Naveen Kumar Arora
Editor

Plant Microbe Symbiosis: Fundamentals and Advances

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Preface

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

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

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

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

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

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About the Editor

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

About the Book

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

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

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

Stefan Shilev

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

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

Introduction

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

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

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

Characteristics of Plant Growth Promoters

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

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

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

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

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

Impact of Rhizobacteria on Plant Nutrition

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

Direct Impact

Nitrogen Fixation

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

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

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

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

Phosphorus Solubilization

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

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

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

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

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

Sequestering Iron by Bacterial Siderophores

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

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

Phytohormone Production

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

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

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

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

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

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

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

Lowering Ethylene Concentration

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

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

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

Indirect Impact

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

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

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

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

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

Case Studies for PGPR-Based Immobilization of Heavy Metals

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

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

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

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

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

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

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

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

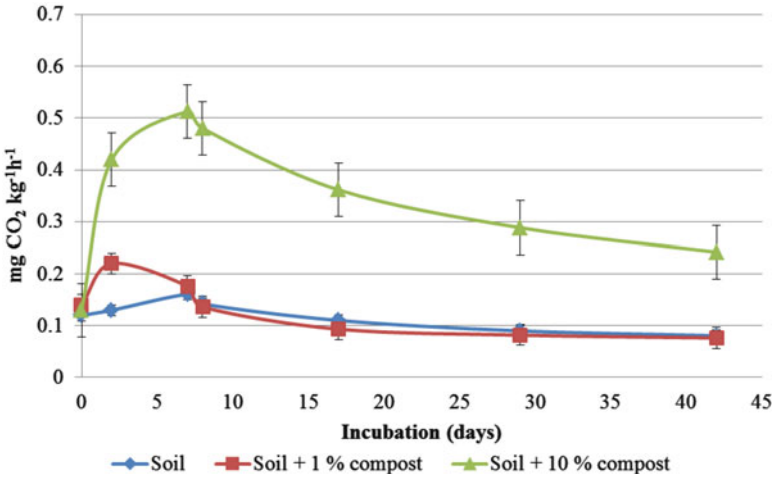

Fig. 5.1 Dynamics of intensity of soil respiration expressed per milligrams of CO_2 per kilogram of soil per hour. Results represent the mean value of three repetitions and the standard error

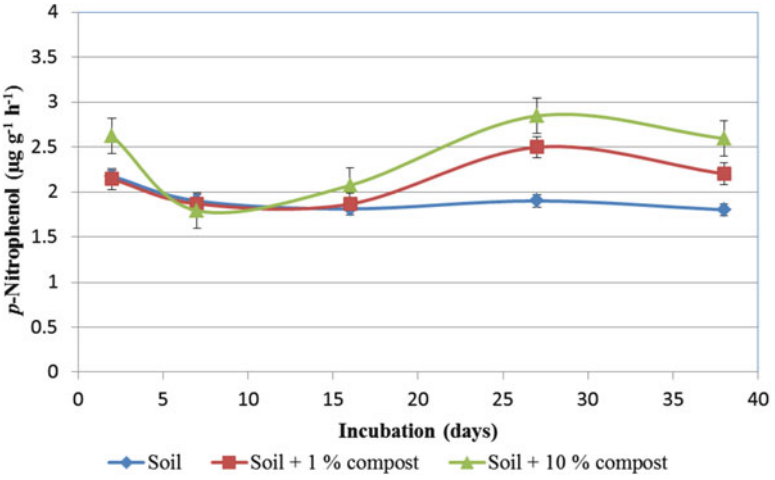

Fig. 5.2 Dynamics of β-glucosidase activity in soil expressed in milligrams of *p*-nitrophenol secreted per gram of soil per hour for each treatment. Results represent mean of three repetitions and the standard error

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

Fig. 5.3 Aspects of plants at the end of experiment

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

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

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

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

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

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

The results represent the mean ± standard error of three replicates

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

Conclusion

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

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