterized two PSB isolates as *Ps. fluorescens* and *B. megaterium* based on several cultural (colony size, surface, margin, elevation, optical features, pigment production, and growth in liquid medium), microscopic (Gram staining, cell shape, and endospore formation), and biochemical features (amylase, catalase, gelatinase, nitrate reduction, and caseinase test).

Additional functions of PSB can be assessed by analyzing some characteristics such as growth curve in Pikovskaya's agar, King's B agar, and Nfb semisolid medium, carbon source utilization, Indole acetic acid (IAA) production, intrinsic antibiotic resistance profile (IARP), nitrogenase activity, and in screening experiments for growth promotion of crop plants (Thakuria et al. 2004). Additional plant-growth-promoting effects of PSB *R. leguminosarum* bv. *phaseoli* strains P31 and R1 have been determined as P-solubilization, siderophore, and HCN and IAA

production (Chabot et al. 1996). However, morphological and biochemical tests have been found not enough for accurate identification of bacteria. Therefore, molecular techniques such as gene sequencing have been used as an acceptable method for the identification of bacteria including PSB (Eisen 1995).

Currently, 16S rRNA gene sequencing has been widely used as phylogenetic marker in microbial ecology of PSB (Ludwig et al. 1998; Chen et al. 2006; Selvakumar et al. 2011). It is a good tool for population fingerprinting, which allows identification of bacteria at genus or even at species level (Islam et al. 2005, 2007a). The 16S rRNA gene sequencing also shows great variability even in very closely related taxonomic groups (Nagpal et al. 1998; Islam et al. 2005). The extent of divergence in the sequence of this gene provides an estimate of the phylogenetic distance existing between different species (Igual et al. 2001). For example, on the basis of 16S rRNA-gene-sequencing phylogenetic analyses, Naik et al. (2008) identified their strains as *Ps. aeruginosa*, *Ps. mosselii*, *Ps. monteilii*, *Ps. plecoglossicida*, *Ps. putida*, *Ps. fulva*, and *Ps. fluorescens*. Ogut et al. (2010) determined the species identification of their PSB isolates as species of *Acinetobacter* (seven), *Pseudomonas* (seven), *Enterobacter* (four), *Enterococcus* (one), *Pantoea* (one), and *Bacillus* (one) genera by the analyses of 16S rRNA gene sequence data of the bacterial isolates. Similarly, Islam et al. (2007a) identified PSB isolates based on phenotypic and 16S rRNA-gene-sequencing data as *Acinetobacter* sp. BR-12, *Klebsiella* sp. BR-15, *Acinetobacter* sp. BR-25, *Enterobacter* sp. BR-26, *Microbacterium* sp. BRS-1, and *Pseudomonas* sp. BRS-2. Polyphasic taxonomical analyses including gene sequencing have been found useful for identification of new PSB species, such as *P. rhizospherae* (Peix et al. 2003), *P. lutea* (Peix et al. 2004), and *Microbacterium ulmi* (Rivas et al. 2004). Other molecular techniques including direct electrophoresis of amplified fragments by PCR, such as RAPD and BOX PCR, are of great use in biodiversity studies of the PSB (Chen et al. 2006; Naik et al. 2008), but at an intraspecific level (Peix and Martinez-Molina 2002).

18.3 Bioassay Methods for Quantitative Evaluation

Qualitative assay is a powerful tool to get initial answer of the question as whether the P-solubilizing trait is present or absent in the target bacteria. However, the information collected through qualitative method can be misleading and must be confirmed with quantitative bioassay method. Quantitative assay reflects the actual picture of P solubilization by PSB and are often applied for comparative analysis of P-solubilizing activity among the PSB selected from a preliminary screening. It is used to select the PSB with highest and persistence performance. A survey of the literature reveals that there are three main quantitative assays, which are commonly used in determining the P-solubilizing efficiency of bacteria and other microorganisms.

18.3.1 Agar Plate Assay

Phosphate-solubilizing capacity of a particular strain can be semiquantitatively determined in terms of phosphorus solubilization index (PSI) by using the following formula: PSI = A/B, where A is the total diameter of the halo zone and B is the colony diameter. Usually, the isolates showing PSI ≥ 2 is considered as PSB (Rahman et al. 2006; Islam et al. 2004a, b, 2007a, b). For example, phosphate solubilization efficiency (SE) of *Ps. fluorescens* and *B. megaterium* was determined to be 2 and 1.29, respectively, by measuring the halo surrounding the colonies of the respective bacterium on Pikovskaya medium (Sharma et al. 2007). Similar PSI based on colony diameter and halo zone was also determined for other PSB on Pikovskaya's medium (Alam et al. 2002; Ahemad and Khan 2010; Duarah et al. 2011). Kumar et al. (2001) determined the P-solubilization efficiency of *A. chroococcum* by measuring the clearance zones on Pikovskaya's and Jensen medium containing 0.125% tricalcium phosphate. The solubilization efficiencies of 130 purified PSB isolates were determined by spotting overnight-grown cultures on top of NBRIP plates supplemented with either $Ca_3(PO_4)_2$ or $FePO_4$ and by calculating the ratio between the diameter of the halo and the diameter of the colony after incubation (Perez et al. 2007).

P-solubilizing ability of *Bacillus* sp. M-13 was semiquantitatively determined on NIRBP-BPP medium under laboratory conditions (Sahin et al. 2004). Ogut et al. (2010) evaluated some phosphate solubilizers by comparing the PSI on TCP agar medium that contained 1% glucose, 0.05% yeast extract, 0.025% $MgSO_4.7H_2O$, 0.01% $CaCl_2$, and 0.5% TCP (pH = 7.8). Earlier, Peix et al. (2001) tested the ability to solubilize TCP of rhizobial isolates in Petri dishes containing yeast extract agar supplemented with a 0.2% of TCP by measuring the diameter of clearing zone surrounding the colonies of each strain. Islam et al. (2007a) estimated the PSI of the 30 rice isolates on NBRIP agar medium, which varied from 1.2 to 6.7. Among the isolates, the isolate BR-25 exhibited the highest PSI (6.7) followed by BR-15 (4.8), when TCP was used as a P source. Despite the popularity of the precipitated phosphate agar assay, the reliability of this halo-based technique has not yet been well established because some strains did not produce any visible halo zone on agar plates that were found capable of solubilizing various types of insoluble inorganic phosphates in liquid medium (Louw and Webley 1959; Gupta et al. 1994).

18.3.2 Liquid Broth Assay

In contrast to the halo-based semiquantitative plate assays, a direct measurement of phosphate solubilization in liquid media is considered more accurate (Nautiyal 1999; Bhadauria et al. 2000; Sangeeta and Nautiyal 2001; Harrris et al. 2006). Moreover, highly efficacious PSBs generally utilize the direct oxidation pathway to produce gluconic and 2-ketogluconic acids, which are necessary to dissolve poorly

soluble calcium phosphates such as TCP or hydroxyapatite (Rodriguez et al. 2006). The first step of this pathway is the oxidation of glucose to gluconic acid (GA) by the holoenzyme glucose dehydrogenase (GDH) (Liu et al. 1992). However, some bacteria lack GDH-dependent phosphate solubilization pathway and never produce visible clearing or halo zone in agar-medium-containing mineral phosphate. Therefore, quantitative assay for the mineral P solubilization in liquid culture by this type of bacteria is particularly very useful. In this assay, bacterial cells are grown in liquid medium for several days and are removed by centrifugation. Soluble phosphate released into the cell-free culture supernatant from the initial insoluble phosphate substrate is measured by molybdophosphoric acid colorimetric method (Jackson 1973). The rate of P solubilization is estimated by subtracting the final culture solution P from the un-inoculated control of P substrate (Rodriguez and Fraga 1999; Islam et al. 2007a, b). Various liquid culture media are used in this assay. For example, quantitative estimation of TCP solubilization by *Pseudomonas putida* B0 was carried out using Erlenmeyer flasks containing Pikovskaya broth inoculated with bacterial suspension (Pandey et al. 2006). The solubilization of insoluble phosphate was examined in cultures of *P. fluorescens* strains (MC07, B16, M45) and *B. megaterium* grown in the Pikovskaya medium supplemented with insoluble phosphate, $Ca_3(PO_4)_2$ or hydroxyapatite (Jeon et al. 2003). Determination of phosphate-solubilizing activity of PSB in Pikovskaya's broth was performed in many other studies (Pal 1998; Naik et al. 2008; Zaidi et al. 2006).

In plate assay, NBRIP medium was comparable to Pikovskaya's medium; however, in broth assay, NBRIP medium consistently resulted in a threefold increase in P solubilization (Nautiyal 1999). It has been suggested that soil microbes should be screened in NBRIP broth assay for the identification of the most efficient phosphate solubilizers (Nautiyal 1999). The growth of bacteria in broth culture is also dependent on pH of the medium. The growth of bacteria is inversely related to the change of pH in the culture medium (Fig. 18.3) (Islam et al. 2010). Quantitative estimation of phosphate solubilization by *Acinetobacter* sp. BR-12, *Klebsiella* sp. BR-15, *Acinetobacter* sp. BR-25, *Enterobacter* sp. BR-26, *Microbacterium* sp. BRS-1, and *Pseudomonas* sp. BRS-2 was carried out using Erlenmeyer flasks containing 100 ml of NBRIP broth medium inoculated with the bacteria at around 10^8–10^9 CFU/ml (Islam et al. 2007a). Optical density of culture medium revealed that the rice rhizoplane PSB exhibited almost similar and rapid growth at pH 5 and 7 and very slow or no growth at pH 3 (Fig. 18.3). The pH of the medium of all rhizoplane bacteria was decreased, corresponded with time, to pH 5 and 7 except for two rice seed endophytes. These results were reasonable because the bacteria were isolated from the rhizoplane of rice cultivated in a high land having the soil pH 6.7.

The phosphate-solubilization efficiency of each of the PSB isolate belonging to genera *Pantoea*, *Erwinia*, *Pseudomonas*, *Rhizobium*, and *Enterobacter* was determined in NBRIP liquid medium inoculated with each isolate (Castagno et al. 2011). The kinetics of $Ca_3(PO_4)_2$ solubilization mediated by different other PSB isolates were also monitored in liquid NBRIP medium (Perez et al. 2007; Vyas and Gulati 2009; Park et al. 2010; Viruel et al. 2011). Several other liquid media have also been used in studying P solubilization by PSB. For example, the P-solubilization

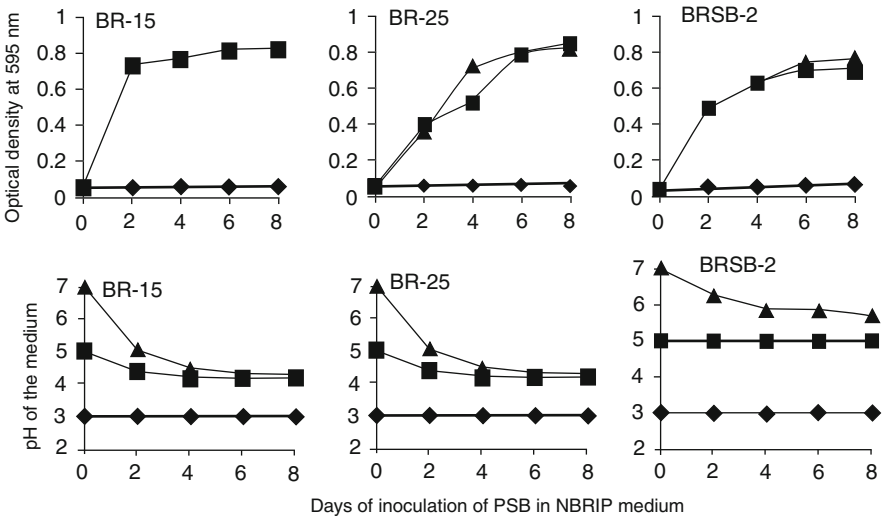

Fig. 18.3 Changes of the growth of some rice PSB represented by the optical density and the pH value of the culture medium with time inoculation in National Botanical Research Institute's phosphate (NBRIP) medium. BR-15, *Klebsiella* sp.; BR-25, *Acinetobacter* sp.; BRSB-2, *Pseudomonas* sp. (adapted from Islam et al. 2007)

efficiency of the six best PSB such as *B. brevis* strains, *B. megaterium*, *B. polymyxa*, *B. sphaericus*, *B. thuringiensis*, and *Xanthomonas maltophilia* were analyzed in RPAM (lacking agar and bromothymol blue) medium (Freitas et al. 1997). On the other hand, determination of mineral P-solubilization efficiency of *Acinetobacter* sp. strains was assessed in TCP broth (Ogut et al. 2010). Although the accuracy of liquid broth media is high however, they are obviously labor intensive and time consuming than that of agar media.

The bacterial phosphate-solubilizing capacity was influenced by specific root exudates of different rhizosphere of plant (Glick 1995). Some RP-solubilizing bacteria such as *Serratia marcescens* EB 67 and *Pseudomonas* sp. CDB 35 have been reported to be able to use root exudates or a broad range of carbon substrates in soil and supply P to plants in the rhizosphere (Hameeda et al. 2006). For this reason, Hamdali et al. (2008) determined the P-solubilizing activity of *St. griseus* BH7 and YH1, *Mi. aurantiaca* KH7, and *St. cavourensis* BH2 by estimating the amount of soluble P released from rock phosphate to a synthetic minimum liquid medium containing rock phosphate and wheat root exudates inoculated with each actinomycete strains.

18.3.3 Plant Assay

In plant assay, the influence of bacteria on plant P acquisition is measured as total P content in plant tissues, giving clue on the putative phosphate-solubilizing

efficiency of PSB in the rhizosphere (Pandey et al. 1998). Here, surface-sterilized seeds inoculated or uninoculated with PSB are sown into soil and allow the developing seedlings to grow for a certain period. Then the plant tissues are sampled, dried, wet digested, and analyzed for total P content (Gamalero et al. 2004). By using plant assay, Duarah et al. (2011) analyzed the effect of *Staphylococcus epidermidis* TP06, *Erwinia tasmaniensis* TP08, *Ps. aeruginosa* TP13, *Ps. aeruginosa* TP14, *Ps. aeruginosa* TP16, *B. subtilis* TP27, and *Ps. aeruginosa* TP373 on P uptake by rice and yard-long bean by estimating total P contents in the leaves of 30-day-old seedlings. The P-solubilizing efficiency of *Bacillus* sp. strains PSB9 and PSB16 was determined by measuring soil available P and P content in the rice plant tissue that were treated with bacterial inoculants (Panhwar et al. 2011b). Similarly, the influences of PSB on P contents in various plant tissues have been measured in tomato inoculated with *Ps. fluorescens* 92rk and P190r and/or the arbuscular mycorrhizal fungus (AMF) *Glomus mosseae* BEG12 (Gamalero et al. 2003); cotton inoculated with *R. meliloti* URM1 (Egamberdiyeva et al. 2003); lettuce inoculated with PSB *R. leguminosarum* bv. *phaseoli* strains P31 and R1 (Chabot et al. 1996); maize inoculated with *Serratia marcescens* EB 67, *Pseudomonas* sp. CDB 35, *P. trivialis* BIHB 745 and BIHB 747, *Ps. poae* BIHB 808, and *Pseudomonas* spp. BIHB 756 (Hameeda et al. 2008; Vyas and Gulati 2009); and chickpea co-inoculated with *P. jessenii* PS06 (a PSB) and *M. ciceri* (Valverde et al. 2006). A spermosphere model to study the ability of the actinomycete (A) strains to mobilize soluble phosphate from RP in the presence of the plant was used by Hamdali et al. (2008).

18.3.4 Formulation

In the past few decades, remarkable advancement on microbial phosphate solubilization research resulted in a good number of bacteria with high P-solubilizing potential. However, only a few of them have been formulated for practical use in agriculture. Figure 18.4 shows major steps involved in commercial development of PSB for crop production. Majority of the PSB is directly applied to the seed as cell suspension (Sharma et al. 2007; Duarah et al. 2011; Panhwar et al. 2011a, b). Formulation of these PSB is necessary to maintain live inoculant cells in a metabolically and physiologically competent state under field conditions. Formulation of PSB is currently carrier-based (Menaka and Alagwadi 2007). In preparation of carrier-based inoculants, solid carriers like peat, talc, vermiculite, perlite, lignite, etc., are used. Hameeda et al. (2008) developed a peat-based formulation for two PSB strains, *Se. marcescens* EB 67 and *Pseudomonas* sp. CDB 35, for plant growth studies in maize, but peat is sufficiently available in tropical countries (Arora et al. 2008). In preparation of these peat-based inoculants, neutralized peat was packed in high-density polyethylene (HDPE) bags, autoclaved, injected with 30 ml of bacterial cells harvested from mid-log phase culture, covered with a label at the injecting point, thoroughly kneaded to ensure uniform adsorption of the bacterial cells into

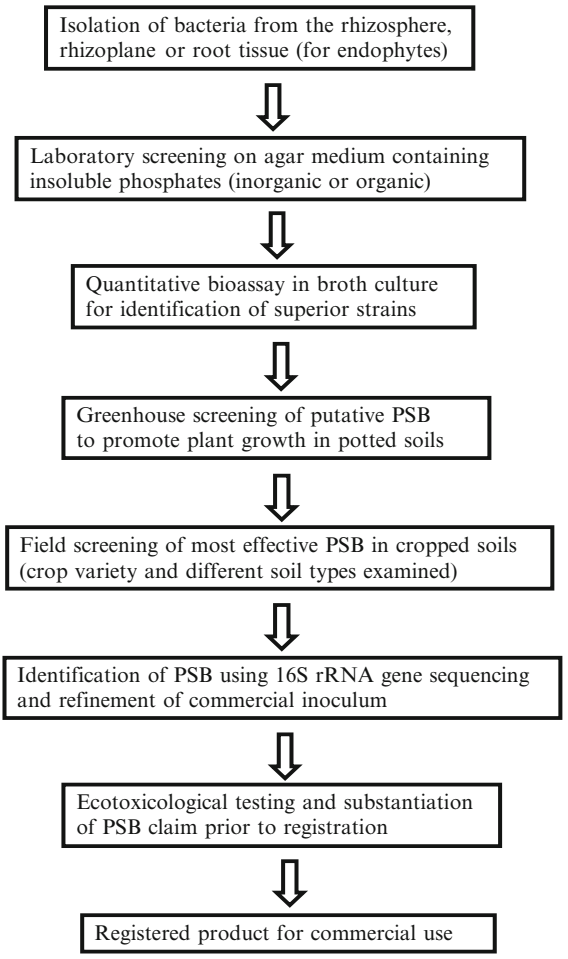

Fig. 18.4 Major steps involved in commercial development of PSB for crop production

the carrier material, and incubated at $30 \pm 1°C$ for a period of 10 days. *Se. marcescens* EB 67 and *Pseudomonas* sp. CDB 35 inoculated in peat had good shelf life up to 180 DAI. Liquid inoculants have also been used and becoming popular. Sridhar et al. (2004) developed a liquid inoculant using osmo/cell protectants for phosphate-solubilizing *B. megaterium*, which supported a higher viable population up to a storage period of 6 months. Liquid formulation of *B. megaterium* containing osmoprotectants, viz., polyvinyl pyrrolidone (PVP) and high quantity of glycerol (12 ml L^{-1}) and glucose, supported higher viable population up to a storage period of 4 weeks at 48°C ($\log_{10}$ 10.62 CFU ml^{-1}) and desiccation stress ($\log_{10}$ 10.04) and also enhanced the P-uptake of cowpea plants significantly (Velineni and Brahmaprakash 2011). Use of cheaper sources like low-grade rock phosphate (RP) in developing P-solubilizing biofertilizer has been found economical. These formulations have the ability of releasing the native soil P as

well as increasing the availability of P from the added RP, thereby increasing growth and yield of different crops (Khan et al. 2007, 2010; Nagaraju et al. 1995; Zaidi et al. 2003; Gull et al. 2004; Hamdali et al. 2008).

Evidence also support the potential use of mixed inoculants-based formulation in improving nutrient uptake and plant growth compared to single strains of bacteria. The combined inoculation of *Rhizobium* and PSB has increased nodulation, growth, and yield parameters in chickpea (Alagawadi and Gaur 1992; Gupta and Namdeo 1997; Jain et al. 1999; Kloepper and Schroth 1981). Co-inoculation of *Pseudomonads* and AMF synergistically increased plant growth compared with singly inoculated plants (Gamalero et al. 2004). In contrast, dual inoculation of N_2-fixing *Bacillus* OSU-140 and P-solubilizing *Bacillus* M-13 did not always significantly increase leaf, root, and sugar yield of sugar beet and grain and biomass yield of barley compared to single applications both with N_2-fixing bacteria (Sahin et al. 2004). The beneficial effects of the bacteria on plant growth varied significantly depending on environmental conditions, bacterial strains, plant species, and soils.

18.4 Effect of PSB on Plant P Nutrition

The solubilization of insoluble phosphate in the rhizosphere is the most common mode of action involved in PSB that enhance nutrient availability to plants (Rodriguez and Fraga 1999; Richardson 2001). Application of PSB alone or in combination with low rate of P fertilizers has been shown to significantly increase P availability in soils as well as high P uptake by major crop plants such as rice, wheat, maize, and beans (Alam et al. 2008a; Ogut et al. 2010; Panhwar et al. 2011a). For example, application of *Bacillus* spp. strains PSB9 and PSB16 alone or in combination with varying doses of P fertilizers significantly increased soluble P and plant P uptake in aerobic rice genotype (Panhwar et al. 2011a). Inoculation with *Acinetobacter* sp. WR922 significantly increased wheat P content by 27% at 15 days after emergence (DAE) compared to the control. The plant P content in inoculated plants at 30 DAE was linearly correlated with soluble P of the bacterial cultures (Ogut et al. 2010).Tomato plants treated with *Ps. fluorescens* 92rk and P190r alone or in combination with *G. mosseae* BEG12 showed significantly higher P content in the leaf compared to controls, respectively. The highest leaf P content was recorded in plants co-inoculated with all three microorganisms (Gamalero et al. 2004). There are numerous examples in which PSB solubilize complex insoluble forms of soil P into plant available forms and enhances the ability of plants to uptake more P (Raj et al. 1981; Toro et al. 1997; Egamberdiyeva et al. 2003; Wani et al. 2007; Afzal and Bano 2008; Oliveira et al. 2009; Vyas and Gulati 2009; Yildirim et al. 2011).

The use of PSB bioinoculants has been shown to reduce the application of P fertilizer significantly in low P soil. Sundara et al. (2002) reported that the application of the PSB *B. megaterium* reduced the required P dosage by 25% for sugarcane.

Young et al. (2003) reported that when different PSB strains were applied with 50% of required chemical fertilizer on lettuce (*Lactuca sativa*), there was a 25% growth increase as compared with only chemical fertilizer treatments, while Kumar et al. (2010) observed that growth and yield of *Cajanus cajan* (L) var. Manak by bacterial combinations amended with chemical fertilizer. There are more evidences that the PSB inoculation with mineral P also increases the efficiency of P fertilizer and decreases about 25–50% of the required P to plants (Attia et al. 2009; Yildirim et al. 2011).

18.4.1 Effect of PSB on Other Nutrient Uptake

A large body of literature suggest that inoculation of some PSB not only enhance P nutrition in plants but also increase uptake of a few other essential nutrient elements such as N and K by crop plants (Gaind and Gaur 1991; Toro et al. 1997; Vyas and Gulati 2009; Yildirim et al. 2011). For example, Vyas and Gulati (2009) demonstrated that treatments of maize with phosphate-solubilizing *Ps. trivialis* strains BIHB 745, BIHB 747, *Pseudomonas* sp. BIHPB 756, and *Ps. poae* BIHB 808 significantly increased total N, P, and K contents in plant tissues over single superphosphate. Moreover, PSB application also influences pH, organic matter, N, P, and K contents in the soils. Yildirim et al. (2011) reported that application of P-solubilizing and N_2-fixing bacterial inoculations with manure significantly increased macro- and micronutrients such as N, K, Ca, S, P, Mg, Fe, Mn, Zn, and Cu uptake by broccoli compared with control (without bacterial inoculants). The use of the N_2-fixing and P-solubilizing bacteria in chickpea (Elkoca et al. 2008), barley (Cakmakci et al. 2007), tomato (Adesemoye et al. 2010), lettuce (Lai et al. 2008), and strawberry (Gunes et al. 2009) also stimulated macro- and micronutrient uptake such as N, P, K, Ca, Mg, Fe, Mn, Zn, and Cu by the plants. Duarah et al. (2011) showed that a reduced application of NPK with PSB can give a better result in NPK use efficiency (Gyaneshwar et al. 2002; Kennedy et al. 2004).

18.4.2 Seed Germination

Seed bacterization with PSB enhances seed germination of plants (Mishra et al. 2009; Sharma et al. 2007; Sapsirisopa et al. 2009; Duarah et al. 2011). For example, Duarah et al. (2011) have shown that the treatments with PSB alone or in the form of consortia of compatible strains with or without the external application of chemical NPK gave more germination index (G. I.) from 2.5 to 5 in rice and 2.7 to 4.8 in bean seeds as compared to the control. During seed germination, amylase plays an important role in hydrolyzing the endosperm starch into sugars, which provide energy for the growth of roots and shoots (Kaneko et al. 2002). The induction of α-amylase also leads to the biosynthesis of phytohormones such as

gibberellins that stimulates the plant growth (Fincher 1989; Kaneko et al. 2002). The induction of amylase activity during germination has been found to increase through the inoculation of PSB with or without the externally applied NPK fertilizer, which helps in seed germination and thereby the better growth of the plant. Further, the amylase activity in seeds and leaves of rice and yard-long bean was found to be significantly higher in treatments containing the PSB than that of the control and the NPK alone. It was also observed that the amylase activity in the seeds ($58.4 \pm 2.2 - 94.4 \pm 3.0$ μg g^{-1} h^{-1}) were much higher than that in the leaves which was recorded from 2.20 ± 1.1 to 6.5 ± 1.9 μg g^{-1} h^{-1} (Duarah et al. 2011). It is because of the more starch content in seed than the leaf which triggers the seed germination. Naik et al. (2008) reported that 16% of phosphate-solubilizing pseudomonad strains produce aminocyclopropane-1-carboxylate (ACC) deaminase. It is reported that the ACC-deaminase-producing bacteria increase root elongation and seed germination by lowering plant ethylene levels (Glick et al. 1995; Belimov et al. 2001).

In addition to enhancement of nutrient uptake and stimulation of growth, inoculation of PSB has been reported to increase resistance of plants against abiotic stresses such as salinity of soils. Generally, increasing salinity impairs seed germination by interfering essential nutrient uptake and direct toxicity effects of salt ions (Flowers and Flowers 2005). Sapsirisopa et al. (2009) demonstrated that rice seeds inoculated by a salt tolerant *B. megaterium* A12ag significantly induce germination over other inoculated and non-inoculated treatments at EC 10 dS/m. Although the exact mechanism of salt tolerance has not been known, however, the increase in seed germination might be a result of bacterial activity in favor of better nutrient uptake by plants. Further studies are needed to understand how PSB inoculants enhance salt tolerance in the treated plants (Haq-Ikram-ul et al. 2005).

18.4.3 Growth and Development

Enhanced growth and development of plant by the application of PSB has been reported in numerous research articles. Panhwar et al. (2011a, b, 2011c) demonstrated that inoculation of PSB *Bacillus* spp. PSB9 and PSB16 significantly increased leaf area index, tiller numbers, plant height, leaf chlorophyll content, photosynthesis rate, root morphology, and plant biomass of aerobic rice genotypes. Similarly, the root length, shoot length, and the total biomass of rice and yard-long bean were significantly increased by the application of PSB (Duarah et al. 2011). In all the PSB-treated yard-long bean, an appreciably higher amounts of fresh weight and dry weight were also recorded compared to the control. Hameeda et al. (2008) showed that bacterial strains with phosphate-solubilizing ability and other plant-growth-promoting traits increased 20–40% of plant biomass. Increase in plant biomass (dry weight) was 99% with *Serratia marcescens* EB 67, and 94% with *Pseudomonas* sp. CDB 35 was recorded under glasshouse conditions. However, increase in plant biomass at 48 and 96 days after sowing was 66% and 50% with EB

67, and 51% and 18% with CDB 35 was found under field conditions. Similarly, the PSB treatments with *Ps. trivialis* BIHB 745, *Ps. trivialis* BIHB 747, *Pseudomonas* sp. BIHB 756, and *Ps. poae* BIHB 808 resulted in significantly higher at par growth parameters such as plant height, root length, shoot dry weight, and root dry weight over single superphosphate treatment in maize (Vyas and Gulati 2009).

The increased plant growth have also been reported by PSB inoculations in numerous crop plants such as *B. megaterium* in rice (Sapsirisopa et al. 2009); *Az. chroococcum* (Kumar et al. 2001), *Acinetobacter* sp. (Ogut et al. 2010), and *Pseudomonas* sp. (Babana and Antoun 2005, 2006) in wheat; *Bacillus* sp. (Canbolat et al. 2006), *Pseudomonas* sp., *Se. marcescens* (Hameeda et al. 2008), *En. agglomerans* (Laheurte and Berthelin 1988), and *Ps. putida* (Pandey et al. 2006) in maize; *Me. mediterraneum* PECA21 in barley and chickpea (Peix et al. 2001); *Enterobacter* sp. and *B. subtilis* in onion (Toro et al. 1997); *Brevibacillus reuszeri* and *R. rubi* in broccoli (Yildirim et al. 2011); and *Ps. fluorescens* in tomato (Gamalero et al. 2004) (Table 18.1). The significantly higher plant growth with some PSB treatments over NPK might be due to the immobilization of applied P by native soil microbiota and physicochemical reactions in the soil (Vyas and Gulati 2009). Despite the enhancement of plant growth, by PSB has been reported in many papers, little is known about the underlying molecular mechanisms of these biofunctional probiotic bacteria. Apart from P solubilization, some of these bacterial inoculants also have ability to fix atmospheric nitrogen (Dey et al. 2004) and/or produce various phytohormones (Kloepper et al. 1989) to promote the growth of inoculated plants.

18.4.4 Yield and Quality

Application of PSB has been found to increase yield and quality of many crops such as rice, wheat, maize, potato, and beans. Generally, 5–30% yield increase has been recorded in various crops by PSB inoculation either through seed bacterization or soil application (Datta et al. 1982). The grain and straw yields of rice increased appreciably due to inoculation of *B. firmus* NCIM-2636 and *Ps. striata*, which were further increased with addition of chemical fertilizers (Datta et al. 1982; Kundu and Gaur 1984). The PSB, *Ps. pieketti* Psd6, increased rice grain yield by 76.9% over the uninoculated control in microplot experiments (Thakuria et al. 2004). Seed treatment with *Se. marcescens* EB 67 and *Pseudomonas* sp. CDB 35 increased the grain yield of field-grown maize by 85% and 64%, respectively, compared to the uninoculated control (Hameeda et al. 2008). Phosphate-solubilizing and phytohormone-producing *Az. chroococcum* enhanced grain and straw yield of wheat (Kumar et al. 2001). Inoculation with PSB *Bacillus* sp. increased yields only by 7.5% and 5.5% in sugar beet and barley, respectively (Sahin et al. 2004).

Quality parameters were also affected by the applications of PSB. In the plots receiving bacterial inoculations, white sugar contents of sugar beet were higher compared to the plots receiving N fertilizer application (Sahin et al. 2004). There is

concern within the sugar industry that too much nitrogen is currently being applied to the beet crop. Excessive use of N reduces the quality of the harvested beet that makes the crop less profitable for both grower and processor. *Stevia rebaudiana* produces two predominant diterpene glycosides, stevioside and rebaudioside A, which are 40–300 times sweeter than sucrose. Mamta et al. (2009) isolated four PSB strains, namely, *Burkholderia gladioli* 10216 and 10217, *En. aerogenes* 10208, and *Se. marcescens* 10238 from the rhizosphere of *Ste. rebaudiana*. Application of these PSB strains alone or in combination not only enhanced the growth of plants but also significantly increased (150–555%) the contents of both stevioside and rebaudioside A in the leaves than that of uninoculated plants. These results indicate that PSB application can influence both yield and quality of the crop plants. Further studies are needed to unravel how PSB inoculation increases the contents of these secondary metabolites in the leaves of *Ste. rebaudiana* plants.

18.5 Mechanism of Phosphate Solubilization and Plant Growth Promotion by Plant Probiotics

Phosphorus release from insoluble phosphates and plant growth promotion by plant probiotics has been attributed to several mechanisms. A number of theories have also been proposed to explain the mechanism of phosphate solubilization.

18.5.1 Organic Acid Secretion

A large body of literature suggests that the underlying molecular mechanism of mineral phosphate solubilization by PSB strains is associated with the production of low-molecular-weight organic acids (Table 18.2) (Goldstein 1995; Kim et al. 1997). The released organic acids chelate the cations bound to phosphate through their hydroxyl and carboxyl groups and thereby convert it into soluble forms (Kpomblekou and Tabatabai 1994). Organic acid production is reported to be involved during solubilization of inorganic phosphates by many efficient PSB such as *Acinetobacter* sp., *Bacillus* spp., *Burkholderia* sp., *Enterobacter* sp., *E. agglomerans*, *Klebsiella* sp., *Microbacterium* sp., *Pseudomonas* sp., *Ps. fluorescens*, *Ps. trivialis*, *Ps. poae*, *Pa. eucalypti*, *Serratia* sp., *Pantoea* sp., *Ralstonia* sp., and *Sinorhizobium meliloti* (Laheurte and Berthelin 1988; Goldstein 1995; Krishnaraj and Goldstein 2001; Chen et al. 2006; Islam et al. 2007a; Pérez et al. 2007; Trivedi and Sa 2008; Vyas and Gulati 2009; Bianco and Defez 2010; Ogut et al. 2010; Panhwar et al. 2011b; Castagno et al. 2011). The amount and type of the organic acid produced varied among different species even in the strains within a species.

Table 18.2 Phosphate-solubilizing bacteria and their mechanisms of solubilization of insoluble phosphorus

PSB strain	Crop	Mechanism of action	Reference
Acinetobacter sp. BR-12 *Klebsiella* sp. BR-15, *Acinetobacter* sp. BR-25, *Enterobacter* sp. BR-26, *Microbacterium* sp. BRS-1, and *Pseudomonas* sp. RS-2	Rice	Secrete organic acids	Islam et al. (2007)
Staphylococcus epidermidis TP06, *Erwinia tasmaniensis* TP08, *Ps. aeruginosa* TP13, *Ps. aeruginosa* TP14, *Ps. aeruginosa* TP16, *B. subtilis* TP27, and *Ps. aeruginosa* TP373	Rice and bean	Urease and phosphatase activities and induction of alpha-amylase activity	Duarah et al. (2011)
Bacillus spp. PSB9 and PSB16	Aerobic rice	Organic acids (oxalic, malic, succinic, and propionic)	Panhwar et al. (2011b)
		Colonize on the surface and interior of rice roots	Panhwar et al. (2011c)
St. anthocysnicus Psd1 *Ps. aeroginosa* Psd5, *Ps. pieketti* Psd6, and *Bacillus* sp. Psd7	Rice	IAA-like substances and nitrogenase activity	Thakuria et al. (2004)
St. griseus BH7 and YH1 *Micromonospora aurantiaca* KH7	Wheat	Produce IAA	Hamdali et al. (2008)
St. cavourensis BH2			
Acinetobacter sp. WR922	Wheat	Gluconic acid production	Ogut et al. (2010)
En. agglomerans	Maize	Organic acid production	Laheurte and Berthelin (1988)
Ps. trivialis BIHB 745, BIHB 747	Maize	Gluconic acid, oxalic acid, 2-ketogluconic acid, lactic acid, succinic acid, formic acid, citric acid, and malic acid secretion	Vyas and Gulati (2009)
Ps. poae BIHB 808 *Pseudomonas* spp. BIHB 756			
Si. meliloti strain RD64 (IAA over producer)	*Medicago truncatula*	(1) Overproduction of indole-3-acetic acid (2) Upregulation of genes coding for the high-affinity P transport system (3) Induction of acid phosphatase activity and increased secretion of malic, succinic, fumaric, and 2-hydroxyglutaric acid	Bianco and Defez (2010)

(continued)

Table 18.2 (continued)

PSB strain	Crop	Mechanism of action	Reference
B. subtilis strain SJ-101	Mustard plant	Production of indole acetic acid (IAA) and solubilization of inorganic phosphates	Zaidi et al. (2006)
Pa. eucalypti	*Lotus tenuis*	Secretion and oxidation of gluconic acid	Castagno et al. (2011)
Burkholderia sp., *Serratia* sp., *Ralstonia* sp., *Pantoea* sp.	–	Secretion of gluconic acid	Pérez et al. (2007)

Gluconic acid was the major organic acid which has been produced during phosphate solubilization by *Acinetobacter* sp. (Ogut et al. 2010), *Ps. trivialis*, *Ps. poae*, and *Pseudomonas* spp. (Vyas and Gulati 2009), *Pa. eucalypti* (Castagno et al. 2011), *Burkholderia* sp., *Serratia* sp., *Ralstonia* sp. and *Pantoea* sp. (Perez et al. 2007), *Azospirillum* spp. (Rodriguez et al. 2004), and *Citrobacter* sp. (Patel et al. 2008). The production of 2-ketogluconic, oxalic, malic, lactic, succinic, formic, propionic, and 2-hydroxyglutaric acid has been reported during phosphate solubilization by *Arthrobacter* sp., *Ar. ureafaciens*, *Bacillus* spp., *B. coagulans*, *B. megaterium*, *Chryseobacterium* sp., *Citrobacter koseri*, *Delftia* sp., *En. intermedium*, *Ps. fluorescens*, *Ps. trivialis*, *Ps. poae*, and *Pseudomonas* spp., *Rhodococcus erythropolis*, *Se. marcescens,* and *Sinorhizobium meliloti* (Illmer and Schiner 1992; Gyaneshwar et al. 1998; Hwangbo et al. 2003; Chen et al. 2006; Vyas and Gulati 2009; Trivedi 2008; Bianco and Defez 2010; Panhwar et al. 2011b). On the other hand, propionic acid was reported to produce by strains of *B. megaterium* (Chen et al. 2006) and *Bacillus* spp. (Panhwar et al. 2011b) during phosphate solubilization.

Vyas and Gulati (2009) demonstrated that the quantity of organic acids produced by *Pseudomonas* strains differed with the nature of the insoluble phosphates (substrates). However, they did not find any significant relationship between the quantity of organic acids produced and the solubilization of rock phosphates by *Pseudomonas* strains. Chen et al. (2006) identified three isolates which did not produce any kinds of organic acids but showed considerable inorganic P solubilization. Similar results were also obtained by several other investigators (Illmer and Schiner 1992; Chen et al. 2006). Therefore, release proton (H^+) from the PSB for P solubilization has been proposed (Illmer and Schinner 1995). However, besides organic acid and protons, there may be other nonorganic acid substances produced by PSB that are probably involved in P solubilization (Zhao et al. 2002). Exopolysaccharide (EPS) production by PSB has been found in many cases as a novel factor for microbial dissolution of inorganic such as *Enterobacter* sp. EnHy-401, *Arthrobacter* sp.ArHy-505, and *Azotobacter* sp. AzHy-510 (Yi et al. 2008). Interestingly, PSB that produce EPS have a stronger ability for P solubilization than those that have no ability to produce EPS. Addition of EPS into growth medium could increase the amount of P solubilized by organic acid, but failed to release phosphorus from TCP alone. The increase of P solubilization brought by EPS is

attributed to the participation of EPS led to the change in homeostasis of P solubilization, pushing it towards P dissolved by holding free phosphorus in the medium, consequently resulting in greater phosphorus released from insoluble phosphate.

18.5.2 Enzyme Activity

Mineralization of most organic phosphorus compounds is carried out by means of phosphatase enzymes. The major source of these enzymes in soil is considered to be of microbial origin. Acid phosphatase enzymes produced by PSB facilitate the hydrolysis of organic P esters and released larger amounts of organic acids, known to be highly effective in mobilizing P from insoluble sources (Dey et al. 2004). Availability of organic phosphate compounds for plant nutrition could be a limitation in some soils resulting from precipitation with soil particle ions (Rodriguez et al. 2006). Therefore, the activity of this enzyme in the rhizosphere is very crucial for plant nutrition. Duarah et al. (2011) reported that in case of the treatments with NPK fertilizer (50% or 100% dose), only a slight increment was seen for phosphatase activity; however, the increment was significantly higher when treated with PSB alone. Similarly, *Si. meliloti* RD64 induced higher levels of acid phosphatase enzymes than the untreated cells (Bianco and Defez 2010). Increase in phosphatase activity in PSB-treated soil is an indication of the increased soil fertility and improvement in the phosphate solubilization. Similarly, release of phytase from the bacteria is involved in solubilization phytates from the soils.

18.5.3 High Plant Colonization

Despite their potential as low-input practical agents of plant growth promotion, application of PSB has been hampered by inconsistent performance in field tests; this is usually attributed to their poor rhizosphere competence (Lugtenberg and Kamilova 2009; Richardson and Simpson 2011). Rhizosphere competence of any plant-growth-promoting bacteria comprises effective root colonization combined with the ability to survive and proliferate along growing plant roots over a considerable time period, in the presence of the indigenous microflora (Lugtenberg and Dekkers 1999; Islam et al. 2005, 2007a). Given the importance of rhizosphere competence as prerequisite of effective plant P nutrition, understanding root-PSB communication, as affected by genetic and environmental determinants in spatial and temporal contexts, will significantly contribute to improve the efficacy of these biofertilizer agents (Compant et al. 2005; Richardson and Simpson 2011).

The presence of flagella and fimbriae on bacterial cell is important for their motility, chemotaxis, and adherence on plants roots. The persistence and proliferation of an applied PSB in the rhizosphere of desired crop plant is critical for its

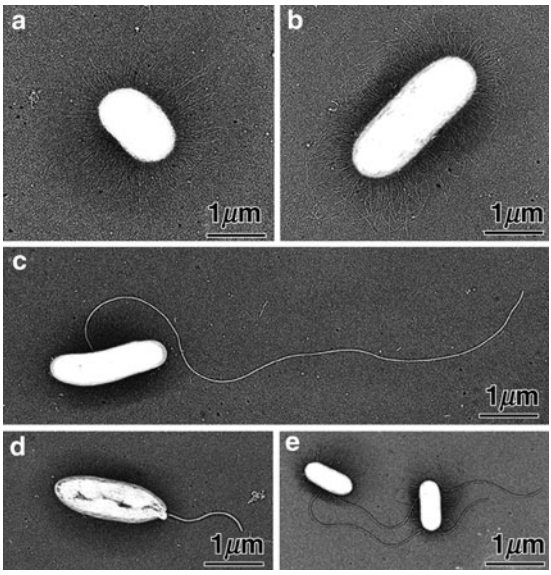

Fig. 18.5 Transmission electron micrographs showing morphological features of some PSB isolated from the rhizoplane of rice (Oryza sativa L.) cv. BRRIdhan 29. (**a**) *Acinetobacter* sp. BR-25; (**b**) *Klebsiella* sp. BR-15; (**c**) *Microbacterium* sp. BRS-1; (**d**) *Pseudomonas* sp. BRS-2; (**e**) *Enterobacter* sp. BR-26

efficacy of supplying soluble P to plant roots from the insoluble sources. Morphological features of PSB and their ability to colonize roots of seedlings previously inoculated with a potential strain can be studied by using transmission and scanning electron microscopy (TEM and SEM) (Islam et al. 2005, 2007a). Although a large volume of literature is available on in vitro screening of phosphate solubilization of bacteria on agar media, studies concerning the fate of applied PSB as seed inoculants in the rhizosphere are very limited. Islam et al. (2007a) investigated the morphology of various PSB isolated from the rhizoplane and surface-sterilized seeds of rice. TEM observation revealed that bacteria isolated from the rhizoplane of field-grown rice possessed dense fimbriae (Fig. 18.5a, b, e), but those isolated from the roots of rice seedlings grown from the surface-sterilized rice seeds (Fig. 18.5c, d) in gnotobiotic Hoagland's gel medium had no fimbriae (Fig. 18.5). Fimbriae are filamentous protein appendages on bacterial cell surface; their known function is adhesion to surfaces of the plants or other objects (Korhonen et al. 1986). Their observation suggests that rhizoplane bacteria generally possessed fimbriae on their cells, while the endophytes (Fig. 18.5c, d) may lack these hairy protein appendages as they remain inside the plant. However, a further study with high number of bacterial endophytes and epiphytes is needed to confirm this hypothesis.

To evaluate ability of *Acinetobacter* sp. BR-25 and *Klebsiella* sp. BR-15 to colonize rice roots, Islam et al. (2007a) inoculated surface-sterilized seeds of rice cv. BR12 with an aqueous suspension of bacterial cells (*ca.* 10^8 CFU/seed), followed by incubation in a test tube (18-cm long and 1.5-cm i.d.) containing one-fifth strength of Hoagland's solution with gellan gum. Using SEM, they observed that roots of seedlings grown from seeds, previously inoculated with

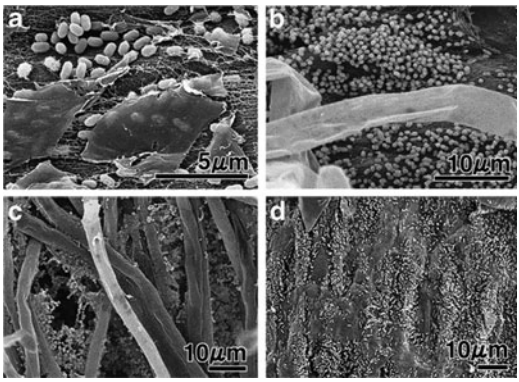

Fig. 18.6 Scanning electron micrographs showing dense colonization of *Acinetobacter* sp. BR-25 (**a**–**c**) and *Klebsiella* sp. BR-15 (**d**) on the surface of roots (cv. BR29) of rice seedlings from seeds previously inoculated with bacteria (Islam et al. 2007)

bacteria, vigorously colonized and attached in an unilaminar fashion (Fig. 18.6). Although bacterial biofilm was observed throughout the rice roots (Fig. 18.6a, b, d), higher density of microcolonies were always found at the root-hair zones (Fig. 18.6c); however, root hairs were almost free from the bacteria in both the cases. These results suggest that inoculation of rice seeds with P-solubilizing rhizoplane bacteria might be a useful way for improving P uptake by rice plants in Bangladesh as both tested bacteria showed high multiplication in the rhizoplane upon seed inoculation.

Colonization ability of an endophytic diazotrophic PSB, *Pa. agglomerans*, isolated from seeds of deep water rice has been investigated by histochemical analysis (Verma et al. 2001). The seedlings grown in hydroponics showed that the tagged (tagged with reporter gene *gus*A) strain colonized the root surface, root hairs, root cap, points of lateral root emergence, root cortex, and the stellar region. Interestingly, treatment of the roots with 2,4-D produced short, thickened lateral roots which showed better colonized by *P. agglomerans*.

18.5.4 Other Functions of PSB (Nitrogen Fixation, Secretion of Phytohormones, Etc.)

In concert with P solubilization, some strains of PSB exert multiple effects to promote plant growth and soil fertility. These effects may include nitrogen fixation, phytohormone production, urease activity, siderophore production, and/or antagonisms against phytopathogens (Thakuria et al. 2004; Zaidi et al. 2006; Hamdali et al. 2008; Duarah et al. 2011; Naik et al. 2008; Yildirim et al. 2011; Bianco and Defez 2010; Islam and Hossain 2012). For example, the positive effects of *R. rubi* on soil fertility and growth and yield of broccoli were found to be associated with N_2-fixation and phosphate solubilization (Yildirim et al. 2011).

The efficiency of PSB in biological nitrogen fixation has also been reported (Kucey et al. 1989; Gyaneshwar et al. 2002).

Urease activity in both rice-cultivated and yard-long bean-cultivated soil was being increased in treatments with PSB compared to NPK fertilizers (Duarah et al. 2011). Increase in urease activity in PSB-treated soil may be defined as the infrequent participation of PSB in the nitrogen fixation (Welch 1981). Production of indole-3-acetic acid (IAA) by PSB has been claimed in many cases such as *St. anthocysnicus*, *Ps. aeroginosa*, *Ps. pieketti*, and *Bacillus* sp. in rice (Thakuria et al. 2004); *Streptomyces griseus*, *Micromonospora aurantiaca*, and *St. cavourensis* in wheat (Hamdali et al. 2008); *Si. meliloti* in *Medicago tranctula* (Bianco and Defez 2010); and *B. subtilis* in mustard (Zaidi et al. 2006). The hormone IAA is known to have dual role in influencing plant growth, by involving in the biocontrol together with glutathione S-transferases in defense-related plant reactions, and inhibits the germination of spore and growth of mycelium of different pathogenic fungi (Brown and Hamilton 1993; Strittmatter 1994). Noel et al. (2001) showed that the IAA supply to excised potato leaves reduced the severity of the disease provoked by *Phytophthora infestans*. Hamdali et al. (2008) reported that two phosphate-solubilizing actinomycete, *St. griseus*-related strain (BH7) and *M. aurantiaca*-related strain (KH7), efficiently limit the growth of two phytopathogenic fungi, *Fusarium* sp. and *Pythium ultimum* on Petri dishes. Naik et al. (2008) found that phosphate-solubilizing fluorescent Pseudomonads showed production of plant-growth-promoting enzyme, hormone, siderophores, antibiotics, and antagonisms against phytopathogenic fungi of various crops. Such PSB strains with innate potential of producing an array of responses may play a vital role in plant growth promotion, disease suppression, and subsequent enhancement of yield.

18.6 Conclusion and Perspective

A wide variety of bacterial genera such as *Pseudomonas*, *Bacillus*, *Klebsiella*, etc., have been reported with phosphate-solubilizing activity, and some of them have shown high promise for P nutrition in crop plants through various mechanisms including secretion of organic acids to solubilize metal-bound phosphates and production of enzymes to degrade organic phosphates in soils. The production of organic acids by PSB is appeared to be independent of their genetic relatedness, and each strain has its own ability to produce organic acids during solubilization of inorganic phosphates (Vyas and Gulati 2009). Although, many bacterial strains show P-solubilizing activity in laboratory culture, a lower number are successful in a laboratory greenhouse, and a much lower number are functioning under practical field conditions (Lugtenberg and Kamilova 2009). In fact, our knowledge on bacterial quorum sensing and ongoing complex molecular cross talks within the rhizosphere after inoculation of a certain PSB is limited. Understanding the reasons for the failures in greenhouses and in the field conditions may lead to the isolation of improved strains. Application of plant probiotics such as PSB for improving P

nutrition in crop plants is strongly connected to our better understanding of bacterial diversity, host specificity, mode of action, appropriate formulation, and method of application. Recent advances on whole genome sequencing of several important crop plants and PSB strains will provide future basis for better understanding of PSB–plant interactions and development of improved strains as effective biophosphorus fertilizer for eco-friendly low-input sustainable agriculture.

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