Mohammad Saghir Khan · Almas Zaidi
Javed Musarrat *Editors*

Phosphate Solubilizing Microorganisms

Principles and Application of Microphos Technology

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Preface

The rapidly increasing human population has placed tremendous pressure on agriculture sector to provide sufficient quantity and better quality foods in a more sustainable manner. In order to achieve food security, artificially developed chemicals (fertilizers/pesticides) have been used over the years in energy-intensive agricultural practices to overcome the nutrient deficiencies of the soils and hence to optimize the food production. Even though the synthetic fertilizers among agrochemicals, for example, single super phosphate, when applied in different production systems, have shown promising results, when used excessively and inadvertently, they cause a profound diminishing impact on soil health (soil fertility) and concurrently diminish the viability and productivity of crops. Phosphorus among soil constituents is one of the most important plant nutrients next to nitrogen. Even though there is no deficiency of phosphorus reserves in agronomic soils worldwide, the availability of soluble phosphorus to plants is a major global problem due largely to its rapid fixation and precipitation ability. This, in effect, leads to severe phosphorus deficit in soils. To mitigate such acute phosphatic problems, especially in resource crunch agricultural sector, chemically synthesized fertilizers are applied on a regular basis and at larger scale. Even though the use of artificial fertilizers in agricultural practices has resulted in some better results, their use and misuse has been questioned due in part to its cost and hazardous impact onto natural environment including soil ecosystems. Considering these challenging threats, the interest and awareness have been generated among scientists to uncover some easy-to-operate options. In this regard, in order to accomplish lab-to-land concepts, the naturally abundant yet functionally divergent phosphate-solubilizing microorganisms (PSM) have attracted greater attention of the farm practitioners due to its low cost and easy-to-apply approach. Indeed, PSM offer a practicable alternative to hugely expensive chemical P fertilizers. Application of PSM involving bacteria, fungi, and actinomycetes in agricultural practices has shown some overwhelming results with different crops like legumes, vegetables, and cereal, etc. Apart from providing phosphorus to plants, these organisms also profoundly increase the plant growth by supplying other major plant nutrients like nitrogen via N_2 fixation, increasing the availability of plant hormones, absolving the lethal impact of pathogenic microorganisms, and secreting

a few enzymes, etc. Thus, PSM possessing numerous multifunctional plant growth-promoting abilities could be of great practical help to both farmers and students/teachers/scientists across different ecological regions of the world.

Phosphate-Solubilizing Microorganisms: Principles and Application of Microphos Technology is an inclusive source of information on numerous useful aspects of phosphate-solubilizing microorganisms which could be applied and practiced for enhancing crop production in distinctly variable agro-ecosystems. This book highlights both fundamental information on the subject and strategies as to how the PSM could be raised to the level of microbial inoculants (microphos), mechanisms, and physiological functions of PSM and factors affecting the growth and phosphate-solubilizing potentials of such microbes. Furthermore, there are separate chapters on the role of phosphate-solubilizing fungi and actinomycetes in the survivability and development of some economically important plants. Discussion on cold-tolerant PSM as elaborated in this book may upgrade and popularize the use of such microbes in enriching the soil P pool and hence increasing the agricultural produce in temperate climatic zones of the world. The ecological diversity and biotechnological implications of PSM and their consequent impact on crops are discussed separately. Special attention is given on to assess the sole/synergistic/additive effects of PSM on some important legumes and cereal crops grown distinctively in different production systems. This book further describes the role of PSM in improving the nutrient uptake and consequently the yield of aerobic rice. The book also highlights a broad and updated view of the management of plant diseases using phosphate-solubilizing microbes. Moreover, the book describes as to how the consortia of plant growth-promoting rhizobacteria other than phosphate solubilizers facilitate the plant growth under stressed environment. The impact of PSM on the growth and development of some notable vegetable crops is also considered and effectively discussed.

The major aim of **Phosphate-Solubilizing Microorganisms: Principles and Application of Microphos Technology** is to compose scientific information available so far in this area and to make this information available to readers and practitioners in a more meaningful and practical way so that maximum benefits of this technology could be achieved. The book gives an extensive and well-organized scientific coverage in the area of microphos and how the use of microphos technology could be exploited and extended to larger section of the agronomic society in an inexpensive and easy way. This book is likely to be of special interest to the postgraduate students, research scholars, teachers, scientists, and professionals working in the field of microbiology, soil microbiology, biotechnology, agronomy, plant sciences, plant physiology, and plant protection sciences. In addition to gratifying the desires of the academicians/professionals, **Phosphate-Solubilizing Microorganisms: Principles and Application of Microphos Technology** also provides information to the policy makers, inoculant making industries and the people practicing agriculture, and microbial biotechnology across the globe. Each chapter presented herein is contributed by highly experienced academicians/professionals, and attempts have been made to emancipate the quality information and updated knowledge on the subject for ultimate use in academics and/or agriculture practices.

We are very much grateful to our experienced and highly professional scientific colleagues who participated in this endeavor and contributed the state-of-the-art information and balanced scientific knowledge to make this book a reality. Chapters contributed by each scientist/teacher are well structured and involve suitable tables and well-formatted figures. The cooperation extended by our research scholars in designing and weaving the manuscripts presented in this book is deeply acknowledged. We are undeniably very appreciative of our family members who provided their full support and affection during the entire period of this book preparation. Above all, AZ and MSK are extremely thankful to their adorable children, Zainab and Butool, for their patient and helpful attitude all through the book project. Further, we appreciate the great efforts of book publishing team at Springer-Verlag, Switzerland, in responding to all our queries very promptly and earnestly. Finally, if someone finds any typographical mistakes or otherwise in this book, they are requested to inform us so that the mistakes can be corrected and improved in subsequent print/edition. We also invite suggestions and healthy criticism from the readers of this book in order to improve the scientific contents in future print/edition.

Aligarh, India — Mohammad Saghir Khan
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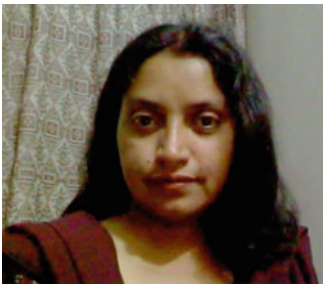
The Editors

Mohammad Saghir Khan, *Ph.D.*, is an Associate Professor at the Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh, India. Dr. Khan received his M.Sc. from Aligarh Muslim University, Aligarh, India, and Ph.D. (microbiology) from one of the premier universities, "Govind Ballabh Pant University of Agriculture & Technology, Pantnagar, India." He has been teaching microbiology to postgraduate students for the last 18 years and has research experience of 20 years. In addition to his teaching, Dr. Khan is engaged in guiding students for doctoral degree in microbiology. He has published over 100 scientific papers including original research articles, review articles, and book chapters in various national and international publication media. Dr. Khan has also edited eight books published by the leading publishers. Dr. Khan is deeply involved in research activities focusing mainly on rhizobiology, microbiology, environmental microbiology especially heavy metals–microbes–legume interaction, bioremediation, pesticide–PGPR–plant interaction, biofertilizers, and rhizo-immunology.



Almas Zaidi received her M.Sc., M.Phil., and Ph.D. (Agricultural Microbiology) degree from Aligarh Muslim University, Aligarh, India, and is currently serving as Assistant Professor at the Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh, India. Dr. Zaidi has been teaching microbiology (Ag.) at postgraduate level for the last 10 years and has research experience of 14 years. She has published over 50 research papers, book chapters, and review articles in journals of national and international repute. Dr. Zaidi has edited six books published by the leading publishers. Her main focus of research

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Javed Musarrat, M.Sc., M.Phil, Ph.D. (biochemistry), formerly the Chairman of the Department of Agricultural Microbiology and the Dean, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, India, has been teaching biochemistry, microbiology, and molecular biology to postgraduate students for the last 28 years and has research experience of about 30 years. He has contributed more than 100 national and international scientific publications. Prof. Musarrat has edited four books, published by the leading publishers. He is associated with several international and national scientific bodies such as DBT, CSIR, UGC, ICAR, UPCST, CCRUM, and NPST (KSA) in various capacities. His major area of interest includes the molecular microbiology, microbial ecology, genetic toxicology, and nanotechnology.

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Chapter 1
Microphos: Principles, Production and Application Strategies

Almas Zaidi, Md. Saghir Khan, and Ees Ahmad

Abstract The increasing demand for inexpensive, ecologically sound and environmentally friendly agricultural practices has warranted the use of microbial fertilizers. The preparation from microbial inoculants (biofertilizers) especially the organisms capable of transforming insoluble phosphorus (P) to soluble and available forms is one of the better choices for enhancing crop production by supplying essential nutrients and other growth regulators in different production systems. Furthermore, the critical interactions between microbial communities with soil constituents and plants have provided some novel clues to better exploit them in agricultural practices. Even though the use of microbial preparation in agriculture is an old practice, the production of efficient inoculants expressing consistent performance under field soil is a major obstacle in their extensive and practical application. Therefore, the variations in the performance of microbial inoculants including microphos have greatly hampered their large-scale application. On the other hand, the selection of the technology for inoculant production and modes of their application are key to their success. We highlight here the various strategies employed to produce the phosphatic microbial inoculants (microphos), and how this inoculants can be applied under different agro-ecological niches is discussed and considered.

Keywords Phosphate-solubilizing microorganisms • Microphos • Rhizosphere • Phylogenetic tree • Plant growth regulators

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1.1 Introduction

The major and most essential macronutrient, phosphorus (P), is required by the plants for vital functions such as cell division, energy transfer, signal transduction, macromolecular formation, nucleic acid synthesis, photosynthesis and respiration, nitrogen fixation and production of oil, sugars and starches (Saber et al. 2005; Zaidi et al. 2009; Eftekhari et al. 2010; Elser 2012). Consequently, acquisition of sufficient concentration of P enhances the growth and development of plants in different production systems (Hayat et al. 2010; Ahemad et al. 2009; Vikram and Hamzehzarghani 2008). However, of the total soil P pool (0.5 %), only 0.1 % is plant available (Scheffer and Schachtschabel 1988) and the remaining soil P is inaccessible to plants (Rodríguez and Fraga 1999). Therefore, the deficiency of P impedes the growth and yields of plants heavily. Such P scarcity in agronomic practices is, however, corrected through the application of synthetic phosphatic fertilizers which indeed is expensive and hazardous. Moreover, greater portion of P applied exogenously to soils is rapidly fixed into soil constituents (Norrish and Rosser 1983; Borling et al. 2001; Hao et al. 2002) and, hence, becomes unavailable to plants. Even though the organic P constitutes a large fraction of P (as much as 50 % in soils), yet it is not directly used up as nutrient unless degraded by soil enzymes. Considering the high cost of chemical phosphatic fertilizers and ability of P to form a complex with soil constituents, it has become imperative to find an inexpensive and viable alternative to chemical P fertilizers. In this regard, the bio-preparation containing viable and sufficient number of efficient phosphate-solubilizing microorganisms (PSM) quite often called as "microphos" has provided some solution to the P problems (Ahemad and Khan 2010; Hui et al. 2011; Xiang et al. 2011; Khan et al. 2013). When applied to seed, plant surfaces or soil, PSM colonize the rhizosphere or the interior of the plant (endophytes) and facilitate growth by providing P to growing plants (Khan et al. 2006). Several PSM inhabiting the soils (Behbahani 2010; Ahemad and Khan 2011a; Marra et al. 2011; Sanjotha et al. 2011; Yadav et al. 2011; Abd El-Fattah et al. 2013; Saxena and Sharma 2007) include bacteria (Khan et al. 2010; Yasmin and Bano 2011; Oves et al. 2013), fungi (Khan et al. 2010) and actinomycetes (Franco-Correa et al. 2010; Kaviyarasi et al. 2011; Balakrishna et al. 2012; Hamdali et al. 2012). Several authors attribute the solubilization of inorganic insoluble P by PSM to the production of organic acids and chelating oxo acids from sugars (Gulati et al. 2009; Khan et al. 2010). Mechanistically, when applied to seeds and soils, PSM facilitates plant development by (i) supplying hugely important nutrients to plants (Sashidhar and Podile 2010); (ii) releasing phytohormones, for example, IAA (Naz et al. 2009; Kavamura et al. 2013), gibberellins (Dey et al. 2004; Cassan et al. 2009) and cytokinin and ABA (Zahir et al. 2004; Cassan et al. 2013); (iii) alleviating the stress induced by ethylene on plants by synthesizing 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce ethylene level (Ahmad et al. 2012); (iv) producing siderophores for iron sequestration (Roca et al. 2013) and cyanogenic compounds (Ghyselinck et al. 2013); (v) releasing antimicrobial compounds

capable of inhibiting the growth of phytopathogens (Khan et al. 2002; Guo et al. 2004; Saravanakumar et al. 2007; Khan et al. 2009; Sambanthamoorthy et al. 2012); and (vi) providing resistance to drought, salinity, waterlogging and oxidative stress (Alvarez et al. 1996; Stajner et al. 1997; Saleem et al. 2007). Therefore, the use of microphos in crop production is considered as an environment-friendly alternative to further applications of mineral P fertilizers. However, in order to produce microphos, the organisms with P-solubilizing ability must be isolated and characterized. Subsequently, the microphos are tested both under pot soil or field environment prior to their transfer to the practitioner/farmers for application in agricultural practices.

1.2 Rationale for Using Microphos in Sustainable Agriculture

In contemporary agricultural practices, millions of tons of agrochemicals including P fertilizers are frequently but indiscriminately used to achieve optimum crop yields. Such synthetic chemicals are, however, not completely used up by plants and, hence, persist in different forms in soil. From here, they leach deep into the grounds and disrupt the composition and functions of beneficial rhizosphere microorganism (Ai et al. 2012), soil matrix (Ai et al. 2013; Lemanski and Scheu 2014) and via food chain, the human health (Ayala and Rao 2002). Furthermore, the chemical fertilizers are used either alone, for example, single super phosphate (Maheshwari et al. 2011), or as mixture (Malhi et al. 2007), for example, diammonium phosphate (DAP), for enhancing crop production in different soil ecosystems. The excessive use of agrochemicals is, however, posing some serious threats to the very sustainability of the environments and is being considered as one of the major problems around the world. So, due to the alarmingly very high costs of fertilizers and some acute environmental hazards associated with the use of synthetic fertilizers (López-Bellido et al. 2013), it has become increasingly important to find some low-cost alternative like the use of renewable resources which could both be inexpensive and could minimize the environmental threats (Bashan 1998; Vessey 2003; Adesemoye and Kloepper 2009). In this context, the discovery of plant growth-promoting rhizobacteria (Kloepper et al. 1986; Ahemad and Khan 2011b; Ahmad et al. 2013; Oves et al. 2013) and the preparation from PSM (microphos) have provided some relief to the poor agronomic practitioners largely due to: (i) low-cost technology with a high cost-benefit ratio, (ii) easy and abundant availability of PSM, (iii) enhances plant growth and crop yields through increased P supply and other growth regulators, (iv) reduces the environmental pollution caused from the manufacturing of the fertilizers and chemicals used, (v) improves soil health and conditioning, (vii) protects plants from pathogens damage and (viii) helps plant to grow under stressed conditions. Therefore, the discovery of PSM and, hence, the production of microphos have attracted greater attention of agronomists

than microbiologists in recent times because they can reduce/minimize the dependence on synthetic P fertilizers and, hence, can protect soil from chemical toxicity. During the last couple of decades, there has been some practical progress in this direction where some new and functionally exciting/novel PS microbes have been identified and used for enhancing agriculture productivity in a more sustainable manner (Khan et al. 2007, 2010).

1.3 Rhizosphere and PSM Colonization

Heterogeneously distributed microbial communities play an important role in the acquisition and transfer of various nutrients in soil. For P, soil microorganisms are involved in a range of processes that affect P transformation and thus influence the subsequent availability of P (as phosphate) to plant roots. The rhizosphere indeed is the narrow region of soil that is directly influenced by root secretions (Sørensen 1997) and associated soil microorganisms (Fig. 1.1) and plays some critical roles in plant growth and consequently in soil fertility (Avis et al. 2008). According to Bringhurst et al. (2001), the rhizosphere includes the region of soil bound by plant roots, often extending a few mm from the root surface. This region of soil is much richer in bacteria than the surrounding bulk soil (Hiltner 1904). In soil, microbes are often limited by energy, and hence, root exudates such as organic acids, sugars and amino acids provide energy to them and stimulate their growth and metabolic activities which in turn influence biogeochemical cycling of nutrients in soils (Cardoso and Freitas 1992; Stevenson and Cole 1999; Fontaine and Barot 2005). Studies based on molecular techniques have estimated more than 4,000 microbial species per gram of soil (Montesinos 2003). Of these, about 10^7–10^9 colony-forming units of culturable bacteria have been found in per gram of rhizosphere soil (Benizri et al. 2001), whereas the population densities in the rhizoplane have been reported to range from 10^5 to 10^7 colony-forming units per gram of fresh weight (Benizri et al. 2001; Bais et al. 2006). Furthermore, the microbial populations first colonize the rhizosphere following soil inoculation (Gamalero et al. 2003) as shown by many techniques like microscopic tools, immuno-markers or by fluorescence in situ hybridization (FISH) and by using gnotobiotic conditions. Following colonization, bacterial cells are visualized as single cells attached to the root surfaces and subsequently as doublets on the rhizodermis, forming a string of bacteria (Hanson et al. 2000). From here onwards, the whole surface of some rhizodermal cells are colonized, and bacteria can establish even as microcolonies or biofilms (Benizri et al. 2001). In a similar manner, rhizoplane colonization has been studied using both in vitro-grown plants and plants grown in natural soil inhabiting a high microbial diversity. In order to provide benefits to plants, such microorganisms (inoculated one/natural inhabitants of soils) thus must be rhizosphere and/or rhizoplane competent (Elliot and Lynch 1984; Compant et al. 2005) for an extended period of times (Whipps 2001). Many factors can be involved in rhizosphere and rhizoplane competence by PGPB (Albareda et al. 2006). However,

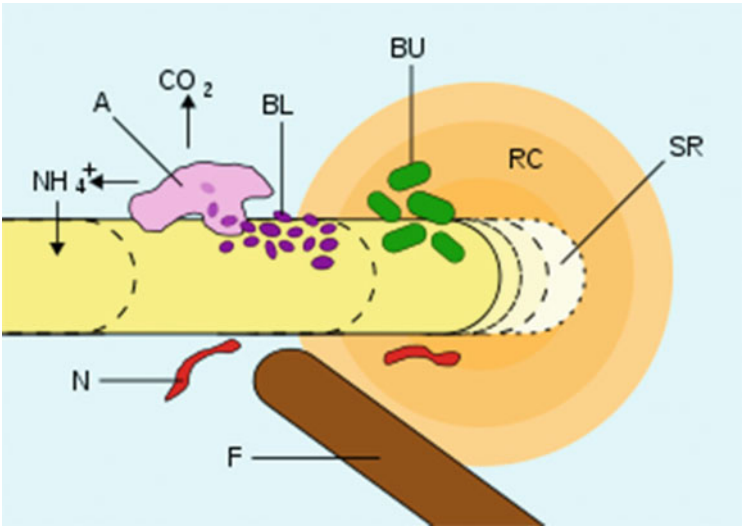

Fig. 1.1 An illustration of the rhizosphere. *A* amoeba-consuming bacteria, *BL* energy-limited bacteria, *BU* non-energy-limited bacteria, *RC* root-derived carbon, *SR* sloughed root hair cells, *F* fungal hyphae, *N* nematode worm (adapted from http://en.wikipedia.org./wiki/rhizosphere)

the competence of bacteria varies among different rhizospheres/rhizoplane (Gamalero et al. 2003) which has been described to be linked to root exudation (Lugtenberg et al. 2001). For instance, carbon fixed by plant photosynthesis is known to be partly translocated into the root zone and released as root exudates (Bais et al. 2006). Moreover, various carbohydrates, amino acids, organic acids and other compounds, which provide a source of nutrients for root-associated bacteria, are released in the rhizosphere (Walker et al. 2003). Such exudates act as chemoattractants towards which the bacterial population moves and in effect allow them to colonize and multiply in both the rhizosphere and the rhizoplane (Lugtenberg and Kamilova 2009). Plant exudates thus provide a rich source of energy and nutrients for the bacteria in rhizosphere, resulting in more microbial populations in the region than outside the region (Haas and Defago 2005). The colonization of plant rhizosphere by *Bacillus subtilis* sp. and *Pseudomonas* sp. has been well studied (Trivedi et al. 2005; Steenhoudt and Vanderleyden 2000). Rhizobacteria may depend on other microbes for nutrient sources as one microbe may convert plant exudates into a form that can be used by another microbe. Thus, rhizosphere has appeared as a versatile and dynamic ecological environment of intense plant–microbe interactions (Mayak et al. 2004) harnessing essential micro- and macronutrients affecting plant growth, although the process of root colonization is under the influence of various parameters such as bacterial traits, root exudates and several other biotic and abiotic factors (Benizri et al. 2002). Broadly, chemotaxis is generally considered to play an important role for successful rhizosphere/rhizoplane colonization (Andrews and Harris 2000; Walsh

et al. 2001). Recently, it has been reported that soil microorganisms, including free-living as well as associative and symbiotic rhizobacteria belonging to the genera *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Proteus*, *Pseudomonas*, *Rhizobium*, *Serratia* and *Xanthomonas* in particular, are the integral parts of rhizosphere biota (Glick 1995; Kaymak 2011) and have shown successful rhizosphere colonization.

1.4 Occurrence of Phosphate-Solubilizing Microbes

Soil is a dynamic system that harbours numerous microbial communities, and it is reported that one gram of fertile soil contains 10^1 to 10^{10} bacteria (Hayat et al. 2010). In soil ecosystems, bacteria are found in different forms such as bacilli, spiral and cocci. Of these, the rod-shaped bacilli are common in soil and have been found as prominent P solubilizers among various bacteria (Khan et al. 2010). However, the composition and structure of PSM within soil varies greatly and are influenced largely by the physico-chemical characteristics of soil (Kim et al. 1997; Khan et al. 2007). Besides soils, PSM have also been reported in various rhizosphere soils of different crops, for example, wheat [(*Triticum aestivum*) Rawat et al. 2011; Babana et al. 2013], maize [(*Zea mays*) Ranjan et al. 2013], rice [(*Oryza sativa*) Panhwar et al. 2012], sugar cane (*Saccharum officinarum*), onion [(*Allium cepa*) Ranjan et al. 2013], garlic (*Allium sativum*), betel vine plant [(*Piper betel* L.) Tallapragada and Seshachala 2012], chickpea (*Cicer arietinum* L.), pea (*Pisum sativum*), green gram [*Vigna radiata* (L.) Wilczek)], lentil (*Lens esculentus*), mentha (*Mentha arvensis*), potato (*Solanum tuberosum*), tomato [(*Lycopersicon lycopersicum)* Ranjan et al. 2013], chilli (*Capsicum annuum*), cabbage (*Brassica oleracea* var. *capitata*), mustard (*Brassica campestris*), jasmine (Ranjan et al. 2013), rhizoplane (Compant et al. 2013), phyllosphere (Ryan et al. 2008; Vorholt 2012), rock phosphate deposit area soil (Richardson et al. 2009), marine environment (Zhu et al. 2011) and polluted soils (Luo et al. 2011). Due to variation in PSM populations in different agro-ecological habitat and considering their functional diversity, it has become extremely important to search PSM with varied biological and chemical properties so that the soil microbial diversity, mechanistic basis of nutrient transformation and plant growth promotion by PSM could be revealed.

1.5 Production Strategies of Microphos (PSM Inoculants)

The production of efficient microbial inoculants involving P-solubilizing activity (microphos) broadly includes (a) collection of samples and determination of microbial diversity; (b) isolation, screening and selection of PSM from heterogeneous

microbial populations; (c) bioassay of P-solubilizing activity of the microbial strains; (d) characterization and identification of PSM; (e) bioassay of plant growth-promoting activities; (f) selection of suitable carriers, mixing of inocula with selected carriers and development of microbial inoculants; and (g) pot/field trials of prepared microphos before commercial recommendation for agricultural practices.

1.5.1 Collection of Samples and Assessment of Microbial Diversity

The soil samples are collected generally in sterile polythene bags from a depth of 15–12 cm^2 from conventional/polluted non-rhizosphere and rhizosphere soils, mixed thoroughly and are used for determining microbial diversity. The total bacterial, fungal, actinomycetal populations, phosphate-solubilizing micro-organisms (PSM) and asymbiotic nitrogen fixers, for example, *Azotobacter*, can be isolated using standard media and microbiological methods (Holt et al. 1994). For this, soil samples are serially diluted in sterile normal saline solutions (NSS), and 100 µl of diluted suspension is spread plated (Buck and Cleverdon 1960) on nutrient agar [g/l:beef extract 3; peptone 5; agar 15; pH 7], Martin's medium [g/l: dextrose 5; potassium dihydrogen orthophosphate 1; magnesium sulphate 0.5; streptomycin 0.006; Rose Bengal 2 part in 3,000 part of medium; 1 g of chloramphenicol/nalidixic acid can be dissolved in 100 ml of sterile water and 0.3 ml of this solution is added to 100 ml of Rose Bengal medium after it is cooled to 45 °C], Kenknight's medium [g/l: dextrose 1; potassium dihydrogen phosphate 0.1; sodium nitrate 0.1; potassium chloride 0.1; magnesium sulphate 1.50] or starch casein agar (SCA) medium [g/l: starch 10; casein 0.3; KNO_3 2; NaCl 2; K_2HPO_4 2; $MgSO_4 \cdot 7H_2O$ 0.05; $CaCO_3$ 0.02; $FeSO_4 \cdot 7H_2O$ 0.01 agar 18; pH 7.2; tetracycline (100 µg/ml) and amphotericin B (50 µg/ml) are added to medium after autoclaving to prevent bacterial growth and fungal growth, respectively (Williams and Davies 1965; Porter and Tresner 1960)], Pikovskaya (Table 1.1) medium, Ashby's medium (Table 1.1) and yeast extract mannitol (YEM) agar medium (Table 1.1) for total bacterial counts, fungal populations, actinomycetes, phosphate solubilizers, *Azotobacter* and rhizobia, respectively.

Each sample should be replicated at least three times and incubated at 28 ± 2 °C for 2, 3, 5, 5 and 5 to 7 days for quantifying the populations of bacteria, fungi, actinomycetes, PSM and *Azotobacter*, respectively. Where microbiological assay is not done immediately, the samples are kept in sterile polythene bags and stored at 4 °C for a short period of time. Standard culture medium and growth conditions should be used for isolation and enumeration of microbial populations as given in Table 1.2.

Table 1.1 Chemical composition of media used for assessment of microbial diversity in soil

Media component	Amount (g/l)			
	Pikovskaya medium	NBRIP medium	Ashby's medium	Yeast extract mannitol agar
Dextrose	10.0	10.0	–	–
Mannitol	–	–	20.0	10.0
Yeast extract	–	–	–	1.0
$Ca_3(PO_4)_2$	5.0	5.0	–	–
$CaCO_3$		–	5.0	2.0
$MgCl_2{\cdot}6H_2O$	5.0	5.0	–	–
$MgSO_4{\cdot}7H_2O$	0.25	0.25	0.2	0.2
KCl	0.2	0.2	–	–
$(NH_4)_2SO_4$	0.1	0.1	–	–
K_2HPO_4	–	–	0.2	0.5
K_2SO_4	–	–	0.1	–
NaCl	–	–	–	–
Bromophenol blue (BPB)	–	0.025	–	–

Table 1.2 Culture medium and growth conditions used for isolation and enumeration of microbial populations

Microbes	Medium	Incubation temperature (°C)	pH of medium	Incubation period (days)
Bacteria	Nutrient agar	28 ± 2	7 ± 0.2	1–2
Fungi	Martin's agar	28 ± 2	7 ± 0.2	3–5
Actinomycetes	Kenknight's agar	28 ± 2	7 ± 0.2	5–7
PSM	Pikovskaya agar	28 ± 2	7 ± 0.2	5–7
Azotobacter spp.	Ashby's agar	28 ± 2	7 ± 0.2	5–7
Rhizobia	YEM agar	28 ± 2	7 ± 0.2	2–5

1.5.2 Isolation, Screening and Selection of PSM

Gerretsen (1948) initially demonstrated that microbial activity in the rhizosphere could dissolve sparingly soluble inorganic P and increase plant growth. Subsequently, Pikovskaya (Pikovskaya 1948) devised a medium (Table 1.1) for the isolation and screening of PSM. Later on, a modified Pikovskaya medium using bromophenol blue dye as suggested by Gupta et al. (1994) and National Botanical Research Institute P [NBRIP] medium (Table 1.1) developed by Nautiyal (1999) are used for the isolation and selection of P solubilizers. However, there are conflicting reports on the performance of these media. For example, the bromophenol blue method used to improve the clarity and visibility of the yellow-coloured halo has not necessarily improved the plate assay (Nautiyal 1999). Moreover, the Pikovskaya medium contains yeast extract, and it is desirable

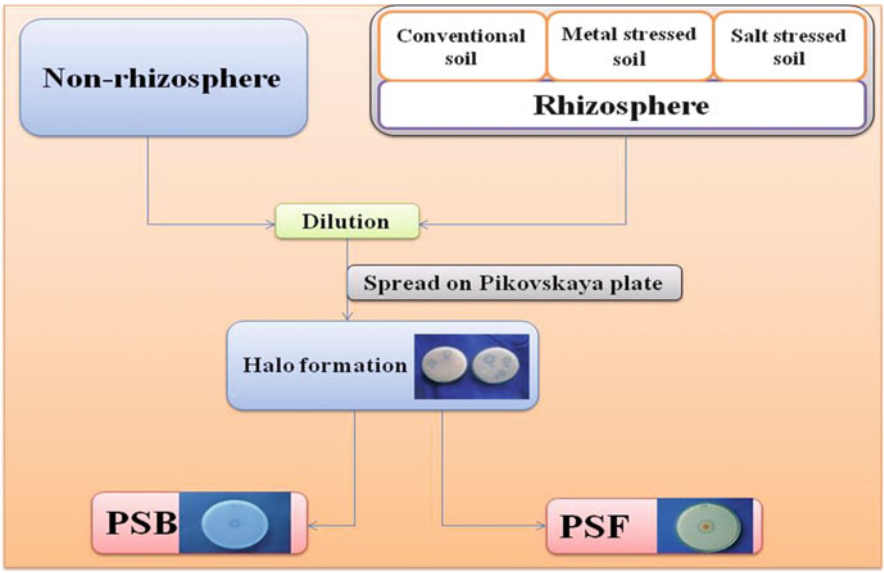

Fig. 1.2 Isolation and selection of P-solubilizing microorganism from different sources

to formulate a defined medium to elucidate the role of microorganisms in P mineralization. On the contrary, the NBRIP medium has several advantages over other media (Nautiyal 1999). For instance, the NBRIP medium can be used as a defined medium because it excludes the use of yeast extract. Secondly, NBRIP is more efficient in a broth assay compared to the Pikovskaya medium.

Despite the variation in the effectiveness of different media, first of all, PSM is isolated from soils/any source using serial plate dilution method or by enrichment culture technique (Fig. 1.2). The serially diluted rhizospheric or non-rhizospheric soil samples are then spread (100 µl) or streaked or spot (10 µl) inoculated on solid Pikovskaya plates or any plates containing insoluble P (e.g. tricalcium phosphate) and incubated. After proper incubation of inoculated solid Pikovskaya plates for 5–7 days (bacteria) and 3–5 days (fungi and actinomycetes) at 28 ± 2 °C, the P-solubilizing microbes are detected by the formation of clear halo around their colonies (Plate 1.1). The development of a clear zone around the colony on the culture plates are taken as an index of P solubilization. However, the reliability of this halo-based technique is questioned as many isolates in other studies did not produce any visible halo/zone on agar plates but could solubilize insoluble inorganic P in the liquid medium (Gupta et al. 1994; Louw and Webley 1959). The phosphate solubilizers are then maintained on medium, for example, Pikovskaya, which is used for PSM isolation until use. Since P-solubilizing organisms exhibit many-fold variations in P-dissolving activity (Khan et al. 2007) and instability with regard to their P-solubilizing activity (Illmer and Schinner 1992), they are repeatedly subcultured to test the persistence of P-solubilizing potential. Once the efficient PSM are selected, they are tested for their ability to solubilize insoluble P

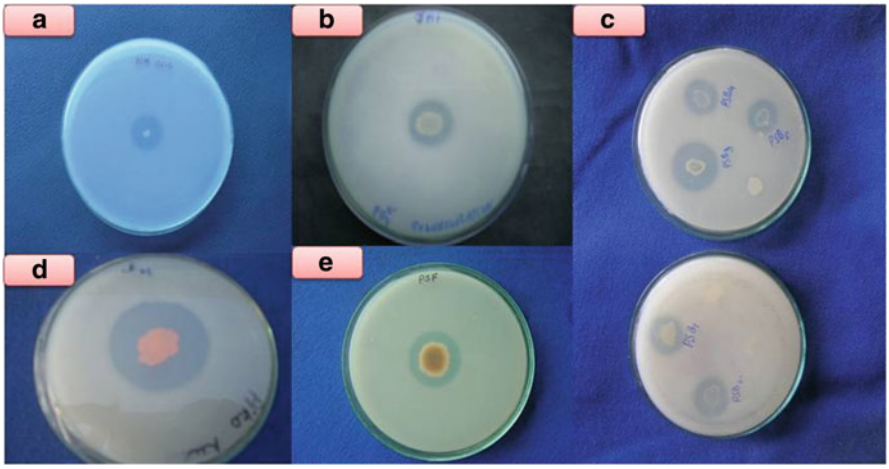

Plate 1.1 Phosphate solubilization on Pikovskaya plate by some notable P solubilizers. (**a**) *Bacillus*, (**b**) *Azotobacter*, (**c**) *Serratia*, (**d**) Fungi, (**e**) *Pseudomonas* sp.

under liquid culture medium. Finally, the efficient P-solubilizing organisms are selected and used for the development of inoculants whose performance is tested under pot/field environments against various crops of economic importance.

1.5.3 Bioassay of P-Solubilizing Activity

The microbial strains expressing PS activity during screening process are further enriched by inoculating into the Pikovskaya medium, incubated at 28 ± 2 °C for 7 days and then observed on solid plates for halo formation. The solubilization index (SI) and solubilizing efficiency (SE) of such microbes are calculated by the formula suggested by Premono et al. (1996) as

$$\text{Solubilization Index (SI)} = \left(\text{colony diameter} + \text{zone of halo}\right)/\text{colony diameter}$$
$$\text{Solubilizing Efficiency (SE)} = \left(\text{zone of halo}/\text{colony diameter}\right) \times 100$$

The colonies forming clear halo around microbial growth indicating P solubilization are counted and further used to determine the relative P-solubilizing efficiency [RPSE] in liquid Pikovskaya medium. The clear halo around bacterial growth is measured, and cultures are further used to determine the extent of P solubilization in liquid Pikovskaya medium. For quantitative measurement, 100 ml of Pikovskaya broth containing 5 g TCP is inoculated with 1 ml of 10^8 cells/ml of each culture. The flasks are incubated for 5, 10 and 15 days with shaking at 120 rpm at 28 ± 2 °C. A 20 ml culture broth from each flask is removed and centrifuged ($9{,}000 \times g$) for 30 min, and the amount of water-soluble P released into the

supernatant is estimated by the chlorostannous-reduced molybdophosphoric acid blue method (King 1932; Jackson 1967). To 10 ml of supernatant, 10 ml chloromolybdic acid (ammonium molybdate 15 g; distilled water 400 ml and 10 N HCl 400 ml. These materials are mixed slowly with rapid stirring and cooled, and the volume is made to one litre with distilled water) and 5 drops of chlorostannous acid (stannous chloride 10 g; concentrated HCl 25 ml; the stock solution is kept in airtight bottle and one ml of stock solution is mixed in 132 ml of distilled water at the time of experiment) are added, and the volume is adjusted to 50 ml with distilled water. The absorbance of blue colour developed is read at 600 nm. The amount of P solubilizer is calculated using the calibration curve of KH_2PO_4. The change in pH following TCP solubilization is also recorded. Each independent experiment should be repeated three times after several subcultures to ensure the reproducibility of the results. Solubilization index and SE of the bacterial isolates showing greater solubilization on both solid and liquid media and persistence of PS activity after several subcultures are the criteria for the selection of efficient PS strains for further studies.

1.5.4 Microbiological and Biochemical Characterization of PSM

The phosphate solubilizers are identified firstly by microbiological and biochemical tests. The microbiological tests may include the assessment of colonial morphology [shape, margin (serrated or smooth)], colour and characteristics such as the secretion of watery or mucoid/gummy substances from colonies, Gram reaction and shape of microbes. The biochemical reaction may involve indole reaction, citrate utilization, methyl red test, Voges–Proskauer, catalase, oxidase test, starch, gelatin, lipid hydrolysis, mannitol salt utilization test and sugar fermentation test, etc. The resulting characteristics are compared with those given in *Bergey's Manual of Determinative Bacteriology* (Holt et al. 1994), and strains are identified to generic level only.

1.5.4.1 Antibiotic Sensitivity Behaviour of Isolated Cultures

Antibiotic sensitivity behaviour of the isolated P solubilizers is determined using the antibiotic discs of known potency by disc diffusion method of Bauer (1966) in order to find antibiotic markers for the PSM strains. For this, freshly prepared and autoclaved nutrient broth is inoculated by isolated bacterial cultures and incubated for 24 h at 28 ± 2 °C. 100 µl of overnight grown test culture is taken on nutrient agar plates and is evenly spread with sterile glass rod spreader. Plates are then mounted with individual antibiotic (e.g. amoxicillin, chloramphenicol, ciprofloxacin, cloxacillin, nalidixic acid, nitrofurantoin, norfloxacin, novobiocin, doxycycline

hydrochloride, erythromycin, etc.) disc using a sterile forceps. Each antibiotic-mounted plate is incubated at 28 ± 2 °C for 24–48 h. After incubation, the zone of inhibition is measured, and the strains are scored as resistant (R) and susceptible (S). Following the standard antibiotic disc sensitivity testing method (Margalejo et al. 1984), the plates are recorded for comparing the zone of inhibition (diameter in mm) with chart provided by the disc manufacturers.

1.5.4.2 Identification of Phosphate-Solubilizing Organisms

Microbial cultures showing greater P-solubilizing activity in vitro, when grown on Pikovskaya medium, and exhibiting optimum solubilization of insoluble P in liquid culture medium are selected and presumptively identified to the genus level using morphological and biochemical test. Such organisms are then identified to the species level using whole-cell fatty acid methyl ester (FAME) profile and 16S rDNA sequence analysis (Chung et al. 2005; Chen et al. 2006). For 16S rDNA sequence analysis, partial 16S rRNA gene sequences of selected strains are done using universal primers, 518 F (5′CCAGCAGCCGCGGTAATACG3′) and 800R (5′TACCAGGGTATCTAATCC3′). All nucleotide sequence data should then be deposited in the public domain (e.g. GenBank sequence database). There are various agencies which are providing molecular sequencing for identifying bacterial cultures to species level, for example, Macrogen Inc., Seoul, South Korea. The online programme BLASTn is then used to find related sequences with known taxonomic information in the databank at the NCBI website (http://www.ncbi.nml.nih.gov/BLAST) to accurately identify and compare the isolates with nearest neighbour sequence available in the NCBI database.

1.5.4.3 Construction of Phylogenetic Tree

The sequence obtained from nucleotide-sequencing agencies is initially estimated by the BLASTn online programme facility of NCBI (http://www.ncbi.nml.nih.gov/BLAST) and then aligned with all related sequences obtained from GenBank by ClustalW (Thompson 1994). Phylogenetic tree is then reconstructed by neighbour-joining method (Saitou and Nei 1987). Bootstrapped neighbour-joining relationships are estimated with MEGA4 software (Tamura et al. 2007).

1.5.5 Bioassay of Plant Growth-Promoting Activities of PS Bacteria

1.5.5.1 Screening for 1-Aminocyclopropane-1-Carboxylate (ACC) Deaminase Activity

Using the spot inoculation method, 5 µl of each isolated PS bacterium is placed on a section of plate (marked in 16 equal parts) containing DF (Dworkin and Foster 1958) salt minimal medium [g/l: KH_2PO_4 4; Na_2HPO_4 6, $MgSO_4{\cdot}7H_2O$ 0.2, glucose 2.0, gluconic acid 2.0; citric acid 2.0; trace elements, 1 mg $FeSO_4{\cdot}7H_2O$, 10 µg H_3BO_3, 11.19 µg $MnSO_4{\cdot}H_2O$, 124.6 µg $ZnSO_4{\cdot}7H_2O$, 78.22 µg $CuSO_4{\cdot}5H_2O$, 10 µg MoO_3, pH 7.2 and 2.0 g $(NH_4)_2SO_4$ as nitrogen source] supplemented with three mM ACC instead of [$(NH_4)_2SO_4$)] and incubated at 28 ± 2 °C for 72 h. The bacterial growth should be checked daily as suggested by Penrose and Glick (2003). At least one ACC deaminase-positive bacterial strain should be used as a control in this type of study (Nascimento et al. 2011), and all the samples should be tested in duplicate, and experiments must be repeated at least three times to ensure the reproducibility of the results.

Quantitative Assay of ACC Deaminase Activity

The ACC deaminase activity of P solubilizers (Ahmad et al. 2013) can be assayed following the method of Honma and Shimomura (1978) later modified by Penrose and Glick (2003). According to this method, the amount of α-ketobutyrate is measured which is produced by reaction of the enzyme ACC deaminase which cleaves ACC to α-ketobutyrate and NH_3. The number of mmol of α-ketobutyrate produced by this reaction is determined by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate ranging between 0.1 and 1 mmol. A stock solution of 100 mM α-ketobutyrate (Sigma-Aldrich) is prepared in 0.1 M Tris–HCl, pH 8.5, and stored at 4 °C. Just prior to use, the stock solution is diluted with the same buffer to make a 10 mM solution from which a standard concentration curve is generated. Each in a series of known α-ketobutyrate concentrations is prepared in a volume of 200 ml, 300 ml of the 2,4-dinitrophenylhydrazine reagent (0.2 % 2,4-dinitrophenylhydrazine in 2 M HCl) (Sigma-Aldrich) is added and the contents are vortexed and incubated at 30 °C for 30 min during which time the α-ketobutyrate is derivatized as a phenylhydrazone. The colour of the phenylhydrazone is developed by the addition of two ml 2 M NaOH; after mixing, the absorbance of the mixture is measured at 540 nm. Using this method, the ACC deaminase activity can be measured in bacterial extracts prepared in the following manner. The ACC deaminase-positive bacterial strains, for example, P solubilizers (10^8 cells/ml) are inoculated in Luria–Bertani broth (g/l: tryptone 10; yeast extract 5; NaCl 10; pH 7.5) and incubated in a shaking incubator at 200 rpm for 24–48 h at 28 ± 2 °C. Then, cultures are centrifuged at $8{,}000 \times g$ for 10 min at 4 °C, and the

biomass of P solubilizers is harvested. The supernatant is removed, and the cells are washed with 5 ml DF salts minimal medium. Following an additional centrifugation for 10 min at 8,000 × *g* at 4 °C, the cells are resuspended in 7.5 ml DF salts minimal medium in a fresh culture tube. Just prior to incubation, the frozen 0.5 M ACC solution is thawed, and an aliquot of 45 ml is added to the cell suspension to obtain a final ACC concentration of 3.0 mM. The bacterial cells are re-shaken in the incubator to induce the activity of ACC deaminase at 200 rpm for 24 h at the same temperature as is done for overnight-incubated cultures. The bacteria cultures are harvested by centrifugation at 8,000 × *g* for 10 min at 4 °C. The supernatant is removed, and the cells are washed by resuspending the cell pellets in 5 ml 0.1 M Tris–HCl at pH 7. Each bacterial cell pellet, prepared as described above are suspended in 1 ml of 0.1 M Tris–HCl, pH7.6 and transferred to a 1.5-ml micro-centrifuge tube. The contents of the 1.5-ml micro-centrifuge tube are spun at 16,000 × *g* for 5 min, and the supernatant is removed. The pellet is suspended in 600 ml of 0.1 M Tris–HCl, pH 8.5. A 30 µl of toluene is added to the cell suspension and vortexed at the highest setting for 30 s. At this point, a 100-ml aliquot of the "toluenized cells" is set aside and stored at 4 °C for protein assay by Lowery et al. (1951) method at a later time. The remaining toluenized cell suspension is immediately assayed for ACC deaminase activity. All sample measurements should be carried out in duplicate. 200 µl of the toluenized cells are placed in a fresh 1.5-ml micro-centrifuge tube; 20 ml of 0.5 M ACC is added to the suspension, briefly vortexed and then incubated at 30 °C for 15 min. Following the addition of 1 ml of 0.56 M HCl, the mixture is vortexed and centrifuged for 5 min at 16,000 × *g* at room temperature. One ml of the supernatant is vortexed together with 800 ml of 0.56 M HCl. Thereupon, 300 ml of the 2,4-dinitrophenylhydrazine reagent (0.2 % 2,4-dinitrophenylhydrazine in 2 M HCl) is added to the glass tube; the contents are vortexed and then incubated at 30 °C for 30 min. Following the addition and mixing of 2 ml of 2 N NaOH, the absorbance of the mixture is measured at 540 nm.

1.5.5.2 Quantitative Assay of Indole Acetic Acid

Indole-3-acetic acid (IAA) synthesized by P solubilizers (Wani and Khan 2010; Ahemad and Khan 2012) is quantitatively evaluated by the method of Gordon and Weber (1951), later modified by Brick et al. (1991). For this, the PS bacterial strains are grown in Luria–Bertani (LB) broth. Luria–Bertani broth (100 ml) having 0, 50, 100, 200, 400 and 500 µg/ml tryptophan is then inoculated with 1 ml culture (10^8 cells/ml) of PS cultures and incubated for 3, 6, 9 and 12 days at 28 ± 2 °C with shaking at 125 rpm. After incubation, 5 ml of culture of each treatment is spun (9,000 × *g*) for 15 min, and an aliquot of 2-ml supernatant is mixed with 100 µl of orthophosphoric acid and 4 ml of Salkowski' reagent (2 % 0.5 M $FeCl_3$ in 35 % perchloric acid) and incubated at 28 ± 2 °C in darkness for 1 h. The absorbance of developed pink colour is read at 530 nm. The IAA concentration in the supernatant is determined using a calibration curve of pure IAA as a standard. The experiment should be repeated three times on different time intervals.

1.5.5.3 Qualitative and Quantitative Estimation of Siderophores

The PS bacterial strains are further tested for siderophore production using Chrome Azurol S (CAS) agar medium following the method of Alexander and Zuberer (1991). Chrome Azurol S (CAS) agar medium is prepared from four solutions as (i) *Solution 1*, Fe-CAS indicator solution: A 10 ml of 1 mM $FeCl_3 \cdot 6H_2O$ [in 10 Mm HCl} is mixed with 50 ml of an aqueous solution of CAS (1.21 mg/ml). The above solution is then added to 40 ml of HDTMA (1.82 mg/ml) and cooled to 50 °C. (ii) *Solution 2*, buffer solution: A 30.24 g of PIPES is dissolved in 750 ml of a salt solution containing 0.3 g KH_2PO_4, 0.5 g NaCl and 1 g NH_4Cl, pH 6.8, with 50 % KOH, and water is added to bring the volume to 800 ml. (iii) *Solution 3*: (in 70 ml water) 2 g glucose, 2 g mannitol, 493 mg $MgSO_4$. $7H_2O$, 11 mg $CaCl_2$, 1.17 mg $MnSO_4$. H_2O, 1.4 mg H_3BO_3, 0.04 mg $CuSO_4 \cdot 5H_2O$, 1.2 mg $ZnSO_4$. $7H_2O$ and 1 mg $Na_2MoO_4 \cdot 2H_2O$. Autoclaved, cooled to 50 °C, then added to the buffer solution along with 30-ml filter-sterilized 10 % (W:V) casamino acids (Solution 4). The indicator solution is added last with sufficient stirring to mix the ingredients without forming bubbles. Chrome Azurol S agar plates are then prepared separately and divided into equal sectors and spot inoculated with 10 µl of 10^8 cells/ml and incubated at 28 ± 2 °C for 5 days. Development of yellow-orange halo around the bacterial growth is considered as positive for siderophore synthesis. Each individual experiment should be repeated three times to ensure the reproducibility of results. The production of siderophore by the PSB strains are further detected quantitatively using Modi medium (K_2 HPO_4 0.05 %; $MgSO_4$ 0.04 %; NaCl 0.01 %; mannitol 1 %; glutamine 0.1 %; NH_4NO_3 0.1 %). Modi medium is inoculated with 10^8 cells/ml of PSB and incubated at 28 ± 2 °C for 5 days. Catechol-type phenolates are measured on ethyl acetate extracts of the culture supernatant using a modification of the ferric chloride–ferrocyanide reagent of Hathway. Ethyl acetate extracts is prepared by extracting 20 ml of supernatant twice with an equal volume of solvent at pH 2. Hathway's reagent is prepared by adding 1 ml of 0.1 M ferric chloride in 0.1 N HCl to 100 ml of distilled water, and to this, 1 ml of 0.1 M potassium ferrocyanide is added (Reeves et al. 1983). For the assay, one volume of the reagent is added to one volume of sample, and absorbance is determined at 560 nm for salicylates with sodium salicylate as standard and at 700 nm for dihydroxy phenols with 2, 3-dihydroxy benzoic acid (DHBA) as standard.

1.5.5.4 Assay of Hydrogen Cyanide and Ammonia

Hydrogen cyanide (HCN) production by PS cultures is detected by the method of Bakker and Schipper (1987). For HCN production, PS bacterial strains are grown on an HCN induction medium (g/l: tryptic soy broth 30; glycine 4.4; agar 15) for 3–4 days at 28 ± 2 °C. For each bacterial strain, a 100 µl of 10^8 cells/ml is spread on the Petri plates. A disc of Whatman filter paper no. 1 dipped in 0.5 % picric acid and 2 % Na_2CO_3 is placed at the lid of the Petri plates. The plates are then sealed with

Parafilm, and after 4 days of incubation at 28 ± 2 °C, an orange-brown colour of the paper indicating HCN production is observed. For ammonia assessment, the bacterial strains are grown in peptone water (g/l: peptone 10; NaCl 5; pH 7) and incubated at 28 ± 2 °C for 4 days. One ml of Nessler's reagent [potassium iodide 50 g; distilled water (ammonia free) 35 ml; add saturated aqueous solution of mercuric chloride until a slight precipitate persists; potassium hydroxide 400 ml; dilute the solution to 1,000 ml with ammonia-free distilled water. Allow to stand for 1 week, decant supernatant liquid and store in a tightly capped amber bottle] is added to each tube and the development of yellow colour indicating ammonia production is recorded following the method of Dye (1962).

1.5.5.5 Bioassay of Exo-Polysaccharides

The exo-polysaccharides (EPS) produced by the PS bacterial strains are determined under in vitro conditions as suggested by Mody et al. (1989). For this, PS bacterial strains are grown in 100-ml capacity flasks containing basal medium supplemented with 5 % sucrose. Inoculated flasks are then incubated for 5 days at 28 ± 2 °C on rotary shaker (100 rpm). Culture broth is spun (5,433 g) for 30 min, and EPS is extracted by adding three volumes of chilled acetone (CH_3COCH_3) to one volume of supernatant. The precipitated EPS is repeatedly washed three times alternately with distilled water and acetone, transferred to a filter paper and weighed after overnight drying at room temperature. In a study, Ashraf et al. (2004) have shown that inoculating wheat seedlings with EPS-producing bacteria restricts sodium uptake and stimulates plant growth under salt stress.

1.5.5.6 Determination of Antifungal Activity

Antifungal activity of the PSB against plant pathogenic fungi, for example, *Rhizoctonia* sp., *Penicillium* sp. and *Alternaria* sp., can be assessed on agar plates as described by Weller and Cook (1986) and Wong and Baker (1984). Fungal pathogens maintained on potato dextrose agar (PDA) are transferred to Petri dishes containing fresh PDA (g/l: potato infusion 4; dextrose 20; agar 15; pH 5.4) to produce fungal mycelium plugs. The PS bacteria are grown in YEM broth (N_2 PSB), Ashby's broth (*Azotobacter* with PS activity) and Luria–Bertani (other PSB like *Pseudomonas*/*Bacillus*) broth, respectively. A 1-ml stationary cell of each PSB (10^8 cells/ml) is inoculated into 100 ml YEM broth, Ashby's broth and Luria–Bertani broth, for rhizobia, *Azotobacter* and PS bacteria, respectively. The samples (1.8 ml) of each broth is removed in eppendorf and centrifuged at 3,875 × *g* for 10 min, and the supernatants are filtered through sterile Millipore filter. A 200-µl sample of each strain is then placed in an 8-mm well cut into the centre of pre-inoculated fungal plates. Inoculated plates are incubated at 28 ± 2 °C for 2 days (PS bacteria) and 5 days (rhizobia and *Azotobacter*), and the zone of growth

inhibition (mm) is recorded. Each individual experiment should be replicated three times at different time intervals.

1.5.6 Production of Microphos

The main objective of microphos production strategy is to enhance the survival of PS organisms during the period between its production and application to the soil and the rhizosphere it colonizes. The microphos is prepared commonly by adding flask-grown cultures (small scale) or fermenter-grown broth (large scale) containing a large population (about 10^8–10^9 cells/ml) of PSB to finely powdered carrier followed by a period of incubation. Here, both the evaluation of broth media at regular intervals for the presence of specific and viable PSB and selection of a suitable carrier for inoculant preparation are vital. Generally, the microphos production involves (i) inoculum preparation, (ii) processing of the raw material and (iii) mixing of PSM broth with carrier materials and inoculant packaging.

1.5.6.1 Inoculum Preparation

After proper selection of potential PS organisms, high-quality microphos is prepared. For this, the starter PS organisms are first grown in specific medium, for instance, Pikovskaya/NBRIP medium, in a small capacity (50 ml) flask, and then the inoculum are transferred to a larger capacity flasks or a fermenter at the rate of 1–5 % of the medium. However, conditions for PS growth in both flasks and fermenter of different capacities, like the pH of the medium and nutrient supply (C and N source), must be at the optimal level. Culture should be incubated at temperature as required by each specific PS bacterium. The broth in flasks or fermenter should be checked at regular intervals for both contamination and microbial density. If at any stage the broth becomes contaminated, it should immediately be discarded. When the bacterial density reaches to 10^9 cells/ml, culture growing in flasks/fermenter can be withdrawn and added to sterilized carrier materials. Incorporation of microorganisms in carrier material enables easy handling, long-term storage and high effectiveness of microphos.

1.5.6.2 Processing of Carrier Material

Various types of materials are used as carrier for seed or soil inoculation (Table 1.3) to improve the survival and biological effectiveness of inoculants by protecting bacteria from biotic and abiotic stresses (Malusá et al. 2012). Carrier is a delivery vehicle which is used to transfer live microorganism in a good physiological condition from an agar slant of laboratory to a seed/rhizosphere (Smith 1992). Since a suitable carrier plays a major role in formulating microbial inoculants, the

Table 1.3 Different carriers used for inoculant production

Carrier material	Inoculant bacterium	Characteristics
Sterilized oxalic acid industrial waste	*Rhizobium*	Seed inoculation; *Rhizobium* multiplication in carrier in ambient temperature up to 90 days; carrier sterilization resulted in significant increase in grain yield, nodule number and N content
Alginate-perlite dry granule	*Rhizobium*	Soil inoculation; *Rhizobium* strains survived in dry granules beyond 180 days; the inoculant can be stored in a dry state without losing much viability
Composted sawdust	*Bradyrhizobium, Rhizobium* and *Azospirillum*	Seed inoculation; good growth and survival of the inoculant strains
Agriperlite, expanded clay, kaolin, Celite, Diatom, porosil MP, MicroCel, vermiculite	*Agrobacterium radiobacter* K84	Crown gall control. Screening was performed to find improved formulation of K84 cells; effect of carrier storage temperature and carrier water content on survival of K84 was examined
Cheese whey grown cells in peat	*Rhizobium meliloti*	Seed inoculation; better survival at various temperature during storage even under desiccation
Mineral soils	*Rhizobium*	Seed inoculants; *Rhizobium* survived better at 4 °C than at higher temperature
Coal/charcoal	*Rhizobium*/PS bacteria	Seed inoculants
Granular inoculants amended with nutrients	*B. japonicum*	Soil inoculants; bentonite granules, illite and smectite granules, silica granules amended with glycerol, Na glutamate and inoculated with either peat or liquid *B. japonicum* inoculants; enhanced early nodulation of soybean and increased N content of grain
Soybean oil or peanut oil added with lyophilized cells	*Rhizobium*	Seed inoculants; provide more protection than peat-based inoculants when rhizobia are inoculated on seeds and exposed to condition of drought and high temperature
Perlite	*Rhizobium, Bradyrhizobium, Bacillus*	Seed inoculants; combination of a sucrose adhesive with the perlite carrier gave better survival of bacteria on seeds; produced similar number of nodules, nodule dry weight, crop yield and nitrogen content as peat-based inoculants

(continued)

Table 1.3 (continued)

Carrier material	Inoculant bacterium	Characteristics
Wastewater sludge	*Sinorhizobium meliloti*	Seed inoculants; result showed the suitability of using sludge as a carrier because it had the same or a higher potential than peat to support survival of *S. meliloti*
Wheat bran, sugar cane bagasse	*Rhizobium*/*Bradyrhizobium* and PS fungus, *A. niger*	Soil inoculants; the number of microorganisms was the highest with peat, followed by bran and sugarcane bagasse
Nutrient-supplemented pumice	*Rhizobium*	Seed inoculants; good storage and handling properties and could be mixed directly with the seeds during the sowing process

use of any ideal carrier material is important in the production of good quality microbial inoculants including microphos. Among various materials, peat soil, lignite, vermiculite, charcoal, press mud, wastewater sludge, cow dung cake powder, sawdust, farmyard manure (FYM) and soil mixture have been used by many workers as carrier materials for producing the microbial inoculants (Ben Rebah et al. 2002; Trivedi et al. 2005; Maheshwari 2008; Khan et al. 2010, 2013). Of these materials, the neutralized peat soil/lignite has been found as the better carrier material for inoculant production. However, an ideal carrier should have these properties: (i) it should be inexpensive, mixable, packageable and locally available in powder or granular form in adequate quantities; (ii) the carrier must permit gas exchange, particularly oxygen, and have high organic matter content (Bashan 1998) and water holding and retention capacity and it should be more than 50 %; (iii) it should be easy to process (mixing, curing and packaging operations) and free of lump-forming materials; (iv) it should be easy to sterilize by autoclaving or gamma-irradiation; (v) it should have good adhesion to seeds (Hegde and Brahmaprakash 1992) and good pH buffering capacity (Keyser et al. 1993); (vi) it should be non-toxic to inoculant bacterial strain and plant and easily biodegradable and non-polluting; (vii) it should nearly be sterile and uniform; (viii) it should support growth and survival of bacteria; and (ix) rapid release of bacteria in soil. For preparation of seed inoculant, the carrier material is milled to fine powder with particle size of 10–40 μm. The selected carriers are then sterilized before mixing with inoculum so that high number of inoculant bacteria can be maintained on carrier for long storage period. Furthermore, carrier is sterilized to prevent undesirable spreading of pathogenic bacteria to agricultural field. Different methods have been adopted to sterilize the carrier materials in order to find the most suitable one without any effect on their quality. In this context, gamma-irradiation has been found as the most suitable way of sterilization since gamma-irradiation does not alter the physical and chemical characteristics of the carrier materials. However, there are other ways by which carrier materials can also be sterilized. Of these,

autoclaving is the most commonly used and has the superiority among all employed methods due to low cost and its ability to allow absolutely pure culture of inocula to be prepared. For autoclaving, carrier material is packed in partially opened, thin-walled polypropylene bags and autoclaved for 60 min at 121 °C. However, during autoclaving, some materials change their properties and produce toxic substances which could be toxic to some bacterial strains. Once carrier is sterilized, it is ready for mixing with PS inocula.

1.5.6.3 Mixing of Carrier with Inocula and Inoculant Packaging

The most suitable carrier material is first spread in clean, dry, sterile metallic or plastic trays, and the bacterial culture developed at small scale (flasks) or large scale (fermenter) is added to the sterilized carrier and mixed well manually by wearing sterile gloves or mechanically by mixer. The culture suspension is added at a level of 40–50 % WHC of the carrier. After proper mixing, the inoculant is kept in a polythene bag (low density and thickness of the bag should be around 50–75 μm) of 200 g capacity, sealed with electric sealer and allowed for curing for 2–3 days at room temperature. Curing of carrier-based microphos can be done by spreading the inoculant on a clean floor/polythene sheet by keeping in open shallow tubs/trays with polythene covering for 2–3 days at room temperature before packaging. After packaging, the packet containing microphos should legibly be marked with the name of the manufacturer, name and cost of the product, batch number and strain number, the mode of application, date of manufacture and expiry, full address of the manufacturer and storage information. The microphos bags are now ready for pot/field application or can be stored for later use. The microphos packets can, however, be stored for about 3 months at 25 ± 2 °C. Similarly, the two cultures of the same groups or different groups [one or two fungi/AM fungi together or one PSM and other PGPR] can be mixed together in order to produce a mixed/co-inoculant. However, before the two organisms, identical or different, are used, their compatibility towards each other and the persistence of P-solubilizing activity under in vitro conditions must be ascertained (Khan et al. 2007). If the two organisms show any kind of antagonisms under laboratory conditions, they should not be used together for developing a mixed or co-culture of the microphos. Approaches used in the production and application of phosphate-solubilizing microbes are shown in Fig. 1.3.

1.5.6.4 Instruction for Microphos Storage

Carrier-based microphos packet should be stored in a cool place away from heat or direct sunlight. The microphos packets may also be stored at room temperature or in cold storage conditions. However, microphos should regularly (at least at 15 days interval) be checked very carefully to evaluate the number of viable cells using plate count method or serological methods (if available). The PSM density in

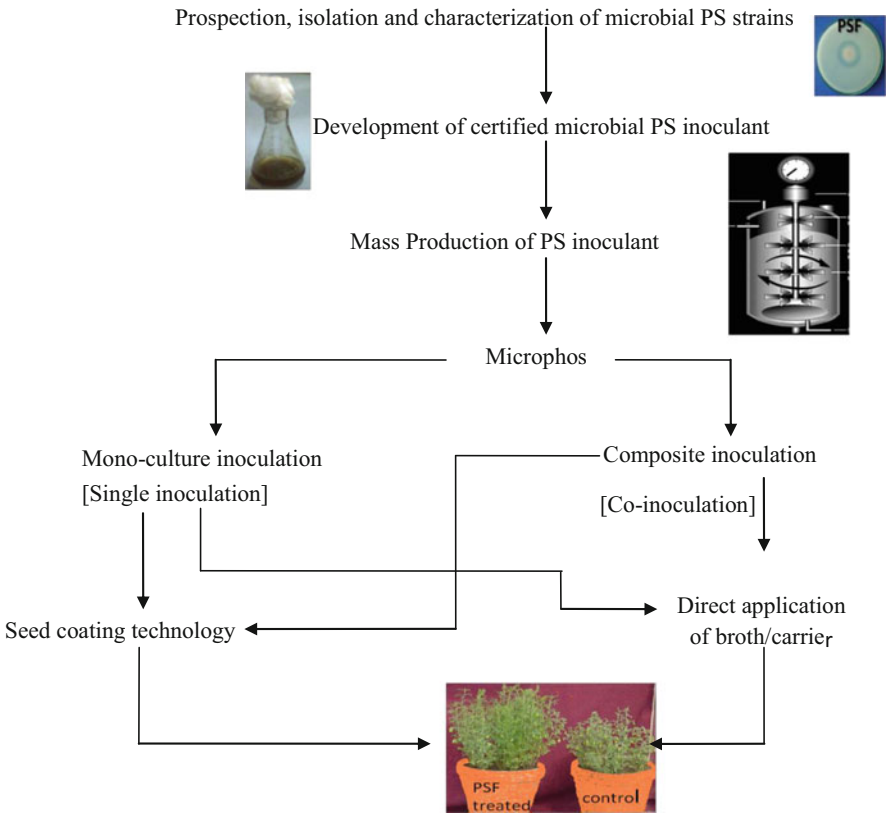

Fig. 1.3 Approaches used for production and application of microbial phosphatic inoculants [adapted from Khan et al. (2009)]

prepared microphos packet should not be less than 10^9 cells/g of inoculant at the time of preparation and 10^7 cells/g on dry weight basis before expiry date. However, this microbial load in carrier-based microphos may vary from organisms to organisms.

1.5.7 Method of Microphos Application

The microphos containing potential PS organism can be applied in different ways, for example, (i) seed treatment or seed inoculation, (ii) seedling root dip and (iii) main field/soil application.

1.5.7.1 Seed Treatment

Coating/bacterization of plant seeds by microbial cultures involving microphos has traditionally been the most common and widely used method in agricultural practices for enhancing crop production across different ecological niches (Khan et al. 2007, 2010). In this method, one packet containing 200 g of microphos is sufficient to treat 10 kg of healthy and good quality medium-sized seeds of groundnut, wheat, cotton, maize, etc., whereas 100 g per acre inoculant is required for priming small-sized seeds. For seed treatment, one packet of the microphos is cut open and the inoculants (bacteria–carrier mixture) are mixed with water to make slurry and then mixed with seeds. To achieve the firm and sufficient coating of inoculant on seed surface, use of adhesive/sticker solution such as 10 % gum arabic, methylethylcellulose, sucrose solutions and vegetable oils is recommended. Furthermore, any locally available sticky material (e.g. jaggery solution) which is non-toxic to bacteria and seeds can also be used as adhesive. The seeds intended for one-acre lands are then dipped in the slurry for about 3 h so as to have a uniform coating of the culture over the seeds. The uniformly bio-primed seeds are then dried under shade for 30 min, and the shade-dried seeds are sown in pot or field soils within 24 h. Even though this method allows the adequate loading of bacterial cells on to the seeds [10^8 cells/seed], this method has certain limitations, for example, the bacterized seeds may come in direct contact with any seed applied with chemicals, which, in turn, may adversely affect the survivability of the inoculated strains. Secondly, the bacterial cultures after application may move away from rooting zones and hence could be exposed to agrochemicals after planting.

1.5.7.2 Seedling Root Dip

Here, contents of the microphos packets are mixed in water, and the root portion of the seedlings required for an acre is dipped in the mixture for 5 to 10 min; seedlings are then removed from the suspension and transplanted as early as possible. Suspension of one kg microphos in 10–15 l of water is sufficient for treating seedlings for one acre. This method is generally used for transplanted crops, for instance, vegetable crops.

1.5.7.3 Main Field/Soil Application

Seed inoculation technique in agricultural practices may not always be successful. For example, if PS organisms belonging to N_2 fixing (e.g. rhizobia) are applied, it may result in poor nodulation and hence depressed nitrogen fixation by legumes. Secondly, there may be low colonization and weak establishment of the inoculated rhizobacterial strains. This situation might be due to low population and/or low survival of the inoculated bacterial strain on the seed surface and in the soil.

So, in such cases, "soil inoculation" method should be adopted wherein a large population of a bacterial strain can be introduced directly into the soil. For soil inoculation, in general, granular inoculant is placed into the furrow under or alongside the seed. This enhances the chance for the inoculated strain to be in contact with plant roots. Alternatively, four packets of microphos are mixed with 20 kg of dried and powdered farmyard manure (FYM) and then broadcasted in one acre of main field just before transplanting. This method allows a rapid and greater colonization of P-solubilizing organisms per unit area. In addition, the direct contact of inocula with chemically treated seeds is minimized. This method also offers advantages like (i) it is quick compared to seed inoculation technique which requires mixing of seeds with inoculants, (ii) inoculants can withstand low-moisture conditions better than carrier-based inoculants and (iii) it is less expensive compared to other inoculation methods. Thus, in accordance with these considerations, two approaches can be applied for microphos applications: (i) the monoculture approach [MCA] where P-solubilizing microorganisms can be used alone and (ii) the co-culture or multiple culture approach [CCA], where microphos prepared from two or more identical or different microbial strains can be mixed together and then applied under natural field/pot house conditions. The bacterial inoculants, however, should not be mixed with insecticide, fungicide, herbicide and fertilizers. When seeds are treated with fungicides, the seeds should be treated first with pesticides and then with microphos.

1.6 Conclusion

In high-input agricultural practices, the deficiency of soil P is circumvented mainly through the use of chemical phosphatic fertilizers, the excessive and continued use of which results in loss of soil fertility and, hence, the crop productivity. Microphos in this context might play a pivotal and practicable role in enhancing the soil P pool without adversely disturbing the soil microflora and the processes mediated by them. Since majority of microbial inoculants developed so far are used for enhancing legume, cereal and some vegetable production, there is an increasing demand from fruit and vegetable production sector, where use of chemical fertilizers is either not allowed or is restricted for human health reasons. In this regard, the development of microphos could serve a viable option for such crops, and using microphos, some success has been achieved over in different production systems. The challenge, however, is to find some novel phosphate-solubilizing microorganism expressing multiple growth-promoting activities that could be applied under diverse agroecosystems. Moreover, there is a need to develop some simple techniques for mass production of microphos and its delivery systems so that the use of microphos could be popularized and increased across different regions in a sustainable manner. The commercialization of microphos is, however, a challenging task which requires full-scale, cost-effective manufacturing, packaging and quality control systems. Furthermore, the large-scale field trials for microphos are needed

to ascertain the potentiality and functionality of PSM. Thus, the search for identifying new PS microbes and fine-tuning the production strategies of microphos requires the continuous efforts of scientists working in different disciplines across the countries.

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Chapter 2
Mechanism of Phosphate Solubilization and Physiological Functions of Phosphate-Solubilizing Microorganisms

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Abstract Phosphorus (P) is the second important key plant nutrient after nitrogen. An adequate supply of P is therefore required for proper functioning and various metabolisms of plants. Majority of P in soils is fixed, and hence, plant available P is scarcely available despite the abundance of both inorganic and organic P forms in soils. A group of soil microorganisms capable of transforming insoluble P into soluble and plant accessible forms across different genera, collectively called phosphate-solubilizing microorganisms (PSM), have been found as best eco-friendly option for providing inexpensive P to plants. These organisms in addition to supplying soluble P to plants also facilitate the growth of plants by several other mechanisms, for instance, improving the uptake of nutrients and stimulating the production of some phytohormones. Even though several bacterial, fungal and actinomycetal strains have been identified as PSM, the mechanism by which they make P available to plants is poorly understood. This chapter focuses on the mechanism of P-solubilization and physiological functions of phosphate solubilizers in order to better understand the ecophysiology of PSM and consequently to gather knowledge for managing a sustainable environmental system. Conclusively, PSM are likely to serve as an efficient bio-fertilizer especially in areas deficient in P to increase the overall performance of crops.

Keywords Phosphate solubilization • Mineralization • Organic acids • Plant growth regulators • ACC deaminase

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2.1 Introduction

Phosphorus (P) is one of the most essential plant nutrients which profoundly affect the overall growth of plants (Wang et al. 2009) by influencing various key metabolic processes such as cell division and development, energy transport, signal transduction, macromolecular biosynthesis, photosynthesis and respiration of plants (Shenoy and Kalagudi 2005; Ahemad et al. 2009; Khan et al. 2009). On the contrary, unlike N, the atmosphere does not provide soluble P to plants. And hence, the source of P is largely the primary and secondary minerals and/or organic compounds. In comparison to other nutrients, P concentration in soil solution is much lower and ranges from 0.001 to 1 mg/l (Brady and Weil 2002). Broadly, P compounds in soil can be placed into three categories: (i) inorganic compounds, (ii) organic compounds of the soil humus and (iii) organic and inorganic P compounds associated with the cells of living matter. Mineral compounds of P usually contain aluminium (Al), iron (Fe), manganese (Mn) and calcium (Ca) and vary from soils to soils. For example, P forms a complex with Al, Fe and Mn in acidic soils, while in alkaline soils it reacts very strongly with Ca. However, under all conditions, the types of soil P compounds are determined mainly by soil pH and by the type and concentrations of soil minerals. Some of the most common P minerals are presented in Table 2.1. Of the total soil P pool, about 50 % of P is in the organic forms (Richardson 1994), which varies between 4 and 90 % in most soils (Yadav and Verma 2012). The organic P in plants includes (i) inositol phosphate (10–50 % in soil) which represents a series of phosphate esters ranging from monophosphates up to hexaphosphates. Phytic acid (inositol hexakisphosphate) is the main compound that plants use to store P in seeds to support early seedling growth following germination. Phytin (a Ca–Mg salt of phytic acid) is the most abundant of the known organophosphorus compounds in soils. Other organic P in soils occur as sugar phosphates, nucleotides (0.2–2.5 %), phosphoprotein (trace), phosphonates (Tate 1984) and phospholipids (1–5 %) (Yadav and Verma 2012). Of the various forms of P, plants take up only negatively charged primary and secondary orthophosphate ions ($H_2PO_4^-$ and HPO_4^{2-}) as nutrient. Indeed, the amount of plant available P is very low relative to the total soil P. Moreover, majority of the soil P is fixed, and only a small fraction of P is available for uptake by plants. Therefore, P deficiency results in stunted growth, dark leaves, and inhibition of flowering and root system development. In most plants, these symptoms will appear when P concentration in the leaves goes below 0.2 %. And, hence, in many cases, phosphatic fertilizers which are quite soluble and manures that also contain P (soluble P, organic P and inorganic P) are applied to overcome P deficiency in soils and to provide adequate P to plants. The P of the phosphatic fertilizers or the manure reacts very strongly with soil constituents and becomes unavailable to plants. The insoluble and inaccessible forms of P are hydrolysed to soluble and available forms through the process of solubilization (inorganic P)/mineralization (organic P). The immobilization in contrast is the reverse reaction of mineralization. During immobilization, microorganisms convert inorganic forms to organic phosphate,

Table 2.1 Common phosphorous (P) minerals found in acid, neutral and calcareous soils

S.No.	Minerals	Chemical formula
	Acid soils	
	Strengite	$FePO_4.2H_2O$
	Variscite	$AlPO_4.2H_2O$
	Neutral and calcareous soils	
	B-tricalcium phosphate	$Ca_3(PO4)_2$
	Dicalcium phosphate	$CaHPO_4$
	Dicalcium phosphate dihydrate	$CaHPO_4{\cdot}2H_2O$
	Fluorapatite	$Ca_5(PO4)_3$ F
	Hydroxyapatite	$Ca_5(PO4)_3$ OH
	Octacalcium phosphate	$Ca_4H(PO4)_3{\cdot}2–5\ H_2O$

Adapted from Yadav and Verma (2012)

which are then incorporated into their living cells. Mineralization and immobilization of P occur simultaneously and are influenced by structure and compositions of microbes and physico-chemical characteristics of soils besides the exudates of various plant genotypes.

2.2 Phosphate Solubilization by Microbes: Current Perspective

The insoluble forms of P such as tricalcium phosphate ($Ca_3PO_4)_2$, aluminium phosphate (Al_3PO_4), iron phosphate (Fe_3PO_4), etc. may be converted to soluble P by P-solubilizing organisms inhabiting different soil ecosystems (Gupta et al. 2007; Song et al. 2008; Khan et al. 2013; Sharma et al. 2013). Soil microorganisms in this regard have generally been found more effective in making P available to plants from both inorganic and organic sources by solubilizing (Toro 2007; Wani et al. 2007a) and mineralizing complex P compounds (Bishop et al. 1994; Ponmurugan and Gopi 2006), respectively. Several workers have documented their findings in order to better understand as to how the microbial populations cause the solubilization of insoluble P (Illmer and Schinner 1995; Khan et al. 2007, 2009; Buch et al. 2008). Of the various strategies adopted by microbes, the involvement of low molecular mass organic acids (OA) secreted by microorganisms has been a well-recognized and widely accepted theory as a principal means of P-solubilization, and various studies have identified and quantified organic acids and defined their role in the solubilization process (Maliha et al. 2004; Khan et al. 2010; Marra et al. 2012). The OA produced by many P-solubilizers, for example, bacterial cultures (Table 2.2) or fungi (Table 2.3), in the natural environment or under in vitro conditions chelate mineral ions or decrease the pH to bring P into solution (Pradhan and Shukla 2005). Consequently, the acidification of microbial cells and their surrounding leads to the release of P-ions from the P-mineral by H^+ substitution for Ca^{2+} (Goldstein 1994; Mullen 2005; Trivedi and Sa 2008). The

Table 2.2 Organic acid production and P-solubilization by PS bacteria

PS bacteria	Organic acid produced	Initial pH	Final pH	Amount of P solubilized (μg/ml)	Time (h)	References
Enterobacter sp. Fs-11	MA, GA	7.0	4.5	43.5	240	Shahid et al. (2012)
Pseudomonas trivialis (BIHB 769)	GA, 2-KGA, LA, SA, FA, MA	7 ± 0.2	3.70	806.4 ± 2.3	120	Vyas and Gulati (2009)
P. poae (BIHB 808)	GA, 2-KGA, SA, CA, MA	7 ± 0.2	3.58	821.4 ± 1.7	120	Vyas and Gulati (2009)
Pseudomonas spp. (BIHB 751)	OA, GA, 2-KGA, FA, MA	7 ± 0.2	4.20	318.7 ± 2.0	120	Vyas and Gulati (2009)
Enterobacter Hy-401	OA, GA, MA, LA, CA, SA, FuA	7–7.5	4.32 ± 0.02	623.6 ± 23.0	120	Yi et al. (2008)
Arthrobacter Hy-505	OA, GA, LA, CA	7–7.5	5.50 ± 0.04	428.9 ± 15.3	120	Yi et al. (2008)
Azotobacter Hy-510	OA, GA, TA, LA, SA, FuA	7–7.5	4.69 ± 0.05	229.03 ± 15.2	120	Yi et al. (2008)
Enterobacter Hy-402	OA, GA, TA, CA, SA, FuA	7–7.5	4.51 ± 0.02	111.73 ± 8.07	120	Yi et al. (2008)
Rhodococcus erythropolis (CC-BC11)	GA	7–6.8	5.3	186.9	72	Chen et al. (2006)
Bacillus megaterium (CC-BC10)	CA, LA, PA	7–6.8	5.1	270.2	72	Chen et al. (2006)
Arthrobacter sp. (CC-BC03)	CA, LA	7–6.8	4.9	519.7	72	Chen et al. (2006)
A. ureafaciens (CC-BC02)	CA	7–6.8	5.0	316.1	72	Chen et al. (2006)
Serratia marcescens (CC-BC14)	CA, GA, SA, LA	7–6.8	4.9	421.8	72	Chen et al. (2006)
Delftia (CC-BC21)	SA	7–6.8	4.9	346.1	72	Chen et al. (2006)
Chryseobacterium (CC-BC05)	CA	7–6.8	6.0	298.9	72	Chen et al. (2006)
Phyllobacterium myrsinacearum (CC-BC19)	GA	7–6.8	5.2	201.2	72	Chen et al. (2006)

GA gluconic acid, *2-KGA* 2α-ketogluconic acid, *LA* lactic acid, *SA* succinic acid, *FA* formic acid, *MA* malic acid, *CA* citric acid, *OA* oxalic acid, *FuA* fumaric acid, *TA* tartaric acid, *PA* propionic acid, *AA* acetic acid, *IBA* isobutyric acid, *IVA* isovaleric acid, *VA* valeric acid, *ISA* isocaproic acid, *ND* not determined [modified from Khan et al. (2013)]

Table 2.3 Some examples of organic acids produced by P-solubilizing fungi

Organism	Predominant acids	References
Aspergillus niger FS1, *Penicillium canescens* FS23, *Eupenicillium ludwigii* FS27, *Penicillium islandicum* FS30	Citric, gluconic, oxalic	Mendes et al. (2013)
Aspergillus awamori S19	Oxalic, malic, citric, succinic, fumaric	Jain et al. (2012)
T. flavus, *T. helicus*, *P. purpurogenum*, *P. janthinellum*	Acetic, butyric, citric, fumaric, gluconic, glucuronic, lactic, oxalic, propionic, succinic, valeric	Scervino et al. (2010a, b)
Aspergillus niger, *Penicillium bilaiae*, *Penicillium* sp.	Oxalic, citric	Arwidsson et al. (2010)
Aspergillus flavus, *A. candidus*, *A. niger*, *A. terreus*, *A. wentii*, *Fusarium oxysporum*, *Penicillium* sp., *Trichoderma isridae*, *Trichoderma* sp.	Lactic, maleic, malic, acetic, tartaric, citric, fumaric, gluconic	Akintokun et al. (2007)
A. flavus, *A. candidus*, *Penicillium oxalicum*	Glutaric, malic, gluconic, oxalic	Shin et al. (2006)
Aspergillus flavus, *A. niger*, *P. canescens*	Oxalic, citric, gluconic, succinic	Maliha et al. (2004)
Penicillium rugulosum	Citric, gluconic	Reyes et al. (2001)
A. niger	Succinic	Vazquez et al. (2000)

efficiency of solubilization, however, depends on the kind of organic acids released into the medium and their concentration. Furthermore, the quality of the acid is more important for P-solubilization than the total amount of acids produced by phosphate solubilizing (PS) organisms (Scervino et al. 2010a, b). Additionally, the simultaneous production of different organic acids by the PS strains may contribute to the greater potential for solubilization of insoluble inorganic phosphates (Marra et al. 2012).

There are also reports which suggest that insoluble P could be transformed into soluble forms of P without OA production by microbes (Asea et al. 1988; Illmer and Schinner 1992; Chen et al. 2006). For example, Altomare et al. (1999) while investigating the P-solubilizing ability of plant growth-promoting and biocontrol fungus *Trichoderma harzianum* T-22 did not produce OA under in vitro condition suggesting that the insoluble P could be solubilized by mechanisms other than acidification process also. The fungal-solubilizing activity was credited both to chelation and to reduction processes, which may be useful in the management of phytopathogens. Apart from the OA theory, some of the inorganic acids (Reyes et al. 2001; Richardson 2001) such as HCl (Kim et al. 1997), nitric acid and sulphuric acids (Dugan and Lundgren 1965) produced by chemoautotrophs and the H^+ pump, for example, in *Penicillium rugulosum*, have also been reported to solubilize the insoluble P (Reyes et al. 1999). The inorganic acids so released

convert TCP to di- and monobasic phosphates with the net result of an enhanced availability of the element to plants.

2.2.1 *Mineralization: Enzymatic Degradation of Complex Organic P Compounds*

Organic P compounds undergo mineralization, and the resulting P is taken up as nutrient by plants. In this regard, numerous soil microbes or rhizosphere microflora possess the ability to transform organic P into soluble forms of P (Tarafdar and Claassen 1988; Rodriguez et al. 2006). This mineralization process is mediated by the enzymes especially phosphatases (Tarafdar et al. 1988; Yadav and Tarafdar 2003; Aseri et al. 2009) and phytases (Maougal et al. 2014), released by the soil microbes. The enzyme phosphatases (e.g. acid and alkaline phosphatases) released exterior to the cell (exo-enzymes) are non-specific in nature and use organic P as a substrate to convert it into inorganic form (Beech et al. 2001). Of the two phosphatases, acid phosphatases (To-O et al. 2000), a widely distributed enzyme and commonly found in fungi (To-O et al. 1997; Abd-Alla and Omar 2001), for example, *Colletotrichum graminicola* (Schadeck et al. 1998a, b), are considered as the principal mechanism for mineralization of soil organic P (Hilda and Fraga 1999) where it catalyses the release of inorganic P from organic P compounds such as inositol hexaphosphate (Nozawa et al. 1998; Tarafdar and Gharu 2006; Yadav and Tarafdar 2007, 2011). However, the degradation of organic P mediated by phosphatases varies greatly among different fungi (Guimarães et al. 2006). Another attractive application of P-dissolving enzymes is the mineralization of soil organic P through phytate degradation mediated by the enzyme phytase, which specifically causes release of P from phytic acid. Phytate is a major component of organic P in soil. Though the ability of plants to obtain P directly from phytate is very limited, the growth and P nutrition of *Arabidopsis* plants supplied with phytate was improved significantly when they were genetically transformed with the phytase gene [phyA] derived from *Aspergillus niger* (Richardson 2001). This led to the increase in P nutrition to such an extent that the growth and P content of the plant was equivalent to control plants supplied with inorganic P. Similar increase in utilization of inositol P by plants in the presence of microbial communities including P-solubilizing fungus (*A. niger*) capable of producing phytase is reported (Richardson 2001; Vassilev et al. 2007). Phosphonatases (Kumar et al. 2013) and C–P lyases (Salimpour et al. 2010) are the other enzymes that cleave the C–P of organophosphonates. Once the inorganic or organic P compound is changed to soluble P, it can now easily be used up as P nutrient by plants, algae, cyanobacteria and autotrophic bacteria and thereafter could be immobilized into organic cellular macromolecules, for example, DNA, RNA and ATP. Considering the critical impact of such enzymes in dissolution of complex organic compounds into usable form of P, it is highly desirable to develop the bacterial/fungal inoculants with high

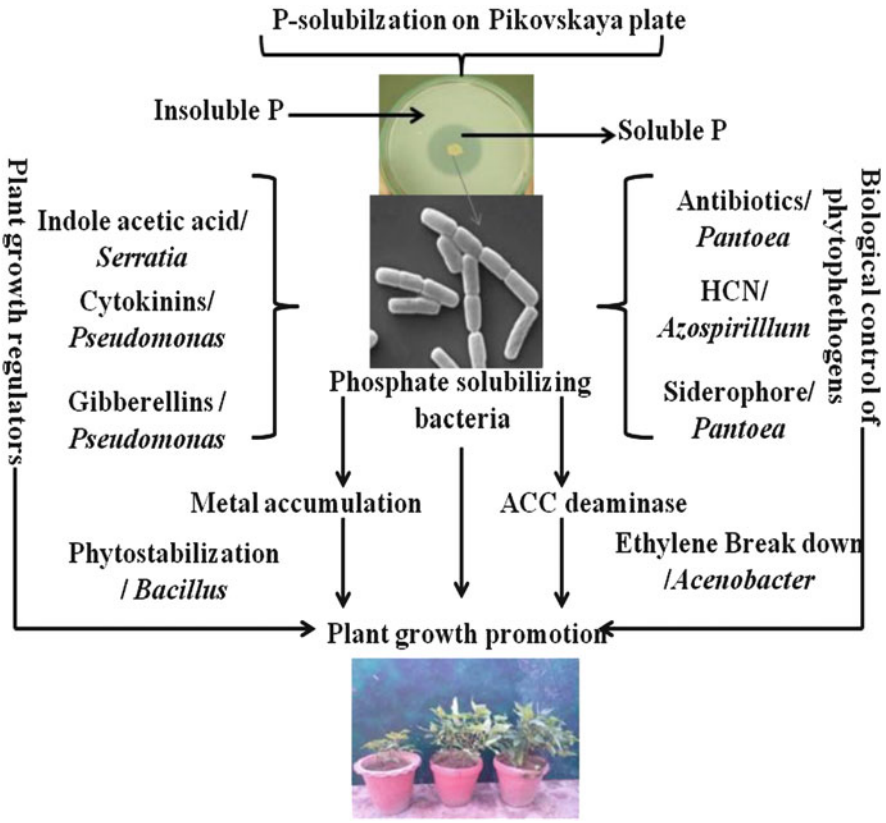

Fig. 2.1 An illustration depicting functional diversity among PS bacteria [Adapted from Khan et al. (2013)]

phosphatase and phytase activity which in turn could possibly be of great practical value in sustainable crop production.

2.3 Physiological Functions of Phosphate-Solubilizing Microorganisms

Phosphate-solubilizing microorganisms increase the overall performance of plants by providing mainly soluble P to plants in different production systems. However, they also benefit plants by other mechanisms (Fig. 2.1). Indeed, PSM exhibit multifunctional properties (Vassileva et al. 2010; Yadav et al. 2011; Khan et al. 2013); for example, they are known to synthesize siderophores (Hamadali et al. 2008; Wani et al. 2008a; Tank and Saraf 2003; Viruel et al. 2011) and IAA and gibberellic acid (Sattar and Gaur 1987; Souchie et al. 2007; Viruel et al. 2011).

Phosphate-solubilizing bacteria such as Gram-negative *P. fluorescens*, *P. aeruginosa* and *Chromobacterium violaceum* also secrete antibiotics (Lipping et al. 2008; Taurian et al. 2010) and provide protection to plants against soilborne pathogens (biocontrol) (Khan et al. 2002; Vassilev et al. 2006; Singh et al. 2010). Other physiological traits of PSM involve the release of cyanide, a secondary metabolite which is ecologically important (Wani et al. 2007b) and gives a selective advantage to the producing strains (Rudrappa et al. 2008; Badawi et al. 2011). Besides strict P-solubilizers, a few genera of rhizobia, for example, *Bradyrhizobium* and *Rhizobium*, have also been found to solubilize P and secrete IAA (Pandey and Maheshwari 2007; Badawi et al. 2011). There are numerous PS bacteria that possess the ability to synthesize a key enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick et al. 2007), which hydrolyses ACC [the immediate precursor of plant hormone ethylene (C_2H_4)] to NH3 and α-ketobutyrate and thus mitigate the inhibitory effects of C_2H_4. Some of the compounds synthesized by PS bacteria with possible effect on plant growth are listed in Tables 2.4 and 2.5.

2.3.1 Some Examples of Positive Plant Growth Regulators Synthesized by PSM

Plant growth regulators (PGRs) are the substances that influence physiological processes of plants at very low concentrations and modify or control one or more specific metabolic events of a plant (Danova et al. 2012; Sane et al. 2012). According to the Environmental Protection Agency (EPA), the plant regulators have been defined as "any substance or mixture of substances intended, through physiological action, to accelerate or retard the rate of growth or maturation, or otherwise alter the behaviour of plants or their produce". Such compounds produced by the plant or by PGPR are called plant hormones (Davies 1995; Karadeniz et al. 2006). Broadly, on the basis of chemical structures and their subsequent effects on plants, plant growth-regulating substances have been divided into five general groups: (1) auxins, (2) gibberellins, (3) cytokinins, (4) ethylene and (5) a group called inhibitors, which includes abscisic acid (ABA), phenolics and alkaloids (Frankenberger and Arshad 1995; Ferguson and Lessenger 2006). The production of auxins (Glick 1995; Wani et al. 2007b, 2008b; Ahemad and Khan 2012) and ethylene (Sasek et al. 2012), for example, by PSM, is considered a common microbiological trait, while the synthesis of cytokinins by bacteria, for example, *Paenibacillus polymyxa* (Raza et al. 2008), is less common (Timmusk et al. 1999). The gibberellin secretion at high concentrations is, however, very rare (Solano et al. 2010). Generally, majority (>80 %) of the soil bacteria are capable of secreting auxins especially IAA, indole butyric acid or similar compounds via tryptophan metabolism (Solano et al. 2010; Legault et al. 2011). A few examples of the phytohormones secreted by PGPR including PS bacteria (Table 2.4) and PS

Table 2.4 Growth-promoting substances produced by plant growth-promoting rhizobacteria

Organisms	Growth regulators	References
Pseudomonas putida	ACC deaminase, IAA, siderophore, ammonia, HCN, P-solubilization	
Pseudomonas sp., *Pseudomonas fluorescens*, *Burkholderia glumae*	ACC deaminase, IAA, siderophore, ammonia, HCN, P-solubilization	Rashid et al. (2012)
Bacillus	ACC deaminase, IAA, siderophore, P-solubilization, lytic enzyme, HCN	Kumar et al. (2012)
Azotobacter	IAA, siderophore, P-solubilization	Farajzadeh et al. (2012)
Klebsiella	IAA, siderophore, P-solubilization, HCN	
Azotobacter, Fluorescent *Pseudomonas*, and *Bacillus*	IAA, siderophore, ammonia, HCN, P-solubilization	
Pantoea dispersa strain 1A	P-solubilization, IAA, siderophore, HCN	Selvakumar et al. (2008a, b)
Bacillus spp.	IAA, siderophore, HCN	Wani et al. (2007d)
Pseudomonas, *Bacillus*	Siderophore, IAA, P-solubilization	Rajkumar et al. (2006)
Brevibacillus sp.	IAA	Vivas et al. (2006)
Xanthomonas sp. RJ3, *Azomonas* sp. RJ4, *Pseudomonas* sp. RJ10, *Bacillus* sp. RJ31	IAA	Sheng and Xia (2006)
Bacillus sp.	P-solubilization	Canbolat et al. (2006)
Brevibacterium sp.	Siderophore	Noordman et al. (2006)
Bacillus subtilis	IAA and P-solubilization	Zaidi and Khan (2006)
Variovorax paradoxus, *Rhodococcus* sp. and *Flavobacterium* (Cd tolerant)	IAA and siderophore	Belimov et al. (2005)
Pseudomonas fluorescens	IAA, siderophore, P-solubilization	Gupta et al. (2005)
Pseudomonas putida	Siderophore	Tripathi et al. (2005)
Azotobacter, fluorescent *Pseudomonas*	IAA	

rhizobia (Table 2.5) and other compounds and their direct or indirect impact on plant growth and development are reviewed and discussed briefly in the following section.

Table 2.5 Examples of plant growth-promoting substances synthesized by symbiotic nitrogen fixers

Symbiotic N_2 fixer	Crop enhancer	References
Rhizobium leguminosarum RP2	ACC deaminase, IAA, HCN, siderophore, ammonia, EPS	
Bradyrhizobium MRM6	IAA, HCN, siderophore, ammonia, EPS	
Rhizobium MRL3	IAA, HCN, siderophore, ammonia	
Sinorhizobium strain	Chitinase	Qing-xia et al. (2011)
Rhizobium leguminosarum var. *Phaseoli*	IAA	Stajković et al. (2011)
Rhizobium spp.	IAA, siderophore	Mehboob et al. (2010)
Sinorhizobium meliloti	IAA, P-solubilization	Bianco and Defez (2010)
Bradyrhizobium	IAA, gibberellic acid	Afzal et al. (2010)
Mesorhizobium	IAA	
Rhizobium spp.	IAA	Chakraborty et al. (2009)
Rhizobium leguminosarum	IAA, siderophore	
Mesorhizobium	IAA, HCN, siderophore, ammonia, P-solubilization	
Rhizobium strain TAL 1145	ACC deaminase	Tittabutr et al. (2008)
Rhizobium spp.	IAA, gibberellic acid, zeatin	Boiero et al. (2007)
Mesorhizobium loti MP6	IAA, HCN, siderophore, P-solubilization	Chandra et al. (2007)
Rhizobium etli USDA9032	Phenazine, antibiotic	Krishnan et al. (2007)

2.3.1.1 Synthesis and Physiological Functions of Phytohormones

Synthesis of IAA

The synthesis of IAA by microbes (Fig. 2.2) involves one of the three pathways: (1) Indoleacetic acid formation via indole-3-pyruvic acid and indole-3-acetic aldehyde is found in the majority of bacteria like *Erwinia herbicola*; saprophytic species of the genera *Agrobacterium* and *Pseudomonas*; and certain representatives of *Bradyrhizobium*, *Rhizobium*, *Azospirillum*, *Klebsiella* and *Enterobacter*. (2) The conversion of tryptophan into indole-3-acetic aldehyde may involve an alternative pathway in which tryptamine is formed. This pathway is believed to operate in pseudomonads and azospirilla. (3) IAA biosynthesis via indole-3-acetamide formation is reported for phytopathogenic bacteria *Agrobacterium tumefaciens*, *Pseudomonas syringae* and *E. herbicola* and saprophytic pseudomonads like *Pseudomonas putida* and *P. fluorescens*. The genes controlling IAA synthesis via this pathway are also reported in symbiotic bacteria like *Rhizobium* spp.,

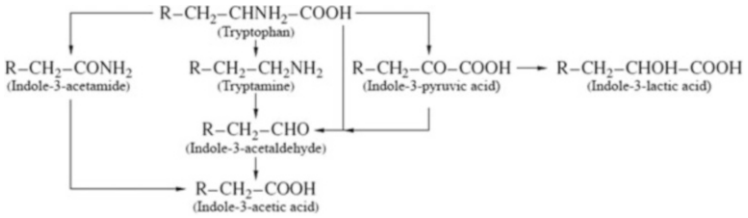

Fig. 2.2 Biosynthetic pathways of IAA in bacteria [Adapted from Patten and Glick (1996)]

Bradyrhizobium spp. and *Azospirillum* spp., although the activity of the corresponding enzymes is either negligible or not detectable. Indoleacetic acid biosynthesis that involves tryptophan conversion into indole-3-acetonitrile is found in plants, *Alcaligenes faecalis*, and possibly the cyanobacterium *Synechocystis* sp., and the tryptophan-independent pathway, more common in plants, is also found in microorganisms (azospirilla and cyanobacteria). However, the synthesis of IAA using this pathway is reported to be insignificant, and the mechanisms are largely unknown. Many bacteria are known to synthesize auxins using such pathways and help the plants to grow better. Bacteria in general form maximum amount of IAA during the steady-state stage of their growth while ammonium ions and glutamine inhibit IAA biosynthesis (Tsavkelova et al. 2006). The genes involved in IAA synthesis in bacterial strains may be plasmid or chromosomal borne. For example, pathogenic bacteria contain Ti plasmids that control the formation of the phytohormone, whereas in saprophytic microorganisms, auxin biosynthesis is governed by chromosomal genes (Tsavkelova et al. 2006). It is reported that 80 % of microorganisms isolated from the rhizosphere of various crops possess the ability to synthesize and release auxins as secondary metabolites (Loper and Schroth 1986). Of the various PGPR strains, bacteria belonging to the genera *Azospirillum*, *Pseudomonas*, *Xanthomonas* and *Rhizobium* as well as *Alcaligenes*, *Enterobacter*, *Acetobacter* and *Bradyrhizobium* have been shown to produce auxins which help in stimulating plant growth (Egamberdieva et al. 2007; Wani et al. 2007c; Kumar et al. 2008; Poonguzhali et al. 2008). However, the extent of IAA production by bacterial strains could be different due in part to the involvement of biosynthetic pathways, location of the genes, regulatory sequences and the presence of enzymes to convert active free IAA into conjugated forms. Moreover, the synthesis of IAA is also influenced by environmental factors (Patten and Glick 1996). Synthesis of IAA by *Rhizobium* spp. in the presence and absence of tryptophan has also been demonstrated (Wani et al. 2007c). In a similar study, Bent et al. (2001) reported that the concentration of indole compounds by three different strains, *Paenibacillus polymyxa* (L6), *P. poly*myxa (Pw-2) and *Pseudomonas fluorescens* (M20), increased with increasing rate of tryptophan (0–200 mg/ml) at different incubation interval.

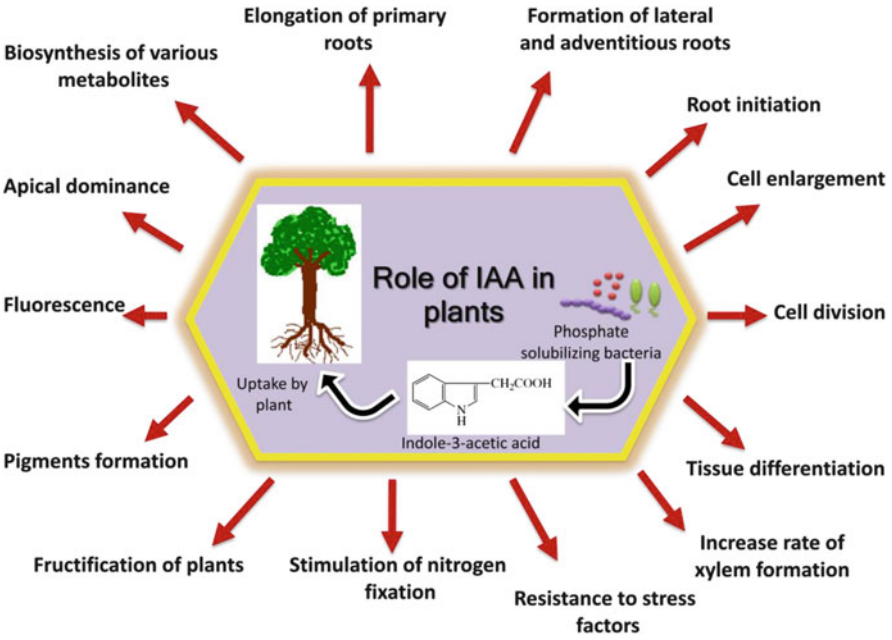

Fig. 2.3 Indoleacetic acid affecting various stages of plant development

Physiological Functions of IAA

The production of phytohormones such as auxins by microbial communities has been reported by various workers over the last 20 years (Giordano et al. 1999a, b; Rajkumar and Freitas 2008; Singh 2008; Ahemad and Khan 2012). Among plant hormones, auxins are the major plant growth regulators produced by PSM (Oves et al. 2013) and exhibit many physiological functions as shown in Fig. 2.3. Apart from varying physiological functions of IAA, the role of IAA in legume–*Rhizobium* symbiosis is briefly discussed in the following section.

*Role of Indoleacetic Acid in Legume–*Rhizobium *Symbiosis*

Among nodule bacteria, rhizobial strains have been reported to produce auxins in variable amounts. For example, Vargas et al. (2009) in a study reported considerably lower frequency of auxin producers (23 %) among populations of clover nodulating *R. leguminosarum* bv. *trifolii*. The auxins so released by nodule bacteria are reported to affect nodulation, and accordingly, IAA synthesizing rhizobia have been found to produce more nodules than IAA-negative mutants (Boiero et al. 2007). The IAA produced by rhizobia may also induce root morphogenesis and consequently enhance its (1) size and weight, (2) branch numbers and patterns and (3) the surface area of roots as reported in non-legumes (Dazzo and Yanni

2006). Inoculation with auxin-producing bacteria may also result in the formation of adventitious roots (Solano et al. 2010). Furthermore, Noel et al. (1996) observed that the inoculation with IAA-producing strains of *R. leguminosarum* accelerated the germination of canola and lettuce. Similarly, Biswas et al. (2000) concluded that the inoculation of rice with *R. leguminosarum* bv. *trifolii* increased dry matter and grain production, besides an increment in N, P, K and Fe content in plant tissue. All these effects were ascribed due to the accumulation of IAA in the rhizosphere following rhizobial inoculation leading to some physiological changes in the root systems with consequent increase in nutrient uptake. In contrast, the overproduction of IAA in some cases by PGPR has been found to have deleterious impact on to plants (Schlindwein et al. 2008). For example, *R. leguminosarum* bv. *trifolii* strain TV-13 produced 171.1 mg/ml IAA in media enriched with tryptophan (Schlindwein et al. 2008), while strains of *Bradyrhizobium* sp. isolated from black wattle roots produced between 1.2 and 3.3 mg/ml IAA and increased the seedling vigour in relation to un-inoculated control plants. The variation in the amount of IAA produced by PGPR was, however, suggested due to differences in the composition of the growth medium and tryptophan concentration. In a follow-up study, Sridevi et al. (2008) observed that IAA production by rhizobia occurred only when tryptophan was added to YM and that the isolates produced the maximum amount of IAA in medium supplemented with 2.5 mg/ml tryptophan concentration.

Other Phytohormones

Like auxins, cytokinins influence both cell division and cell enlargement and also affect seed dormancy, flowering, fruiting and plant senescence (Ferguson and Lessenger 2006). Cytokinin production by PGPR (Boiero et al. 2007) is, however, less obvious compared to the production of auxins. This is probably due to the lack of methods used for cytokinin detection, and hence, reports on cytokinin synthesis by PGPR in general are scarce. Gibberellin is yet another growth regulator which (1) affects seed germination (Miransari and Smith 2009), (2) stimulates growth of plants (Guo et al. 2011) and (3) delays ageing (Ferguson and Lessenger 2006). The production of gibberellins at high concentrations is considered very rare and has been reported for two strains of *Bacillus*, isolated from the *Alnus glutinosa* rhizosphere (Solano et al. 2010). The concentration of gibberellins in nodules is, however, generally higher than in nearby root tissue as supported by the fact that rhizobia have the capacity to produce some amount of gibberellin-like substances. However, it is not known whether bacteria contribute significantly to the amount of gibberellins within the nodule or it is just imported from some remote host plant tissue (Dobert et al. 1992; Hedden and Thomas 2012). Despite all these contrasting facts, the role of gibberellin in *Rhizobium*–legume symbiosis that may have important implications in the endophytic colonization of non-legumes by rhizobia is adequately described. For example, *A. caulinodans* infects the semi-aquatic legume *Sesbania rostrata* via the intercellular crack entry, a process mediated by gibberellins. Considering that crack entry is the main process of endophytic colonization

of non-legumes by rhizobia, the production of gibberellins by the bacterium is reported to facilitate this process (Lievens et al. 2005).

2.3.2 Negative Plant Growth Regulator

Abscisic acid is one of the strong inhibitor of growth and germination and promotes seed dormancy (Miransari and Smith 2009; Yang et al. 2009). Apart from these, ABA also helps plants to tolerate abiotic stresses. When plants are exposed to drought stress, the hormonal balance of plants changes and ABA content in the leaves increases, which reduce the level of cytokinin. This in turn elicits stomata closure (Yang et al. 2009). Cohen et al. (2009) in a similar study suggested that ABA produced along with gibberellins by PGPR strain significantly contributed to water stress alleviation of maize plants. Some rhizobial strains such as *B. japonicum* USDA110 also produce ABA (Boiero et al. 2007) and function in the same way as do the other PGPR (Zhang et al. 2012).

2.3.3 Growth Modulation Enzyme ACC Deaminase

Ethylene is a plant hormone which under normal conditions regulates many physiological processes, such as (1) seed germination, (2) root hair development and root elongation, (3) leaf and organ senescence, (4) leaf and petal abscission, (5) epinasty and (6) fruit ripening (Abeles et al. 1992; Frankenberger and Arshad 1995; Arshad and Frankenberger 2002; Siddikee et al. 2011). Also, ethylene regulates nod factor signalling and nodule formation and has primary functions in plant defence systems. Besides its physiological role in different developmental processes of plants, ethylene is also considered as a stress hormone, whose synthesis in plants is increased substantially by a number of biotic and abiotic stresses. At higher concentrations, ethylene, however, inhibits growth and development of plants (Grichko and Glick 2001). The enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (E.C. 4.1.99.4) which however mitigates the ethylene stress was first purified to homogeneity from *Pseudomonas* sp. strain ACP (Honma and Shimomura 1978), later on partially purified from *P. chlororaphis* 6G5 (Klee et al. 1991) and *P. putida* GR12-2 (Jacobson et al. 1994) and then purified to homogeneity from *P. putida* UW4 (Hontzeas et al. 2004). Enzyme ACC deaminase (a multimeric enzyme) has been found thereafter to be synthesized by a variety of PGPR (Belimov et al. 2005; Rajkumar et al. 2006; Madhaiyan et al. 2007; Mellado et al. 2007).

2.3.3.1 How the Bacterial ACC Deaminase Works

Mechanistically, the ACC deaminase-producing plant growth-promoting bacteria first bind to the surface of a plant (usually seeds or roots), although these bacteria may also be found on leaves and flowers or within a plant's internal tissues, i.e. as an endophyte (Glick et al. 1998). Along with other small molecular components of root exudates, some of the plant ACC (a non-ribosomal amino acid) is exuded from seeds, roots or leaves (Penrose et al. 2001) and may be taken up by the bacteria associated with these tissues and subsequently cleaved by ACC deaminase (Penrose and Glick 2003). The ACC, the immediate precursor of C_2H_4, when hydrolysed by ACC deaminase results in NH_3 and α-ketobutyrate formation (Glick et al. 1998; Penrose and Glick 2003; Reed et al. 2005; Safronova et al. 2006), and hence, it strongly alleviates the stress induced by ethylene-mediated impact on plants by lowering the C_2H_4 levels in plants (Glick et al. 2007; Sessitsch et al. 2005; Sun et al. 2009). The bacteria utilize the NH_3 so evolved from ACC as a source of N and thereby restrict the accumulation of C_2H_4 within the plant, which otherwise inhibits plant growth (Belimov et al. 2002). Thus, the decreased levels of C_2H_4 in turn allow the plants to grow better (Zahir et al. 2008). A model to explain how ACC deaminase promotes plant growth is depicted in Fig. 2.4. It has been observed that plants inoculated with PGPR containing ACC deaminase were dramatically more resistant to the deleterious effects of stress ethylene, synthesized under stressful conditions such as flooding (Grichko and Glick 2001), heavy metals (Burd et al. 1998; Grichko et al. 2000), presence of phytopathogens (Wang et al. 2000), drought and high saline conditions (Mayak et al. 2004). The net result of the cleavage of exuded ACC by bacterial ACC deaminase is that the bacterium is de facto acting as a sink for ACC. Additionally, plants growing in association with ACC deaminase-containing plant growth-promoting bacteria generally have longer roots and shoots and are more resistant to growth inhibition by a variety of ethylene-inducing stresses. Furthermore, the reduction of ethylene levels in plant tissues following ACC deaminase activity can cause significant morphological changes in root tissue, such as changes in root hair length and increases in root mass, accompanied by the consequent improvement in nutrient uptake. The morphological changes in plants are further increased when ACC deaminase action is coupled with the production of auxins by PGPR. The question arises, how bacterial ACC deaminase selectively reduces the deleterious ethylene levels (the second ethylene peak) without affecting the small first peak of ethylene that is thought to activate plant defence responses. In this regard, ACC deaminase is generally present in bacteria at a relatively low level until it is induced, and the induction of enzyme activity is a rather slow and complex process. Immediately following an abiotic or biotic stress, the pool of ACC in the plant is low as is the level of ACC deaminase in the associated bacterium. Stress induces the induction of ACC oxidase in the plant so that there is an increased flux through ACC oxidase resulting in the first (small) peak of ethylene that in turn induces the transcription of protective/defensive genes in the plant. At the same time, bacterial ACC deaminase is induced by the

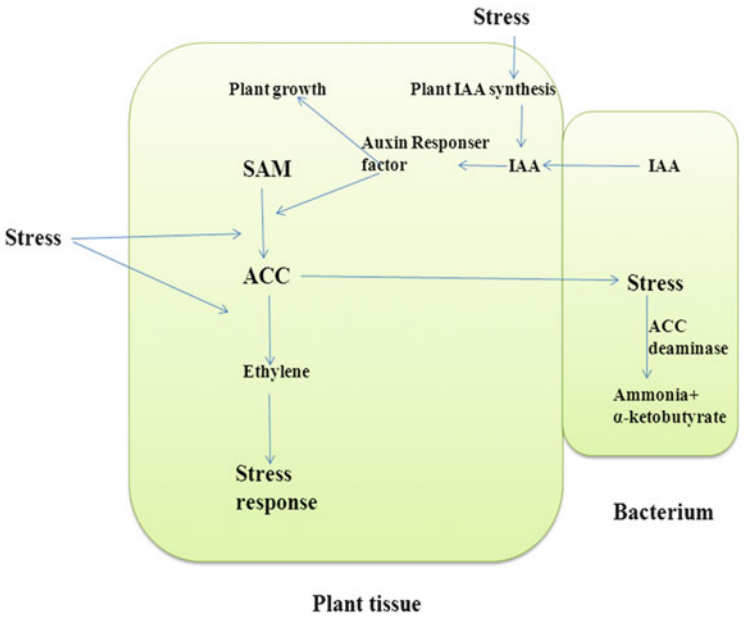

Fig. 2.4 A schematic model of how plant growth-promoting bacteria that both produce ACC deaminase and synthesize IAA may facilitate plant growth. The only enzyme shown in this scheme is ACC deaminase. SAM is converted to ACC by the enzyme ACC synthase; ACC is converted to ethylene by ACC oxidase. IAA biosynthesis, both in bacteria and in plants, is a complex multi-enzyme/protein process as is IAA signal transduction. *ACC* 1-aminocyclopropane-1-carboxylate, *IAA* indole-3-acetic acid, *SAM* S-adenosyl methionine [Adapted from Glick (2014)]

increasing amounts of ACC that ensue from the induction of ACC synthase in the plant so that the magnitude of the second, deleterious, ethylene peak is decreased significantly (typically by 50–90 %). Because ACC oxidase has a greater affinity for ACC than does ACC deaminase, when ACC deaminase-producing bacteria are present, plant ethylene levels are dependent upon the ratio of ACC oxidase to ACC deaminase. That is, to effectively reduce plant ethylene levels, ACC deaminase must function before any significant amount of ACC oxidase is induced. Thus, in the absence of some other mechanism, IAA-producing bacteria might all be expected to ultimately be inhibitory to plant growth. However, this is in fact not the case because as plant ethylene levels increase, the ethylene that is produced through feedback mechanism inhibits IAA signal transduction thereby limiting the extent that IAA can activate ACC synthase transcription (Pierik et al. 2006; Prayitno et al. 2006; Czarny et al. 2007; Stearns et al. 2012). In plants inoculated with PGPR that secrete both IAA and ACC deaminase, the level of ethylene does not increase compared to the plants inoculated only with IAA-secreting bacteria. In the presence of ACC deaminase, there is much less ethylene and subsequent ethylene feedback inhibition of IAA signals transduction so that the bacterial IAA can continue to promote both plant growth and increase ACC synthase

transcription. However, in this case, a large portion of the additional ACC which is synthesized is hydrolysed by the bacterial ACC deaminase. Therefore, the use of such plant growth-promoting bacteria containing ACC deaminase may prove useful in developing strategies to facilitate plant growth in stressed soil environments.

2.3.3.2 Role of ACC Deaminase in Nodulation

ACC deaminase-containing bacteria are relatively common in soil and have been found in a wide range of environments across the world. Indeed, the ability of bacteria to hydrolyse ACC has a competitive advantage over other soil inhabitants because it can use ACC as an N source (Jacobson et al. 1994). This hypothesis suggests that ACC may act as a unique/novel source of N for some soil bacteria. While searching for ACC deaminase positive rhizobial strains, it was found that amongst 13 different rhizobial strains, five strains displayed enzyme activity while seven strains had the *acdS* gene (Ma et al. 2003). Conclusively, it was reported that the *Mesorhizobium* strain only expressed this activity when the bacterium was present within a root nodule. In other investigation conducted in southern Saskatchewan, Canada, of the total 233 rhizobial strains isolated from soil samples collected from 30 different sites, nearly 12 % (27 strains) displayed the ACC deaminase activity (Duan et al. 2009). Similarly, ACC deaminase genes have been reported in chickpea *Mesorhizobium* isolates (Nascimento et al. 2012), *B. japonicum* E109, USDA110 and SEMIA5080 (Boiero et al. 2007). Rhizobial strains that express ACC deaminase are up to 40 % more efficient at forming nitrogen-fixing nodules than strains that lack this activity (Ma et al. 2003, 2004). However, strains of rhizobia that express ACC deaminase have only a low level of enzyme activity compared with free-living plant growth-promoting bacteria, i.e. typically around 2–10 %. Thus, free-living bacteria bind relatively non-specifically to plant tissues (mainly roots) and have a high level of ACC deaminase activity that can protect plants from different abiotic and biotic stresses by lowering ethylene levels throughout the plant. On the other hand, (symbiotic) rhizobia that generally bind tightly only to the roots of specific plants have a low level of enzyme activity which facilitates nodulation by locally lowering ethylene levels. It is not known whether the large differences in enzyme activity that are observed when comparing free-living bacteria with rhizobia are a consequence of differences in the amount of enzyme synthesized by one type of bacteria versus the other or of differences in the specific catalytic activity of the enzymes from the different types of bacteria. It has also been observed that some rhizobia reduces the plant ethylene levels mediated by ACC deaminase activity and enhances nodulation in host legumes (Zahir et al. 2008; Belimov et al. 2009) or modifies root system of non-legumes. For instance, strains of *R. leguminosarum* bv. *viciae* and *Mesorhizobium loti* increased the number of lateral roots in *Arabidopsis thaliana* because of this plant growth-promoting mechanism (Contesto et al. 2008). In addition to the more common mode of *acdS* transcriptional regulation, *acdS* genes from various strains of *M. loti* have been found to be under the transcriptional control of the *nifA* promoter that is

normally responsible for activating the transcription of nif, nitrogen fixation genes (Kaneko et al. 2000; Sullivan et al. 2002; Uchiumi et al. 2004; Nukui et al. 2006; Nascimento et al. 2012). The consequence of this somewhat unusual mode of regulation is that, unlike ACC deaminases from other rhizobia, the *M. loti* ACC deaminase does not facilitate nodulation but, rather, is expressed within nodules. The result of this unusual regulation is, in *M. loti*, ACC deaminase may act to decrease the rate of nodule senescence. This is particularly important because of the fact that nitrogen fixation, a process that utilizes a very high level of energy in the form of ATP, could (perhaps inadvertently) activate stress ethylene synthesis resulting in premature nodule senescence.

2.3.4 Physiological Functions of Siderophores

Iron is essential for almost all life for processes such as respiration and DNA synthesis. Despite being one of the most abundant elements in the Earth's crust, the bioavailability of iron in many environments such as the soil is limited by the very low solubility of the Fe^{3+} ion. In the aerobic environment, iron accumulates in common mineral phases such as iron oxides and hydroxides and hence becomes inaccessible to organisms. Microbes (e.g. bacteria and fungi) have, therefore, evolved a strategy to acquire iron by releasing siderophores (Greek: "iron carrier"), small (generally less than 1,000 molecular weight) high-affinity iron-chelating compounds, which scavenge iron from the mineral phases by forming soluble Fe^{3+} complexes that can be taken up by active transport mechanisms. Broadly, siderophores act as solubilizing agents for iron from minerals or organic compounds under conditions of iron starvation (Miethke and Marahiel 2007; Indiragandhi et al. 2008). There are more than 500 different siderophores which are produced mainly by Gram-positive and Gram-negative bacteria. Siderophores are highly electronegative and bind Fe (III), preferentially forming a hexacoordinated complex. The iron ligation groups have been tentatively classified into three main chemical types: (1) hydroxamate (e.g. aerobactin and ferrichrome), (2) catecholates/phenolates (e.g. enterobactin) and (3) hydroxyl acids/carboxylates (e.g. pyochelin). Some siderophores contain more than one of the three iron-chelating groups (Table 2.6). Siderophores are, however, usually classified by the ligands used to chelate the ferric iron. Citric acid can also act as a siderophore. The wide variety of siderophores may be due to evolutionary pressures placed on microbes to produce structurally different siderophores (Fig. 2.5). Siderophores are important for some pathogenic bacteria for their acquisition of iron. The strict homeostasis of iron leads to a free concentration of about 10^{-24} mol/l, and hence, there are great evolutionary pressures put on pathogenic bacteria to obtain this metal. For example, the anthrax pathogen *Bacillus anthracis* releases two siderophores, bacillibactin and petrobactin, to scavenge ferric iron from iron proteins.

Table 2.6 Some examples of siderophores produced by various bacteria and fungi

S.No.	Siderophores	Producing organisms
1	Hydroxamate	
A	Ferrichrome	*Ustilago sphaerogena*
B	Desferrioxamine B (deferoxamine)	*Streptomyces pilosus*, *Streptomyces coelicolor*
C	Desferrioxamine E	*Streptomyces coelicolor*
D	Fusarinine C	*Fusarium roseum*
E	Ornibactin	*Burkholderia cepacia*
2	Catecholate	
A	Enterobactin	*Escherichia coli*
B	Bacillibactin	*Bacillus subtilis*, *Bacillus anthracis*
C	Vibriobactin	*Vibrio cholera*
3	Mixed ligands	
A	Azotobactin	*Azotobacter vinelandii*
B	Pyoverdine	*Pseudomonas aeruginosa*
C	Yersiniabactin	*Yersinia pestis*

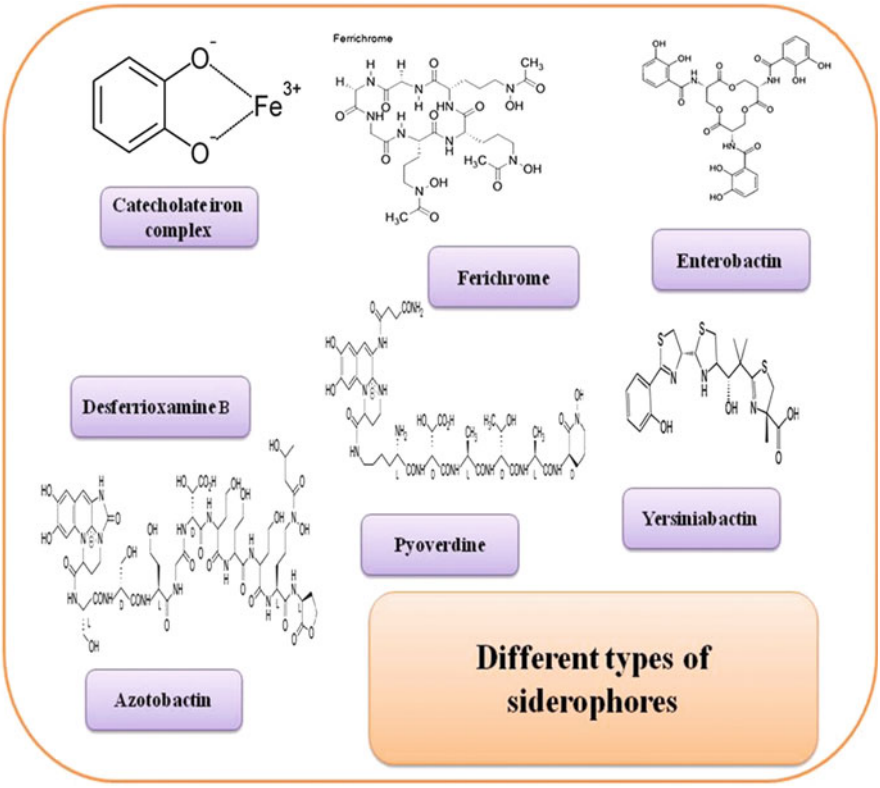

Fig. 2.5 Different types of siderophores

2.3.4.1 Role of Siderophores in Biological Nitrogen Fixation

Siderophore produced by majority of PGPR (Rajkumar et al. 2010) including rhizobia (Ahemad and Khan 2012) has been suggested as one of the modes of growth promotion of nodulated legumes under field conditions wherein siderophores facilitate the uptake of iron (assimilation) from the environment (Kloepper and Schroth 1978; Katiyar and Goel 2004). The iron enzymes involved include nitrogenase, leghemoglobin, ferredoxin and hydrogenase with nitrogenase and leghemoglobin constituting up to 12 % and 30 % of total protein in the bacterial and infected plant cells, respectively (Verma and Long 1983). A nodulated legume has been found to have an increased demand for iron compared to that of a non-nodulated plant (Derylo and Skorupska 1993). For example, *Pseudomonas* sp. strain 267 enhanced symbiotic N_2 fixation in clover under gnotobiotic conditions, produced fluorescent siderophores under low-iron conditions and secreted B group vitamins (Marek-Kozaczuk and Skorupska 2001). However, Tn5 insertion mutants of strain 267 defective in siderophore production did not differ from the wild type in promoting the growth of clover suggesting that the siderophore production had no effect on stimulating nodulation. In contrast, Gill et al. (1991) demonstrated that mutants of *R. melioti* that were unable to produce siderophores were able to nodulate the plants, but the efficiency of N_2 fixation was less compared to the wild type, indicating the importance of iron in N_2 fixation. In a similar study, *Kluyvera ascorbata*, a siderophore-producing PGPR, was able to protect plants from heavy metal toxicity (Burd et al. 1998).

2.3.5 Cyanogenic Compounds

Cyanide is yet another secondary metabolite produced during the early stationary growth phase (Knowles and Bunch 1986) by several PGPR, notably *Pseudomonas* spp. and *Bacillus* (Wani et al. 2007d), *Chromobacterium* (Faramarzi and Brand 2006) and *Rhizobium* spp. (Wani et al. 2008a, b) by oxidative decarboxylation pathway using glycine, glutamate or methionine as precursors (Curl and Truelove 1986). The cyanide so released by microbial communities in solution acts as a secondary metabolite and confers a selective advantage onto the producer strains (Vining 1990). Although cyanide is a phytotoxic agent capable of disrupting enzyme activity involved in major metabolic processes, its role as a biocontrol substance is overwhelming (Devi et al. 2007; Voisard et al. 1989). Hydrogen cyanide (HCN) among cyanogenic compounds effectively blocks the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations. However, producer microbes, mainly pseudomonads, are reported to be resistant (Bashan and de-Bashan 2005).

2.3.6 *Production of Lytic Enzymes*

In high input modem agricultural practices, pesticides are frequently and inappropriately used to protect crop plants from damage by insects, disease and so on which today still destroy almost 33 % of all food crops. The use of pesticides in this respect is considered effective if they provide the desired biological results and are inexpensive. However, the indiscriminate use of pesticides has resulted in the adverse impact on soil fertility, human health and the environment. Therefore, one of the safest strategies involving microorganisms for controlling/managing plant pests often called as "biocontrol" holds great promise as an alternative to the use of synthetic agrichemicals. Biological control agents are generally considered more environmentally sound than the pesticides and other antimicrobial treatments. In this context, the antagonistic potential of microbes in particular has formed the base for effective applications of such organisms as an alternative to the chemical control measures against a range of fungal and bacterial plant pathogens. And hence, a variety of microbial compounds have been identified/extracted that have been found to inhibit/suppress the phytopathogenic growth leading thereby to the reduction in damage to plants (Helbig 2001; Yang et al. 2005; Raza et al. 2008). These microbially synthesized compounds include defence enzymes, such as chitinase, β-1,3-glucanase, peroxidase, protease and lipase (Bashan and de-Bashan 2005; Karthikeyan et al. 2006). Chitinase and β-1,3-glucanase degrade the fungal cell wall and cause lysis of fungal cell. Furthermore, chitin and glucan oligomers released during degradation of the fungal cell wall by the action of lytic enzymes act as elicitors that elicit various defence mechanisms in plants. Such enzymes produced by *Pseudomonas stutzeri* have demonstrated the lysis of the pathogen *Fusarium* sp. (Bashan and de-Bashan 2005). Peroxidase (PO) represents another component of an early response in plants to pathogen attack and plays a key role in the biosynthesis of lignin which limits the extent of pathogen spread (Bruce and West 1989). In bean, rhizosphere colonized by various bacteria induced PO activity (Zdor and Anderson 1992). In a study, a rapid increase in PO activity was recorded in coconut (*Cocos nucifera* L.) treated with a mixture of *P. fluorescens*, *T. viride* and chitin which contributed to induced resistance against invasion by *Ganoderma lucidum*, the causal agent of *Ganoderma* disease (Karthikeyan et al. 2006). These findings suggest that PGPR possessing the ability to synthesize hydrolytic enzymes can effectively be utilized for managing the plant diseases and can help to reduce the pesticide usage.

2.4 Conclusion

In intensive agricultural practices, P is supplied to plants through synthetic phosphatic fertilizers, which indeed is expensive and environment disruptive. Application of phosphate-solubilizing microorganisms as an alternative to chemically

synthesized phosphatic fertilizer is therefore an urgent requirement in crop production systems. The success of this microbiological approach, however, depends on identification, preparation and delivery of multifunctional phosphate solubilizers to farm practitioners. Understanding the mechanistic basis of phosphate solubilization by soil dwellers is critical and requires multifaceted approach to uncover the hidden phosphate-solubilizing potentials of functionally diverse yet naturally abundant soil microflora. Moreover, the functional variations among phosphate solubilizers need to be identified. Once identified and physiologically characterized, phosphate-solubilizing microbes are likely to provide benefits to crops in sustainable agriculture.

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Chapter 3
Factors Affecting Phosphate-Solubilizing Activity of Microbes: Current Status

Javed Musarrat and Md. Saghir Khan

Abstract Phosphorous (P) plays an important role in regulating the vital metabolism and concomitantly the health of plants. The use of phosphate-solubilizing microorganisms (PSM) in P-deficient soils has been found effective in transforming insoluble P into soluble forms and, hence, enriching the soil P pool. The structure, composition, and physiological functions of soil dwellers depend, however, on the variable soil constituents and other environmental factors. Moreover, the establishment and performance of these microbes are affected severely by environmental stressors such as high temperature, pH, and salt, etc. prevalent in degraded ecosystems such as alkaline/saline soils. Therefore, any alteration in normal environmental factors leads to poor growth and survival of PSM. Also, PSM, when introduced exogenously into soil as inoculant, encounter a furious competition from the indigenous soil microflora. The success of the inoculants, therefore, depends on how quickly and efficiently such microbes overcome the stressful environmental variables. This chapter focuses on the effects of different factors on the overall functioning of the PSM, which is likely to help in developing environment-friendly bio-inoculants, especially for P acquisition by plants under environmentally challenged conditions.

Keywords PSM • Temperature • Salts • Alkalinity • pH

3.1 Introduction

Phosphorus is one of the 16 known major plant nutrients that plays a significant role in plant metabolism (Vikram and Hamzehzarghani 2008; Padmavathi and Usha 2012). On global basis, 40 % of the arable soil is P deficient (Vance 2001) because

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most of the P remain in insoluble forms (Omar 1998; Zaidi et al. 2009). Furthermore, a large portion of the phosphatic fertilizers applied to soil is rapidly fixed/immobilized and becomes unavailable to plants (Xiao et al. 2011). The insoluble and fixed forms of P, therefore, alter the fertility of soil (Bhattacharyya and Jha 2011) and limit plant growth (Prejambda et al. 2009; Victoria et al. 2009; Plassard and Dell 2010). Due to this P fertility problems, growers often apply severalfold excess P than required by plants (Goldstein 1986) which after accumulation/deposition gradually results in soil pollution and pollution of other water resources such as lakes, streams, and groundwater (Del Campillo et al. 1999; Reddy et al. 2002). On the contrary, the rate of replenishment and availability of P in soil is determined largely by so many environmental factors, for example, soil pH, temperature, humic substances, soil P concentration, its fixation by soil, microbial composition including PSM (Hameeda et al. 2008; Henri et al. 2008; Srividya et al. 2009) and their functions, and various plant exudates (Hoffland et al. 1989; Ae et al. 1990; Gillespie and Pope 1990; Hartwig et al. 1991; Cook et al. 1995). Apart from these, the P solubilization process, a complex phenomenon, also depends on many other factors such as the nutritional richness of soils and growth dynamics and physiological functions of the organisms involved in solubilization of complex organic P (mineralization) or inorganic P (solubilization) (Cunningham and Kuiack 1992; Reyes et al. 1999; Chen et al. 2006). Moreover, the performance of PSM has also been found to be severely affected by stressors and vegetation (Yoon et al. 2001; Gupta et al. 2007; Sánchez-Porro et al. 2009; Yadav et al. 2010), while for proper growth, establishment, and normal functioning of PSM in soils, the organisms should be provided with a healthy and nutrient-rich environment (Vassileva et al. 1999). Despite conflicting reports on the P solubilization efficiency of PSM in fluctuating environment (Kern et al. 2012), such microbes, when coated onto seeds or applied in soils, have shown a variable yet profound increase in P uptake by plants and in turn enhance the crop yields (Zaidi et al. 2003; Afzal and Bano 2008; Hamdali et al. 2012; Ahmad et al. 2013) under changing/stressed environmental conditions. The impact of various environmental variables on structural and functional diversity of PS microbes is reviewed and discussed in the following section.

3.2 Factors Affecting Inorganic P Solubilization

3.2.1 Hydrogen Ion Concentration (pH)

Among the various environmental factors affecting the growth and metabolic activities of microbial populations including PSM (Table 3.1; Fig. 3.1) is the pH of the medium in which organisms are growing (Narsian and Patel 2000; Reyes et al. 2002; Khan et al. 2007). Functionally, the optimum pH for maximum solubilization of inorganic P by bacteria has been found to be neutral or slightly

Table 3.1 Effect of different environmental variables on phosphate-solubilizing activity of microorganisms

Phosphate-solubilizing microbes	Medium used	pH	Temperature (°C)	C sources	N sources	Types of substrates	Incubation periods (days)	P solubilization			References
								Broth (μg/ml)	Zone size (mm)	Change in pH	
Streptomyces thermonitrificans NTU-88	PVK	7	50	Glucose	$(NH_4)_2SO_4$	TCP	5	57 ± 27	ND	5.2 ± 0	Chang and Yang (2009)
B. coagulans C45	PVK	7	25	Glucose	$(NH_4)_2SO_4$	TCP	5	370.2 ± 1	ND	4.4 ± 0	Chang and Yang (2009)
		7	50	Glucose	$(NH_4)_2SO_4$	TCP	10	466.8 ± 2	ND	4.0 ± 0	Chang and Yang (2009)
	$AlPO_4$	7	50	Glucose	$(NH_4)_2SO_4$	$AlPO_4$	10	77.5 ± 6	ND	5.0 ± 0	Chang and Yang (2009)
	$FePO_4$	7	50	Glucose	$(NH_4)_2SO_4$	$FePO_4$	10	8.3 ± 0.4	ND	5.9 ± 0	Chang and Yang (2009)
	RP	7	25	Glucose	$(NH_4)_2SO_4$	RP	10	95.2 ± 1	ND	5.0 ± 0	Chang and Yang (2009)
Burkholderia glathei MB14	NBRIP	7	23	Glucose	Arginine	TCP	7	5 mg/ml	ND	ND	Kim et al. (2005)
B. coagulans		8	–	Glucose	NH_4Cl	DCP	3	–	3.8	–	Gyaneshwar et. al (1998)
Citrobacter koseri		8	–	Glucose	NH_4Cl	DCP	3	–	3.5	–	Gyaneshwar et. al (1998)
P. aeruginosa	PVK	6	35	Glucose	$(NH_4)_2SO_4$	TCP	7	209	–	4.61	

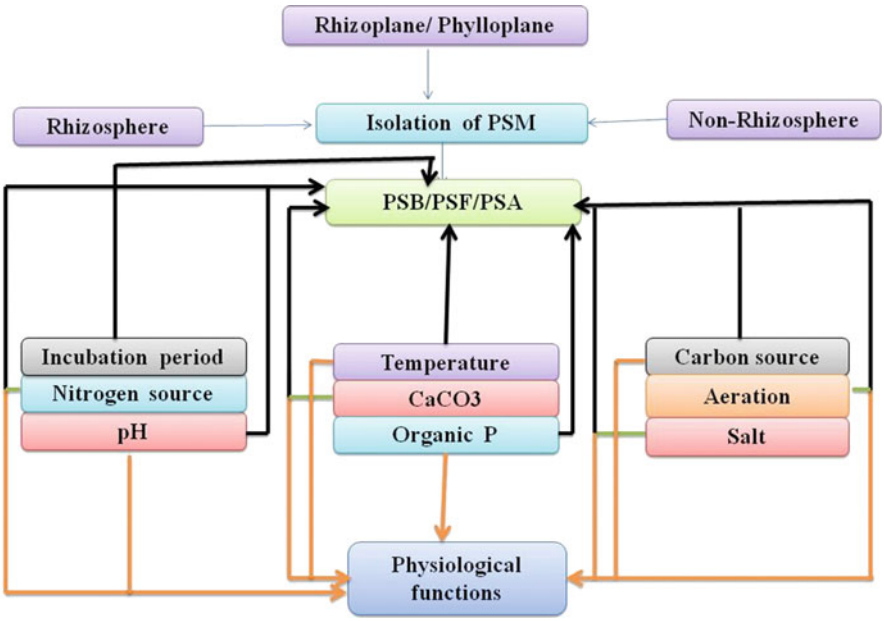

Fig. 3.1 Factors affecting the survival and physiological functions of PSM

acidic (Bajpai and Sundara 1971). The phosphate-solubilizing (PS) activity of PSF and yeast in contrast occurs best in the pH range 4–6 (Ahmad and Jha 1968). Moreover, the activity of PSF is considerably reduced when pH values increases to pH 7.0 to pH 8.0 because fungi in general prefer slightly acidic conditions for growth than do the bacterial cultures. This indeed is true because in most of the cases, acidification has been reported as the principal mode of P solubilization by microbes (Khan et al. 2007, 2009, 2010, 2013). Therefore, high P solubilization by microbes is quite often associated with a consequent decrease in the pH of the medium in which they are growing. Concomitantly, a significantly negative correlation between pH and solubilization of inorganic P, for example, calcium phosphate, has been reported (Wani et al. 2008). Also, a significant correlation between final pH value and titratable acidity and between titratable acidity and soluble P has been observed when 42 bacterial isolates were tested for their ability to solubilize rock phosphate (RP) and Ca-P in culture medium (Nahas 1996). Recently, the liquid Pikovskaya (PVK) medium inoculated with PSF and PSB showed greater reduction in pH, and it has been observed that more the phosphate solubilized, the greater was the reduction in pH values (Reena et al. 2013). Interestingly, both bacteria (*P. aeruginosa* and *B. subtilis*) and fungi (*A. niger*, *Penicillium* sp., and *Micrococcus* sp.) showed maximum P solubilization at pH 3.0 at 28 °C and 37 °C, respectively. Furthermore, such bacterial and fungal cultures produced carboxylic acids, which solubilized the insoluble Tri-calcium phosphate (TCP) efficiently in the medium. The titratable acidity also confirmed the release of carboxylic acid in

the medium. The converse correlation observed between the pH and soluble P concentration indicated that organic acid production by these PS strains might have played a significant role in the acidification of the medium facilitating the P solubilization as also reported by Hwangbo et al. (2003).

3.2.2 Temperature

Temperature is one of the important factors that immediately affect the interior of the cell and the biological activity of soils. Generally, normal temperature has been employed in soil biological research. On the contrary, the soil temperature remains seldom constant under field conditions because of the diurnal temperature and seasonal fluctuations. Therefore, bacteria responds differently to varying temperatures and hence are capable of growing at elevated temperature (thermophiles, thermotolerant) and also at extremely downshifted temperatures such as at or below 0, 15, and 20 °C (psychrophiles, psychrotolerant) by synthesizing a group of heat and cold shock proteins, respectively. These temperature-induced proteins are highly significant for the survival of bacteria at higher or lower temperatures (Negi et al. 2009). Moreover, temperature has bewildering effects onto the "P"-solubilizing abilities of naturally occurring thermotolerant PSB, for example, *B. subtilis* (Moussa et al. 2013), *Acidithiobacillus caldus* (Xiao et al. 2011), and *B. smithii* (Chang and Yang 2009), and psychrotolerant strains of bacteria, for instance, *Pseudomonas fragi* (Selvakumar et al. 2009). At low temperature, the biological activity remains low which, however, improves further with increasing temperature towards optimum range, beyond which microorganisms are either desiccated or show variable responses. Generally, the PS microbes identified and considered so far belong to mesophilic group (Khan et al. 2007, 2010), suggesting that they could only be utilized under mesophilic environment. However, a few thermotolerant (Chang and Yang 2009; Rao et al. 2009; Maheswar and Sathiyavani 2012; Panda et al. 2013) and psychrophilic PS organisms have also been reported exhibiting P-solubilizing activity (Katiyar and Goel 2003; Negi et al. 2009; Pallavi and Gupta 2013). In this regard, bacterial cultures particularly *B. subtilis* and *B. circulans* showed consistent P solubilization even at 45 °C which was due to the ability of their enzyme systems to tolerate higher temperatures. Such a situation of higher soil temperature during summer is generally found in tropics where temperature may reach up to 50 °C. Therefore, the inoculant designed for growth and survival at higher temperatures in soil and happening during storage and transport is one of the most craving characteristics. It is thus urgently needed to isolate PS organisms which can tolerate high temperatures of tropics. To fulfil such demands, Gaind and Gaur (1991) tested several PSM for their P-solubilizing efficiency while growing them at 35, 40, and 45 °C temperatures. Even though there was a marked variation in PS activity of PSM, the effect was more pronounced at 45 °C. The thermotolerant bacterial strains exhibiting PS activity at higher temperatures were identified as *B. subtilis* and *B. circulans,* while fungal

strain was identified as *A. niger* (TT10). Later on, seed inoculation of green gram revealed a better establishment of temperature-tolerant strains which was evident by the high rhizosphere microbial populations. In a similar investigation, Panda et al. (2013) reported that *P. fluorescence* exhibited enhanced P solubilization at 35 °C, while *P. corrugata* isolated from a temperate region in Sikkim (Himalaya) solubilized TCP both at psychrophilic and mesophilic temperature ranges (Pandey et al. 2002, 2006). The maximum P solubilization occurred at 21 °C but the test culture could also solubilize more TCP at 4 °C than at 28 °C. Phosphate solubilization by *P. lurida* M2RH3 determined at three incubation temperatures in other studies revealed a steady increase in the soluble P levels across the incubation temperatures coupled with a steady drop in pH of the culture supernatant (Selvakumar et al. 2010a, b). Likewise, cold-tolerant species of *Pantoea dispersa* and *Exiguobacterium acetylicum* very effectively solubilized P at lower temperatures (Selvakumar et al. 2008a, 2010a, b). *Serratia marcescens* strain SRM (MTCC 8708) could solubilize about 28 mgP/ml in National Botanical Research Institute's Phosphate (NBRIP) broth at 4 °C (Selvakumar et al. 2008a, b), whereas *Pseudomonas* sp. PGERs 17 (MTCC 9000) solubilized P at various temperatures (Mishra et al. 2008). Gulati et al. (2008) also screened various *Pseudomonas* strains from the cold region which could solubilize TCP, Mussoorie rock phosphate (MRP), Udaipur rock phosphate (URP), and North Carolina rock phosphate (NCRP). Thermotolerant multifunctional PS microbes with six types of enzyme activities and three types of inorganic P-solubilizing efficiencies expressing at 25 °C and 50 °C were isolated from composts and biofertilizers. Microbial inoculation accelerated the decomposition of agricultural and animal wastes and resulted in high soluble P content. Of the microbial inoculations, *B. smithii* (F18) had the shortest maturity period, the highest soluble P content, and the highest microbial populations which were followed by *B. coagulans* (C45) and *B. licheniformis* (A3). Inoculation with thermotolerant PSB released more soluble P than did the actinomycetes or fungi. However, all isolates could solubilize calcium P and Israel RP, aluminium P, iron P, and hydroxyapatite (Chang and Yang 2009).

3.2.3 Carbon and Energy

Development of growth and phosphate-solubilizing activity of PSM has also been affected by the presence of various carbon sources, for example, glucose, galactose, fructose, starch, and mannitol, present in the rhizospheres, discharged as photosynthates by many plants (Derrien et al. 2004; McRae and Monreal 2011) which are used as C and energy source by many soil microbes including PSM (Yadav et al. 2010; Khan et al. 2013). Such carbon sources has been reported to affect the production of enzymes involved in dissolution of organic P and (Qureshi et al. 2010) and solubilization of inorganic P by *A. niger*. In another report, sucrose was found as the best C source for *P. rugulosum* for solubilization of hydroxylapatite and $FeSO_4$ (Reyes et al. 1999). According to Nautiyal (1999) when glucose

was used as C source, microorganisms produced higher amounts of organic acids which causes more insoluble P solubilization. The influence of C on the solubilization of insoluble P was determined further by Song et al. (2008) who used different sugars such as glucose, sucrose, or maltose in order to assess the P solubilization activity of *Burkholderia cepacia* (DA23). Among all sugars, P solubilization was found to be twofold lesser in a medium containing sucrose relative to glucose which generally resulted in the most extensive solubilization of calcium P (Panda et al. (2013). Mechanistically, the P-solubilizing efficiency of microorganisms is associated with its ability to secrete organic acids (Maliha et al. 2004; Khan et al. 2009, 2010), the nature and quantity of which, however, varies between different sugars and microbes (Rodriguez et al. 2004; Hu et al. 2006; Perrig et al. 2007). For example, glucose, galactose, maltose, and sucrose used as single C source were metabolized by *Bacillus* (strain T-34) and produced citric, malic, acetic, and lactic acids in considerably higher concentrations compared to those secreted by *Azospirillum* (WS-1) and *Enterobacter* T-41 strains (Tahir et al. 2013). Similar results have also been reported by others (Chen et al. 2006; Archana et al. 2012) where *Azospirillum*, *Bacillus,* and *Enterobacter* produced variable amounts of citric, oxalic, gluconic, and 2-keto-gluconic acids. Also, the concentrations of the preferred energy source plays a pivotal role in enhancing PS activity of microbes, and generally the PS activity increases with increasing concentration of sugars added to the growth medium. Increasing the concentration of glucose, for instance, from 1 to 3 % resulted in profound increase in RP solubilization by PSM because higher concentration of glucose led to increased production of acidity, an important factor in P solubilization (Song et al. 2008). In other reports, a further increase in glucose concentrations from 1 to 5 % (w/v) enhanced the P solubilization considerably, and the most obvious increment has been recorded up to 3 % glucose which, however, decreases at 5 % glucose level (Son et al. 2006; Stephen and Jisha 2011).

3.2.4 Nitrogen Source

Nitrogen (N), like many other nutrients, influences the growth and functionality of soil-inhabiting PSM. Nitrogen is involved in various metabolisms such as it participates in synthesis of amino acids, proteins, and nucleotides. Microorganisms take up N in ammonical, nitrite, nitrate, or amino form, depending upon the enzyme present in their system and greatly influencing P solubilization activity (Bar-Yosef et al. 1999; Habte and Osorio 2012). Recently, an in vitro experiment was carried out to evaluate the effect of different nitrogen (N) forms (NH_4^+ and/or NO_3^-) on the dissolution of rock phosphates (RP) by the PSF *Mortierella* sp. (Habte and Osorio 2012). It has been reported that in the presence of NH_4Cl or NH_4NO_3, the solution of pH following *Mortierella* sp. application significantly decreases from an initial value of 7.6 to 3.4 and 3.7, respectively, while KNO_3 reduces the pH to 6.7 only. Due to greater decrease in pH, there was significantly more P solubilized in the

presence of NH_4Cl (129.65 mg/l) than in the presence of NH_4NO_3 (109.25 mg/l) as reported by Habte and Osorio (2012). The concentration of P solubilized by *Mortierella* sp. in the presence of KNO_3 was only 0.08 mg/l. Moreover, the excess of NH_4^+ adversely affected the growth of *Mortierella* sp. In the presence of NO_3^- as the only source of N, *Mortierella* sp. not only dissolves a small amount of inorganic phosphate (Pi) from the RP but also immobilizes most of it into its mycelia. In contrast, in the presence of NH_4Cl, *Mortierella* sp. has been effective in dissolving RP, and the Pi released remained in solution, while only a little portion was immobilized by the fungal mycelia (Habte and Osorio 2012). In yet another investigation, ammonium significantly decreased mineral phosphate solubilization (mps) in a wild-type Mps^+ strain IR94-MF1 and superpositive Mps^{++} of *Penicillium rugulosum* mutants (Reyes et al. 1999). Also, ammonium in most of the studies has been found as a better N source than nitrate (Wenzel et al. 1994; Asea et al. 1988), and *P. fluorescence,* for example, utilized $(NH_4)^2SO_4$ most efficiently and significantly decreased the pH of the medium during P solubilization. According to Sulbaran et al. (2009), *P. agglomerans* MMB051, when grown in the presence of KNO_3, as an alternative N source, instead of $(NH_4)^2SO_4$, changed the final pH of the culture supernatant which was almost two units higher (pH 5.1 ± 0.15) than that recorded for $(NH_4)_2SO_4$ (pH 2.86 ± 0.21). The final concentration of soluble P by *P. agglomerans* MMB051 was however lower in supernatant prepared from cells grown in medium treated with KNO_3 (58.15 mg/l) relative to those recovered from $(NH_4)_2SO_4$-grown cells (95.75 mg/l). In bacteria, although different NO_3^- transporter systems have been identified, nitrate/proton symporter is the main transporter for NO_3^- (Rowe et al. 1994; Kucera and Kaplan 1996). Consequently, there may be an increase in extracellular pH due to NO_3^- uptake by bacterial cells (Crawford and Glass 1998) which possibly could explain the differences in the P-solubilizing abilities of *P. agglomerans* MMB051 cells grown in the presence of NH_4^+ or NO_3^- ions (Sulbaran et al. 2009). Similar effect of different N sources on PS activity has been reported (Roos and Luckener 1994; Relwani et al. 2008).

3.2.5 *Effect of $CaCO_3$ and Aeration*

Acidic soils are generally limed to adjust the pH of the soil, while pyrite and gypsum are used to amend alkaline/saline soils. However, addition of $CaCO_3$ to the medium markedly reduces P solubilization by bacteria and fungi in liquid media. For example, *Enterobacter intermedium*, isolated from grass rhizosphere, even though had a strong ability to solubilize insoluble P, the concentration of soluble P was significantly decreased to 200–250 mg/l when grown in medium treated with 1 % $CaCO_3$ compared to medium without $CaCO_3$ (1,000 mg/l). Furthermore, the bacteria oxidize glucose to gluconic acid and sequentially to 2-keto-gluconic acid (2-KGA) (Hwangbo et al. 2003). Similarly, calcium added as $CaCl_2$, $CaCO_3$, and $Ca(OH)_2$ to the medium reduces the P solubilization by *Rhizobium* and *Bradyrhizobium* from RP because $CaCO_3$ enhances the pH of the

medium towards alkalinity, which inhibits the growth of bacteria, resulting in little solubilization (Halder et al. 1990). Aeration is yet another important factor that contributes hugely to P solubilization by microbes. Strains of *P. striata* and *A. awamori* improve the P solubilization in shake culture as compared to stationary cultures. The increased P solubilization in shake culture has been attributed to enhanced aeration following shaking of cultures. Under aeration, the P concentration has been reported to increase from 349 ppm to 1,675 ppm, while in the non-aerated environment, it increases from 242 ppm to 1,164 ppm (Jung et al. 2002). However, the P removal by *Burkholderia* spp. from sediments containing mineral phosphate is facilitated in the absence of aeration (Kim et al. 2005).

3.2.6 Kinds of Microorganisms and Incubation Periods

The extent of P solubilization, naturally by microbes, also depends on the composition of microorganisms since heterogeneously distributed microbial communities exhibit varying capacity to solubilize P (Khan et al. 2007). Among microbial communities, the genera *Bacillus* (*B. polymyxa*) and *Pseudomonas* (*P. striata*) have shown maximum P-solubilizing activity (Khan et al. 2009), followed by *Penicillium* and *Aspergillus* (Khan et al. 2010), while *Streptomyces* is the least effective one. Generally, fungi have more PS activity in liquid media than bacteria, actinomycetes, and yeast (Khan et al. 2010). P-solubilizing fungi show greater P-solubilizing activity both in precipitated agar and in liquid media than do bacteria because the hyphae of fungi remain attached to P mineral particles and fungi in soil are able to traverse longer distances more easily than bacteria and are thus more important to P solubilization in soils. Even there is difference in P solubilization among pigment-producing and pigment-non producing bacterial strains (Jayashree et al. 2011). As an example, pink-pigmented facultative methylotrophic (PPFM) strains isolated from Adyar and Cooum rivers in Chennai and forest soils in Tamil Nadu, India, along with *Methylobacterium extorquens*, *M. organophilum*, *M. gregans*, and *M. komagatae* showed phosphate solubilization activity on NBRIP-BPB plates after 7 days of growth. The growth of PPFMs in TCP-amended medium has been reported to be directly proportional to the concentration of glucose oxidized. Higher P solubilization has been observed in four strains MSF 32 (415 mg/l), MDW 80 (301 mg/l), *M. komagatae* (279 mg/l), and MSF 34 (202 mg/l), after 7 days of incubation. A drop in pH from 6.6 to 3.4 has been found coupled with an increase in titratable acidity. Furthermore, acid phosphatase activity has been found to be more pronounced in the culture filtrate than alkaline phosphatase activity (Jayashree et al. 2011). Many P-solubilizing bacteria lose their ability to solubilize P on regular sub-culturing (Khan et al. 2007), while fungi in contrast retain their P-solubilizing activity even after several sub-culturing and could continue actively solubilizing P for many years (Khan et al. 2010).

The incubation period also plays an important role in production of organic acids which in turn affect the P solubilization process (Maliha et al. 2004; Khan et al. 2007). Experiments have shown that P solubilization in culture medium progressively increases with gradual increase in incubation periods (Ahmad 2014). The decrease in P solubilization, however, occurs after certain period of incubation which could be due to the depletion of nutrients, production of certain toxic metabolites in the growth medium, or autolysis of cells (Khan et al. 2013). In a study, Stephen and Jisha (2011) reported maximum solubilization of P (30.44 mg/100 ml) by *Burkholderia* sp. (MTCC 8369) after 18 days of incubation which decreased thereafter with subsequent incubation days. This decrease in P solubilization in some cases has been found to be due to increase in pH of the medium caused by utilization of P and organic acids to run the various metabolic reactions of the PSB (Tripura et al. 2007). Similar variation in P solubilization with change in time has been reported by others (Zaidi 1999; Balakrishnan et al. 2012; Panda et al. 2013).

3.2.7 Humic Substances and Organic Matter

Humic substances in soils are the dark brown, extremely versatile, and fully decomposed remains of plant or animal organic matter. Humic compounds consist of humic acid, fulvic acid, and humin fraction. In soils, humic substances play some important roles in (i) soil conditioning and plant growth (Benedetti et al. 1996); (ii) improving nutrient uptake, especially P, S, N, and Zn; (iii) removing toxins from both soils and animals; (iv) stimulating soil biological activity; (v) solubilizing minerals; (vi) improving soil structure; (vii) protecting soil from degradation; and (viii) enhancing water-holding capacity (WHC) for better drought resistance and reduction in water usage. Humic substances also improves the effectiveness of RP by causing the release of PO_4 from hardly soluble rock minerals because of high total acidity and its ability to complex and chelate the resulting solutions and to stimulate microbial metabolism. Of the different humic compounds, humic acid (HA), a naturally occurring polymeric organic compound (Schnitzer and Khan 1972; Sposito 1989) is an active constituent of organic humus which improves soil structure and enhances the WHC of the soil. Apart from these, HA affects the growth of useful soil organisms and serves as an adsorption and retention complex for inorganic plant nutrients (Brannon and Sommers 1985). Moreover, HA can convert soil constituents into forms that are suitable for uptake by plant due to its ability to form complexes (Vaughan and McDonald 1976). Since HA contains 51–57 % organic C, 4–6 % N, and 0.2–1 % P, it improves crop yields by supplying N and P to the plants together with the improvement in the physico-chemical and biological characteristics of the soils (Hajra and Debnath 1987).

The application of humic compounds in the presence of PSB increases the pH and available P and decreases the exchangeable ions. The presence of sodium

humate and fulvic acid, for example, has been found to improve P solubilization by *B. megaterium* var. *phosphaticum* from insoluble TCP, and the amounts of P solubilized were in proportion to the quantities of the humic substances added (Khan et al. 2009). Humic and fulvic acids react with the insoluble TCP and release soluble P. Of these, fulvic acid releases more P than sodium humate. Humic substances on the other hands act as strong complex forming and chelating agents. The chelation property of humic substances is thus of great use in increasing the efficiency of P fertilizers which are rendered insoluble through fixation mechanisms. In a study, a pot experiment was conducted at green house (LRRI) NARC, Islamabad, on loamy soil during kharif season of year 2012, to investigate the interactive effect of HA, PSB, and varying level of P on P use efficiency in chickpeas. The N, P, and K contents were found to be significantly increased by the application of HA (50 mg HA/kg) and PSB inoculation, and maximum N (4.5 %), P (36 %), and K (2.15 %) contents were recorded in chickpea grown in the presence of 50 mg P_2O_5/kg. Also, the nutrient availability in soil was also improved following single or mixed application of HA, PSB, and P (Sarwar et al. 2013). From this study it has been suggested that the combined application of HA, P, and PSB inoculation improves nutrient use efficiency and could help in reducing the use of P fertilizer by 25 % for chickpea production (Sarwar et al. 2013). In a similar study the combined effects of humic compounds and PSB (*Pseudomonas putida*) were investigated to increase the yields of soybean, grown under glasshouse experiment. The humic compounds were extracted from rice straw compost, the PSB were obtained from Bogor Agricultural University, and the soils (Typic Paleudult) for this experiment were collected from Kentrong Banten, Indonesia. The results showed that the application of humic compounds together with PSB inoculation increased the pH and available P, while it decreased the exchangeable Al of an ultisol (Winarso et al. 2011). The improved soil characteristic, however, did not lead to any significant difference in the uptake of macronutrients by soybean plant (Winarso et al. 2011). The application of organic matter has been found to improve the physical, chemical, and biological properties of soil, which in turn provide a better environment for the growth and activity of the indigenous/introduced PSM.

3.2.8 Kinds of Substrate

Phosphate-solubilizing microorganisms have been found to solubilize a variety of P compounds including DCP and TCP, Fe and Al-P, bone meal, apatites, and different types of RP. In a study, the production of soluble P by *Burkholderia cepacia* DA23 with TCP and hydroxylapatite was higher compared to Al-P, and the production also increased following increase in amounts of the insoluble P (Song et al. 2008). The reactivity of PRs, the main constituent of which is mineral apatite ($Ca_5(PO_4)^3X$) where X is predominantly fluorine, is determined by the rate of dissolution in acid and the amount of P recovery. The reactivity also depends on

the composition of the apatite mineral, presence of impurities, and particle size. Increasing the degree of substitution of carbonate for P and of magnesium and sodium for Ca in the apatite structure and decreasing particle size enhance the reactivity of PRs (Chien and Menon 1995). The solubilization of apatites and RP depends upon the chemical composition and arrangements of minerals, which increase their resistance to solubilization as compared to DCP and TCP. The maximum RP solubilization has been reported with particle size ranging between 30 and 99 mesh. The efficiency of solubilization, however, gets reduced when the size of RP is finer compared to coarser particles. The solubilization of RP of varying particle sizes, due to inoculation with efficient solubilizing *A. awamori* and *P. striata,* is reported to be maximum when RP size is between 30 to 59 and 60 to 99 (Gaur 1986). Microbial conversion is less obvious with finer RP particles than with coarser particles. However, complete solubilization of RP never occurs because a part of RP is so strongly bound that even concentrated HCI or H_2SO_4 cannot solubilize it (Gaur 1986). Other P sources are Udaipur rock phosphate (UPR), Mussoorie rock phosphate (MRP), and North Carolina rock phosphate (NCRP) which have fluorapatite structure with the highest substitution of P with carbonate in NCRP (Narayanasamy and Biswas 1998). The higher solubilization and lowered quantities of organic acids detected in the presence of NCRP could be due to the higher reactivity and greater diversion of organic acids in the neutralization of free carbonates in the solubilization of NCRP as compared to MRP and URP (Bolland 2007). Likewise, the higher solubilization and production of organic acids in the presence of TCP could be attributed to its amorphous nature with simple structure and absence of any free carbonates as compared to the crystalline lattice structure of the RP (Kumari et al. 2008). The decreasing soil pH also increases PR effectiveness (Rivaie et al. 2008; Chien et al. 2010) and dissolution, which has been shown to be linearly correlated with the reserve acidity of the soil.

3.2.9 Effect of Salt Concentrations

Soils containing salts have various ions that may obstruct the uptake of water and concurrently be toxic to numerous soil microflora (Zahran 1997; Rietz and Haynes 2003; Tripathi et al. 2006; Yuan et al. 2007; Vanessa et al. 2008; Li et al. 2011). However, phosphobacteria among microbes have been found in even highly saline environments, for example, marine habitat (Chookietwattana and Maneewan 2012; Promod and Dhevendaran 1987). Phosphate-solubilizing microorganisms when grown in salt-affected environments exhibit variable responses (Table 3.2) to different concentrations (Srinivasan et al. 2012) and compositions of salts (Yadav et al. 2011). For example, *Pseudomonas aeruginosa*, *P. putida*, *P. cepacia,* and *P. fluorescens* when grown in the presence of varying concentrations of salts (NaCl) displayed optimum P solubilization at 0–1.25 % NaCl, but the higher concentrations of NaCl delayed the P solubilization process (Deshwal and Kumar 2013). In a similar study, the effect of salt concentrations (0 %, 2 %, 4 %, 6 %, and 8 %) on

Table 3.2 Effect of salt on P solubilization by different microorganisms

PSM	Medium	P source	pH	Temperature (°C)	Salt	Concentration (g/l)	Incubation period (days)	Solubilization		References
								Broth (mg/l)	Change in pH	
P. aeruginosa	MPVK	TCP	5.9	35	NaCl	4	7	0.30	4.74	
			5.6			5		0.24	4.32	
			5.75			6		0.20	4.39	
			5.85			7		0.23	4.47	
B. megaterium (AIIy)	MPVK	TCP	7.5	30	NaCl	0.2 M	5	119.8	–	Chookietwattana and Maneewan (2012)
						0.4 M		121.2		
						0.8 M		97.2		
B. megaterium (A12 ag)	MPVK	TCP	7.5	30	NaCl	0.2 M	5	137.9	–	Chookietwattana and Maneewan (2012)
						0.4 M		141.5		
						0.8 M		113.1		
Aspergillus awamori	PVK	TCP	7	28 ± 2	NaCl	0.4 M	7	14.75 %	–	Srinivasan et al. (2012)
						0.8 M		14.08 %		

the PS ability of *Bacillus* strains isolated from the rhizosphere of wheat from three areas in arid and semi-arid regions in Algeria was assayed (Cherif-Silini et al. 2013). The bacterial cultures grown in NBRIP medium treated with varying rates of salts had variable solubilization activity which decreased with gradual increase in salinity. However, the response of *Bacillus* to salt was strain dependent where D1 (121.84 μg/ml) and D13 (112.83 μg/ml) strains showed maximum P solubilization compared to other strains. Interestingly, the strains B8 (58.8 μg/ml), BA5 (78.7 μg/ml), and BA11 (88.5 μg/ml) could solubilize more P at 2 % NaCl and demonstrated a high solubilization capacity with concomitant drop in pH of the medium at higher NaCl concentrations. Some of the strains like B14 (31.94 μg/ml), B18 (78.54 μg/ml), BA7 (75.80 μg/ml), and BA12 (35.26 μg/ml) produced the same results even at 4 % NaCl. *Bacillus* sp. in other investigation has also shown optimum P solubilization at 2.5 % salt concentration (Banerjee et al. 2010). On the contrary, among fungi, the P solubilization has been reported even from 2 % NaCl (Srividya et al. 2009) in *Aspergillus niger* F7 to 10 % NaCl (Rosado et al. 1998); however, the activity declines with any further increase in the concentration of NaCl (Johri et al. 1999). The control treatment (without salt) showed luxuriant growth, but the drop in pH and P solubilization was quite low. Moreover, Zhu et al. (2011) have isolated a high P-solubilizing bacterium *Kushneria* sp. (YCWA18) from the sediment of a saltern. Being a halotolerant and capable of growing on solid media at a very high (20 % w/v) NaCl concentration, the *Kushneria* sp. (YCWA18) showed a declining trend of P solubilization with increasing rates of NaCl (Cabrera et al. 2007). The reduction in P activity following microbial growth in high-salt environment can thus be explained as follows: (i) salts adversely affect the growth and cell proliferation resulting in a loss of solubilization efficiency or (ii) chloride ions (Cl^-) sequester or neutralize protons or acids produced in the media and hence reduce the P-solubilizing activity. The decrease in PSM population with increasing concentration of NaCl can be attributed to the exposure of organisms to the conditions of hyper osmolarity resulting in a decrease in their cytoplasmic water activities.

Solutes (NaCl) increase the osmolarity of the medium which in turn cause the loss of intracellular water with a concomitant increase in the osmolarity of the intracellular contents (Botsford 1984). It appears likely that proteins (enzymes) and other biological macromolecules have evolved to function only within certain normal ranges of water activities, outside which some essential cellular functions become impaired (Csonka 1989). In a study, the nodule bacteria, for instance, *Rhizobium* strains, isolated from alkaline soils tolerated high salt concentrations (up to 5 %), but 1,290 mM (7.5 %) salt was inhibitory to the growth of *Rhizobium* strains (Surange et al. 1997). The ability of the selected PSB and PSF to grow and solubilize TCP under salt stress has also been examined by Srinivasan et al. (2012). It has been reported that after 15 days growth, *Aerococcus* sp. (strain PSBCRG1-1) irrespective of NaCl concentrations showed maximum P solubilization compared to other strains. The amount of Pi released increases with incubation period irrespective of strains and salt concentrations. The percent Pi release, in general, increased with an increase in NaCl concentration, up to 0.8 M for bacterial

solubilization, and declined thereafter. The amount of Pi released among PSF in general declined with enhancing NaCl concentration at all incubation periods (Srinivasan et al. 2012). According to Kumar et al. (2010) P solubilization also increased with an increase in NaCl concentration. Realizing the variable impact of salts on microbial structure and function, the impact of salts on P-solubilizing organisms and barley crops inoculated with PSB was conducted (El-Din and Saber 1983). A significant increase in P uptake by plants due to inoculation was observed, but this increase was negatively correlated with increasing salinity levels. The strong promotion of growth of plants, percent Pi release by PSM, and P uptake by plants due to combined interactions of plants and microbes provide evidence that crop productivity could be improved in P-deficient soils affected by salinity. The tomato (*Lycopersicon esculentum*) seeds inoculated with halotolerant PSB strains and grown in different saline conditions exhibited a significant increase in germination percentage of the seeds at salt concentration between 0 and 60 mM, suggesting that the isolated halotolerant PSB may provide P to the growing plants under saline conditions (Soni et al. 2013).

3.2.10 Factors Affecting Organic P Mineralization

The availability of organic P depends on microbial activity to break down the organic matter (OM) and release this P into available forms. The organic P availability depends on physico-chemical characteristics of soils such as (i) soil conditions and weathering process, which influence microbial activity; (ii) soil pH, temperature, and warm moist conditions; and (iii) nutrient levels of soils. Of these factors, temperature above 30 ° C has the maximum positive impact on mineralization of organic P, while the optimum temperature supporting P solubilization is 35 °C. Below 30 °C net immobilization of P occurs. The moisture range of 50–75 % of total WHC is considered optimum for mineralization of organic P although it may also occur in flooded conditions. Alternate wetting and drying favour mineralization of P as it breaks up water stable soil aggregates and exposes for decomposition of otherwise inaccessible humic matter to microorganisms. Other factors that affect organic P mineralization are the cultivation practices (Hedley et al. 1982; Miguel and Wright 2008) which stimulate microbial activity following aeration and facilitate faster decomposition of OM. Hence, cultivated soils generally contain less organic P than virgin soils. Also, cultivation decreases phytate, phospholipids, and nucleic acid P compounds but increases non-hydrolysable residues. Although the effect of aeration is not always consistent, but due to poor aeration, the organic matter decomposition decreases particularly at O_2 levels below 1 % of partial pressure of O_2 in atmosphere. The mineralization of organic P is mediated through certain enzymes which indeed are influenced by several factors. For example, the availability of phosphatases, one of the several enzymes involved in the mineralization of organic P in soil, is enhanced by organic residue addition because P ties up as insoluble Fe, Al, and Ca. Addition of inorganic P may also increase the

mineralization of organic P as a consequence of enhanced solubility of organic P and hence its insusceptibility to mineral. The rate of organic P mineralization has, however, been found to be greatly influenced by the activity of microorganisms in the soil. Species of *Penicillium* (Gawas-Sakhalkar et al. 2012), *Aspergillus* (Qureshi et al. 2013), *Rhizopus* (Acikel and Erşan 2010), *Mucor* (Boyce and Walsh 2007), *Bacillus* (Mahesh et al. 2010), and *Pseudomonas* (Cho et al. 2005; Infantes et al. 2012) produce phosphatase that degrades nucleic acids, glycerophosphates, and phytin. Since carbohydrate is required as C and energy source for mineralization of organic P by soil microorganisms, the organic P mineralization in soil/rhizospheres occurs very rapidly.

3.3 Conclusion

Optimal microenvironmental parameters and metabolizable C compounds must be applied as energy source to the microbial solubilizers to ensure their growth, organic acid production, and, simultaneously, P solubilization. However, low and high temperatures, pH, and salinity, among other factors, are considered the most important abiotic environmental variables that affect both plant physiology and growth and the activity of plant beneficial microbes including PSM. Understanding the impact of such environmental factors on structure and functions of PSM is therefore extremely important for developing and modelling these microphosphatic fertilizers for ultimate transfer to consumers. In order to obtain PSM with high PS activity, it is important to analyse samples from different sources/locations including extreme environments so that a better suited PSM could be identified. A constant exploration of the natural microbial biodiversity of soil and the optimization and fine-tuning (manipulation) of PS microbes are, therefore, required for developing more proficient microbial P inoculants. No doubt, manipulating PSM to acclimatize well to extreme environment is likely to hold the key to improved plant nutrition under stressed environmental conditions and hopefully increased crop yields in the sustainable crop production practices.

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Chapter 4
Phosphate-Solubilizing Fungi: Impact on Growth and Development of Economically Important Plants

Hruda Ranjan Sahoo and Nibha Gupta

Abstract Phosphorus (P) is an important mineral macronutrient required for proper growth and development of plants. It is involved in the synthesis of a series of fundamental cellular molecules such as phospholipids, nucleic acids, nucleotides, etc. Since it is deficient in soils, only a minute amount is available for plant acquisition. Moreover, soluble chemical fertilizers are quickly immobilized in soil and thus are not available for uptake by plants. So, alternative and cleaner agricultural practices have to be developed. In this regard, P supply through biological systems is considered a viable alternative, and inoculation of P-solubilizing microorganisms, especially fungi to soil, is a reliable source for increasing soluble P in soil. Phosphate-solubilizing fungi have been reported from different ecological niches such as agricultural fields, arctic region, forest, mangrove, mine areas, volcanic areas, vermicompost, etc. Following inoculation, phospho-fungi have shown to improve the growth of different group of plants such as cereals, legumes, oilseed and fibre crops, vegetables and horticultural crop, etc. Overall, the use of microbial inoculants particularly the phospho-fungi as a substitute to synthetic phosphatic fertilizers has been found effective in plant-growth promotion and inexpensive vis-à-vis maintaining the natural integrity and fertility of soil.

Keywords Phosphate-solubilizing fungi • Mineral solubilizers • Biofertilizers • Agricultural crops

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4.1 Introduction

Phosphorus is one of the major plant nutrients and plays some important physiological and biochemical roles in plant growth and development (Bagyaraj et al. 2000). However, a greater part of soil P, approximately 95–99 %, is insoluble and, hence, cannot be utilized by the plants. The rapid fixation of P by soil constituents leads to soil P deficiency. This necessitates the application of P fertilizer to the P-deficient soils regularly in agricultural practices in order to fulfil the phosphatic demands of crops. However, most of the available P added to calcareous soils may become unavailable within a short period of time (Tisdal et al. 1995). Considering the brisk P-fixing ability of calcareous soils and increasing cost of P fertilizers, scientists suggested adding P to the soils as raw material (e.g. rock P) after pulverizing it (Antonio and David 1997). In the last decades, several strategies were applied to reduce the P fixation through the following: (i) use of high rates of P fertilizers, (ii) selection of fertilizers, (iii) time and method of application, (iv) combination with amendments and other fertilizers, (v) use of soil tests, etc. (Engelstad and Terman 1980). However, the efficiency of P fertilizers is still low and range between 5 and 10 % (Havlin et al. 1999). In modern agronomic practices, enormous amounts of synthetic P fertilizers are applied, which however, adversely affects the environment (Brady and Weil 1999). Therefore, primary approach in agronomic management of P is to scavenge the native/fixed P and also to overcome the fixation of applied P fertilizer. In this respect, the use of microorganisms capable of mobilizing P into available/soluble forms as biofertilizers is feasible, particularly in sustainable agriculture production systems. Hence, there is enormous interest in isolating PSM, including P-solubilizing and P-mineralizing saprophytic fungi (phospho-fungi), due to their large biomass-producing ability, high metabolic activity and ability to maintain solubilizing capacity for longer periods. Such phospho-fungi have been isolated from various soils (Pandey et al. 2008; Morales et al. 2011). The rationale for using such P-solubilizing fungi as an alternative to synthetic phosphatic fertilizer in agriculture is highlighted.

4.2 Rationale for Mineral P Solubilizers

For sustained agricultural production, use of efficient fertilizer to maintain the soil and plant quality is critical. The application of synthetic fertilizers has been practised for more than a thousand years in many countries since it provides essential nutrients to plants, improves soil structure, helps in the moisture-retaining capacity of various soils and increases microbial activities (Chen et al. 2006). In developing countries like India, the stress on agriculture is increasing day by day. The land under farming is declining and this has posed an extra pressure on agriculture. There are reports that most of the agricultural lands are deprived of

one or more minerals required for the growth and development of plants (Gyaneshwar et al. 2002). In order to maintain a good health, plants require these minerals sufficiently and regularly. And hence, the plant nutrients in the form of chemical fertilizers are applied from external sources. Such chemical fertilizers, however, pose health hazards and cause pollution problems, when applied excessively in soil. Besides, chemical fertilizers are quite expensive. Moreover, the usage of chemical fertilizers alters the structure, composition and function of beneficial soil microorganisms (Whitelaw 2000; Reena et al. 2013).

One of the most important problems in tropical agriculture is the low-soil-P availability. Many of the tropical soils are highly weathered and have a high P fixation capacity that makes their management more difficult. Sanchez and Logan (1992) in a study estimated that 1,018 million ha in the tropics have a high P fixation capacity. In tropical America, there are 659 million ha affected, 210 in Africa and 199 in Asia. The term "P fixation" is used in reference to a series of complex reactions that remove bioavailable soil P from the soil solution, where roots directly take up plant nutrients (Barber 1995). Additionally, P is one of the essential nutrients and is classified as macronutrient because it is required in large amounts by the plants (Bushman et al. 2009). On the contrary, about 98 % soils have inadequate supply of available P (Hansan 1996) and hence plants suffer heavily from P deficiency. Most of the soils contain the substantial reserves of total P; large part of it relatively remains inert and only <10 % of soil P enters the plant–animal cycle (Kucey and Leggett 1989). When P is added as fertilizer to the soil, it gets rapidly fixed. Therefore, P is one of the three major nutrients which are generally added to soil in agronomic practices.

4.3 Mechanism of P Solubilization: A Brief Account

The major microbiological means by which phosphate compounds are mobilized is the production of low molecular weight organic acids (Goldstein 1995) accompanied by acidification of the medium. These organic acids are the source of biotical generated H + ions, which dissolves the mineral phosphate and make it available for the plants (Bhattacharya and Jain 2000). The type of organic acid produced and their amounts, however, differ with different organisms. Among them, glucuronic and α-ketogluconic acids are the most frequently secreted organic acids causing mineral phosphate solubilization (Song et al. 2009). Other organic acids such as acetic, citric, succinic, propionic, glycolic, oxalic, malonic, fumaric and tartaric acid have also been identified among P solubilizers (Ivanova et al. 2006). Ryan et al. (2001) in a study reported that the ability of different carboxylic anions to desorb P decreased with a decrease in the stability constants of Fe- or Al-organic acid complex in the order: citrate > oxalate > malonate/malate > tartrate > lactate > gluconate > acetate > formate. Tri- and dicarboxylic acids are more effective as compared to monobasic and aromatic acids–aliphatic acids which have also been found significant in P solubilization compared to phenolic, citric and fumaric acids

(Mahidi et al. 2011). The organic acids produced by PSM acidify the microbial cells and their surroundings (Richardson et al. 2009) and the release of P ions from the phosphate mineral by H^+ substitution for Ca^{2+} through induction of metabolic processes that are effective in directly solubilizing and mineralizing P from sparingly available forms of P (Illmer and Schinner 1995). In soil, organic acids further reduce the pH of their surroundings and can either dissolve the P directly by lowering the pH of soil or they can chelate heavy metal ions such as Ca, Al and Fe and release associated P with them (Awasthi et al. 2011). The mechanism of P solubilization also involves lowering of pH by release of proton/bicarbonate, gaseous exchange, chelation of cations and by competing with P for the adsorption sites in soil (Nahas 1996). Some of the inorganic acids (e.g. HCl) are also helpful in solubilizing P, but they are less effective as compared to organic acids (Kim et al. 1997). There are other mechanisms by which microorganisms solubilize inorganic P other than the secretion of organic acids, for example, by producing siderophores (Vassilev et al. 2006) and secretion of phenolic compounds and humic substances (Patel et al. 2008).

4.4 Types of Phosphate Solubilization

Mainly two forms of P, namely, organic and inorganic forms, occur in soils and are important for plants as a specific source of P. The relative amounts of P in both forms, however, vary from soil to soil. Most inorganic P compounds in soil belong to one of the two groups: (i) those in which calcium is the most dominant controlling cation (calcium phosphate) and (ii) those in which iron and aluminium are the controlling cations (iron and aluminium phosphate). Calcium phosphates, including rock phosphate ores (fluorapatite, francolite), are insoluble in soil with respect to the release of inorganic P (Pi) at rates necessary to support agronomic levels of plant growth (Goldstein 2000). Phosphate-solubilizing microorganisms increase the P nutrition of plants through enhanced solubility of Ca phosphates (Vassilev et al. 2006) and their solubility increases with a consequent decrease in soil pH. Phosphate solubilization is mainly due to the combined effect of pH decrease and organic acids production (Khan et al. 2010). Microorganisms through secretion of different types of organic acids and pH lowering mechanisms dissociate the bound forms of P like $Ca_3(PO_4)_2$. Nevertheless, buffering capacity of the medium reduce the effectiveness of PSMs in releasing P from tricalcium phosphates (Stephen and Jisha 2009). Carboxylic anions produced by PSMs have high affinity to calcium and solubilize more P than acidification alone (Staunton and Leprince 1996). Complexing of cations is an important mechanism in P solubilization if the organic acid structure favours complexation (Fox et al. 1990). It is controlled by nutritional, physiological and growth conditions of the microbial culture (Reyes et al. 2007), but it is mostly due to the lowering of pH alone by organic acids or production of microbial metabolites (Abd-Alla 1994). Calcium phosphate (Ca-P) release results from the combined effects of pH decrease and

carboxylic acids synthesis, but proton release cannot be the single mechanism (Deubel et al. 2005).

4.4.1 Solubilization of Iron Phosphate/Aluminium Phosphate

Solubilization of Fe and Al by PSMs occurs via proton release accompanied by decrease in the negative charge of adsorbing surfaces to facilitate the sorption of negatively charged P ions. Proton release can also decrease P sorption upon acidification which increases $H_2PO_4^-$ in relation to HPO_4^{2-} having higher affinity to reactive soil surfaces (Whitelaw 2000). Carboxylic acids mainly solubilize Al-P and Fe-P (Khan et al. 2007; Henri et al. 2008) through direct dissolution of mineral P as a result of anion exchange of PO_4^{3-} by acid anion or by chelation of both Fe and Al ions associated with phosphate (Omar 1998). Root-colonizing *Pseudomonads* with high-affinity iron uptake system based on the release of Fe^{3+}-chelating molecules, i.e. siderophores (Altomare et al. 1999), have been reported to solubilize bound Fe. Moreover, carboxylic anions replace P from sorption complexes by ligand exchange and chelate both Fe and Al ions associated with P, releasing P available for plant uptake after transformation. Ability of organic acids to chelate metal cations is greatly influenced by its molecular structure, particularly by the number of carboxyl and hydroxyl groups. Type and position of the ligand in addition to acid strength determine its effectiveness in the solubilization process (Kpomblekou and Tabatabai 1994).

4.4.2 Mineralization of Organic Phosphate

Mineralization of soil organic P plays an imperative role in P cycling of a farming system. Organic P may constitute 4–90 % of the total soil P. Approximately half of the soil and rhizosphere microorganisms possess P mineralization potential which is catalysed by enzymes, for instance, phosphatases (Tarafdar and Claassen 1988). Phosphatase enzymes are present in all organisms but only bacteria, fungi and some algae are able to secrete them outside of their cells. There are two different kinds of phosphatases such as acid phosphatases and alkaline phosphatases which use organic P as a substrate to convert it into inorganic form (Beech et al. 2001). Principal mechanism for mineralization of soil organic P is indeed the production of acid phosphatases (Hilda and Fraga 1999). Acid phosphatase by plant roots/microbes (Yadav and Tarafdar 2001) or alkaline phosphatase (Tarafdar and Claassen 1988) enzymes hydrolyse the soil organic P or split P from organic residues. Soil organisms such as *Bacillus and Streptomyces* sp. have been reported to mineralize very complex organic P by producing extracellular enzymes and

phospholipases (Kannahi and Umaragini 2013). Many PS fungi, for example, *Aspergillus fumigatus* (Yadav and Tarafdar 2003) and *Trichoderma harzianum* (Aseri et al. 2009), have also been reported to produce acid and alkaline phosphatase enzymes, respectively (Yadav and Tarafdar 2003). In addition, PS fungi, for example, *A. terreus* and *P. simplicissimum*, produced phytase, an enzyme which releases soluble inorganic P from organic P compound (inositol hexaphosphate) (Yadav and Tarafdar 2007).

$$\text{Inositol hexaphosphate} + \text{water} \rightarrow \text{Inositol} + \text{phosphate} \quad (\text{catalyzed by phytase})$$

4.5 Groups of Mineral Solubilizers

4.5.1 Fungi

Fungi are important component of soil microbiota constituting more of the soil biomass than bacteria, depending on soil depth and nutrient conditions. Wide ranges of soil fungi are reported to solubilize insoluble P. *Aspergillus* and *Penicillium* among fungi are the most common P-solubilizing fungi (Seshadri et al. 2004; Wakelin et al. 2004). Other fungal species like *Talaromyces* and *Eupenicillium* are considered "key organisms" in the P cycle (Whitelaw 2000). Most of the fungi, for example, *A. terreus*, *A. flavus*, *A. awamori*, *A. niger*, *A. tubingensis*, *A. aculeatus*, *Penicillium digitatum*, *P. simplicissimum*, *Eupenicillium parvum*, *Sclerotium rolfsii* and species of *Fusarium*, *Rhizoctonia*, etc., have been found to solubilize inorganic Ca-P, for example, tricalcium phosphates (Das et al. 2012; Vyas et al. 2007; Reddy et al. 2002), but had poor Al-P- or Fe-P-solubilizing ability (Illmer and Schinner 1995).

4.5.2 Occurrence of Phosphate-Solubilizing Fungi

Phosphate-solubilizing fungi have been isolated from different habitats such as agricultural soil, arctic region, husk waste, coffee plantation, forest soil, hill soil, mangrove area, mine soil, rhizosphere of different crop plants, saline soil, terrestrial soil, vermicompost, volcanic soils, etc., and are listed in Table 4.1.

Table 4.1 Distribution and occurrence of phosphate-solubilizing fungi

Habitat	Fungi	References
Arecanut husk waste	*Aspergillus niger*, *A. terreus*, *Botrytis cinerea*	Naveenkumar et al. (2012)
Coffee plants	*Cylindrocarpon obtusisporum*, *C. didymum*, *Paecilomyces marquandii*, *Penicillium janthinellum*	Posada et al. (2013)
Forest soil	*Aspergillus flavus*, *A. niger*	Das et al. (2012)
Hawaiian soil	*Mortierella* sp.	Habte and Osorio (2012)
Hills soil	*Penicillium citrinum*, *P. islandicum*, *P. mellini*, *P. olivicolor*, *P. restrictum*, *P. rugulosum*	Sharma et al. (2010), Sharma (2011)
Himalayan soil	*Aspergillus glaucus*, *A. niger*, *A. sydowii*	Rinu et al. (2013)
Rhizospheric soil and roots	*Aspergillus niger*, *Penicillium notatum*	Malviya et al. (2011)
Rhizosphere of banana	*Aspergillus sp.*, *Penicillium sp.*	Reena et al. (2013)
Rhizosphere of chick pea	*Aspergillus niger*	Yadav et al. (2011c)
Rhizosphere of melon	*Aphyllophorales*, *Aspergillus*, *Penicillium*, *Rhizopus*	Coutinho et al. (2011)
Rhizosphere of sugarcane and sugar beet	*Alternaria alternata*, *Aspergillus awamori*, *A. fumigates*, *A. niger*, *Curvularia pallescens*, *Penicillium oxalicum*, *P. rubrum*, *Trichoderma viride*	Mahamuni et al. (2012)
Rhizosphere soil of green gram	*Aspergillus niger*, *Fusarium oxysporum*	Kannahi and Umaragini (2013)
Rhizosphere soil of leguminous plant	*Aspergillus* sp.	Selvi (2013)
Saline soil	*Aspergillus clavatus*, *A. fumigatus*, *A. nidulans*, *A. niger*, *A. sydowii*, *A. terreus*, *A. ustus*, *Fusarium* sp., *Penicillium* sp.	Singh et al. (2012), Sanjotha et al. (2011)
Tea leaves	*Penicillium* sp.	Nath et al. (2012)
Vermicompost	*Emericella nidulans*	Bhattacharya et al. (2013)

4.5.3 Examples of Some Notable Phosphate-Solubilizing Fungi

About 62 different species of PS fungi belonging to different genera such as *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, *Talaromyces*, *Trichoderma*, etc. have been recovered from different habitats and are listed in Table 4.2. Some of the fungi with greater P-solubilizing abilities are discussed briefly in the following section.

Table 4.2 Phosphate-solubilizing fungi recovered from various sources

P-solubilizing fungi	Habitat	References
Aspergillus species		
Aspergillus awamori	Rhizosphere of sugarcane and sugar beet	Mahamuni et al. (2012)
A. clavatus	Agricultural soil; saline soil	Chakraborty et al. (2010); Singh et al. (2012)
A. flavus	Forest soil; agricultural soil; rhizosphere soil	Das et al. (2012); Gomashe et al. (2012); Priya et al. (2013)
A. fumigatus	Rhizosphere of sugarcane and sugar beet, agricultural soil; saline soil	Mahamuni et al. (2012); Priya et al. (2013); Singh et al.(2012)
A. glaucus	Himalayan soil	Rinu et al. (2013)
A. melleus	Agricultural soil	Chakraborty et al. (2010)
A. nidulans	Saline soil	Singh et al. (2012)
A. sydowii	Himalayan soil; saline soil	Rinu et al. (2013); Singh et al. (2012)
A. terreus	Arecanut husk waste; saline soil	Naveenkumar et al. (2012); Singh et al. (2012)
A. ustus	Saline soil	Singh et al. (2012)
Penicillium species		
P. albidum	Volcanic soils	Morales et al. (2011)
P. chrysogenum	Agricultural soil	Naik et al. (2013)
P. citrinum	Hill soil; rhizosphere of sugarcane; rhizospheric soils	Sharma et al. (2010, 2011); Yadav et al. (2011a, b)
P. frequentans	Volcanic soils	Morales et al. (2011)
P. islandicum	Hill soil	Sharma et al. (2010, 2011)
P. janthinellum	Agricultural soil; coffee plantations	Scervino et al. (2010); Posada et al. (2013)
P. mellini	Hill soil	Sharma (2011)
P. nigricans	Mangroves	Kanimozhi and Panneerselvam (2010)
P. notatum	Rhizospheric soil and roots	Malviya et al. (2011)
P. olivicolor	Hills soil	Sharma et al. (2010)
P. oxalicum	Rhizosphere of sugarcane and sugar beet; mine soil	Mahamuni et al. (2012); Singh et al. (2011)
P. purpurogenum	Agricultural soil	Scervino et al. (2010)
P. restrictum	Volcanic soils; hills soil	Morales et al. (2011); Sharma et al. (2010, 2011)
P. rubrum	Rhizosphere of sugarcane and sugar beet	Mahamuni et al. (2012)
P. rugulosum	Hills soil	Sharma et al. (2010, 2011)

4.5.3.1 Penicillium

Among the various PSF, *Penicillium radicum*, a PSF isolated from the rhizosphere of wheat, has shown P-solubilizing ability when grown in liquid culture medium containing either ammonium or nitrate as the sole source of N. Insoluble or sparingly soluble P (1,000 mg P/l) was supplied as calcium monohydrogen

phosphate ($CaHPO_4$), calcium orthophosphate ($Ca_3(PO_4)_2$), crystalline ferric phosphate ($FePO_4 \cdot 4H_2O$), crystalline aluminium phosphate ($AlPO_4$), colloidal ferric phosphate or colloidal aluminium phosphate. Phosphate solubilization was highest for $CaHPO_4$ (475 mg P/l), $Ca_3(PO_4)_2$ (360 mg P/l) and colloidal aluminium phosphate (207 mg P/l). Phosphate solubilization was generally higher with ammonium than the nitrate (Whitelaw et al. 1999). Wakelin et al. (2004) also found *Penicillium* spp. exhibiting P-solubilizing activity both on and in the roots of wheat plants grown in southern Australian agricultural soils. Of the different fungal species, *P. bilaiae* strain RS7B-SD1 was the most effective, mobilizing 101.7 mg P/l. Other effective strains included *P. simplicissimum* (58.8 mg P/l), *P. griseofulvum* (56.1–47.6 mg P/l) and *Talaromyces flavus* (48.6 mg P/l) and two unidentified *Penicillium* spp. (50.7 and 50 mg P/l). A newly identified strain of *P. radicum* (KC1-SD1) could mobilize 43.3 mg P/l. Reyes et al. (2007) in a similar study found six fungal strains belonging to the genus *Penicillium* endowed with high hydroxyapatite dissolution capacities. Five of them had similar phenotypes to *P. rugulosum* IR94MF1 but they solubilized hydroxyapatite at different degrees with both N sources. On the contrary, Vyas et al. (2007) showed high solubilization of TCP, aluminium P, MRP and North Carolina RP by *E. parvum*. The organism also exhibited tolerance against desiccation, salinity, acidity, aluminium and iron. Similar reports on P solubilization by *P. restrictum*, *P. rugulosum*, *P. citrinum*, *P. islandicum*, *P. olivicolor*, *P. mellini* (Sharma et al. 2010, Sharma 2011), *P. citrinum* (Yadav et al. 2011a, b) and *P. oxalicum* (Singh et al. 2011) are available in the literature. In a recent study, the TCP-solubilizing activity of two different endophytic *Penicillium* species isolated from tea leaves was reported by Nath et al. (2012). Both the isolates had remarkable PS activity up to 8 days with consequent increase in the acidity of the medium. Tricalcium P-solubilizing activity of species 1 ranged between 39.22 ± 1.17 and 86.1 ± 1.2 μg/ml, while that of species 2 varied between 32.57 ± 1.41 and 84.25 ± 1.5 μg/ml following 2 to 10 days incubation (Figs. 4.1 and 4.2).

4.5.3.2 Aspergillus

Barroso et al. (2006) studied the solubilization of $CaHPO_4$ and $AlPO_4$ *by A. niger* using several C and N sources. Solubilization of Ca-P was enhanced when the C sources were mannitol, maltose, galactose and glucose (in that order), while Al-P was solubilized in the order: galactose > sucrose > maltose. More extensive growth, acid production and decrease in pH were recorded in the Al-P medium than in the Ca-P medium. According to Gupta et al. (2007), *Aspergillus* isolated from mangrove plants grown in Bhitarkanika, Orissa, showed good PS activity, while Kang et al. (2008) found a "soil isolate" *Aspergillus* sp. which had excellent potential to solubilize RP with occurrence of high levels of citric acid which also caused a significant drop in pH of the medium. Similarly, *A. niger and A. fumigates* solubilized RP and TCP significantly (Hefnawy et al. 2009). In other study, Singh et al. (2011) showed that *A. niger* strain 1 could solubilize 285 mg P/ml, while

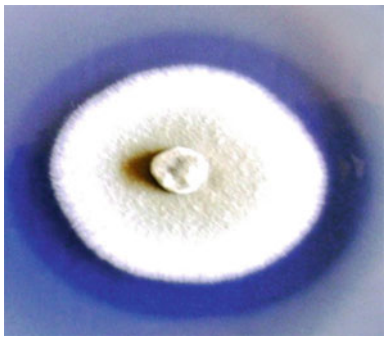

Fig. 4.1 Plate culture showing phosphate solubilization by *Penicillium chrysogenum* Thom

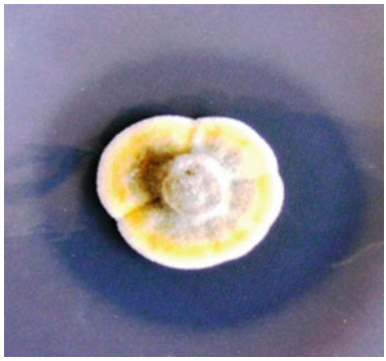

Fig. 4.2 Plate culture showing phosphate solubilization by *Penicillium restrictum* Gilman and Abott (courtesy: Dash, S. Ph.D. Thesis: Characterization and evaluation of biofertilization potential of phosphate and iron solubilizing fungi and rhizobia for tree legumes, Utkal University, 2012)

A. niger strain 2 solubilized 262 mg/ml from 0.5 % TCP after 7 days growth. This was probably the first report of TCP solubilization by any Arctic fungal strains which could be used to prepare fungal P biofertilizer. Zeroual et al. (2012) isolated *A. niger*, from agricultural soil, and tested for its ability to solubilize different P matrixes (TCP, DCP, phosphates rock). Singh et al. (2012) isolated a total of 42 fungal isolates belonging to 12 different species from 40 soil samples of unusual habitats of Agra region, Uttar Pradesh, India. Of these, 27 *Aspergilli* showed PS activity, and 18 from 27 fungi, namely, *A. clavatus*, *A. fumigatus*, *A. nidulans*, *A. niger*, *A. terreus*, *A. ustus and A. sydowii*, showed comparatively more PS activity. While comparing the PS activity of all fungi, *A. niger* (KH-4, KH-6 and CH-2) had the largest PS activity which could be used in the field as biofertilizers for supplying P to field-grown crops and, hence, increasing the crop productivity. Rinu et al. (2013) in a follow-up experiment recovered three species of *Aspergillus*, namely, *A. niger*, *A. glaucus* and *A. sydowii*, from Indian Himalayan Region (IHR), and assayed their aluminium P- and iron P-solubilizing efficiency in the presence of different C and N sources. The P solubilized by fungal cultures varied considerably among C and N wherein *A. niger* solubilized 32 % and 8 % of the supplemented aluminium P and iron P, respectively. This result indicated that the C and N sources influenced the PS efficiency of all the *Aspergillus* spp.

4.5.3.3 Trichoderma, Mortierella and Galactomyces

A total of 14 strains of *Trichoderma* isolated from the forest tree rhizospheres of Pinus, deodar, bamboo, guava and oak using *Trichoderma*-specific medium were evaluated for P-solubilizing activity employing National Botanical Research Institute Phosphate (NBRIP) broth containing TCP as the sole P source and were compared with a standard culture of *T. Harzianum* (Kapri and Tewari 2010). Even though all fungal cultures could solubilize TCP, the degree of solubilization differed among fungal isolates which varied between 111.5 μg/ml and 404.07 μg/ml in the culture filtrates of *Trichoderma*. In a similar study, Habte and Osorio (2012) evaluated the effect of N (NH_4^+ and/or NO_3^-) on the dissolution of RP by a PSF *Mortierella* sp. In the presence of NH_4Cl or NH_4NO_3, the pH of the medium was significantly decreased from an initial value of 7.6 to 3.4 and 3.7, respectively. In the presence of KNO_3, the pH went down only to 6.7. As a result, significantly more P was detected in the presence of NH_4Cl (129.65 mg/l) than in the presence of NH_4NO_3 (109.25 mg/l), while the concentration of P in the presence of KNO_3 was only 0.08 mg/l. Yingben et al. (2012) observed that *Galactomyces geotrichum* P14, isolated from soil of phosphate mines, demonstrated maximum solubilization of insoluble P (1252.13 mg/l) within 40 h in a modified phosphate growth medium supplemented with TCP.

4.5.3.4 Mixed Group

Pradhan and Sukla (2005) tested two fungal isolates for their TCP-solubilization efficiency on solid and in liquid medium. The fungal Isolates were identified as *Aspergillus* sp. and *Penicillium* sp. on the basis of their colony morphology and microscopic characteristics. Phosphate solubilization was coupled to pH decrease mediated by growth of fungus in medium containing glucose as C source. *Aspergillus* sp. solubilized 480 g P/ml, while *Penicillium* sp. solubilized 275 g P/ml from 0.5 % TCP after 4 and 3 days of growth, respectively. High P-solubilization activity of *Aspergillus* sp., *Penicillium* sp. *and Fusarium* sp. isolated from saline area of Purna river basin was reported by Rajankar et al. (2007). Of the total 107 soil samples collected from saline affected area, 31 % samples had P-solubilizing fungi wherein 87 % were identified as *Aspergillus* spp., 8.7 % as *Penicillium* spp. and 4.3 % as *Fusarium* spp. Xiaoa et al. (2008) conducted a similar experiment for RP solubilization by PS fungi, *Candida krissii*, *P. expansum and Mucor ramosissimus*, isolated from phosphate mines (Hubei, PR China). The content of soluble P was the highest when the initial pH for RP solubilization was 5.5 in the medium inoculated with *C. krissii*, which was different from that of 7 in the medium inoculated with *P. expansum* and 7.5 in the medium inoculated with *M. ramosissimus*. Mittal et al. (2008) isolated 6 PSF (2 strains of *A. awamori* and 4 of *P. citrinum*) from various rhizosphere. The P-solubilizing activity of PSF in liquid varied from 38 to 760 μg/ml for TCP and 28–248 μg/ml for MRP among all isolates. Phosphate-

solubilizing bacteria and fungi associated with Salix alba Linn from Lahaul and Spiti valleys of Himachal Pradesh were recovered using PVK, modified Pikovskaya (MPVK) and NBRIP media. The PSF belonged mainly to *Penicillium* sp., *A. fumigatus*, *A. niger*, *A.* spp. and non-sporulating sterile groups. Amongst the PSF, 7 fungal isolates dissolved higher amounts of P from North Carolina RP than MRP and Udaipur rock phosphate (URP). However, the organisms solubilized higher P in NBRIP broth than PVK broth. FC28 (*Penicillium sp.*) isolate could solubilize 52.3 µg/ml amongst fungi, and while solubilizing URP, FC28 and FC39 displayed maximum decrease in pH of medium from 6.8 to 5.96 in NBRIP broth (Chatli et al. 2008).

According to Srividya et al.(2009), *A. niger* (F7), *A. niger* (F4), *A. niger* and *Penicillium* sp. showed 107.7, 108.3, 112.7 and 110.3 % PS efficiency on PVK medium with 0.5 % (w/v) TCP and 285, 187.5, 258 and 70.5 µgP/ml, respectively, from 0.5 % (w/v) TCP in liquid broth after 5 days of growth. The fungal isolate F7 however showed a varied level of PS activity both on solid and in liquid culture medium treated with different C and N sources. In a similar study, Jayaraman and Ilyas (2010) assessed the PS activity of *Aspergillus* and *Penicillium* isolated from the paddy rhizosphere in Tamil Nadu, India. Phosphate solubilization efficacy of the fungal strains followed the order: *A. niger* > *Penicillium* sp. > *A. fumigatus*. Coutinho et al. (2011) isolated a total of 318 filamentous fungi from areas cultivated with melon and determined their PS ability. Of these, 52 fungal isolates were able to solubilize P and were identified *as Aphyllophorales* (*2*), *Aspergillus* (*34*), *Penicillium* (*10*) *and Rhizopus* (6). Yadav et al. (2011a) tested the P-solubilization potential of *Aspergillus niger* strain BHUAS01, *P. citrinum* strain BHUPC01 and *T. harzianum* in vitro which showed 328 µg P/ml, 301 µg P/ml and 287 µg P/ml, respectively. Noor et al. (2013) demonstrated that microorganisms are the most prominent entities for solubilization of P in various soils of different areas of Sindh Province including Tando Muhammad Khan, Tando Allahyar, Nawabshah, Ratodero-Larkana, Shikarpur and Umerkot, Pakistan. These soils had varying concentrations of chemicals, variable climatic conditions, pH and microbial populations especially the PSA. The isolated fungi expressing PSA included species of *Fusarium*, *Aspergillus*, *Penicillium* and *Rhizopus*. Among PSF, *Aspergillus* sp. showed greatest PSA as compared to other fungi. Naveenkumar et al. (2012) isolated fungal species from the *Areca catechu* husk waste and determined the PSA. The zone of clearance was higher in *A. terreus* (0.8 ± 0.03 cm), medium in *B. cinerea* (0.5 ± 0.08 cm) and very low PS activity was detected for *A. niger* (strain 2) and unidentified 3 (0.1 ± 0.03 cm). The P-solubilizing activity in broth was higher for unidentified 2 (550 ± 8.5 µg/ml), medium in unidentified 1 (530 ± 10 µg/ml) and very low activity in *A. niger* (strain 2) (40 ± 2.52 µg/ml). Mahamuni et al. (2012) isolated PSF from the sugarcane and sugar beet rhizosphere of Western Maharashtra region of India on the basis of clear zones on Pikovskaya agar medium and solubilization indices. The PSF recovered from both rhizospheres were identified as *A. niger* (NFCCI 1991), *A. awamori* (NFCCI 1992), *A. fumigatus* (NFCCI 1993), *Alternaria alternata* (NFCCI 1994), *Curvularia pallescens* (NFCCI 1996), *P. oxalicum* (NFCCI 1997), *P. rubrum* (NFCCI 1998) and *T. viride* (NFCCI

1999). The percent P solubilized in medium containing TCP and RP by the fungi ranged from 34.2 to 58 % and from 16.6 to 36.6 %, respectively. Among PSF, *C. pallescens* (NFCCI 1996) produced the highest soluble P while *A. alternata* (NFCCI 1994) had the lowest PSA when grown in medium supplemented with TCP. *Trichoderma viride* (NFCCI 1999) showed a variable PSA. Posada et al. (2013) isolated fungal isolates from 8 coffee plantations in Columbia and Mexico. *Cylindrocarpon didymum* and *C. obtusisporum* (both from Columbia) could solubilize 9.9 and 6.4 mg PO_4^{3-} P/l and accumulated 8.6 and 11.6 mg P in biomass. However, *Penicillium janthinellum* and *Paecilomyces marquandii* (both from Mexico) solubilized 7 and 1.9 mg PO_4^{3-} P/l and accumulated 11.3 and 17.3 mg P in biomass.

4.6 Some Examples of Impact of PSF on Plant Growth

Several plants have shown dramatic increase in growth following PSF application. The list of groups of plants positively influenced by PSF application is given in Table 4.3.

4.6.1 Cereals

4.6.1.1 Maize and Corn

In a study, Richa et al. (2007) showed that *A. tubingensis and A. niger* inoculation improved the growth of maize (*Zea mays*) and the level of P in shoots. In a similar investigation, the single and mixture of 3 genera [*Aspergillus* (*2 species*), *Penicillium*, (*2 species*) *and Cephalosporium* sp.] of RP-solubilizing fungi were used in the presence and absence of organic fertilizer (chicken manure) to assess their impact on corn (Kassim and Al-Zandinany 2011). Rock phosphate was added at a fixed rate (88 kg/ha). Vegetative growth of corn was used as an indicator and plants were grown for 60 days, during which, P availability was measured at a 2-week interval. Dry matter accumulation in shoots and roots of inoculated/uninoculated plants and the uptake of P by the corn plant was measured. Results indicated that the total amount of P solubilized, expressed as percentage of the added RP in the presence of organic fertilizer, was 41.5 % in soil inoculated with the mixture of fungi which was followed by single application of *Penicillium* (37.3 %), *Aspergillus* and *Cephalosporium* (36.6 %), resulting in an increase of 31.8 %, 22.5 % and 20 % over control plants, respectively. Recently, Patil et al. (2012) evaluated the ability of *P. bilaiae and Penicillium* spp. and different P levels on growth, yield and nutrient content in maize grown in calcareous soil. Seed inoculation with PSF along with P_2O_5 significantly influenced plant height, number of leaves per plant, dry matter production, cob length, grain weight per cob, 1,000 grain weight, grain

Table 4.3 List of host plants benefitted by phosphate-solubilizing fungi

Plant group	Host plant	PSF	Effect	References
Cereals	*Amaranthus cruentus L.*	*Aspergillus niger*	Number of leaves, dry weight of shoot, total P content increased	Reena et al. (2013)
	Maize and corn	*Aspergillus* sp., *Penicillium* sp. and *Cephalosporium* sp.	Amount of phosphorus absorbed and plant dry weight	Kassim and Al-Zandinany (2011)
	Wheat	*P. bilaji*, *Penicillium* spp.	Plant height, number of leaves per plant, dry matter production, cob length, grain weight per cob,1,000 grain weight, grain yield	Patil et al. (2012)
	Wheat	*P. oxalicum*	Increased the growth and yield	Singh et al. (2011)
	Wheat	*A. awamori*, *A. niger*	Increased the grain and straw yield	Sharma et al. (2012)
Leguminous crops	Chick pea	*Trichoderma harzianum* and *A. niger*	Shoot length, root length, dry weight of shoot and root increased	Yadav et al. (2011a)
	Cowpea	*Aspergillus sp.*	Wet and dry weight of the shoot, root and pod were higher	Manivannan et al. (2012)
	Soybean	*A. niger*, *A. melleus* and *A. clavatus*	Root phosphate content showed an increase	Chakraborty et al. (2010)
Oilseed and fibre crops	Anise	PSF	Highest seed yield, essential oil content in seeds	Zand et al. (2013)
	Groundnut	*A. niger*, *P. notatum*	Increased dry matter, yield, percentage of protein; oil. N and P content percentage increased	Malviya et al. (2011)
Vegetable and horticultural crops	Cucumber	*Aspergillus* spp.	Increase in shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, root dry weight, plant length, leaf area and leaf chlorophyll content	Islam et al. (2014)
	Lettuce	*P. albidum*	Increase in the gross weight	Morales et al. (2011)

(continued)

Table 4.3 (continued)

Plant group	Host plant	PSF	Effect	References
	Tomato	*A. awamori*, *Trichoderma viride*	Improved yield	Sibi (2011)
	Tomato	*A. niger*	Increased the yield and dry matter content	Anwer and Khan (2013)
Forest trees	Bamboo	*AM* fungi and *A. tubingensis*	P increased in shoot tissues	Giridhar Babu and Reddy (2010)
	Dalbergia sissoo	*P. chrysogenum* and *Aspergillus* sp.	Maximum biomass production	Dash et al. (2013)

yield and tissue nutrient content (N, P, K, Zn and Fe) at tasseling of leaves and at harvest. Higher growth and yield of maize were achieved when P-solubilizing fungi were used with 100 % P_2O_5 compared to 0 and 50 % P_2O_5. It was concluded that single and dual inoculation of PSF along with P fertilizer gave 20–23 % higher maize yield over control.

4.6.1.2 Rice

Ferreira et al. (2008) performed an experiment in greenhouse conditions with three P-solubilizing isolates involving two bacteria and one fungi and four increasing inocula concentrations (106–109 cfu/ml). The height, root dry matter, length and volume of rice (*Oryza sativa*) increased following PSF inoculation (PSF-8) in the first experiment. This inoculant (PSF-8) also enhanced the rice growth at the highest inoculum concentration in the second experiment.

4.6.1.3 Wheat

In a study, Xiaoa et al. (2008) isolated 3 PSF from phosphate mines of Hubei, People's Republic of China, and identified them as *Penicillium expansum*, *Mucor ramosissimus and Candida krissii*. Subsequently, these PSF were tested under pot trial to evaluate their growth-promoting abilities using wheat as a test crop. Generally, all fungi enhanced the growth of wheat plants and increased soil-available P and P and N uptake of wheat seedling in soil containing RP under pot conditions. This study thus demonstrated the capability of the fungal isolates to convert insoluble form of P into plant-available P form from RP, and therefore, such fungal cultures hold great potential for developing biofertilizers to enhance soil fertility and promote wheat plant growth. Similarly, P-solubilizing fungus *P. oxalicum* isolated from the rhizosphere soil of rock phosphate mine landfills

was tested for its efficacy to solubilize RP and effect on the growth of wheat and maize plants grown in soil amended with RP. Field experiments clearly showed that the plants inoculated with *P. oxalicum* had significantly higher growth and resulted in maximum yield of wheat and maize compared to the control plants. The P content was also significantly increased in the plants (Singh et al. 2011). Sharma et al. (2012) in a follow-up study assessed the influence of P levels and PSF on yield and nutrient uptake by wheat. The dry matter production by wheat at tillering, ear emergence and harvest was significantly higher. Application of *A. awamori* gave the highest dry matter accumulation at tillering, at ear emergence and at harvest. Increasing levels of P also increased the grain and straw yield significantly. Increasing level of P and inoculation with *A. awamori* and *A. niger* significantly increased the uptake of N, P and K in wheat at all stages of growth. The maximum N, P and K uptake was, however, recorded in grain and straw at harvest which was followed by ear emergence and tillering stage when seeds were inoculated with *A. awamori*.

4.6.2 Leguminous Crops

The effect of 6 PS fungi including two strains belonging to *A. awamori* and four to *P. citrinum* was tested for their growth-promoting efficiency against chickpea plants (*Cicer arietinum* L. cv. GPF2) grown in pot experiments (Mittal et al. 2008). A maximum stimulatory effect on chickpea growth was observed following inoculation of two *A. awamori* strains which resulted in 7–12 % increase in shoot height, nearly threefold increase in seed number and twofold increase in seeds weight as compared to the control (uninoculated) plants. Inoculation of four strains of *P. citrinum* however exhibited lesser stimulatory effect and showed only 7 % increase in shoot height, twofold increase in seed number and 87 % increase in seed weight relative to the control plants. However, a consortium of all the 6 fungal isolates showed no stimulatory effect on chickpea plants growth. Later on, Kapri and Tewari (2010) reported a significant increase in biological properties of *Trichoderma*-inoculated chickpea plants grown in P-deficient soil under glasshouse conditions. The dry matter accumulation in above-the-ground plant organ (shoot) was increased by 23 % and 33 % due to inoculation with the *Trichoderma* (DRT-1) in the soil amended with 100 and 200 mg TCP kg^{-1} soil, respectively, after 60 d of sowing. In a similar investigation, Yadav et al. (2011a) showed that co-inoculation of T. *harzianum and A. niger* demonstrated a significant increase in the growth of chickpea plants. Manivannan et al. (2012) conducted an experiment using *Aspergillus* sp. to enrich the total P content of vermicompost. The fungus was grown in mass and the spore count was done periodically. Pre-prepared vermicompost was mixed with a spore suspension of *Aspergillus* sp. at 1×108 spores/g. Treated vermicompost was then mixed with soil, for pot culture studies. The composition

of PS fungi with vermicompost showed higher productivity of *Vigna unguiculata* (*L.*) *Walp*. (cowpea). Pot culture studies also showed that the wet and dry weight of the shoot, root and pod were higher in vermicompost enriched with microbial inoculants than in vermicompost alone and in control plants. The maximum growth performance and yield of a cow pea was observed in the *Aspergillus* sp.-enriched vermicompost. This is probably due to more phosphorus content in the soil formulation. Saber et al. (2009) demonstrated that the inoculation of green gram (*Vigna radiata*) seeds with *A. niger and Penicillium* in the presence of RP and calcium superphosphate (CSP) increased significantly the growth, seed yield and P uptake and also improved the nodulation status and population of total and P-dissolving fungi in the rhizospheric soil of green gram and could save about 1/3 P fertilizer dose. Chakraborty et al. (2010) observed a significant increase in soybean growth following *A. niger*, *A. melleus and A. clavatus* application under in situ. However, a decrease in soil P content was recorded following fungal application.

4.6.3 Oilseed and Fibre Crops

The PSF has also been found to have positive effects on growth and development of oil seed and fibre crops. As an example, Zand et al. (2013)in a study investigated the effects of PSM and plant density on seed yield and essential oil content of anise (*Pimpinella anisum*). Treatments consisted of control (P1), seed inoculation (P2) and seed inoculation + spraying on the plant base at stem elongation stage (P3) and plant density at four levels (67, 34, 23 and 17 plants/m^2). The PS organisms had positive effects on all the measured traits especially when it was used two times (at seed inoculation + spraying on the plant base at stem elongation stage). Highest seed yield and essential oil content in seeds were obtained at plant density of 17plants/m^2. Greenhouse and field experiments were conducted to evaluate the effect of a PSF isolate of *P. bilaiae* on the yield and P uptake by canola (*Brassica napus* L.). Under greenhouse conditions, *P. bilaiae* inoculation did not affect canola pod or straw dry matter production, but it did increase straw and pod P concentrations and resulted in increased P uptake over uninoculated plants. Addition of P (20 mg/kg soil) as Florida rock phosphate (FRP) together with *P. bilaiae* enhanced P uptake by canola which was at par with those resulting from the sole application of mono-ammonium phosphate (MAP), used at the same rate of P. Addition of FRP had inferior effect on plant P uptake. However, the addition of *P. bilaiae* generally increased dry matter yields and P uptake by canola growing in two fields, suggesting that *P. bilaiae* might have accounted for increase in the P pool and hence greater uptake by plants (Kucey and Leggett 1989). Likewise, *A. niger and P. notatum* solubilized TCP in vitro and promoted the growth of groundnut (*Arachis hypogaea*) plants grown in soil amended with TCP (Malviya et al. 2011). From pot experiments it was clear that the dual inoculation of *A. niger*

and P. notatum significantly increased dry matter and yield of groundnut plants as compared to the control plants. Also, a significant increment in percentage of protein and oil as well as an increase in the percentage of N and P content of the plant was noticed. The increase in N levels of groundnut plants was, however, nonsignificant with the percentage of total P, under the experimental conditions.

4.6.4 Vegetable and Horticultural Crop

4.6.4.1 Cucumber

Aspergillus PPA1 significantly increased shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, root dry weight, plant length, leaf area and leaf chlorophyll content of cucumber (*Cucumis sativus*) plants compared to non-treated control plants. The growth rate of plants increased with the increasing concentration of fungal inoculum applied to soil. The fungus was re-isolated from the roots of cucumber plants at higher frequencies. These results suggest that *Aspergillus* spp. PPA1could be used as a root-colonizing plant-growth-promoting fungus for enhancing the overall performance of cucumber (Islam et al. 2014).

4.6.4.2 Lettuce and Tomato

Kohler et al. (2007) observed that a joint inoculation of *P. albidum* with native mycorrhizae had a synergistic effect on the growth of lettuce (*Lactuca sativa*). Also, *Penicillium albidum* possessed with the capacity to solubilize both inorganic and organic P enhanced the growth and mineral nutrition of lettuce plants growing in a volcanic soil (Morales et al. 2011). In other experiments conducted by Sibi (2011), the co-inoculation of *A. awamori and T. viride* significantly increased the nutrient value of the compost. A maximum P content (64.3 %) was observed in co-inoculation treatment which was followed by single inoculation of *A. awamori* (62.2 %). The present findings revealed that PS fungi can interact positively in promoting nutrient content of compost and tomato (*Solanum lycopersicum*) growth leading to improved yield. A similar increase in the yield and dry matter content of tomato plants due to inoculation of nursery root-dip culture of *A. niger* is reported (Anwer and Khan 2013). Salicylic acid, total phenolic and chlorophyll contents of plant, and lycopene, ascorbic acid (Vitamin C), Brix index, diameter of fruit skin, and rate of pressure tolerance of tomato fruit were increased compared to untreated control plants. Among all isolates, *A. niger* SkNAn5 was found to be the most efficient and increased yield by 54 % and dry matter of tomato plants by 59.8 %. *A. niger* SkNAn5 also significantly increased the salicylic acid of root and fruit quality of tomato, having increased amount of vitamin C (35.59 g/100 g against

control 23.9 g/100 g), lycopene (9.8 mg/100 g against control 8.3 mg/100 g) and rate of pressure tolerance of fruits (2.84 kg/cm against control 1.35 kg/cm). These results suggest that nursery application of *A. niger* SkNAn5 may improve quantity and quality of tomato fruits.

4.6.5 Forest Trees

4.6.5.1 Bamboo and *Dalbergia sissoo*

Co-inoculation of arbuscular mycorrhizal (AM) fungi and PS fungus *A. tubingensis* fungi significantly increased the P (150 %), K (67 %), Ca (106 %) and Mg (180 %), whereas the Al and Fe content were significantly reduced by 50 and 60 %, respectively, in shoot tissues of bamboo (*Dendrocalamus strictus*) plants grown in fly ash as compared to control plants (Giridhar Babu and Reddy 2010). A significant increase in biomass production of *D. sissoo* was recorded when seedlings were inoculated with *P. chrysogenum and Aspergillus* sp. and grown in pot culture under polyhouse misting facility. Growth analysis revealed that NAR (net assimilation rate) and LAR (leaf area ratio) differed with RGR (relative growth rate) in the treatments. Application of selected microbes can lead to a successful establishment of *D. sissoo* in nurseries, even in pot soils, and help in producing quality planting material (Dash et al. 2013) (Figs. 4.3 and 4.4).

4.7 Conclusion and Assumption

The use of biofertilizers or microbial inoculants for replacing the efficacy of chemical fertilizers has been found to be effective in reducing the cost of cultivation and maintaining the natural fertility of soil. Therefore, utilization of PS fungi as biofertilizer has gigantic potential for making use of fixed P present in the soil in crop production without causing any harmful effects on aerial and soil environment. Biofertilizers are more economical due to their low market prices compared to synthetic fertilizers, helpful in improving soil structure and the restoration of environment for leveraging agriculture. Research efforts are, therefore, required for exploring new and better agronomic effectiveness of biofertilizers application for profitable crops such as orchards, flowers and vegetables. Although PSMs are abundant in many of the soils, isolation, identification and selection of PSMs have yet not been successfully commercialized, and thus, its application is still limited. There is therefore an urgent need to popularize the use of inexpensive and more powerful biofertilizers, especially the use of PSM in sustainable crop production across different agro-climatic regions of the world.

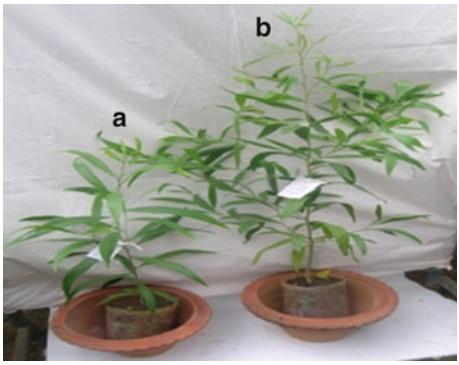

Fig. 4.3 Effect of *Penicillium chrysogenum* Thom. on growth of *Acacia auriculiformis*. (**a**) Uninoculated control, (**b**) inoculated plants (courtesy: Dash, S. Ph.D. Thesis: Characterization and evaluation of biofertilization potential of phosphate and iron solubilizing fungi and rhizobia for tree legumes, Utkal University, 2012)

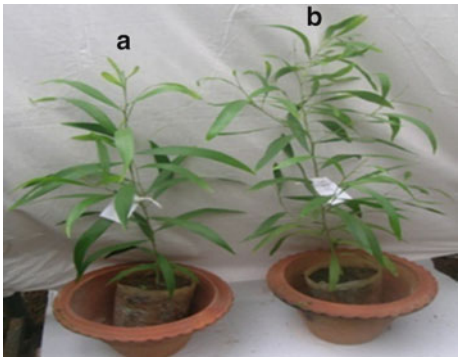

Fig. 4.4 Effect of *Penicillium restrictum* Gilman and Abott on growth of *Acacia auriculiformis*. (**a**) Uninoculated control, (**b**) inoculated plants (courtesy: Dash, S. Ph.D. Thesis: Characterization and evaluation of biofertilization potential of phosphate and iron solubilizing fungi and rhizobia for tree legumes, Utkal University, 2012)

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Chapter 5
Cold-Tolerant Phosphate-Solubilizing Microorganisms and Agriculture Development in Mountainous Regions of the World

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Abstract In many mountainous regions of the world, agriculture faces several major challenges. Of these, cold temperatures and low fertility of soils are of particular importance. For instance, in the Indian Himalayas and in the High Andes, soils are acidic and phosphorus deficient, severely limiting crop production. In the contemporary world, the use of biofertilizers-especially nitrogen fixers and phosphate solubilizers-to enhance crop yields has become an attractive alternative for farmers, due to low costs and no environmental hazards. Nevertheless, use of commercially available biofertilizers in cold climates like the ones prevailing in mountainous regions has been found ineffective. To overcome these challenges, numerous biotechnological/microbiological approaches have been assayed during the last two decades, involving the use of cold-loving (psychrophilic) or cold-tolerant (psychrotrophic) organisms. However, despite their great potential, the development of cold-tolerant biofertilizers based on the rational use of psychrophilic and psychrotrophic microorganisms is still in its infancy. The most important achievements in the field of cold-tolerant phosphate-solubilizing bioinoculants documented so far are reviewed and highlighted in this chapter.

Keywords Cold-tolerant biofertilizers • Psychrophilic microorganisms • Tropical Andes • Phosphate solubilization • Mountain agriculture • Plant growth promotion

5.1 Introduction

According to the United Nations Universal Declaration of Human Rights, "everyone has the right to a standard of living adequate for the health and well-being of himself and of his family, including food, clothing, housing..." (Article 25)

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M.S. Khan et al. (eds.), *Phosphate Solubilizing Microorganisms*,
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(UN General Assembly 1948). Hence, freedom from hunger is a fundamental human right. Alas, the most recent estimates of the Food and Agriculture Organization of the United Nations (FAO) indicate that, even though the proportion of the world's population suffering from undernourishment has declined to 12.5 %, 868 million people still remain undernourished in terms of energy intake, most of them in rural areas of poor countries. Besides, an estimated two billion people suffer from one or more micronutrient deficiencies (FAO Report 2013). Furthermore, considering the pace of human populations, which is likely to reach 7.4 billion by 2017 and 9.3 billion in 2050 (FAO Report 2005), there is an urgent need to increase food production by at least 50 % in the next 20 years. To overcome hunger problems around the world, agriculture has traditionally played a fundamental role in producing food and generating income. Since 1947, significant improvements in agriculture technologies have unleashed processes of productivity growth, economic development, and social transformation, which are witnessed all around the world (FAO Report 2013). However, there is still much to do and agriculture in this context can contribute further to eradicate the malnutrition problems.

In the mountainous regions of the developing world, agriculture faces several major challenges. Two of them are of particular importance: (1) a dramatic growth in mountain populations which is causing an unprecedented pressure on natural resources and (2) an accentuated demand on commercial products (crops and livestock) that intensifies the rapid resource use to ensure high productivities. However, for enhancing the production of many mountain crops, either there are no improved technologies available or, even if available, they are not affordable to small farmers. Because of these and some other related problems like deforestation, soil erosion, and frequent, excessive, and non-judicious use of chemical fertilizers and pesticides, there is greater pressure on land resources, some of which may have been damaged irreversibly (Johda et al. 1992). When considering agriculture in mountainous regions, special attention must be paid to two fundamental aspects: the climatic context, mainly characterized by low soil and atmospheric temperatures especially during the winter months, and the fertility of soils, which is generally low to very low. Indeed, the soils of many mountainous regions of the world are acidic and severely nutrient limited. Of the most important macronutrients for plant growth, phosphorus (P) can be either "occluded" within or else strongly fixed at the surface of soil minerals like Fe and Al hydrous oxides (sesquioxides). Free Al and Fe cations react readily with inorganic forms of P, leading to relatively insoluble precipitates. This reversible process is known as "P fixation" (Johnson and Loeppert 2006). Additionally, intensive weathering of soils removes nutrient cations and leaves behind more stable materials rich in Fe and Al oxides. Besides, human intervention on soils through fertilization, irrigation, and long-term monocropping contributes to increased land degradation and loss of productivity (Tilman et al. 2002). In the following section, two distant mountainous regions of the world, yet sharing striking similarities and challenges when considering their agricultural productivity, are highlighted.

5.2 Indian Himalayan Region

The Indian Himalayan Region (IHR) occupies a special place in the mountain ecosystems of the world. This vast mountain region, which encompasses a total geographical area of about 530.795 km^2 (about 16.2 % of the country's total geographical area), is inhabited by >64 million people (around 6.2 % of the country's population) (SARDM Report 2006). Like elsewhere in other mountainous regions, most IHR people are marginalized and highly dependent on natural resources, agriculture, and livestock. Agriculture is the basis of the livelihood of over 80 % of the rural population in some areas of the IHR (FAO Report 2003). Food grain crops dominate the agriculture sector, despite a slight drop in area due to diversification toward horticultural crops, but even the Green Revolution has failed to climb the Himalayan heights (SARDM Report 2006). For many people in the IHR, which do not have the financial means to improve their yields, crop production is just a matter of subsistence. A series of climatologic, topologic, pedologic, and socioeconomic factors severely limit crop production in the IHR. For example, many agroecosystems are poorly irrigated, subjected to soil erosion, and very often inadequately managed. On the other hand, forest and cropped soils in the IHR are frequently acidic (Rinu et al. 2013) and characterized by poor availability of some essential mineral nutrients. Finally, cold temperatures, sometimes reaching subzero values, are not uncommon in these mountains. In some regions, snowfall is frequent during the winter season, and hence, the soil may remain frozen for several days, limiting growth of crops and increasing the risk of damage incurred by frost and hail.

5.3 Tropical Andes

The Tropical Andes spans 1,542,644 km^2, from western Venezuela to northern Chile and Argentina, and includes large portions of Colombia, Ecuador, Peru, and Bolivia. This region is considered as the richest and most diverse region on Earth. As in the IHR, the rural Andean population, particularly those inhabiting the highlands (>2,500 m), depends largely on agriculture and related activities for their livelihoods. Many agroecosystems, managed by smallholder farmers, are characterized by the exploitation of a mix of old and new world crops including potato, grains (e.g., maize, quinoa, barley, oats), legumes (e.g., fava beans, peas), and a wide array of vegetables (e.g., carrots, garlic, onions, cabbage). In some countries, it is common to find small plantations of native tubers like oca, mashwa, and ullucu (Fonte et al. 2012). Several of these cropping systems are pre-Columbian and, in some cases, date back several millennia (Sandor and Eash 1995; Stanish 2007). However, many farmers in the Andean region have limited access to a number of basic agricultural inputs such as fertilizers, pesticides, improved crop varieties, mechanized tillage, and irrigation. Despite the diversity of

agroecosystems in the Tropical Andean highlands, agriculture is also limited by a familiar set of environmental constraints throughout the region, with climate playing the most important role at high altitudes (Stadel 1991). On the other hand, with a few exceptions, soils in the high Andes are generally thin, fragile, and highly nutrient limited. For example, non-allophanic Andosols, which dominate the Ecuadorean highlands (Poulenard et al. 2001), can have severe problems of P deficiency, acidity, and aluminum toxicity (Dahlgren et al. 2004).

5.4 The Need to Develop Biofertilizers for Mountainous Regions

The land quality in the mountain regions of the developing world is deteriorating, leading to a declining in soil fertility and productivity. That is why many mountain families are facing food shortages which contribute to the chain reaction process of poverty-resource deterioration-scarcity-poverty (Jodha and Shrestha 1993). It is, therefore, imperative to explore new options for increasing the productivity and carrying capacity of farms, in order to improve the livelihoods of marginal mountain households (Partap 1999). This will also allow the mountain people to compete favorably in the modern world. One of these options is the cultivation of high value cash crops, such as fruits and vegetables, a tendency which is slowly increasing in the IHR (FAO Report 2003). Adoption of organic farming practices largely excluding the use of synthetic agro-chemicals and fertilizers and rescue of native species, varieties, and breeds which have played an important role in the human diet and traditional cultures and which are threatened by extinction are other promising alternatives. A complementary and very promising approach for improving the agricultural productivity of farmers without serious economic or environmental impacts is the development and correct use of biofertilizers, well suited to perform efficiently under the particular conditions of mountainous agroecosystems. A biofertilizer can be defined as a product containing live or latent cells of agriculturally beneficial strains of microorganisms that are applied to seeds or soils to build up the numbers of such microorganisms and accelerate certain microbial processes to augment nutrient acquisition by plants (Motsara and Roy 2008). Biofertilizers consist of nitrogen fixers (N-fixers) (*Rhizobium*, *Azotobacter*, *Azospirillum*, cyanobacteria/blue-green algae, *Azolla*), phosphate-solubilizing bacteria (PSB), and mycorrhizal fungi. In the contemporary world, the use of biofertilizers to enhance crop yield particularly of N-fixers and PSB is widespread and well documented (Banerjee et al. 2006). However, use of commercially available biofertilizers in colder climates like the ones prevailing in mountainous ecosystems has proven ineffective (Pandey et al. 1998). Indeed, low temperatures impose a serious threat to the metabolic activities of microorganisms. For instance, it has been estimated that lowering the temperature by 10 °C induces a two- to fourfold decrease in enzyme activity (Feller and Gerday 2003). Therefore, it has become

extremely important to find microorganisms with varying plant growth-promoting (PGP) abilities and potential to adapt well to low temperatures [=cold-tolerant (CT) or cold-loving], in order to develop proficient CT-biofertilizers for use in mountain agriculture.

5.5 Plant Growth-Promoting Rhizobacteria, Plant Growth-Promoting Bacteria, and Phosphate-Solubilizing Bacteria

Soil microorganisms do play a significant role in the biogeochemical cycling of elements, regulating the dynamics of organic and inorganic matter and increasing the availability of plant nutrients in the rhizosphere. The beneficial effects of some primitive "environmentally friendly" agricultural practices on plant growth and development were indeed observed and recorded centuries ago by the Ancient Greeks and Romans. At the time, it was proposed that mixing different soil samples or adding organic manures to the farmland might improve soil fertility and, consequently, crop yields (Tisdale and Nelson 1975). Subsequently, Hellriegel and Wilfarth (1888) and Beijerinck (1888) reported that some soil bacteria may convert atmospheric nitrogen (N) into plant usable forms of N. Thereafter, Kloepper and Schroth (1978) introduced the term "rhizobacteria" referring to the soil bacterial community competent in colonizing plant roots and able to stimulate plant growth. Later on, Kloepper and Schroth (1981) termed such beneficial rhizobacteria as plant growth-promoting rhizobacteria (PGPR). Considering the fact that not all plant-beneficial bacteria are inhabitants of the rhizosphere, sometimes the term PGPB (for plant growth-promoting bacteria) is used instead of PGPR (Andrews and Harris 2003).

Mechanistically, some PGPB enhance crop growth and development indirectly: for example, many of them inhibit plant pathogens (these are termed "bioprotectants," "biocontrollers," or "biopesticides"); others degrade toxic xenobiotics ("bioremediators") or trigger the induced systemic resistance (ISR) in plants. Additionally, some PGPB act directly and promote plant growth by releasing phytostimulators ("biostimulants") or by providing essential nutrients, such as N and P ("biofertilizers") (Glick 2012). Phosphate-solubilizing bacteria (or PSB) belong to the latter group. This heterogeneous group of PGPB is characterized by their ability to readily and efficiently solubilize mineral forms of inorganic P (P_i). The mechanisms employed by PSB to perform this Pi solubilization are as diverse as their phylogeny (see below). Another group of PGPB includes organic-P (P_o) mineralizing bacteria, which hydrolyze organic forms of P (phosphate esters, phosphonates, and anhydrides) through the action of specific enzymes (mainly phosphatases) (Turner et al. 2006; Richardson and Simpson 2011). This process, usually called "substrate mineralization," is of fundamental importance because it releases plant-available orthophosphate (PO_4^{2-}). Many PSB have been tested both

in greenhouse and in field trials and have been found to efficiently promote plant growth in P-deficient soils (Bashan et al. 2013). Concurrently, numerous commercial PSB-based biofertilizers have been developed and are being marketed on a global scale. However, these biofertilizers are not used as commonly as N-fixing bacteria (NFB) in crop production systems, and even in many cases their effectiveness in the soil-plant system has been reported quite uncertain, particularly under field conditions. Still, they remain a very attractive alternative to chemical phosphatic fertilizers, particularly in poor regions of the world.

5.5.1 Mechanisms of Inorganic-P Solubilization: A Brief Account

It is widely agreed that the most important mechanism evolved in bacteria to solubilize sparingly soluble forms of P_i in the soil is related to the abundant production and excretion of organic acids (Rodriguez and Fraga 1999; Goldstein 2007; Khan et al. 2010). This is mainly accomplished by bacterial transformation of sugars exuded through the roots into their respective sugar acids, whose amount and nature varies with the type of available sugars (Deubel et al. 2000). Even though several organic acids including citric, glutamic, succinic, lactic, oxalic, glyoxalic, maleic, fumaric, tartaric, and a-ketobutyric acids have been shown to efficiently mobilize P_i from soils (Khan et al. 2006), two of them are particularly effective: gluconic and 2-ketogluconic acids, probably due to their extremely low pKa (s) (~3.4 and ~2.6, respectively) (Goldstein 1995; Rodriguez and Fraga 1999). Gluconic and 2-ketogluconic acids are produced through direct oxidation (or non-phosphorylating oxidation) of glucose, an alternative aldose-utilization pathway which is expressed in a number of rhizobacteria (Goldstein 2007). Nevertheless, other mechanisms have also been proposed to explain PO_4^{2-} release from P-containing minerals, in the absence of a noticeable acidification of the extracellular milieu. The **sink theory** considers that continuous microbial uptake of P from water solution would disturb the chemical equilibrium between soluble and insoluble forms of P, favoring thus the solubilization process (Halvorson et al. 1990). The **acidification by H^+ excretion theory**, proposed by Illmer and Schinner in 1995, claims that ammonium ion assimilation by bacteria would release protons that will act, in turn, as agents for P solubilization. This would occur in the absence of any organic acid production. The **bacterial surface adsorption theory** is based on the fact that bacterial interaction with P-containing minerals, through exopolymeric substances, will cause both an increase in the extent of mineral dissolution and an inhibition of secondary mineral formation (Wightman and Fein 2004; Rong et al. 2008). The **chelating theory** proposes that the chelating property of the organic acid anions is as important as the proton effect. In such a context, any effective biological chelator—siderophores, for example—would enhance both mineral dissolution and P solubilization (Campbell and Eick 2002; Hamdali

et al. 2008). The **reductive dissolution theory** affirms that the reduction of hydrous ferric oxides under anaerobic conditions may release occluded P and ferrous iron (Fe^{2+}) to the soil solution (Stemmler and Berthelin 2003). Finally, the **inorganic acid production theory** is based on the fact that many acidophilic bacteria (extremophiles), able to oxidize reduced sulfur compounds (such as pyrite) to sulfuric acid (H_2SO_4), may participate in the solubilization of P in soils (Muchovej et al. 1989).

5.6 Cold-Tolerant Microorganisms

The ability of some microorganisms to tolerate (and even proliferate) at low temperatures was first reported by Forster in 1887. This ability is related to the strategies these organisms have evolved to face the challenges imposed by permanently cold environments. Many of these strategies, dependent on mechanisms which are far from universal (Casanueva et al. 2010), are quite well known; others still wait to be elucidated. Some of these are regulation of membrane fluidity, the synthesis of specialized molecules (e.g., cold-shock proteins, cryoprotectors, and antifreeze molecules), the regulation of ion channels permeability (osmoregulation), seasonal dormancy, and perhaps the most important adaptation to freezing temperatures, the modification of enzyme kinetics (Georlette et al. 2004; D'Amico et al. 2006). Although they are both able to grow in cold places, there is an important difference between cold-tolerant (=psychrotrophs or psychrotolerant) and cold-loving (=psychrophilic) microorganisms: psychrotrophs are able to resist suboptimal temperatures of growth (<20 °C) without being seriously compromised; on the contrary, psychrophiles are adapted to grow efficiently at low temperatures and strongly depend on this abiotic factor to successfully colonize cold habitats (Margesin and Miteva 2011). However, distinguishing between these two kinds of microorganisms is not always easy, and there has been a debate concerning such distinction. The classical definition of a psychrophile is related to its growth rate, which is said to be maximal at the so-called optimal temperature. Indeed, Morita (1975) defined psychrophiles as organisms having an optimal temperature for growth at about 15 °C or lower, a maximal temperature for growth at about 20 °C, and a minimal temperature for growth at 0 °C or below. However, some authors have contradicted this definition in the past few years mainly because when considering efficient colonization of cold environments, the growth rate may not be as relevant as growth yield (Bakermans and Nealson 2004). In a paramount work in the field, Margesin (2009) clearly demonstrated that slow growth rates of psychrophiles at the so-called suboptimal, lower temperatures are compensated by high growth yields and maximized cellular fitness. Nevertheless, it is important to consider here that psychrotolerant microorganisms (able to grow between 20 and 40 °C, but also at lower temperatures with much lower rates) are the organisms most frequently found in cold environments (Hoover and Pikuta 2010).

5.6.1 Cold-Tolerant Phosphate-Solubilizing Bacteria

During the past 15 years, the search for cold-tolerant PSB has followed two main routes. The first one was the isolation of native cold-tolerant phosphate-solubilizing bacteria (CT-PSB) from natural soils collected in mountainous regions (including alpine and sub-alpine environments, mainly in the IHR); the second path was the development of cold-tolerant mutants from well-known PS bacterial strains, irrespective of their ability of colonizing the roots of crops and/or to survive in natural rhizospheric environments under the prevailing climatic conditions. Paradoxically, one of the first attempts in this field followed the second approach. During the mid- to late 1990s, Goel and her team at the G.B. Pant University of Agriculture and Technology (India) decided to produce cold-tolerant mutants of *Pseudomonas fluorescens*, a well-known PGPR species able to effectively solubilize P_i, and then to determine their effect on plant growth promotion at low temperatures. The mutants were developed by nitrosoguanidine treatment of three different strains of *P. fluorescens*, namely, GRS1, PRS9, and ATCC13525 (Mishra and Goel 1999). In their first report, Das et al. (2003) showed that some of these *P. fluorescens* mutants were able to solubilize much more P than their respective native strains, at 10 °C. This primary work was complemented by another study which revealed that two of these mutants could enhance growth of wheat and mung bean at 10 °C under in vitro (gnotobiotic system) and in situ conditions (Katiyar and Goel 2003). In a follow-up experiment, Trivedi and Sa (2008) isolated a psychrotrophic strain of *P. corrugata* from IHR soils and generated mutants with high P-solubilizing abilities using nitrosoguanidine. Of the total 115 mutants initially identified, only 2 were chosen to further test their PGPR and P-solubilizing abilities. These mutants were indeed able to solubilize more P at 4, 9, and 28 °C than their native counterpart; this P-solubilizing activity was concomitant to a drastic acidification of the culture broth, related to organic acid production (both gluconic and 2-ketogluconic acids). Subsequently, following bacterization of seeds, the growth of wheat and mung bean increased significantly under in vitro and greenhouse conditions at 10 and 15 °C and in the presence of rock phosphate (as the sole source of P). Also, the bacterial inoculation had a positive effect on soil enzymatic activities, especially acid and alkaline phosphatases.

Even though the mutagenesis strategy has shown some promising results, the alternative approach isolating soil-borne CT-PSB is by far the most popular practice among researchers. This easy and inexpensive experimental approach is based on the premise that for isolating competitive and effective bacterial strains, it is the pool of indigenous soil bacteria that must be screened in the first place. It is therefore assumed that such microorganisms would be well adapted to the particular climatic conditions of the particular site (Paau 1989). The arguments beneath such an assumption are that (1) dominant, competitive indigenous strains are specific to a particular geographical region as a result of natural selection by various biotic and abiotic pressures (e.g., low temperatures, heavy rainfall and snowfall, food and non-food crop species commonly used in the area), (2) important

physiological plant-promotion characteristics (e.g., N-fixation, P-mobilization, antagonism, phytohormone production, and others) may vary with soil and weather parameters, and (3) indigenous strains, when isolated, added to the seed in high numbers, and planted in the same geographical region, under favorable moisture and temperature conditions, will establish themselves in the rhizosphere of crops (Höflich et al. 1994; Pandey et al. 2006a). Nevertheless, in apparent contradiction with these premises, Pandey et al. (1998) tested the effectiveness of well-known mesophilic PGPR inoculants—such as *Azotobacter chroococcum* and *Azospirillum brasilense*—on the promotion of maize growth in colder (=higher) locations of Sikkim Himalaya. A statistically significant positive effect on the *A. chroococcum* inoculated plants was observed when they were grown under subtropical conditions (1,200 m altitude). On the contrary, bacterial inoculations were found to be ineffective at the temperate site (1,900 m altitude). In a similar investigation, Egamberdiyeva and Höflich (2003) assessed the impact of mesophilic PS-PGPR on wheat plants grown at low temperatures (16 °C) and under greenhouse conditions (pot experiments). The bacteria used previously isolated from the phyllosphere, rhizosphere, or soil of the root zone of different crops were not described as psychrophilic or even psychrotolerant and were isolated instead from two distant regions of the world (i.e., Germany and Uzbekistan). Inoculation of seedlings with *P. fluorescens* PsIA12, *Pantoea agglomerans* 050309, and *Mycobacterium* sp. 44 (the three of them isolated from Müncheberg, a German region with semi-continental climate) was found to significantly increase the root and shoot growth of winter wheat at 16 °C compared to 26 °C in loamy sand. On the other hand, *Mycobacterium phlei* MbP18 and *Mycoplana bullata* MpB46, both isolated from the semiarid region of Tashkent in Uzbekistan, significantly increased root and shoot growth of wheat in nutrient-poor Calcisol at 38 °C and in nutrient-rich loamy sand at 16 °C. Furthermore, the inoculated wheat plants had significantly higher N, P, and K contents. In addition, some of these bacterial isolates could survive and establish better at 16 °C than at 26 °C, in the rhizosphere of winter wheat and in the bulk soil.

Apart from these pioneer experiments, later efforts were directed toward isolating indigenous CT-PSB from natural environments of cold mountainous regions and subsequently testing them as potential PGPB under different conditions. With the exception of one study conducted with bacterial isolates obtained from ginseng rhizospheric soil in South Korea (Park et al. 2010) and another one conducted with a bacterial strain naturally colonizing the rhizosphere of Antarctic hair grass (Berrıos et al. 2013), all the other studies conducted so far have involved bacterial strains isolated from either rhizospheric or bulk soil fractions collected at different alpine and sub-alpine locations in the IHR. Not surprisingly, major findings on this subject have come mainly from several Indian research groups. Besides, one of the main outcomes of this effort was the establishment of a culture collection of native "high altitude bacteria," allowing characterization of some selected isolates for plant growth promotion and biocontrol, with special reference to their adaptability to low temperatures (Pandey et al. 2004, 2006a) (Fig. 5.1).

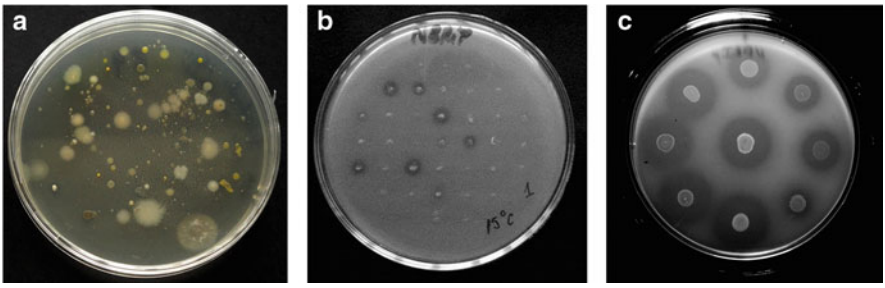

Fig. 5.1 Initial steps toward identification of candidate CT-PSB. (**a**) Primary isolation of bacteria at cold temperatures; (**b**) initial screening of PSB in the chemically defined medium supplemented with insoluble forms of P_i; (**c**) confirmation of P-solubilizing activity at low temperature by selected isolates

The most notable bacterial species isolated so far from natural soils of alpine and sub-alpine regions and tested both for their tolerance to low temperatures and their ability to efficiently dissolve inorganic phosphates (=CT-PSB) include *Pseudomonas fluorescens* (Egamberdiyeva and Höflich 2003), *P. putida* (Pandey et al. 2006b), *P. lurida* (Selvakumar et al. 2011), *P. corrugata* (Pandey and Palni 1998), *P. fragi* (Selvakumar et al. 2009a), *Pantoea agglomerans* (Egamberdiyeva and Höflich 2003), *P. dispersa* (Selvakumar et al. 2008), *Burkholderia vietnamiensis* (Park et al. 2010), *Rahnella* sp. (Vyas et al. 2010), *Serratia marcescens* (Selvakumar et al. 2007), *Tetrathiobacter* sp. (Kumar et al. 2013), *Mycoplana bullata* (Egamberdiyeva and Höflich 2003), *Achromobacter* sp. (Kumar et al. 2013), *Acinetobacter rhizosphaerae* (Gulati et al. 2009), *Bacillus subtilis* (Rinu and Pandey 2009; Malviya et al. 2012), *B. megaterium* (Trivedi and Pandey 2008a), *Mycobacterium phlei* (Egamberdiyeva and Höflich 2003), and *Exiguobacterium acetylicum* (Selvakumar et al. 2009b).

In general, the CT-PSB is a heterogeneous group, which includes species belonging to distant related genera of both Gram-negative and Gram-positive bacteria. Of these, Pseudomonads are by far the most relevant CT-PSB identified and tested so far, followed by *Bacillus* species. In addition to their P_i-solubilizing abilities, CT strains of *Pseudomonas* and *Bacillus* also exhibit other "desirable traits" such as N fixation and antagonism against phytopathogens (Pandey et al. 2006b; Mishra et al. 2008). In accordance with this, growth promotion abilities of CT *Pseudomonas* isolates have been demonstrated using a variety of crops such as mung bean (Katiyar and Goel 2003), wheat (Egamberdiyeva and Höflich 2003; Trivedi and Pandey 2007; Mishra et al. 2008, 2009a; Trivedi and Sa 2008; Selvakumar et al. 2009a, 2011), maize (Pandey et al. 2006b; Kumar et al. 2007), rice (Trivedi et al. 2007), and lentil (Mishra et al. 2011). Similar results were obtained when using CT *Bacillus* strains to promote growth of lentil (Rinu and Pandey 2009; Mishra et al. 2009b), rice, millet (Malviya et al. 2012), and Indian mustard (Kumar et al. 2013). In addition to their P-solubilizing activities, some CT-PSB strains have also shown some other interesting properties (Fig. 5.2). For

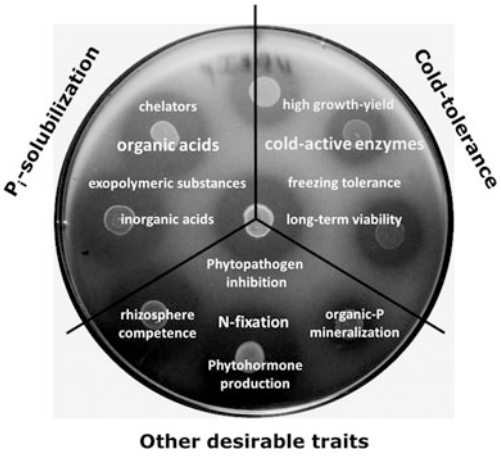

Fig. 5.2 Most prominent traits to look for when searching for proficient CT-PSB

example, *Burkholderia vietnamiensis* M6 has been found to tolerate several environmental stressing factors simultaneously, including high salt concentrations (up to 3 % KCl and 5 % NaCl) as well as low and high pHs (range 2–11 pH units), still solubilizing P_i rapidly (Park et al. 2010). On the other side, both *Rahnella* sp. BIHB 783 and *Acinetobacter rhizosphaerae* BIHB 723 exhibited similar broad-spectrum plant growth-promoting abilities (i.e., both could enhance the growth of four different crops) and high rhizospheric competence, without a significant effect on the resident microbial population (Vyas et al. 2010; Gulati et al. 2009, 2010). The latter attribute has been considered very important in the screening of new isolates for the development of plant growth-promoting formulations (Lottmann et al. 2000; Castro-Sowinski et al. 2007). It has to be mentioned, however, that plant growth promotion experiments with *Rahnella* sp. and *A. rhizosphaerae* were carried out at normal temperatures (25 °C).

Among the CT-PSB, *Exiguobacterium* strains are emerging as very promising candidates to develop efficient inoculants for mountainous regions. *Exiguobacterium* strains have been isolated from an impressive diversity of extreme environments, including the Siberian permafrost, a glacial ice core sample in Greenland, and hot springs in Yellowstone National Park (Vishnivetskaya et al. 2009). Selvakumar et al. (2009b) reported the isolation of a strain identified as *E. acetilycum* 1P (MTCC 8707) from the rhizosphere of apple trees (*Malus domestica*) growing at 2,200 m.a.s.l. in Uttarakhand state, in the N.W. IHR. Strain 1P was able to grow from 4 to 42 °C (optimal growth temperature 30 °C) and to tolerate a wide pH range (4–10 pH units) and also high salt concentrations (up to 8 % NaCl). Besides producing indoleacetic acid (IAA), hydrogen cyanide (HCN), and siderophores at 4 °C, the strain retained its P-solubilizing ability at this suboptimal temperature. More importantly, the PGP ability of *E. acetylicum* 1P was confirmed using wheat as test crop under non-sterile soil conditions at cold temperatures (18 °C) (Selvakumar et al. 2010). Indeed, a significant increase in root and shoot biomass was observed in bacterized seedlings. Additionally, increased

nutrient uptake was also observed in the bacterized plants, when compared to uninoculated controls. Very recently, phytase-producing CT-PSB, able to mineralize P_o as well as to solubilize P_i, were isolated from Himalayan soil samples collected at Uttarakhand region, northern India (Kumar et al. 2013). The isolates were identified as *Achromobacter* sp. PB-01 and *Tetrathiobacter* sp. PB-03, both members of the Burkholderiales, and *Bacillus* sp. PB-13. Despite growing at a wide range of pH (5–11 pH units), temperature (10–42 °C), and salt concentrations (from 0 to 8.5 % NaCl), these strains also exhibited diverse PGPR activities, such as production of IAA and siderophores. Their PGP abilities were confirmed using Indian mustard as test crop grown under greenhouse conditions at 20–25 °C. Bacterization of seeds with *Tetrathiobacter* sp. PB-03 and *Bacillus* sp. PB-13 significantly increased the biomass and P content of 30-day-old plants. Also, *Tetrathiobacter* sp. PB-03 and *Bacillus* sp. PB-13 inhibited the growth of the phytopathogen *Rhizoctonia solani*.

Recently, Berrıos et al. (2013) used an Antarctic bacterial strain to promote growth and development of Antarctic hair grass (*Deschampsia antarctica*). The *Pseudomonas* sp. Da-bac TI-8 strain, previously isolated from the rhizosphere of *D. antarctica*, naturally growing in the Antarctic Peninsula (Barrientos-Díaz et al. 2008), grows both at 4 °C and 20 °C (doubling times of 4.31 h and 1.31 h, respectively) but not at 30 °C. Even though it grew slower at 4 °C, the biomass yield at the end of the exponential phase in LB medium was almost the same as that recorded at 20 °C. Its P-solubilizing activity at 4 °C, attributed to gluconic acid production, was demonstrated in the presence of calcium phosphate dehydrate, calcium hydrogen phosphate, and phosphate rock. When *D. antarctica* seedlings were inoculated with strain Da-bac TI-8, a significant effect on the shoot dry weight/root dry weight ratio of plants was recorded at 22 °C—but not at 13 °C—as compared to uninoculated controls. Interestingly, *Pseudomonas* sp. Da-bac TI-8 was included in the formulation of a microbial bioinoculant developed to efficiently solubilize P at low temperatures and which was submitted to the US Patent and Trademark Office (Gidekel et al. 2010).

5.6.2 Cold-Tolerant Fungi (CTF)

Among the P-solubilizing microorganisms, filamentous fungi occupy a prominent position. Indeed, some fungal species belonging to *Aspergillus* and *Penicillium* genera have been shown to exhibit high P-solubilizing activities. As in the case of PSB, release of organic acids (e.g., citric, gluconic, lactic, oxalic, and succinic) is the main mechanism responsible for this solubilization (Khan et al. 2010). Besides, fungi produce larger amounts of organic acids than bacteria and consequently generally exhibit greater P-solubilizing activities. For example, under certain culture conditions, *A. niger* can convert glucose to citric acid with more than 80 % efficiency and at final concentrations of hundreds of grams per liter (Magnuson and Lasure 2004). Some other advantages in using fungi, instead of bacteria, for

biofertilization purposes have been claimed. For example, fungal hyphae are able to traverse longer distances in the rhizosphere and bulk soil and to firmly attach to P-containing particles (Kucey 1983; Chabot et al. 1993). Also, fungi do not lose the P-dissolving activity upon repeated subculturing under laboratory conditions. Moreover, some fungal species are able to mobilize P_i from sparingly soluble minerals, like iron oxides (Delvasto et al. 2007).

Under certain circumstances, the combined inoculation of more than one species may benefit plants better than either group of organisms alone. Dual-inoculation assays have clearly shown that field effects of PSB may be enhanced upon their mixed inoculation with P-solubilizing fungi (PSF) (Gull et al. 2004; Khan et al. 2006). Not surprisingly then, combined inoculation of PSF and PSB resulted in enhanced growth, nutrient uptake, and yield in several crops. For example, dual inoculation of wheat with an arbuscular mycorrhizal fungus (AMF), *Glomus etunicatum*, and PSB augmented all monitored plant growth and yield parameters, in experiments conducted in pots containing P-deficient soil (Saxena et al. 2014; Minaxi et al. 2013). These are among the main reasons explaining why mixed populations of soil bacteria and fungi are currently prepared and sold as commercial biofertilizers for improving P nutrition of plants (Richardson 2007).

In the context of mountain agriculture, however, only a few studies have been published concerning the potential use of CT-PSF as biofertilizers. The first report concerning the isolation, characterization, and identification of CT-PSF was published in 2008 (Pandey et al. 2008). From soil samples collected in the IHR, a total of 246 fungal isolates, representing 36 genera and 72 species, were isolated. After a thoroughly screening procedure, eight species of PS *Penicillium* were finally selected for further investigation. These isolates solubilized P in vitro after 15–21 days at 21 °C, and this ability was correlated with acidification of the culture medium. They also produced acid and alkaline phosphatases. Additionally, some isolates showed a wide range of tolerance for temperature, pH, and salt concentration. Very similar results were also obtained by this research group when studying ten *Aspergillus* species, isolated from the same IHR soil samples (Rinu and Pandey 2010). Among the species tested, *A. niger* exhibited the highest P-solubilizing activity at low temperatures (9 and 14 °C), after 5–6 weeks of incubation in vitro. This preliminary study demonstrated the potential of CT *Aspergillus* species to be developed as "bioinoculants" for application in cold mountainous regions. The ability of *A. niger* to solubilize and release P_i was further confirmed by Singh et al. (2011). This time, two strains of *A. niger* were isolated not from mountainous ecosystems but from Spitsbergen, the largest island of the Svalbard Archipelago in the Arctic region. Both isolates showed maximum PS activities at pH 7.2 and 20 °C in bioassays conducted in vitro, but they were not studied further. To confirm their potential as good candidates for developing biofertilizers to be used in acidic soils, containing sparingly soluble Al and Fe phosphates, three CT *Aspergillus* species, namely, *A. niger*, *A. glaucus,* and *A. sydowii*, were tested in vitro in the presence of different carbon sources (Rinu et al. 2013). Even though all the three species mobilized P from the P-containing minerals tested, *A. niger* gave the best results: it solubilized 32 % and 8 % of the supplemented Al and Fe phosphate, respectively.

This activity was significantly influenced by C and N sources. In pot-based assays, conducted under greenhouse conditions at 25 °C, all the three species enhanced maize and wheat production. Similarly *Paecilomyces hepiali*, a psychrotolerant fungal species isolated from rock soil of a cold desert site in Indian Himalaya, was also shown to slowly and steadily solubilize P at low (suboptimal) temperatures (i.e., 14 °C) (Rinu and Pandey 2011). This ability was attributed to the production of organic acids and the consequent acidification of the culture medium.

5.7 Cold-Tolerant Bioinoculants

Application of PSB in agronomic practices started about 60 years ago, when a large proportion of the former Soviet Union's agricultural soils were inoculated with a biofertilizer consisting of kaolin-impregnated *Bacillus megaterium* var. *phosphaticum* spores to increase their fertility (Mishustin and Naumova 1962). The results were so spectacular that, in some cases, up to 70 % increases in crop yields were recorded and this was mainly related to P mobilization. The success of Phosphobacterin, as this bioinoculant was called, reflected not only the importance in selecting an appropriate PGPB strain but also the paramount role of a good carrier (kaolin) for the biofertilizer preparation. Indeed, from both a commercial and agricultural point of view, the success of a biofertilizer strongly depends on the development of appropriate formulations, easy to be handled and stored for long periods of time. As highlighted by Bashan (1998), a good carrier for PGPB should have the capacity to deliver the right number of viable cells, in appropriate physiological condition and at the right time. It should also protect bacterial cells from the various biotic and abiotic stresses they will face once applied to the soil. Furthermore, bacteria carried this way must retain their PGP abilities after long periods of storage. Lastly, when considering developing countries, an ideal carrier should be of low cost and locally available. Alas, even though many formulations have been tested, no universal formulation for CT-PSB inoculants is presently available.

Again, efforts have been extensive in this field and mostly made by Indian researchers, aimed at using locally available, low-cost organic raw materials as carriers. Charcoal, for instance, has been employed in many formulations because it is inexpensive and easily available in plenty everywhere. However, the low quality of charcoal may sometimes affect badly its water holding capacity, bulk density, and porosity (NIIR Board 2012). Only the use of good quality charcoal is therefore recommended for bioinoculant formulations. A series of greenhouse and field experiments have been conducted in the last 10 years using charcoal-based CT-PSB inoculants (more appropriately CT-PGPB) (Kumar et al. 2007; Trivedi et al. 2007; Mishra et al. 2009b; Rinu and Pandey 2009; Vyas et al. 2010). In general, the bacterial cultures were grown first in appropriate culture media, mixed with sterilized activated charcoal (usually in combination with a gluing and stabilizing agent like raw sugar or carboxy-methyl cellulose) and, then, applied to seeds

in order to coat them with the biofertilizer. The results obtained confirmed the suitability of these formulations to increase crop growth, nutrient content of various plant components, and other yield attributing parameters. Other carriers have also been tested. For example, Negi et al. (2005) prepared PSB inoculants by mixing bacterial suspensions of four strains of CT *Pseudomonas fluorescens* with a mixture of talc powder and carboxy-methyl cellulose. Talc is considered an excellent coating agent, preventing caking (often occurring during both storage and transportation of biofertilizers), improving fertilizer flow, and reducing water pickup and dust. The formulation developed by Negi et al. was air-dried, packed in autoclaved polybags, and stored at −20 °C. When pea seeds were treated by this formulation, its effectiveness in promoting plant growth and inhibiting phytopathogens was confirmed. Alternatively, formulations based on natural, hydrophilic polymers like sodium alginate have also been proposed and tested for their potential as CT bacterial carriers (Trivedi et al. 2005; Trivedi and Pandey 2007, 2008a, b). Sodium alginate, for instance, a highly porous polymer when appropriately prepared, was used by Bashan in 1986 to develop a new inoculant carrier, capable of slowly releasing the entrapped PGPB in the rhizosphere of crops. Since then, it has been widely used as a suitable carrier for biofertilizer development, even though it is more expensive than charcoal. In 2005, Trivedi et al. tested five carrier-based preparations of plant growth-promoting bacterial inoculants suitable for use in cool regions, namely, (1) alginate beads, (2) alginate beads supplemented with skim milk, (3) alginate-coated seeds, (4) charcoal-based, and (5) broth-based preparations. Two well-known PGPR were included in the formulations: *Bacillus subtilis* (NRRLB-30408) and *Pseudomonas corrugata* (NRRL B-30409) using maize (var. QPM-1) as test crop in plant growth promotion assays conducted at 22 °C. Even though all the formulations tested increased the measured parameters of maize relative to untreated control, alginate-based formulations were the most effective, followed by charcoal- and broth-based formulations, respectively. Long-term rhizosphere colonization was also shown to be more efficient when applying alginate-based formulations as compared to charcoal- and broth-based formulations. Similar growth promotion effects were obtained for wheat when an alginate-based formulation of *P. putida* (MTCC6842) was applied to the soil at the time of sowing in a pot assay conducted at temperatures ranging between 10 and 15 °C (Trivedi and Pandey 2007). The success of alginate-based formulations was further confirmed using *B. megaterium* B388—as PGPB—and maize or wheat as test species (Trivedi and Pandey 2008a). Again, a maximum increase in the growth parameters of both plant species was observed in the case of alginate-based formulations followed by coal- and broth-based formulations, respectively. On the other hand, viability of bacterial inoculants after 180 days of storage at 4 °C was confirmed in formulations containing alginate beads and alginate beads supplemented with skim milk (Trivedi et al. 2005). Trivedi and Pandey (2008b) further reported the survival, viability, and plant growth-promoting ability of *B. subtilis* (NRRLB-30408) and P. *corrugata* (NRRL B-30409) immobilized in sodium alginate beads after 3 years of storage at 4 °C. When using coal or broth for the same purpose, the decrease in bacterial viability for the same period was much

higher, reaching three to four orders of magnitude. Plant-based bioassays-conducted at 22 °C and using wheat as test crop under greenhouse conditions-indicated that the bacterial isolates did not lose their plant growth promotion abilities. Furthermore, the bacterial isolates retained the root colonization, antifungal, and enzyme activities in the alginate-based formulation during storage. The authors noticed, however, some liquefaction at the bottom of the flasks containing *P. corrugata* after 3 years of storage, possibly due to organic acid production. No such loss of integrity was observed in the case of beads entrapping *B. subtilis*. From all these studies, it became apparent that sodium alginate might be the best choice for the large scale production of high-quality CT bacterial inoculants, for commercialization and field application of this microbe-based technology.

5.8 What Lies Ahead?

Even though much work has been done, many aspects concerning the development of CT-PS bioinoculants require further scientific attention. For instance, almost nothing is known about the specificities of bacterial-mediated phosphate dissolution at low temperatures (e.g., enzymes involved, enzyme kinetics, genetics, and regulation). It is widely accepted that the mechanisms adopted by CT bacteria for mobilizing P from sparingly insoluble P-containing minerals are similar to those adopted by mesophilic PS organisms. This assumption, which might prove incorrect, has been biased in many cases in the search for efficient CT P solubilizers toward microorganisms only capable of solubilizing tri-calcium phosphate in agarized media (e.g., Pikovskaya's Medium). Alas, as recently shown by Bashan et al. (2013), this form of reasoning can be misleading and certainly yields many false PS-microorganisms. Therefore, a different experimental approach-including the use of a combination of two or three metal-P compounds together or in tandem according to the end use of these bacteria-has been invoked. In the case of acidic soils, Fe-P and Al-P compounds should be included in the preliminary tests; for alkaline soils, Ca-P compounds (including rock phosphates) would be the right choice. Once a potential candidate is identified following these guidelines, appropriate tests must be performed to confirm direct contribution to P plant nutrition and not to general growth promotion, as commonly done. Indeed, as we have previously seen, in many cases growth promotion of plants by PS microorganisms can be the consequence of other-direct or indirect-mechanisms (e.g., phytohormone production). According to Bashan and his coauthors, isolates that do not comply with this general sequence of testing should not be declared as PSB. Also, it is important to mention here that there are still numerous unexplored natural environments which can be targeted for isolating potential CT P solubilizers. Perhaps one of the most evident choices in this regard is glacier ice. Indeed, as shown by a great number of scientific reports, glaciers are repositories of an almost unknown diversity of microorganisms (Miteva 2008). Many of these are true psychrophiles and possess unexpected metabolic repertoires which, in some cases, have been exploited to

develop a series of biotechnological products (Cavicchioli et al. 2011). Therefore, it would not be a surprise to obtain encouraging results when looking at these environments.

5.9 Conclusion

There is an urgent need to develop cold-tolerant biofertilizers to improve crop production in developing mountainous regions of the world. The obvious impact of such a biotechnology in terms of increasing agricultural productivity of small farmholders, without severely affecting either their economies or the environment, is beyond any doubt. Serious efforts have been made by the scientific community to address this issue, with many bacterial and fungal species already identified and tested, both in greenhouse and in the field. The results obtained so far indicate that cold-tolerant P-solubilizing microorganisms may represent a real alternative to improve agriculture productivity in mountainous regions of the developing world. But still much work is needed to finally achieve the desired bioinoculant formulations which could perform efficiently under diverse conditions, at low cost and with the smallest possible environmental impact.

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Chapter 6
Role of Phosphate-Solubilizing Actinomycetes in Plant Growth Promotion: Current Perspective

Saima Saif, Mohammad Saghir Khan, Almas Zaidi, and Ees Ahmad

Abstract Phosphorus (P), an essential plant nutrient, is a nonrenewable resource whose availability depends exclusively on mined rock phosphates. Deficiency of P in soil results in reduction in food production since all plants require an adequate supply of P for its growth and development. Even though synthetic phosphatic fertilizer has played some major roles in enhancing crop production, its excessive use has also dark sides to it where it has been found to damage the environment, destruct soil fertility, and, via food chain, seriously affect the human health. Considering the nuisance of overuse of P, there is an urgent demand by the agriculture practitioners to find nonhazardous strategy that can overcome/reduce the use of agrochemicals in agricultural practices and, hence, may preserve the very integrity of soil ecosystems. In this context, actinobacteria, a group of Gram-positive bacteria, ubiquitous in soils, are likely to play some important roles in supplying soluble P to plants by solubilizing/mineralizing complex P resources of soils. Additionally, the extracellular metabolites produced by actinomycetes may inhibit phytopathogens and, sometimes such metabolic compounds may also act as plant growth regulators. These qualities, among others, make actinobacteria an ideal candidate for developing as microbial inoculants for ultimate use in agriculture production system. The potential roles of actinomycetes as phosphate solubilizers in enhancing crop production are discussed.

Keywords Actinomycetes • P solubilizers • Plant growth • Biocontrol

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6.1 Introduction

Phosphorus, identified as an essential element for all life forms (Smil 2000), is the second most important plant macronutrient after N (Donahue et al. 1990). Phosphorus accounts for the biomass buildup, the energy transfer, signal transduction, macromolecular biosynthesis, photosynthesis, and respiration chain reactions (Vance et al. 2003; Fernández et al. 2007). Sadly, this highly vital element is one of the least accessible (only 0.1 % of the total soil P reserve) nutrients to the plants (Zou et al. 1992; Takahashi and Anwar 2007). Owing to the suboptimal levels of P, it is often considered a limiting factor to primary production across a diverse range of ecosystem (Elser et al. 2007) and may cause a 5–15 % loss in plant yields (Hinsinger 2001). The low availability of P in soil is primarily due to its highly reactive nature as it readily forms insoluble complexes with the soil ions. Thus, to fulfill the growing P demands for food production and to maintain a balanced fertility in soils and, hence, to achieve a low-input, sustainable eco-friendly agriculture targets, a better management of the soil P reserve is urgently needed. Many strategies have been introduced by the scientists across different regions where the role of phosphate-solubilizing microorganisms involving bacteria (Zaidi et al. 2009; Ahmad et al. 2013), fungi (Khan et al. 2010), and actinomycetes (Gangwar et al. 2012) in increasing plant yields and soil quality via P supply has been recognized. Among these, actinomycetes as P solubilizers (de Vasconcellos et al. 2010; Hamdali et al. 2012) have been less explored despite showing a better genetic and greater biodiversity potential (Pathom-Aree et al. 2006; Thangapandian et al. 2007). Also, they represent heterogeneous and abundant microbial populations and hugely affect cycling of nutrient in soil ecosystems (Elliot and Lynch 1995; Figueiredo et al. 2010). Recently, the role of actinomycetes in sustainable agriculture (Johansson et al. 2004; Strap 2011) via P supply to plants has been identified. Some other actinomycetes, for example, endophytic actinomycete (Araújo et al. 2000; Kunoh 2002; Lee et al. 2008; Qin et al. 2008), which fix atmospheric N into NH_3 and export the fixed N to the host plants, have also shown PS activity (Gangwar et al. 2012). In a study, almost 44 % of the endophytic actinomycetes isolated from rice had PS activity, among which *S. lavendulae* R22 solubilized the maximum (26.5 mg/100 ml) amount of P, while *Micromonospora* R19 isolate could solubilize the minimum amount of P. Realizing the plant-growth-promoting potentials of actinomycetes which involve one or simultaneous mechanisms, here, the recent advances in P solubilization by actinomycetes and its impact on crop production are highlighted.

6.2 Phosphorus Status and P Dynamics in Rhizosphere: Current Perspective

Globally, rock phosphate (RP) is an abundantly available and inexpensive form of P, and therefore, about 80 % of mined RP is used for agricultural fertilizer (Tirado and Allsopp 2012). Worldwide, there is a huge RP deposit in countries such as Morocco and China, while other regions particularly Asian countries depend heavily on import of phosphates for intensive agriculture production systems. From a consumption point of view, China is the largest consumer of P fertilizers accounting for about 34 % of the total world consumption, whereas India ranks second with 19 % of global consumption (FAOSTAT 2012). Similarly, the uptake of P by plants varies greatly (Fig. 6.1). Regrettably, mining of RP and its use as fertilizers pose a serious human health risk (Zhang and Shan 2008; Pan et al. 2010; Tirado and Allsopp 2012). Considering the declining P resources, cost of P-fertilizer production, and direct or indirect human health problems due to their excessive application, scientists around the world have directed their attention toward exploring the natural and inexpensive P resources which could serve as an eco-friendly and economical alternative for chemical fertilizers in sustainable agriculture. In this context, the phosphate-solubilizing actinomycetes have provided some solutions to the expensive P problems.

Further, the chemical and biological processes in the rhizosphere play an important role in soil nutrient availability and crop productivity (Zhang et al. 2010) as presented in Fig. 6.2. Plants acquire P in the form of orthophosphate anions (mainly $H_2PO_4^-$ and $H_2PO_4^{-2}$) which they obtain from different soil P reserves including inorganic P (Pi) and organic P (Po). Broadly, on the basis of plant accessibility, soil P has been grouped as follows: (a) solution P, present in the soil solution and immediately available for plant uptake; (b) active P, adsorbed on active sites of soil yet readily available and in equilibrium with solution P; and (c) fixed P, strongly adsorbed and least soluble with slow conversions to active P over a vast period, even years (Syers et al. 2008; Shen et al. 2011). Generally, concentration of plant-available P in soil is below the critical level required for plant growth and yields (Raghothama 1999). Thus, the synthetic phosphatic fertilizer or organic manure is applied to overcome the P deficiency to plants. Of the total P applied to soils, only 15–25 % of it is available for uptake by plants and the remainder is fixed (Shen et al. 2011). The P dynamics and availability in soil is, therefore, controlled by several factors: (1) the ability of plants to form extensive root systems, (2) impact of microbial colonization onto the development of plants, (3) soil microflora affecting biogeochemical cycling of elements, and (4) physicochemical properties of soils supporting plants and microbial life in soils.

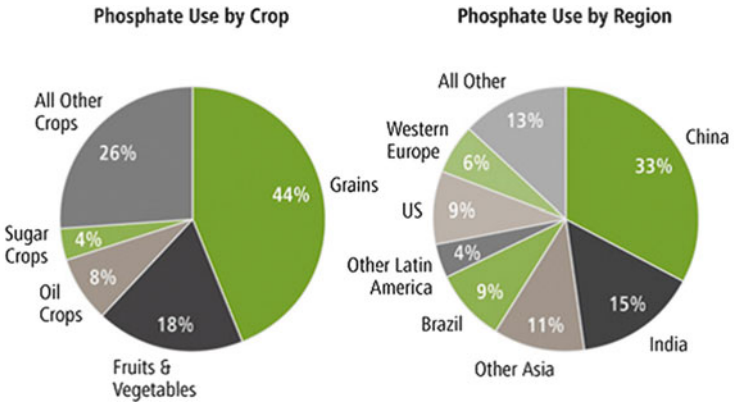

Fig. 6.1 Crop-wise use of phosphorus and its distribution in different countries (Source: CRU, Fertecon, IFA (Potash Corp. 2013)

6.3 Actinomycetes: An Ideal Candidate for Sustainable Crop Production

Actinomycetes are a ubiquitous, saprophytic, and heterogeneous group of microorganisms belonging to an extensive and diverse group of Gram-positive, aerobic, mycelial bacteria that have DNA with high %G + C content (51–73 %) with the exception of freshwater actinobacteria which has low GC content (Takisawa et al. 1993; Ghai et al. 2012). Actinomycetes inhabit both the rhizoplane and rhizosphere (Solans and Vobis 2003; Frioni 2006) and are known to play some important ecological roles in soil nutrient cycling (Elliot and Lynch 1995), probiotics (Lievin et al. 2000), bioremediation (Amoroso et al. 2013), nitrogen fixation, and deterioration and decomposition of plant and animal residues and also provide many bioactive compounds such as vitamins, enzymes, and antibiotics (de Boer et al. 2005; Busti et al. 2006; Prapagdee et al. 2008; Genilloud et al. 2011). Apart from their beneficial impact, actinomycetes also cause some harmful effects such as they cause diseases and spoil different useful materials (Waksman 1950). Other properties which make them an ideal candidate for developing inoculants are as follows: (1) their high genetic and metabolic versatility, (2) they can easily be genetically engineered (Pogell et al. 1991), (3) high growth rate and relatively faster colonization efficiency (Hsu and Lockwood 1975), and (4) ability to tolerate high salt concentration (Vassilev et al. 2012). Despite possessing such qualities, actinomycetes in general have been less explored organisms in agricultural technology, among variously distributed soil microflora (Qin et al. 2011).

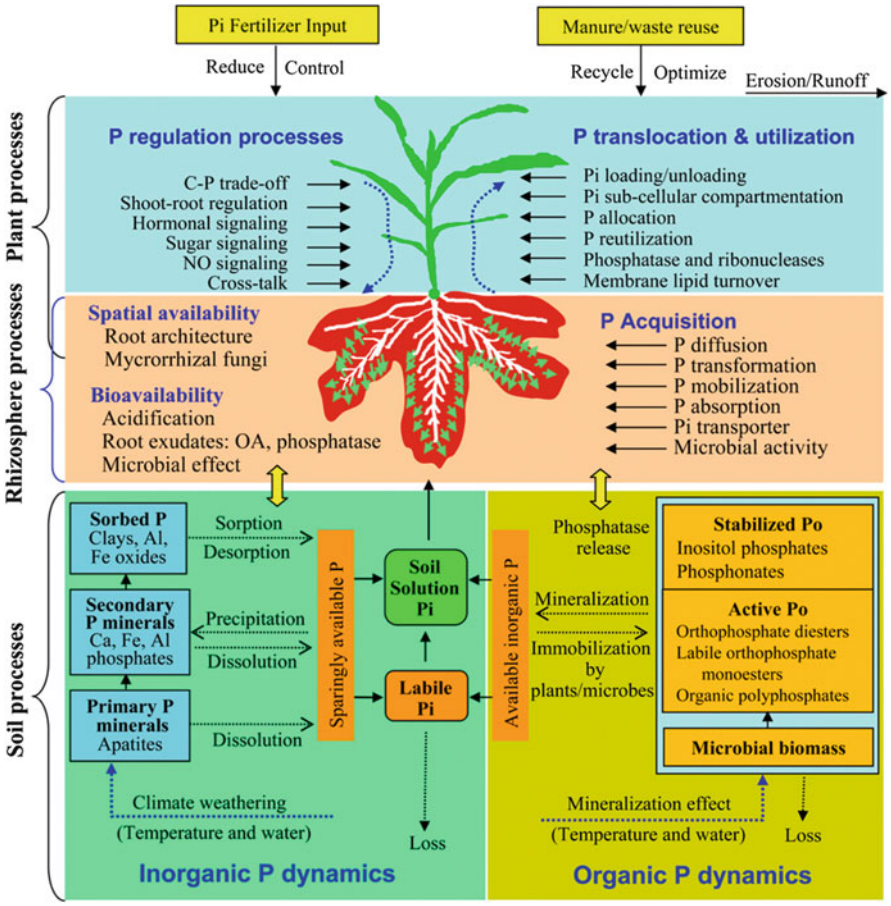

Fig. 6.2 Phosphorus dynamics in soil [Adapted from Shen et al. (2011)]

6.3.1 Isolation and Identification of Actinomycetes

Phosphate-solubilizing actinomycetes have been isolated from diverse environment (Matsumoto et al. 1998; Rai et al. 2007; Lee et al. 2008) such as soil (Xu et al. 1996; Debananda et al. 2009; Salcedo et al. 2014), rhizospheres (Norovsuren et al. 2007), phyllosphere (Gupta et al. 2010), and salt and alkaline environments (Jiang et al. 2005) (Table 6.1). The bioprospecting of actinomycetes from the usual or routine habitat is likely to result in rediscovery or the recovery of the same strain with the similar mode of actions (Jensen et al. 2005).

Actinomycetes represent one of the largest domains of bacteria involving 145 genera and more than 4,000 species (Berdys 2005). Their population has been reported highest at the soil surface which, however, decreases with increasing soil depth (Takahashi and Omura 2003). Prior to isolation, soil is pretreated in order

Table 6.1 Different habitat and varying isolation methods for actinomycetes

Habitat	Actinomycete genus isolated
Cultivated field	*Streptomyces*
Cultivated paddy field	*Micromonospora*
Plant matter	*Microbispora*
Pasture	*Micromonospora*
Lake sediment	*Micromonospora*
Mangrove rhizosphere soil	*Actinobacteria*
Tea field soil	Acidophilic and acid tolerant actinomycetes
Desert soil, marine sediment, seawater, and activated sludge	
Pretreatment	
Yeast extract (6 %, 20 min)	Enrichment of actinomycetes
Heating (100 °C, 1 h)	*Streptosporangium, Microbispora*, *Microtetraspora*
Phenol (1.0–1.5 %, 30 min)	*Dactylosporangium*, Microbispora, *Microtetraspora*
Pre-culture with $CaCO_3$	Enrichment of actinomycetes
Chemotactic method (KCl, γ-collidin, xylose)	Motile actinomycetes, actinomycetes, *Dactylosporangium*
Medium for isolation	
Addition of antibiotics	
Novobiocin	*Actinoplanes*, *Kitasatospora*
Tunicamycin	*Micromonospora*
Rifampicin	*Actinomadura*
Chlortetracycline	*Nocardia*
Macrolide or aminoglycoside	Macrolide or aminoglycoside producer
Addition of humic acid	Rare actinomycetes
Addition of proline	Enrichment of actinomycetes
Gellan gum (substitute for agar)	*Actinobispora*
Conditions for isolation	
High temperature	Thermophilic actinomycetes

Adapted from Takahashi and Omura (2003)

to avoid the emergence of bacterial and fungal contaminants. For this, soil samples are collected bulked, mixed, and allowed to dry heating (at 45 °C for 2 h or 50 °C for 10 min or 60 °C for 30 min) (Goodfellow 1971). The isolation medium is also suspended with antibacterial (penicillin 25 mg/ml) or antifungal (nystatin 0.1 % or cycloheximide 50 mg/ml) agents (Balagurunathan and Radhakrishnan 2007). A 0.1 ml of serially diluted (Nonomura and Ohara 1969) soil sample is spread plated onto different actinomycete isolation media, such as casein starch agar, the Czapek agar, and the oatmeal agar, and incubated at 28 °C for 15–30 days. The resulting colonies are then picked and identified using cultural, morphological (Pridham and Tresner 1974; Nonomura 1989; Sabaou et al. 1998), and physiological (Nonomura and Ohara 1969; Goodfellow 1971) characteristics. The actinomycetes are then identified to species level using fatty acid analysis, mol (%) G + C contents, DNA–DNA hybridization, and 16S rRNA sequencing.

6.3.2 *In Vitro Screening of Phosphate-Solubilizing Actinomycetes*

The phosphate-solubilizing activity of the actinomycetal strains is assessed generally on the Pikovskaya (PVK) medium (Pikovskaya 1948) which contains (g/l): glucose 10; $Ca_3(PO_4)_2$, 5; $(NH_4)_2SO_4$, 0.5; NaCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.1; KCl, 0.2; yeast extract, 0.5; $MnSO_4 \cdot H_2O$, 0.002; and $FeSO_4 \cdot 7H_2O$, 0.002, agar 15. The streaked/spot inoculated plates are incubated at 22 °C for 14 days and observed for halo formation. The development of a clear zone around the colony on the culture plates is taken as an index of P solubilization. Halo size is calculated by subtracting colony diameter from the total diameter (Valverde et al. 2006). Thereafter, the halo-forming actinobacteria is grown on SRSM-1 medium which has the same composition as that of the PVK medium but is supplemented with bromocresol purple as a pH indicator for determining the involvement of organic acid production in PS activity.

6.3.3 *Role of Actinobacteria in Rock Phosphate Solubilization*

Nearly 20 % of the actinomycetes including *Streptomyces* and *Micromonospora* possess profound phosphate-solubilizing potential (Barreto et al. 2008; El-Tarabily et al. 2008; Hamdali et al. 2008b). Actinomycetes as P solubilizer have received greater attention for two reasons: (1) ability to produce agro-active metabolites, for example, phytohormones, siderophores, and antibiotics, etc. (Hoster et al. 2005; Errakhi et al. 2007; Errakhi et al. 2009), and (2) ability to tolerate different stressor molecules (Fabre et al. 1988; Keiser et al. 2000; Hamdali et al. 2008d). Also, the inoculation with actinomycetal strains has been found to increase the plant growth (Hamdali et al. 2008c). Plants take up the orthophosphorus form of P as nutrient, the availability of which depends on soil characteristics (Nath and Borah 1983). However, Fe and Al at high pH and Ca at low pH fix the soluble form into insoluble form rendering it unavailable to the plants (Rengel and Marschner 2005; Johnson and Loepper 2006). The PS actinomycetes, however, through the release of organic acid (acidification) (Hoberg et al. 2005; Abdulla 2009) and by other mechanisms such as chelation, exchange reactions, and polymeric substances formation (Delvasto et al. 2006) convert the insoluble forms of P into soluble forms. The organic anions assist P solubilization mainly in two ways: (a) lowering pH and (b) ligand exchange reaction (Beunemann et al. 2011; Balemi and Negisho 2012). However, the organic anions in some cases have been found not to acidify the medium, probably because once they are released in soil, they already exist in the dissociated form due to their low acid dissociation constants (pKa). In fact, it is assisted by the proton extrusion accompanying respiration and NH^{4+} assimilation (Illmer and Schinner 1992), which compensates for the losses of negative charge

and hence lowers the pH values. Organic anions are also involved in ligand exchange or ligand-enhanced desorption and/or chelation of Fe and Al oxides and Ca phosphates, thereby decreasing the positive surface potential on the metal oxide (Filius et al. 1991) and releasing the inaccessible P from the soil matrix (Raghothama and Karthikeyan 2005). The extent of solubilization by actinomycetes however depends on (1) the source of inorganic P (Pi), (2) the intrinsic PS ability of actinomycetal species, and (3) the types and composition of photosynthates available in the rhizospheres (Banik and Dey 1982). Interestingly, both solubilization (acidification of inorganic P) and mineralization (enzymatic dissolution of organic P) may coexist in the same PS actinomycetal strains (Tao et al. 2008).

Recently, numerous rhizoactinomycetes capable of solubilizing insoluble P have been identified (Franco-Correa et al. 2010; Pragya et al. 2012). For example, apart from soil actinomycetes, many marine actinomycetes have also shown the production of phosphatase which consequently enhanced the P availability (Sahu et al. 2007). In a similar study, Franco-Correa et al. (2010) reported that 20 % of the actinomycetes including *Streptomyces* and *Thermobifida*, isolated from the rhizosphere of *Trifolium repens*, grown in the fields of Sabana de Bogotá, Colombia, had quite active P-solubilizing activity. Even though all isolates produced acid phosphatase wherein 43 % isolates demonstrated alkaline phosphatase, which indicated that all the test actinomycete strains could mineralize the organic P sources (Richardson et al. 2009). Among phosphatases, acid phosphatases, a group of enzymes produced by plants/microbes in response to P stress, catalyze hydrolysis of phosphoric esters in a range of organic P complexes, thereby enhancing plant P uptake (Tarafdar and Claassen 1988; Tarafdar and Claassen 2001; Richardson et al. 2011) from unavailable P resources. There are also some other enzymes secreted by actinomycetes that take part in the dissolution process. As an example, Reza-Ghorbani-Nasrabadi et al. (2012) assessed 97 soil samples collected from different soil ecosystems and showed for the first time that actinomycetes had extracellular phytate-degrading activity. Phytases are a group of enzyme responsible for stepwise dephosphorylation of phytate, the most abundant inositol phosphate in nature. Of the total actinomycetal cultures, 46.3 % showed extracellular phytate-degrading activity in liquid culture medium supplemented with 4 g/l Na-phytate as sole P source. The two more closely studied phytase producers were identified according to 16SrRNA sequencing as *Streptomyces* (sp. isolate No. 43 showed 98 % similarity to *Streptomyces alboniger* and *S. venezuelae*, while isolate No. 63 exhibited 98 % sequence relatedness with *S. ambofaciens* and *S. lienomycini*).

6.3.4 *Actinomycetes as a Potential Candidate for Increased Plant Growth and Yield*

The agronomic use of PGPR at field scale in general has been hampered by poor understanding of mechanisms that facilitate plant growth, inability of bacterial strains to establish in various soils, rhizosphere incompetence, and changing environmental conditions. On the contrary, the actinobacteria with their ability to (1) sporulate, (2) transform various complex soil nutrients into simple and accessible forms, (3) extensively and efficiently colonize plant roots, (4) manage phytopathogens, and (5) secrete other plant-growth-promoting substances make these organisms as preferred choices for developing potential field bio-inoculants. Actinomycetes are metabolically flexible soil/rhizosphere-colonizing microorganisms (Miller et al. 1990; Euanorasetr et al. 2010; Lei et al. 2013) capable of producing a range of compounds of interest, including (1) antifungal compounds which have been found useful in controlling fungal root diseases (Rothrock and Gottlieb 1984; Zucchi et al. 2010; Bungonsiri et al. 2011; Sreevidya and Gopalakrishnan 2012; Francisco et al. 2013) (2) siderophores (Lee et al. 2012; Nakouti et al. 2012; Najwa et al. 2013), ACC deaminase (El-Tarabily 2008), and (3) plant-growth-promoting hormones (Hamdali et al. 2008a; Khamna et al. 2010). Generally, plant root exudates stimulate growth, proliferation, and rhizosphere colonization of actinomycetes that may act as a strong antagonist to fungal pathogens. The root exudates are utilized as a source of carbon and energy by the actinomycetes and, concomitantly, enhance the synthesis of antimicrobial substances (Crawford et al. 1993; Yuan and Crawford 1995). In addition, actinomycetes synthesize an array of biodegradative enzymes which includes chitinases (Blaak et al. 1993; Gupta et al. 1995; Pattanapipitpaisal and Kamlandharn 2012; Sowmya et al. 2012), glucanases (Hopwood 1990; Damude et al. 1993; Mahadevan and Crawford 1996; Harchand and Singh 1997; Thomas and Crawford 1998; Trejo-Estrada et al. 1998; Fayad et al. 2001; Huiling et al. 2014), peroxidases (Ramachandra et al. 1988; Djamila et al. 2011), and other enzymes possibly involved in mycoparasitic activity. Considering the potential role of actinomycetes in the management of plant diseases and plant growth promotion by certain other mechanisms, actinomycetes in recent times are considered as one of the important aspects in sustainable plant production (Palaniyandi et al. 2013) as presented in Table 6.2.

The root-colonizing soil actinomycetes *S. lydicus* WYEC108, for instance, have been reported to influence pea root nodulation and increase the nodulation frequency possibly at the level of infection by *Rhizobium* spp. Following colonization, *S. lydicus* sporulate inside the surface cell layers of the nodules which in turn led to a massive increase in the nodules size. Subsequently, the forms and vigor of bacteroids were greatly improved due to enhanced assimilation of iron and possibly other soil nutrients within nodules. Moreover, bacteroid accumulation of the C storage polymer, poly-β-hydroxybutyrate (PHB), was reduced in colonized nodules (Solans 2007). The co-inoculation of rhizoactinomycetes *Streptomyces* MM40,

Table 6.2 Plant-growth-promoting traits exhibited by phosphate-solubilizing actinobacteria

Actinomycetes	Source of isolation	Test plant	Test condition	Plant-growth-promoting traits	References
Micrococcus sp. NII-0909	Western ghat forest soil in India	Cow pea	In vitro	Auxin production, 1-aminocyclopropane-1-carboxylate deaminase activity, and siderophore production	Dastager et al. (2010)
Microbacterium azadirachtae sp. nov. AI-S262^T	Rhizoplane of neem seedlings	–	Plate assay	IAA production, P solubilization, ACC deaminase activity, and sulfur oxidation	Madhaiyan et al. (2010)
Streptomyces rochei IDWR19, *Streptomyces carpinensis* IDWR53, *Streptomyces thermolilacinus* IDWR81	Wheat rhizosphere	–	Solid-state fermentation	Soil enzyme production (invertase, cellulase alkaline protease, phytase chitinase)	Jog et al. (2012)
Streptomyces rochei IDWR19, *Streptomyces thermolilacinus* IDWR81	Wheat rhizosphere	Wheat	Plant growth experiment	Increase in biomass of 1 × 8- and 2 × 3-fold; increase of shoot length of plants	
Microbacterium sp. F10a	Oil-polluted soil	Wheat	Pot experiment	Significantly increased growth of wheat indoleacetic acid, siderophore, and 1-aminocyclopropane-1-carboxylate deaminase activity and solubilizing inorganic phosphate polycyclic aromatic hydrocarbon degrading (phenanthrene and pyrene removal)	Sheng et al. (2009)
Streptomyces, *Microbacterium*	Inner tissues of a traditional Chinese folk medicine	–	In vitro		Qin et al. (2014)

	Limonium sinense (Girard) Kuntze			Indole-3-acetic acid (IAA), N_2-fixation, ACC deaminase	
Streptomyces griseus related strain BH7	Benguerir phosphate mine, Morocco	Wheat (*Triticum durum* L.)	Test tube and rock phosphate soil experiment	Stimulated aerial growth in more than 70 % of test tube and more than 30 % of RP soil as compared to non-inoculated control treatment	Hamdali et al. (2008c)
Streptomyces tsusimaensis, *Streptomyces caviscabies*, *Streptomyces setonii*, *Streptomyces africanus*, and an identified species of Streptomyces	Herbal vermicomposts	Sorghum	Greenhouse conditions	Enhanced plant height, leaf area, stem weight, leaf weight, root length, root surface area, root volume, and root dry weight over the control	Gopalakrishnan et al. (2013)
		Rice	Field conditions	Enhanced plant height, tillers, primary and secondary panicle number, panicle length, stover and grain yield, total dry matter, and test seed weight over the control. Root length, root volume, and root dry weight significantly enhanced in inoculated plots over the control	
		Chickpea		Biocontrol traits against Fusarium wilt	Gopalakrishnan et al. (2011)
Actinomycetes strains	Rhizosphere of *Araucaria angustifolia*	–	Plate assay	Indoleacetic acid and chitinases	Vasconcellos et al. (2010)

(continued)

Table 6.2 (continued)

Actinomycetes	Source of isolation	Test plant	Test condition	Plant-growth-promoting traits	References
Streptomyces MCR10, *Thermobifida* MCR24, *Nocardia* MCR32, and other unidentified actinomycete strains	Rhizosphere of *Trifolium repens* L.	Clover plants (*T. repens* L.)	Pot experiment	Siderophore production but few unidentified strains also showed growth in N-free media suggesting that they could be nitrogen-fixing bacteria	Franco-Correa et al. (2010)
Streptomyces MCR9, *Thermobifida* MCR24, and *Streptomyces* MCR 26	Rhizosphere of *Trifolium repens* L.	Clover plants (*T. repens* L.)	Co-inoculation with AM fungi *Glomus mosseae* without host; in vitro and in soil	Stimulated mycelial development from *G. mosseae* spores; MCR9 and MCR26 also showed stimulated germination of AM spores	Franco-Correa et al. (2010)
			Pot experiment with host; co-inoculated with AM fungi *Glomus mosseae*	Significant plant growth (particularly shoot biomass at the end of the assay) Increased N and P acquisition by plants due to synergic effect Increased the total mycorrhizal root length of *Glomus* inoculated plants	Franco-Correa et al. (2010)
Streptomyces spp., *Saccharopolyspora* spp., *Actinopolyspora* spp., *Nocardia* spp.	Tissue pieces of leaves, stems, and root of rice (*Oryza sativa*)	–	Plate assays	IAA and siderophore production, antifungal activity against *Aspergillus niger*, *Alternaria brassicicola*, *Chaetomium globosum*, *Fusarium oxysporum*, *Phytophthora dresclea*, *Rhizoctonia solani*, *Botrytis cinerea*	Gangwar et al. (2012)

Actinoplanes ME3, and *Micromonospora* MM18 has been found to promote the growth of *Discaria trinervis* in symbiosis with *Frankia*; however, no plant-growth-promoting effect was observed when rhizoactinomycetes were applied alone to the plant (Solans 2007). In a similar study, Hamdali et al. (2010) isolated a population of PS actinomycetes spp. from Moroccan phosphate mines and tested their growth-promoting efficacy using wheat as a test plant. The most active RP-solubilizing strains had the highest stimulatory effect on the production of plant biomass. Of the various isolated actinomycetes strains, strain BH7 of *Streptomyces griseus* stimulated aerial growth of the plant by 70 % in test tubes and more than 30 % in RP soil compared to the non-inoculated control plants. In another study, the actinomycetes showed P solubilization activity (1,916 mg l^{-1}) and produced phytase (0.68 U ml^{-1}), chitinase (6.2 U ml^{-1}), IAA (136.5 mg l^{-1}), and siderophore (47.4 mg l^{-1}). Furthermore, inoculation of *Streptomyces* mhcr0816 and mhce0811 with *Triticum aestivum* (wheat) significantly improved plant growth, biomass (33 %), and mineral (Fe, Mn, P) content in non-axenic conditions (Jog et al. 2014).

6.4 Conclusion

In order to reduce the environmental and economic stress due to massive use of synthetic fertilizers and to achieve food security goals, the use of actinomycete as biofertilizers in intensive agriculture practices appears to be a sound, inexpensive, and eco-friendly option. Even though the physiological functions and symbiotic roles of actinomycetes have been revealed under in vitro condition, the role of actinomycetes in sustainable crop production is not adequately explored. Considering the importance of actinomycetes in plant growth promotion via disease suppression and some other mechanisms, there is urgent need to popularize and maximize the use of actinomycetes in crop production in order to reduce dependence on chemical fertilizers and hence to preserve soil fertility without damaging the soil dwellers.

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Chapter 7
Ecological Diversity, Mechanism, and Biotechnology of Phosphate-Solubilizing Bacteria for Enhanced Crop Production

Anamika Jha, Sanjay Jha, and Debayan Baidya

Abstract The exponentially growing population has engendered the global need to enhance agricultural production in a sustainable manner. Phosphate solubilization is a vital process that determines plant productivity. Conversely, P availability in soil is highly limited due to chemical reactions that fix it into insoluble forms. Soil naturally has organisms capable of bioameliorating the soil Pi by converting it into orthophosphates that can be taken up by the plants. Addition of such bacteria possessing mineral phosphate-solubilizing (mps) activity has been found to increase plant growth and yield even further. Research evidence has clearly shown that microorganisms apart from possessing P-solubilizing ability also enhance plant growth and development through other mechanisms such as nutrient transformation, nutrient mobilization, and production of biologically active compounds. The understanding of microbial community dynamics, functional variation, the relationship between roots and microbiota and their implications in mps mechanism, and the applications of biotechnological tools need to be interwoven to find efficient bacterial cultures with super plant growth-promoting qualities that can be used to develop effective biofertilizers for enhancing crop nutrition in different agroecological niches.

Keywords Rhizosphere competence • Diversity • DGGE • PSB • Biofertilizer

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7.1 Introduction

Phosphorus (P) is a major growth-limiting nutrient, but unlike nitrogen, there is no large atmospheric source of P that can be made biologically available (Ezawa et al. 2002). Root development, stalk and stem strength, flower and seed formation, crop maturity and production, N_2 fixation in legumes, crop quality, and resistance to plant diseases are some of the important attributes associated with P nutrition. Soil P dynamics is characterized by physicochemical (sorption–desorption) and biological (immobilization–mineralization) processes. Large amount of P applied as fertilizer enters in to the immobile pools through precipitation reaction with highly reactive Al^{3+} and Fe^{3+} in acidic soils and Ca^{2+} in calcareous or normal soils (Gyaneshwar et al. 2002; Hao et al. 2002). Efficiency of P fertilizer throughout the world is around 10–25 % (Isherword 1998), and concentration of bioavailable P in soil is very low reaching the level of 1 mg kg^{-1} soil (Goldstein 1994). Soil microorganisms playing a key role in soil P dynamics and subsequent availability of P to plants (Richardson 2001; Khan et al. 2007) are quite often referred to as phosphate-solubilizing microorganisms (PSMs).

Inorganic forms of soil P are solubilized by PSMs through organic acids production that dissolve P minerals and/or chelate cationic partners of the P ions, i.e., $PO4^{3-}$ directly, releasing P into solution (He et al. 2002). Phosphate-solubilizing bacteria (PSB) among PSM have been used as biofertilizer since 1950s (Kudashev 1956; Krasilinikov 1957). Release of P by PSB from insoluble and fixed/adsorbed P is, therefore, an import aspect of P availability in soils. There are strong evidences that soil bacteria can transform soil P to the forms available to plant. Microbial biomass on the contrary assimilates soluble P and prevents it from adsorption or fixation (Khan and Joergensen 2009). Microbial community also influences soil fertility through other soil processes, for example, decomposition, mineralization, and storage/release of nutrients. Even though microbial inoculants are in use for improving soil fertility since long, research on P solubilization has inadequately been done compared to N_2 fixation. Considering the gap in this area, the ecological perspectives, diversity, mechanism of P solubilization, and role of PSB in plant growth promotion are highlighted here in this chapter. Furthermore, various biotechnological tools currently employed to better understand the plant rhizosphere and its associated microbiota are discussed.

7.2 Distribution, Diversity, and Rhizosphere Competence of Phosphate Solubilizers

Evidence of naturally occurring rhizospheric PSM dates back to 1903 (Khan et al. 2007). Among PSM, fungi more effectively solubilize P than bacteria (Alam et al. 2002). On the contrary, of the whole soil microbial populations, PSB constitute 1–50 %, while PS fungi (PSF) accounts for only 0.1–0.5 % (Chen

et al. 2006). Number of PSB among total PSM in north Iranian soil was found as 88 % (Fallah 2006). Microorganisms involved in P acquisition also include mycorrhizal fungi (Fankem et al. 2006). Among soil bacterial communities, ectorhizospheric strains from *Pseudomonas* and *Bacilli* and endosymbiotic *Rhizobia* have been found as notable P solubilizers (Igual et al. 2001). Additionally, *Enterobacter* sp., *Bacillus megaterium*, *B. circulans*, *B. subtilis*, *B. polymyxa*, *B. sircalmous*, *Pseudomonas striata* (Subbarao 1988; Kucey et al. 1989), *Burkholderia* sp., *Serratia marcescens*, *Klebsiella terrigena*, and *Aeromonas vaga* (Jha et al. 2013) have also demonstrated the ability to solubilize phosphate rocks (RP). Among fungi, *Penicillium* and *Aspergillus* are the most powerful P solubilizers (Whitelaw 2000).

High proportions of ubiquitously found PSM are concentrated generally in the rhizosphere and are metabolically more active than those found in other habitat (Vazquez et al. 2000; Anamika et al. 2007). Usually, one gram of fertile soil contains 10^1 to 10^{10} bacteria, and their live weight may exceed 2,000 kg ha^{-1}. The shape of soil bacteria varies from cocci (sphere, 0.5 μm) to bacilli (rod, 0.5–0.3 μm) to spiral (1–100 μm). However, bacilli are the most dominant and common form in soil whereas spirilli are very rare in natural environments (Baudoin et al. 2002). Population of PSB depends on different soil properties (physical and chemical properties, organic matter, and P content) and cultural activities (Kim et al. 1998a, b). Larger populations of PSB are found in agricultural and rangeland soils (Yahya and Azawi 1998). For instance, in northern part of Iran, the PSB counts ranged from 0 to 10^7 cells g^{-1} soil, with 3.98 % population of PSB among total bacteria (Fallah 2006). Further, zone/site of origin determines the capacity of bacterial isolates to solubilize P. Among various sources, rhizoplanes harbor the bacteria with highest capacity, rhizosphere organisms with intermediate capacity, while those from bulk soils with the least PS activity. The survival of P solubilizers is determined by "chemosequence" and "climosequence" of soils. Inadequate information is, however, available about the diversity of bacterial populations in and around the rhizosphere, possibly due to lack of appropriate techniques required to isolate and accurately identify specific PS strains belonging to the same species. Such limitations hinder the process to explore community dynamics, which leads to the poor understanding of variations in microbial community dynamics in response to soil type, plant type, or stage of plant development (McSpadden Gardener and Driks 2004). In fact, bacterial communities residing in the rhizosphere respond, in particular, with respect to density, composition, and activity, to the plethora and diversity of organic root exudates, resulting in plant species-specific microflora which may eventually vary with the stage of plant growth. The role of plant (largely exudates) in affecting the ability of bacteria to colonize the rhizosphere (Kumar et al. 2011) has been considered as one of the major factors. Among PSB, fluorescent pseudomonads that colonize aggressively the plant roots have been considered as an important group of bacteria due to their biofertilizing and biocontrol properties (Naik et al. 2008; Parikh and Jha 2012; Jha et al. 2012). These strains were taxonomically described as different fluorescent pseudomonad species such as *P. monteilli*, *P. putida*, *P. plecoglossicida*, *P. fluorescens*, *P. fulva*, and

P. aeruginosa on the basis of *16S rRNA* gene sequencing and subsequent molecular phylogeny analysis. Phenotypic analyses as well as 16S rRNA and BOX-PCR-based genotypic analyses revealed a high degree of diversity among PSB as reported by Naik et al. (2008).

7.2.1 Rhizospheric Competence

Rhizospheric competence is a necessary prerequisite for plant growth-promoting rhizobacteria (PGPR). It involves effective root colonization combined with the ability of PGPR to survive and proliferate along the roots of growing plant in the presence of indigenous microbiota over a period of time. Understanding the plant–microbe communication, which is influenced by genetic and environmental factors, can contribute significantly toward revealing the mechanistic basis of PGPR action (Bais et al. 2004). Among soil bacteria, *Bacillus* species are believed to be less rhizosphere competent than *Pseudomonas* species. Eventually, most research even today is focused at the development of biofertilizer and biocontrol agents based on *Pseudomonas* species (Parikh and Jha 2012; Jha et al. 2012). However, studies on the genetic diversity of *Bacillus* inhabiting soil and wheat rhizosphere implied that rhizosphere competence is a characteristic of the strain (genotype) not exclusive to the genus or species. Experiments with different wheat varieties conducted by Milus and Rothrock (1993) have revealed that seeds pelleted with selected strains of *Bacillus* could successfully establish in the rhizosphere. But whether the colonization attained by introduced strains was on the entire root or only on the top few centimeters of root below the seed could not be confirmed.

7.2.2 Rhizospheric Effect and Host Specificity

Though previous studies have proved that plants opt for taxonomic functional groups in the rhizosphere (Mittal and Johri 2007), it is not certain whether plants dynamically select beneficial soil microbial communities in their rhizosphere through rhizodeposition. Although some field studies with mixed plant communities did not find such selections in the rhizosphere, there are reports that suggest a strong correlation between plant and soil microbial communities (Duineveld et al. 2001). The root exudates are believed to be plant specific, and this specificity may reflect the evolution or specific physiological adaptation to conditions of a particular soil habitat. Composition of root exudates has been shown to vary with plant species and stage of plant growth (Mittal and Johri 2007). Concomitantly, the plant is supposed to influence the composition of both indigenous and introduced rhizobacteria. The exudates, for instance, sugar, amino acids, or organic acids, act as chemoattractants and hence affect structure and functions of soil bacteria (Somers et al. 2004). Being a major driving force for microbial root colonization,

plant root exudation could be engineered precisely to stimulate specific microbial colonization on the roots. It has also been observed that genetically engineered plants producing opine, for example, have an altered rhizosphere community compared to their wild counterparts. Furthermore, due to several chemical factors in the rhizosphere of different plants, roots are colonized by microbes out of indefinite pool of soil microbial diversity.

Another important factor that may affect the rhizospheric microbiota has been recognized as the cultivation practices in different production systems. Agriculture management strategies can induce clear shifts in the structures of plant-associated microbial communities. For example, plant genotypes can exert strong effects on the bacterial communities associated with the plants. Growth stage of plant is another important factor that alters the rhizobacterial community structure, and as reported in case of potato rhizosphere, it has been identified as one of strongest factors affecting the bacterial communities (van Overbeek and van Elsas 2008). Besides, land use, soil history, and cultivation practices are some of the other factors which govern the structure of plant-associated microbial communities (Sharma et al. 2013).

7.3 Mechanism of P Solubilization

Phosphorus-solubilizing activity is determined by the ability of microbes to release metabolites such as organic acids, which through their hydroxyl and carboxyl groups chelate the cation bound to phosphate, the latter being converted to soluble forms (Sagoe et al. 1998). General sketch of P solubilization in soil is shown in Fig. 7.1. A wide range of microbial P solubilization mechanisms exist in nature, and much of the global cycling of insoluble organic and inorganic soil phosphates is attributed to bacteria and fungi (Banik and Dey 1982).

Phosphorus solubilization is carried out by a large number of bacteria and fungi acting on sparingly soluble soil phosphates, mainly by chelation-mediated mechanisms (Whitelaw 2000). Inorganic P is solubilized by the action of organic and inorganic acids secreted by PSB in which hydroxyl and carboxyl groups of acids chelate cations (Al, Fe, and Ca) and decrease the pH in basic soils (Kpomblekou and Tabatabai 1994; Stevenson 2005; Jha et al. 2013). The PSB dissolve the soil P through production of low molecular weight organic acids mainly gluconic and keto gluconic acids (Goldstein 1995; Deubel et al. 2000), in addition to lowering the pH of rhizosphere. The pH of rhizosphere is lowered by proton/bicarbonate release (anion/cation balance) and gaseous (O_2/CO_2) exchanges. Release of root exudates such as organic ligands can also alter the concentration of P in soil solution (Hinsinger 2001). Inorganic acids like hydrochloric acid (HCl) can also solubilize phosphate, but they are less effective compared to organic acids at the same pH (Kim et al. 1997). In certain cases phosphate solubilization is induced by phosphate starvation (Gyaneshwar et al. 1999).

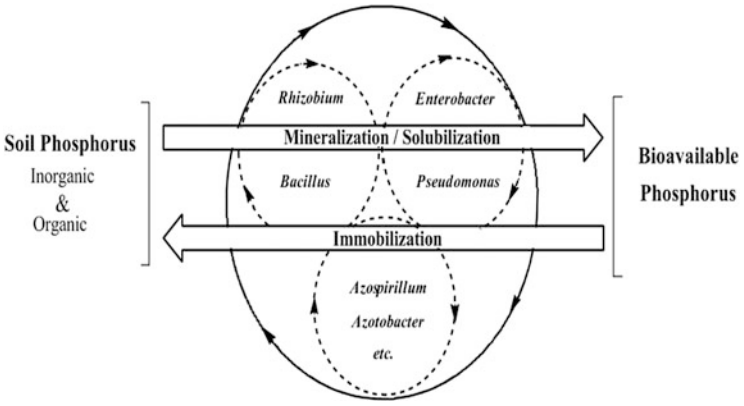

Fig. 7.1 Schematic diagram of soil P mobilization and immobilization in bacteria (Richardson and Simpson 2011)

Soil phosphates mainly the apatites and metabolites of phosphatic fertilizers are fixed in the form of calcium phosphates under alkaline conditions. Many of the calcium phosphates, including rock phosphate ores (fluoroapatite, francolite), are insoluble in soil with respect to the release of inorganic P (Pi) at rates necessary to support agronomic levels of plant growth (Goldstein 2000). Gerretsen (1948) first showed that pure cultures of soil bacteria could increase the P nutrition of plants through increased solubility of Ca-phosphates. Their solubility increases with consequent decrease in soil pH. Microorganisms through secretion of different types of organic acids, e.g., carboxylic acid (Deubel and Merbach 2005), and rhizospheric pH-lowering mechanisms (He and Zhu 1988) dissociate the bound forms of phosphate like $Ca_3(PO_4)_2$. Nevertheless, buffering capacity of the medium reduces the effectiveness of PSB in releasing P from tricalcium phosphates (Stephen and Jisha 2009).

Acidification of the microbial cell surroundings releases P from apatite by proton substitution/excretion of H^+ (accompanying greater absorption of cations than anions) or release of Ca^{2+} (Goldstein 1994; Illmer and Schinner 1995; Villegas and Fortin 2002) (Fig. 7.2), while the reverse occurs when uptake of anions exceeds that of cations, with excretion of OH^-/HCO_3^- exceeding that of H^+ (Tang and Rengel 2003). Carboxylic anions produced by PSB have high affinity to calcium and solubilize more P than acidification alone (Staunton and Leprince 1996). Complexing of cations is an important mechanism in P solubilization if the organic acid structure favors complexation (Fox et al. 1990). It is controlled by nutritional, physiological, and growth conditions of the microbial culture (Reyes et al. 2007), but it is mostly due to the lowering of pH alone by organic acids (Moghimi and Tate 1978) or production of microbial metabolites (Abd Alla 1994). Organic anions and associated protons are effective in solubilizing precipitated forms of soil P (e.g., Fe– and Al–P in acid soils, Ca–P in alkaline soils), chelating metal ions that may be associated with complexed forms of P or may facilitate the release of adsorbed P

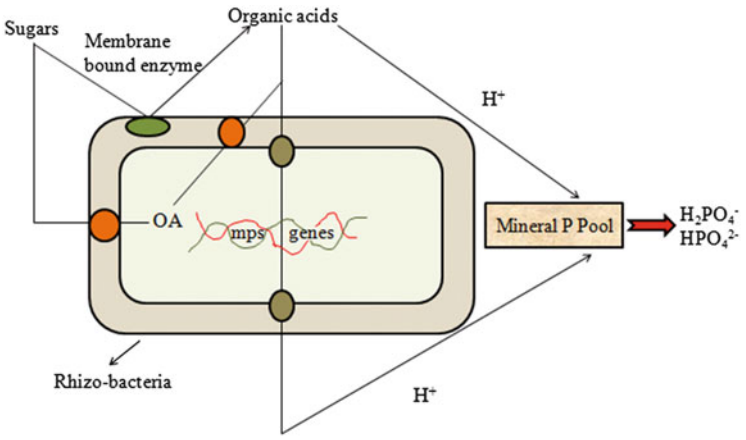

Fig. 7.2 A model of phosphate starvation-inducible mps genes (Bagyaraj et al. 2000)

through ligand exchange reactions (Jones 1998). Dissociation of calcium phosphate (Ca–P) results from the combined effects of carboxylic acids synthesis and subsequent pH decrease involving common mechanism, but proton release is the outcome of several mechanisms (Deubel et al. 2000).

7.4 PSB and Plant Growth Promotion

PSB helps in making the soluble P accessible for uptake by plants and concurrently promotes the growth of plants promotion of plants by PSMs (Khan et al. 2007). Growth promotion can be achieved by production of plant beneficial metabolites, such as phytohormones, antibiotics, or siderophores; however, there are about or more than 20 possible mechanisms by which the plant growth promotion can be mediated (Sharma et al. 2013). Many reports show that bacteria belonging to genera *Bacillus*, *Pseudomonas*, *Serratia*, *Enterobacter*, etc., solubilize inorganic P and aid in plant growth promotion (Rodriguez and Fraga 1999). Use of PSB is reported to increase crop yields up to 70 % (Verma 1993). *Pseudomonas* sp. CDB35, for instance, solubilized P in buffered RP medium (Hameeda et al. 2008) and, when tested, promoted overall growth including the biomass index of the maize. It has, however, been observed that a single PSB strain may not employ several mechanisms at one time; instead simultaneous mechanisms can function in unison and very efficiently enhance the plant growth. The role of a very few select groups of PSB in growth promotion of various crops is listed in Table 7.1 (Patil et al. 2002; Mehrvarz et al. 2008).

Single and dual inoculation along with P fertilizer was 30–40 % better than P fertilizer applied alone in terms of grain yield of wheat. Also, dual inoculation without P fertilizer improved grain yield up to 20 % against sole P fertilization

Table 7.1 Impact of PGPR inoculation on plant growth and yield

Inoculant used	Crops benefited	Experimental soil	Response/effects	References
G. etunicatum + *Burkholderia cepacia BAM 6*	Wheat [*Triticum aestivum*]	Loam soil	Enhanced biomass and phosphorous uptake	Saxena et al. (2013)
Pseudomonas putida	Barley [*Hordeum vulgare*]	Heavy loam	Enhanced chlorophyll content	Mehrvarz et al. (2008)
P. fluorescence + *Bacillus megaterium*	Chickpea [*Cicer arietinum*]	Alluvial soil	Enhanced seedling growth	Sharma et al. (2007)
P. striata	Chickpea, soybean [*Glycine max*]	Sandy alluvial	Increased the number of nodules, weight of nodules, and grain yield	Son et al. (2006)
Bradyrhizobium + *G. fasciculatum* + *B. subtilis*	Green gram [*Vigna radiata* (L.) Wilczek]	Loam soil	Enhanced seed yield	Zaidi and Khan (2006)
B. megaterium + *G. fasciculatum* and *G. fasciculatum*	Banana [*Musa paradisiaca*]	Acidic soil	Biomass and phosphorous intake	Patil et al. (2002)
B. firmus NCIM 2636	Paddy [*Oryza* spp.]	Moist and acidic soil	Increased root biomass and phytohormones	Datta et al. (1982)

(Afzal and Bano 2008). The increase in yield of wheat was attributed to the phosphate-solubilizing potential of PSB applied in this study as reported by others (Kucey et al. 1989; Ponmurugan and Gopi 2006). The seedling length of chickpea (Sharma et al. 2007) was enhanced following PSB inoculation, while in other report, co-inoculation of PSB with certain PGPR reduced P application by 50 % without affecting corn yields (Yazdani et al. 2009). Rhizospheric microorganisms can interact positively in soil and improve plant growth synergistically or additively (Zaidi et al. 2003; Wani et al. 2007) by enhancing N and P uptake by plants. For example, seed yield of green gram was enhanced by 24 % following triple inoculation of *Bradyrhizobium* + *Glomus fasciculatum* + *Bacillus subtilis* (Zaidi and Khan 2006). The synergistic effect of an arbuscular mycorrhizal fungus (AMF), *G. etunicatum*, and an indigenous PSB strain, *Burkholderia cepacia* BAM-6, was determined against wheat plants grown in pots containing soil with low available P to assess their potential to be used as bioinoculants in semiarid regions (Saxena et al. 2013). Seed yield and N concentration were remarkably enhanced by more than 50 and 90 %, respectively, following dual inoculation. Percent root colonization by rhizosphere population of PSB was also increased with time in soil. Integration of half dose of NP fertilizer with biofertilizer resulted in crop yields comparable to those obtained for full rate of fertilizer. This led to reduction in use of

fertilizers, and therefore, the production cost was minimized. The exploitation of P-solubilizing bacteria as biofertilizer thus has enormous potential for making use of ever-increasing fixed P in soil and natural reserves of phosphate rocks.

7.5 Some Examples of Biotechnological Tools to Identify Potential PSB

More than 99 % of soil microorganisms including P solubilizers have not been cultured successfully. Thus, culture-independent methods are needed for evaluating the functional diversity and ecology of PSB involved in P cycling in soils. Molecular approaches for such culture-independent methods have been developed. The molecular techniques based on nucleic acid composition like LMW RNA profiling and PCR-based techniques are excellent tools for this purpose, as they are precise, reproducible, and not dependent on culture media composition or growth phase of microorganisms (Peix et al. 2007). An understanding of coupled biological process at the molecular level is fundamental for assessing the composition and function of microbes which in turn affect the health of soil that eventually could lead to increased soil fertility and consequently the crop production. In this regard, several molecular and cellular techniques are available which in conjunction with biological and chemical indicators help to better understand the functionality of microbes and, hence, the soil health (Gautam and Jha 2011). Some of the techniques used in identifying microbes with varied biological potentials are discussed briefly.

7.5.1 DNA Measurement

Quantification of DNA following its extraction and enrichment (in insoluble P-containing media) from any environmental sample may provide a simple and practicable method for estimating the amount of microbial biomass (Girvan et al. 2004). However, further work on correlating DNA measurements with a particular soil type is required. The total DNA isolation is done, and then it is followed by the amplification of 16S rDNA or the intergenic region (i.e., the region between16S rDNA and 23S rDNA) with the universal primers. The amplicon is then proceeded for sequencing to identify the most abundant type of bacterium present in the sample and to reveal the bacterial diversity.

7.5.2 Fluorescence Microscopy

The number of bacteria in soil, their cell volumes, and the frequencies of dividing cells can be determined by fluorescence microscopy and computerized image analysis (Bloem et al. 1995). Soil microbial biomass can be estimated by staining with fluorescent dyes such as fluorescein isothiocyanate.

7.5.3 Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) is a direct, cultivation-independent technique using rRNA-targeted oligonucleotide probes that is frequently used for the identification of microorganisms in soils. While this technique allows selective visualization of bacterial cells of different phylogenetic groups, it also has some limitations, particularly regarding quantitative analysis of complex samples (Moter and Göbel 2000; Peix et al. 2007).

7.5.4 Stable Isotope Probing

Stable isotope probing (SIP) is a culture-independent technique that allows the identification of microorganisms directly involved in specific metabolic processes. In this method, labeled nucleic acids synthesized during assimilation of an isotopically enriched substrate are isolated and analyzed (Radajewski et al. 2002). The technique has been used to study forest soils. Genetic diversity is most commonly studied by analyzing the diversity of genes encoding 16S rRNA (18S rRNA for eukaryotes). These genes occur in all microorganisms and show species-dependent variations in their base compositions. Three methods are commonly applied to examine the diversity of 16S (and 18S) rDNA sequences in total DNA extracted from soil microbial communities: denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and terminal restriction fragment length polymorphisms (T-RFLP).

7.5.5 RNA Measurement

The composition of soil microbial communities can be estimated by reverse transcriptase polymerase chain reaction (RT-PCR) followed by gel electrophoresis of the amplified cDNA fragments (Duineveld et al 2001). The analysis of specific mRNAs reflects the expression of the corresponding gene in soil. Such measurements can also be done by quantitative real-time RT-PCR, which allows the

detection and quantification of mRNAs present in low amounts in environmental samples including soils (Pfaffl and Hageleit 2001). However, this method requires previous knowledge of the sequence of the mRNA of interest.

7.5.6 Denaturing Gradient Gel Electrophoresis

Differences in the melting behavior of small DNA fragments (200–700 bp) that differ in as little as a single base substitution can be detected by DGGE (Muyzer et al. 1993). The denaturants used are heat (a constant temperature of 60 °C) and a fixed ratio of formamide (ranging from 0 to 40 %) and urea (ranging from 0 to 7 M). The benefit of this approach is that a molecular fingerprint of the community structure is generated for each soil. In fact, each band in each lane of the gel theoretically represents a different bacterial species. In addition, this technique enables the excision and subsequent sequencing of bands, allowing species identification using existing databases. The structure of the bacterial communities associated with the root endosphere and in the plant rhizosphere can be dissected by 16S rRNA gene-based PCR-DGGE (denaturing gradient gel electrophoresis) analysis. Naik et al. (2008) performed molecular phylogenetic analyses by aligning the sequences of 16S rRNA using the multiple sequence alignment program CLUSTAL W. The aligned sequences were then checked for gaps manually, arranged in a block of 600 bp in each row, and saved as molecular evolutionary genetics analysis (MEGA) format in software MEGA v3.0. The pair-wise evolutionary distances were computed using the Kimura 2-parameter model. A DGGE technique has also been developed for analyzing the diversity of the PQQ biosynthetic gene pqqC, a gene which has been found as a good molecular marker for investigations of natural populations of P-solubilizing pseudomonads (Naik et al. 2008).

7.5.7 Temperature Gradient Gel Electrophoresis

Variations are known to exist in the genetic microdiversity within the species of *Bacillus* and *Paenibacillus* (McSpadden Gardener and Driks 2004). Wieland et al. (2001) studied the spatiotemporal variation among the microbial communities from soil, rhizosphere, and rhizoplane with respect to crop species (clover, bean, and alfalfa), soil type, and crop development following a comparative study of 16S rRNA sequences employing TGGE. According to their study, the type of plant species had profound effects on microbial community dynamics, with the effect of soil type typically exceeding that of plant type. Plant development had only minor habitat-dependent effect, and insignificant variations were observed in time-dependent shifts among the microbial communities compared to the soil type or plant type in all the habitats under study. Systematic community shifts could not be

recognized in samples from bulk soil; however, some variations in the TGGE patterns could be correlated to time of development in the rhizosphere and rhizoplane. Nearly, similar findings were reported by Mahaffee and Kloepper (1997) who used fatty acid methyl ester analysis (FAME) to determine the community shifts in the rhizosphere of cucumber. However, only an altered window of observations generated by the use of specific primers could possibly reveal a stronger time-dependent stimulation of certain bacterial groups.

7.5.8 Terminal Restriction Fragment Length Polymorphism

Organisms can also be differentiated according to the patterns derived from cleavage of their DNA. Thus, in T-RFLP, the specific fingerprint of a community is revealed by analyzing the polymorphism of a certain gene. T-RFLP is a high-throughput, reproducible method that allows the semiquantitative analysis of the diversity of a particular gene in a community. It requires the extraction of DNA from a soil sample and its PCR amplification using a fluorescently labeled primer. T-RFLP yields a mixture of amplicons of the same or similar sizes with a fluorescent label at one end. After purification, the amplicon mixture is digested with a restriction enzyme, which generates fragments of different sizes that are separated by gel or capillary electrophoresis. The separated, labeled fragments are then densitometrically detected, and a profile based on fragment lengths is generated. McSpadden Gardener and Driks (2004) studied the population structure of these two groups by T-RFLP using group-specific primers Ba1F and Ba2R and characterized the plant growth-promoting population of PGPR; only minor differences were observed in the number and relative abundance of *Bacillus*-like ribotypes from different sites all the way through Ohio (USA). Despite environmental constraints and interactions with other microorganisms, some bacteria are able to colonize the phylloplane with higher frequency than others. Arias et al. (1999) evaluated the diversity and distribution of *Bacillus* spp. from soybean phylloplane wherein a decline was observed in the population of *Bacillus* spp. from 80 % of total bacterial isolates in early stages to 0 % at the time of harvesting (Kumar et al. 2011).

7.5.9 Microbial Resilience

The ability to estimate the relative abundance of each species of microorganisms in the soil, using the three techniques described above, has led to the suggestion that the "equitability index" (J) of numbers of individual species is an important estimation of the resilience of a soil. The use of statistical packages such as Phoretix enables quantification of both diversity indices and equitability (Girvan et al. 2004). The development of approaches that do not require the establishment of microbial cultures will undoubtedly enhance our knowledge of bio-resources and promote the

discovery of new microorganisms with unique capacities for bioremediation, soil restoration, and therapeutic applications.

7.5.10 BOX-PCR-Based Genotypic Analysis

Naik et al. (2008) evaluated genetic and functional diversity of phosphate-solubilizing fluorescent pseudomonads associated with rhizospheric soils of rice and banana by an array of in vitro assays, gene amplification techniques, fermentation methods, and chromatographic analyses. Taxonomic affiliation of bacteria was done on the basis of *16S rRNA* gene similarity and molecular phylogenetic analyses. These strains were taxonomically described as different fluorescent pseudomonad species such as *P. monteilli*, *P. putida*, *P. plecoglossicida*, *P. fluorescens*, *P. fulva*, *P. monteilli*, and *P. aeruginosa* on the basis of *16S rRNA* gene sequencing and subsequent molecular phylogeny analysis. Phenotypic analyses as well as *16S rRNA* and BOX-PCR-based genotypic analyses revealed a high degree of diversity among PSB reported in this study. Meyer et al. (2013) used MRPP (Multiple Response Permutation Procedure) to examine potential impacts of plant production procedure, plant age, and sampling year on the diversity of the *Pseudomonas* communities colonizing wheat roots, based on their DGGE profiles (presence/absence matrices of individual replicates and frequency matrices of pooled replicates). Additionally, to analyze relationships between diversity-based genotype number and factors such as plant line, replicate (block) effect, plant age, field season, plant production procedures, and damage level (vandals damage), a generalized linear model (glm) was fitted on the number of pqqC genotypes/bands present per plant sample.

7.6 Conclusion and Future Prospects

The fragile agroecosystem is burdened with the responsibility of enhanced agricultural production from a steadily decreasing and degrading land resource in the present global scenario. Current strategies to improve the agricultural productivity via high-input practices have placed considerable emphasis on reliable techniques for each component of the production sequence with little consideration to the integration of these components in a holistic, systems approach. Improvement in agricultural sustainability requires optimal use and management of soil fertility and soil physical properties, both of which rely on soil biological processes and soil biodiversity. In this context, the long-lasting challenges in soil microbiology are development of effective methods to know the types of microorganisms present in soils and to determine functions which the microbes perform in situ. The soil conditions need to be mimicked “in vitro” during isolation and screening of the phosphate solubilizers, and the compatibility and abundance of introduced bacteria

should be checked in soil time to time for optimum crop yields. Key factor in biofertilizer failure is the low colonization and establishment rate of introduced microbial population. Success of this strategy will be very useful in bridging the gap between in vitro and in field biofertilizer applications.

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Chapter 8
Response of PSM Inoculation to Certain Legumes and Cereal Crops

Ees Ahmad, Almas Zaidi, and Md. Saghir Khan

Abstract Phosphate-solubilizing microorganisms (PSM) including bacteria, fungi, and actinomycetes dwelling in soil or other environment, for example, rhizosphere, do play some vital roles in facilitating growth and development of legumes and cereal plants via one or simultaneous mechanisms. Phosphate-solubilizing microbes when applied in agricultural practices provide one of the major plant nutrients, phosphorus, to plants by transforming insoluble P into soluble and plant available forms. This practice of applying PSM for enhancing legumes and cereal production has been found inexpensive and in many cases a successful strategy of reducing fertilizer input in intensive agricultural practices. The advent of such an eco-friendly option in farming system holds greater promise for increasing the productivity of legumes and cereal crops. Here, an attempt is made in this chapter to highlight the role of PSM involving different microbial groups, used either alone or in combination, in the promotion of growth and yield of legumes and cereal crops in different production systems.

Keywords PSM • Cereals • Legumes • AM fungi

8.1 Introduction

An ever-increasing human population has placed tremendous pressure on declining lands under cultivation in different regions of the world. And hence, such challenges need constant efforts to make less fertile soils into fertile ones so that the crops can be provided with sufficient and need-based nutrients for better growth and substantial yields (Rengel 2008). In this context, chemical fertilizers have excessively been used in agriculture worldwide to provide nutrients to support plant

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growth and consequently to boost crop productivity. Since deficiency of P (the second most important plant nutrient after N) is an important chemical factor restricting plant growth, chemical phosphatic fertilizers are widely used to achieve optimum yields (Del Campillo et al. 1999; Shenoy and Kalagudi 2005). Soluble forms of P fertilizer after application are, however, easily and rapidly precipitated as insoluble forms and become inaccessible to plants (Goldstein 1986; Takahashi and Anwar 2007). The deficiency of P in turn can severely limit plant growth and productivity (Fernández et al. 2007), particularly in legumes, where both the plants and their symbiotic bacteria are affected. As a result, this may have a deleterious effect on nodule formation, development, and function (Robson et al. 1981). Undeniably, synthetic fertilizers have resulted in better crop yields but at the cost of deteriorating fertility of soils leading ultimately to human health problems via food chain. There is therefore urgent need to find alternative option to eliminate or at least minimize too much dependence on chemical fertilizers so that the use of biofertilizers on a large scale in agronomic practices could be popularized among farm practitioners. The application of beneficial soil microbes especially phosphate-solubilizing organisms (Krishnaveni 2010; Yu et al. 2011; Zhu et al. 2011; Bashan et al. 2013; Dugar et al. 2013; Sharma et al. 2013) used both alone and in combination with other compatible microbes (Zaidi et al. 2003; Zaidi and Khan 2006; Wani et al 2007a; Awasthi et al. 2011; Khan et al. 2013) has provided some solutions to the ever-increasing use of expensive synthetic fertilizers in farming system. Such microorganisms when used in agriculture practices provide benefits to plants in different ways, for example, they assist in maintaining long-term soil fertility by providing good soil biological activity, suppressing pathogenic soil organisms, and stimulating microbial activity in the rhizosphere (Biswas and Narayanasamy 2006; Ouahmane et al. 2007; Collavino et al. 2010; Parani and Saha 2012). Accordingly, it is reported that the phosphate-solubilizing (PS) bacteria when applied with other plant growth-promoting rhizobacteria (PGPR) could reduce P fertilizer application by 50 % without any significant reduction in crop yields (Jilani et al. 2007; Yazdani et al. 2009) suggesting that PS organisms as inoculant/biofertilizers hold greater promise for sustaining crop production (Wani et al. 2007b; Deepa et al. 2010). Here, we highlight the impact of PS microbes on the growth and yield of certain widely grown legumes and cereal crops in different production systems.

8.2 Synthetic Fertilizers and Soil Microorganisms: Benefits and Deleterious Impact

Synthetic fertilizers are widely used in agricultural practices particularly in developing countries to enhance soil fertility and, hence, crop production. Some argue that fertilizer was as important as seed in the Green Revolution (Tomich et al. 1995) period contributing as much as 50 % of the yield growth in Asia (FAO 1998;

Hopper 1993). Others have found that one-third of the cereal production worldwide is due to the use of fertilizer and related factors of production (Bumb 1995). Fertilizer consumption in many countries including India has increased substantially in recent times, and today India is probably the largest producer and consumer of fertilizers in the world. According to some estimates, the total fertilizer consumption in India was 26.49 million nutrient tonnes in 2009–2010 (Jaga and Yogesh 2012). The importance/use of fertilizers for crop yield is likely to increase further in order to achieve optimum agriculture production and consequently to feed the alarmingly increasing human populations. This is due to two reasons: (1) cultivable land is declining rapidly and there is little scope for bringing more area under cultivation and (2) majority of soils over the world including Indian soils are deficient in many essential nutrients including P. However, the accumulation of such fertilizers in soils which results from the excessive and repeated application and poor uptake by plants significantly affects biological and biochemical properties of soils (Marschner 2003; Yevdokimov et al. 2008; Zhong et al. 2010). Moreover, studies have mainly been conducted at a bulk soil scale or in short-term experiments, and as a result, there is still little information available on rhizosphere effects on extracellular enzyme activities and microbial community structure in agricultural soils, influenced by long-term practices. Among various factors, organic matter (OM) addition has been found to cause a rapid shift in the activities of various enzymes and reactivation of biogeochemical cycles in bulk soil (Madejon et al. 2001; Bastida et al. 2007). It is generally recognized that OM addition tends to increase the total microbial biomass, though the responses of specific groups such as Gram-positive bacteria, Gram-negative bacteria, and fungi vary greatly. For instance, OM additions often result in increased or altered fungal populations (Bastida et al. 2007), variable populations of arbuscular mycorrhizal (AM) fungi (Corkidi et al. 2002), shifts in Gram-positive and Gram-negative bacteria (Peacock et al. 2001; Marschner 2003), and increased fungi/bacteria ratios (Elfstrand et al. 2007). Importantly, the response of the microbial community structure to OM additions tends to be based on differences in the carbon amount or quality of the organic amendments. Inorganic fertilizers such as N, P, and K have also been reported to have a contrasting impact on structure and activities of soil microbes (Goyal et al. 1999; Böhme et al. 2005) especially the P-solubilizers. As an example, Bolle et al. (2013) in a study investigated the adaptation and performance of PS bacteria (three *Bacillus* spp. and two *Pseudomonas* spp.) in conditions of high total P content in soil employing three experiments. In the first experiment, the PS potential of the *Bacillus* and *Pseudomonas* species was determined under fully controlled conditions on several growth media treated with different rates and forms of insoluble P [($FePO_4$, $AlPO_4$) or $(Ca)_3(PO_4)_2$]. All PS bacterial strains survived and proliferate and could solubilize P even after 14 days of incubation. In the second experiment, the same bacterial species were inoculated in pure quartz sand amended with a nutrient solution, and P was added separately in an insoluble form, as Fe–P, Al–P, or Ca–P. The extractable ammonium lactate ranged from 3.2 to 6.9 and 29 to 40.7 mg kg^{-1} sand for the insoluble Al–P and Fe–P treatments, respectively. *Pseudomonas putida* and *B. brevis* performed best as PSB at high P

concentration where the P was fixed with Al or Fe. In the third experiment, *P. putida* and *B. brevis* were inoculated in an acidic sandy P-saturated soil for 4 weeks. The inoculation of the PSB showed promising results in solubilizing P suggesting that the higher concentration of P did not have any negative effect on P-solubilizing activity of microbes. In other study, many hydrolytic enzyme activities of forest soil were increased by the addition of N fertilizer, but the phenol oxidase activity was dropped by 40 % compared to control plots (Saiya-Cork et al. 2002). In yet other investigation, Weand et al. (2010) found that the N addition caused a change in the enzymatic activities in a soil which, however, depends on the nature of the dominant substrates (labile or recalcitrant). Furthermore, the rhizosphere effects on microbial activities and nutrient availability were reduced by fertilizer addition in nutrient-poor forest soil, which was presumed to be due to fertilizer-induced shifts in the belowground C supply (Phillips and Fahey 2008). Similarly, most studies have found obvious changes in soil microbial communities after addition of organic or inorganic fertilizer amendments (Peacock et al. 2001; Marschner 2003; Enwall et al. 2005). Changes in soil microbial community structure were also observed following additions of inorganic N, P, and K fertilizers (Zhang et al. 2007; Yevdokimov et al. 2008). However, the ecological consequences of the application of various fertilizers in the rhizosphere are unclear, because of the poor understanding of how changes in nutrient availability affects the plant and soil microbial processes (Hobbie et al. 2002). Fertilizer additions possibly result in decreased C allocation to roots and subsequent decreases in microbial respiration in the rhizosphere (Phillips and Fahey 2007).

8.3 Pulse Production: A Brief Account

Pulses are the second most important nutritional group of crops after cereals in the dietary system of many countries. India is the largest producer and consumer of pulses in the world accounting for about 25 % of global production, 27 % of consumption, and 34 % of food use (FAO 2009). According to the Indian Council of Agricultural Research (ICAR), an apex body of the National Agricultural Research System, Ministry of Agriculture, Government of India, pulses production in India has been hovering around 13–15 million tonnes during the last decade, while annual domestic demand has risen to 18–19 million tonnes. During 2010–2011, the production of pulses in India, estimated at 17.29 million tonnes, was an all-time high record. According to the ministry of agriculture, the country has, however, achieved all-time high-record pulse production of 18.45 million tonnes (MT) in the 2012–2013 crop year ended June. The previous pulse production record was 14.91 million tonnes during the year 2003–2004. Among kharif pulses (7.3 million tonnes), pigeon pea (3.15 million tonnes) and black gram (1.82 million tonnes) production are all-time higher. A record production of 18.45 million tonnes became possible primarily due to the availability of quality seeds to pulse growers. Apart from the availability of quality seeds of high-yielding varieties, the strong

technology backup, favorable monsoon, increase in minimum support prices, and effective government programs helped for the increase of production of pulses in the country. The projected pulse requirement by the year 2030 is estimated at about 32 million tonnes (ICAR Vision 2030 2011). In India, about dozen of pulse crops, namely, chickpea, pigeon pea, mung bean, urdbean, lentil, field pea, lathyrus, cowpea, common bean, moth bean, horse gram, and rice bean are cultivated on 22.47 million ha area under varied agroecological conditions. About 90 % of the global pigeon pea, 75 % of chickpea, and 37 % of lentil area falls in India (FAOSTAT 2009). Globally, the pulse production in 2009 was 61.5 million tons over an area of 70.6 million ha with an average yield of 871 kg/ha. Of these, beans contributed about 32 % to global pulse production which was followed by dry peas (17 %), chickpea (15.9 %), broad beans (7.5 %), lentils (5.7 %), cowpeas (6 %), and pigeon pea (4 %). Among different nations, developing countries contribute about 74 % to the global pulse production, and the remaining comes from developed countries. India, China, Brazil, Canada, Myanmar, and Australia are the major pulse-producing countries with relative share of 25, 10, 5, 5, and 4 %, respectively. Countries recording annual production growth of more than 4 % are Myanmar (11.48 %), Canada (10.80 %), Germany (8.27 %), Sudan (8.08 %), Spain (7.37 %), Ethiopia (4.92 %), China (4.67 %), and Syria (4.12 %) presented in ICAR Vision 2030 (2011). A few example of top pulse-producing countries are listed in Table 8.1.

8.3.1 Nutritional Value of Important Legumes

Pulses, sometimes called "grain legumes," form an important component of the dietary systems of many countries including India due to their high-protein and essential amino acid content. In addition, pulses provide complex carbohydrates and several vitamins and minerals. Like other plant-based foods, they contain no cholesterol and little fat or sodium. Pulses also provide Fe, Mg, P, Zn, and other minerals. The nutritive value of pulses, however, varies greatly among different legumes (Table 8.2) and plays a variety of roles in maintaining good health (Schneider 2002). Apart from their role in maintaining good human health, pulses also play a key role in crop rotation due to their ability to fix atmospheric nitrogen in association with symbiotic nitrogen fixers like rhizobia. To support the awareness on this matter, the United Nations has declared 2016 the UN International Year of Pulses.

Table 8.1 Top pulse (chickpea and lentil)-producing countries (in metric tonnes)

	Chickpea		Lentil		
Country	2010	2011	2010	2011	2012
India	7,480,000	8,220,000	1,031,600	943,800	950,000
Australia	602,000	513,338	140,000	379,659	463,000
Pakistan	561,500	496,000	DNA	DNA	DNA
Turkey	530,634	487,477	447,400	405,952	438,000
Burma	441,493	473,102	DNA	DNA	DNA
Ethiopia	284,640	322,839	80,952	128,009	151,500
Iran	267,768	290,243	100,174	71,808	85,000
United States	87,952	99,881	392,675	214,640	240,490
Canada	128,300	90,800	1,947,100	1,531,900	1,493,620
Mexico	131,895	72,143	DNA	DNA	DNA
Nepal	DNA	DNA	151,757	206,969	208,201
China	DNA	DNA	125,000	150,000	145,000
Syria	DNA	DNA	77,328	112,470	130,229
World	10,897,040	11,497,054	4,686,673	4,386,870	4,522,097

Source: UN Food and Agriculture Organizations
DNA data not available

8.4 Response of PSM Inoculation to Crops

The discovery of P-solubilizing potentials besides other growth-promoting activities (Table 8.3) among P-solubilizers has been one of the most attractive biological traits that have resulted in reducing the dependence on synthetic P fertilizers and consequently protecting soil fertility and environmental safety from chemical toxicity. And therefore, the use of PS bacteria isolated from different soils (Saha and Biswas 2009; Hui et al. 2011; Xiang et al. 2011; Minaxi et al. 2012) belonging largely to the genera pseudomonads (Behbahani 2010; Bholay et al. 2012), bacilli (Erkovan et al. 2010; Sanjotha et al. 2011), rhizobia (Chandra et al. 2007; Marra et al. 2011), and *Azotobacter* (Yi et al. 2008; Audipudi et al. 2012) etc. as an alternative to chemical fertilizer has generated greater interest among agronomists than microbiologists to employ such microbes in practical field application for enhancing the crop production (Kumari et al. 2009; Erkovan et al. 2010; Yu et al. 2011; Gupta et al. 2012) in different agroecological niches (Khan et al. 2007; Vega 2007). However, direct inoculation of free PS bacteria into soil is not easy to maintain the survival of bacterial cells around roots of plants since they are easily susceptible to a variety of environmental variables such as temperature, humidity, and salt stress (Wu et al. 2012). Also, the variable response of PSB inoculation to plant is mainly due to the differences in the quality of inoculants applied under pot/field soils. Considering the vast and varied activities, researchers around the world have either attempted or included the use of single or mixture of this novel group of economically feasible biological materials in agronomic operation for sustainable pulse and cereal production.

Table 8.2 Nutritional value of important legumes

Nutritional value (per 100 g)	Chickpea	Soybean	Green gram	Pea	Lentil	Pigeon pea
Energy (KJ)	686	1,866	1,452	339	1,477	569
Carbohydrates (g)	27.42	30.16	62.62	14.45	60	23.88
Sugars (g)	4.8	7.33	6.6	5.67	2	3.0
Dietary fiber (g)	7.6	9.3	16.3	5.1	31	5.1
Fat (g)	2.59	19.94	1.15	0.4	1	1.64
Protein (g)	8.86	36.49	23.86	5.42	26	7.2
Water (g)	60.21	8.54	–	–	10	–
Vitamin A equiv. (μg)	1	1	–	38	–	–
Thiamine (vit. B_1) (mg)	0.116	0.874	0.621	0.266	0.87	0.4
Riboflavin (vit. B_2) (mg)	0.063	0.87	0.233	0.132	0.211	0.17
Niacin (vit. B_3) (mg)	0.526	1.623	2.251	2.09	2.60	2.2
Pantothenic acid (B_5) (mg)	0.286	0.793	1.91	–	2.12	0.68
Vitamin B_6 (mg)	0.139	0.377	0.382	0.169	0.54	0.068
Folate (vit. B_9) (μg)	172	375	625	65	479	173
Vitamin B_{12} (μg)	0	–	–	–	–	–
Vitamin C (mg)	1.3	6.0	4.8	40	4.4	39
Vitamin E (mg)	0.35	0.85	0.51	0.13	–	0.39
Vitamin K (μg)	4	47	9	24.8	–	24
Calcium (mg)	49	277	132	25	56	42
Iron (mg)	2.89	15.7	6.74	1.47	7.54	1.6
Magnesium (mg)	48	280	189	33	122	68
Phosphorus (mg)	168	704	367	108	451	127
Potassium (mg)	291	1,797	1,246	244	955	552
Sodium (mg)	7	2	–	05	6	5.0
Zinc (mg)	1.53	4.89	2.68	1.25	4.78	1.04
Manganese (mg)	–	2.517	1.035	0.41	–	0.574

Source: USDA Nutrient Database (http://en.wikipedia.org)

8.4.1 *Phosphate Solubilizers-Legume Interactions: Current Perspective*

The sole or composite application of PS bacteria (Table 8.4) for raising legume production has received considerable attention worldwide (Fernández et al. 2007; Comakli and Dasci 2009; Bianco and Defez 2010) and is discussed and considered in the following section.

8.4.1.1 Impact of Monoculture of PSB on Legume Improvement

The constantly increasing costs of phosphatic fertilizers have generated interest among farming communities toward using microbial phosphatic fertilizers (PSM) for enhancing the legume production (Jha et al. 2011). Considering the importance of PSM in legume improvement, Kannapiran and Sri Ramkumar (2011) assayed the single inoculation effects of PS bacteria *Pseudomonas putida* (Plate 8.1a) and

Table 8.3 Growth-promoting substances released by phosphate-solubilizing bacteria

Phosphate-solubilizing bacteria	Plant growth-promoting traits	References
Advenella sp. and *Cellulosimicrobium* sp.	Siderophore, IAA, ammonia, and antifungal activity	Singh et al. (2014)
Pseudomonas aeruginosa, P. Putida, Pseudomonas cepacia	IAA, HCN, and siderophore	Deshwal and Kumar (2013)
Pseudomonas fluorescens and *Bacillus subtilis*	IAA, gibberellic acid, siderophore	Sivasakthi et al. (2013)
Pantoea agglomerans and *Burkholderia anthina*	IAA, ammonia, siderophore, and HCN	Walpola and Yoon (2013)
Pseudomonas fluorescens	Siderophore, auxin, and ACC deaminase	Alishahi et al. (2013)
Pseudomonas aeruginosa and *Bacillus* sp.	IAA	Kannapiran and Sri Ramkumar (2011)
Pseudomonas aeruginosa and *Bacillus* sp.	IAA, siderophore, antifungal activity	Panhwar et al. (2012)
Enterobacter aerogenes sp. (NII-0907 and NII-0929), *E. cloacae* subsp. *cloacae* sp. (NII-0931), *E. asburiae* sp. (NII-0934)	IAA, HCN	Deepa et al. (2010)
Acinetobacter rhizosphaerae	IAA, siderophores, ACC deaminase	Gulati et al. (2009)
Pseudomonas sp.	ACC deaminase, IAA, siderophore	Poonguzhali et al. (2008)
Bacillus subtilis	IAA, siderophore, antifungal activity	Singh et al. (2008)
Serratia marcescens	IAA, siderophore, HCN	Selvakumar et al. (2008)
Pseudomonas fluorescens	ACC deaminase	Shaharoona et al. (2008)
Acinetobacter sp., *Pseudomonas* sp.	ACC deaminase, IAA, antifungal activity, N_2-fixation	Indiragandhi et al. (2008)
Enterobacter sp.	ACC deaminase, IAA, siderophore	Kumar et al. (2008)
Burkholderia	ACC deaminase, IAA, siderophore, heavy metal solubilization	Jiang et al. (2008)
Pseudomonas jessenii	ACC deaminase, IAA, siderophore, heavy metal solubilization	Rajkumar and Freitas (2008)
Pseudomonas aeruginosa	ACC deaminase, IAA, siderophore	Ganesan (2008)
Pseudomonas sp.	ACC deaminase, IAA, siderophore, heavy metal solubilization	Rajkumar and Freitas (2008)
Azotobacter sp., *Mesorhizobium* sp., *Pseudomonas* sp., *Bacillus* sp.	IAA, siderophore, antifungal activity, ammonia production, HCN	Ahmad et al. (2008)

(continued)

Table 8.3 (continued)

Phosphate-solubilizing bacteria	Plant growth-promoting traits	References
Fluorescent Pseudomonas	IAA, siderophores, HCN, antifungal activity	Shweta et al. (2008)
Pseudomonas vancouverensis	IAA, HCN, siderophore, antifungal activity	Mishra et al. (2008)
Bacillus spp.	IAA, siderophores, ammonia production, HCN, chromium reduction, metal solubilization	Wani et al. (2007a, b)
Pseudomonas PSB5, *Bacillus* PSB9	IAA and siderophores	Wani et al. (2007c)
Klebsiella oxytoca	IAA, nitrogenase activity	Jha and Kumar (2007)
Bacillus subtilis	IAA	Zaidi et al. (2006)

Bacillus pumilus (Plate 8.1b) and N_2-fixing *Azotobacter* sp. (Plate 8.1c) on the growth, chlorophyll content, and P and N content of black gram plants grown in green house and observed a variable impact on the measured parameters. The N contents in roots and shoots of inoculated black gram plants differed considerably among the treatments. The variation in the performance of inoculated plants was attributed to the release of differing amounts of siderophore, HCN, and ammonia by the inoculant bacterial strains. Moreover, the substantial production of IAA by *A. chroococcum* (23.6 μg ml^{-1}) and *A. beijerinckii* (17.6 μg ml^{-1}) and by the P-solubilizers *P. aeruginosa* (26.5 μg ml^{-1}) and *Bacillus* sp. (19.8 μg ml^{-1}) might also have accounted for considerable increase in the black gram plants. Similarly, PS-fluorescent pseudomonads isolated from the groundnut rhizosphere, when used as microbial P biofertilizer, enhanced the groundnut germination by 30 % while it increased the grain yield by 77 %. Further, the PS culture also showed antagonistic activity against plant pathogen *Macrophomina phaseolina*. The increase in yield of groundnut following *Pseudomonas* application, therefore, suggested that this strain has two basic traits: (1) biocontrol activity against phytopathogen and (2) that it provided the available form of P and consequently enhanced the yield of groundnut (Shweta et al. 2008). Dey et al. (2004) in yet another study observed a significantly higher pod yields, haulm yield, and nodule dry weight in *P. fluorescens*-inoculated peanut plants compared to those recorded for uninoculated plants grown in pots and field trials. The seed bacterization also resulted in higher N and P contents in soil. In addition, the pod yields were increased by 23–26 %; other plant characteristics such as root length, pod number, 100-kernel mass, shelling out-turns, and nodule numbers were also increased following bacterial inoculation. Seed treatment with *P. fluorescens* also depressed the incidence of soil-borne fungal diseases, like collar rot and charcoal rot of peanut (Bhatia et al. 2008) caused by *A. niger*. While considering the overall improvement in inoculated peanut, it was inferred that the increase was due to (1) the synthesis of IAA, ACC deaminase, and siderophore and

Table 8.4 Examples of sole and composite inoculation effects of phosphate-solubilizing bacteria on biological and chemical characteristics of certain legume and cereal crops

Phosphate solubilizers	Legumes	Plant attributes	References
Bradyrhizobium japonicum with PSB	Soybean	Plant biomass, grains per plant, and grain yield increased	Shiri-Janagard et al. (2012)
Bacillus sp. with *Rhizobium*	Mash bean	Co-inoculation improved pod and straw yield; increased the root length, root mass, and number of nodule and mass; enhanced the nutrient concentration in mash plant and grains	Qureshi et al. (2012)
Enterobacter sp.	Cowpea	Root and shoot length, dry biomass, seedling length	Deepa et al. (2010)
Citrobacter, Pantoea, Klebsiella, and *Enterobacter*	Pigeon pea	Shoot P content, dry shoot/root ratio, dry weight	Patel et al. (2010)
Bacillus sp.	Chickpea	Root and shoot length, nodulation, dry weight	Wani and Khan (2010)
Pontibacter niistensis	Cowpea	Root and shoot weight, dry weight, seedling growth	Dastager et al. (2011)
P. fluorescens with *Burkholderia cepcia, Aeromonas vaga*	Mung bean	Root and shoot length, dry weight, leaf area, photosynthetic yield, P content in leaf	Jha et al. (2011)
Bacillus, Pseudomonas	Alfalfa	Root and shoot dry weight, root length, N content in shoot	Guiñazú et al. (2010)
Enterobacterium with *Sinorhizobium meliloti*	Chickpea, pea	Increased P uptake and biomass	Hynes et al. (2008)
P. putida and *B. japonicum*	Soybean	Root and shoot dry weight, nodulation	Rosas et al. (2006)
P. putida	Alfalfa	Root and shoot dry weight, nodulation	Rosas et al. (2006)
	Cereals		
P. fluorescens with *S. meliloti*	Wheat	Increased dry matter accumulation in roots and shoots, shoot length, and P uptake	Schoebitz et al. (2013)
Unidentified PSB with farmyard manure	Maize	Phosphate-solubilizing bacteria along with FYM increased the yield attributes and grain and stover yields. PSB inoculation along with FYM enhanced the content of NPK in grain and stover, and their uptake by grain and stover. The inoculation of PSB along with FYM also enhanced the available NPK	Taipodia and Yubbey (2013)

(continued)

Table 8.4 (continued)

Phosphate solubilizers	Legumes	Plant attributes	References
		content in soil at harvest, and protein and carbohydrates in grains.	
Unidentified PSB with triple superphosphate	Rice	Plant height and number of tillers per plant were significantly increased; level of mineral nutrients in rice plant tissues was increased	Sarkar et al. (2012)
P. agglomerans NBRISRM	Maize, Chickpea	Shoot length, leaves, seed, N,P, and K uptake	Mishra et al. (2011)
P. fluorescens, P. Putida	Wheat	Plant height, tillers, number of grains/spike, 1,000-grain weight, grain and straw yield, N, P, and K uptake	Zabihi et al. (2011)
Paenibacillus alvei, Bacillus simplex, Bacillus cereus	Wheat	Shoot and root biomass and total root length	Hassen and Labuschagne (2010)
Pseudomonas sp.	Wheat	Improved grain yield, shoot weight, and plant height	Afzal and Bano (2008)

(2) antifungal activity expressed by the test bacterial strain against various fungal pathogens. Similar increase in the biological and chemical characteristics and quality of pea and chickpea under both controlled conditions and field environment following P-solubilizing, auxin, ACC deaminase, ammonia, and siderophore-producing strains of *Acinetobacter rhizosphaerae* and *Mesorhizobium mediterraneum* (PECA21) has been reported (Gull et al. 2004; Gulati et al. 2009). Likewise, inoculation of green gram seeds with PSB demonstrated an extensive nodulation, and increased shoot dry matter and total dry matter, P content, and P uptake in green gram plants 45 days after sowing relative either to rock phosphate (RP) or single super phosphate (SSP) application (Vikram and Hamzehzarghani 2008).

8.4.1.2 Synergistic Effects of Phosphate-Solubilizing Bacteria with Other Soil Microflora

Phosphate-solubilizing microorganisms while inhabiting rhizosphere are reported to exhibit many fold relationship with other soil microflora especially the PGPR and enhance the overall performance of legumes additively or synergistically both in the presence and absence of chemically synthesized phosphatic fertilizers in different production systems (Plate 8.2). Phosphate-solubilizing organisms in addition to supplying P to plants can also enhance plant growth by improving the efficiency of BNF, by accelerating the availability of other trace elements, and by production of phytohormones (Khan et al. 2009, 2013). Accordingly, increase in the yield of

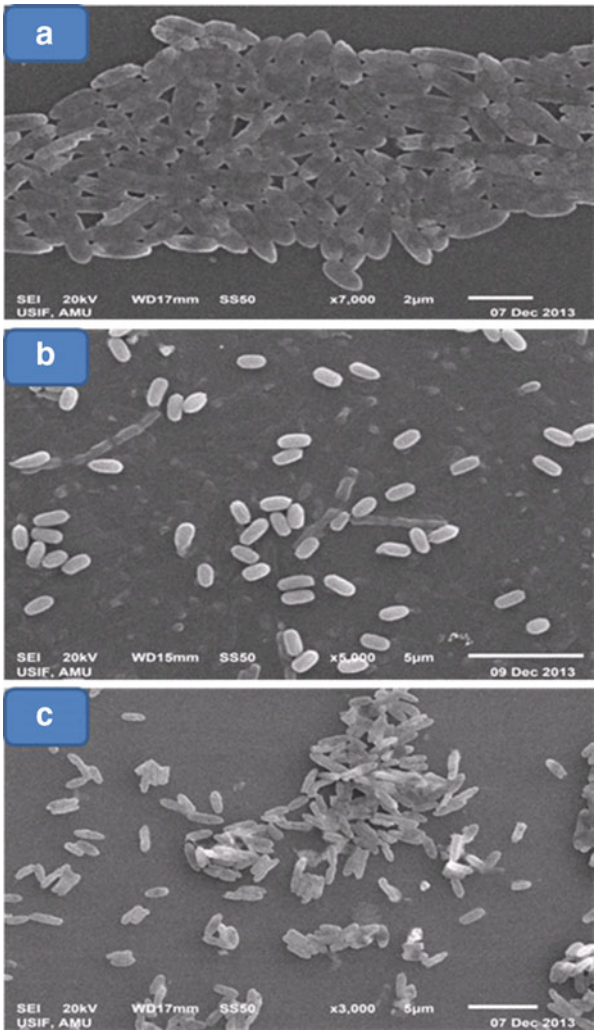

Plate 8.1 Scanning electron microscopy of bacterial strains: (**a**) *P. putida* strain PSE3, (**b**) *B. pumilus* strain ES3, (**c**) *Azotobacter* strain AZ19

various legumes has been observed following seed or soil inoculation of PS organisms with other PGPR (Messele and Pant 2012), fungi (Mittal et al. 2008; Jain et al. 2012), and AM fungus (Zaidi et al. 2003; Zaidi and Khan 2006; Khan and Zaidi 2007). In a study, Walpola and Yoon (2013) observed that the PS bacteria *Pantoea agglomerans* and *Burkholderia anthina* under greenhouse conditions remarkably enhanced shoot and root length, shoot and root dry matter, and P uptake of green gram plants. Growth of the inoculated plants improved further by adding TCP with PSB inoculation. Moreover, the dual inoculation of both *P. agglomerans* and *B. anthina* in the presence of TCP exhibited the highest increase in growth and P uptake by green gram plants suggesting that these bacterial cultures together could act as a promising alternative to minimize the P problem in agricultural soils. Shiri-Janagard et al. (2012), in order to investigate the effects of biological and

chemical fertilizers on soybean yield, carried out an experiment at the University of Tabriz Research Farm, Iran. Biological fertilizer consisting of (1) non-inoculated (NI), (2) PS bacteria (PSB), (3) *Bradyrhizobium japonicum* (BJ), and (4) *B. japonicum* + PSB (BJ + PSB) was in the presence of absence of varying levels of chemical fertilizers: (1) control (0 %), (2) 16.5 kg/ha^{-1} urea + 49.5 kg/ha^{-1} triple superphosphate (33 %), (3) 33.3 kg/ha^{-1} urea + 99 kg/ha^{-1} triple superphosphate (66 %), and (4) 50 kg/ha^{-1} urea + 150 kg/ha^{-1} triple superphosphate (100 %). Grain weight was significantly enhanced following BJ and BJ + PSB application over NI and PSB. Also, grains per pod were more in bio-inoculated plants. Plant biomass, grains per plant, and grain yield per plant in NI- and PSB-inoculated plants were enhanced with increasing chemical fertilizers. The highest plant biomass, grains per plant, and grain yield were recorded for treatment having 16.5 kg/ha^{-1} urea + 49.5 kg/ha^{-1} triple superphosphate fertilizer× BJ + PSB. Inoculation of seeds by BJ and BJ + PSB without chemical fertilizer application had equal or higher performance than non-inoculated seeds with 100 % chemical fertilizer. Therefore, soybean seed inoculation by *B. japonicum* singly or in combination with PSB not only reduced the use of chemical fertilizer but also highlighted the role of synergistic microbes in improving the yield and yield components of soybean. In a follow-up study, Suri and Choudhary (2013) determined the interactive effects of soybean-AM fungi (*Glomus mosseae* and *G. intraradices*)-PS bacterium (*P. striata*) on the productivity, nutrient dynamics, and root colonization in soybean grown in P-deficient Himalayan acidic alfisol. Sole application of PSB or AM fungus considerably enhanced VA-mycorrhizal root colonization and root weight besides crop productivity and nutrient uptake over control. The co-inoculation of PSB and AM fungus also showed a similar stimulatory effect on mycorrhizal root colonization and root weight relative to control. Dual inoculation of *G. mosseae* and *P. striata* significantly increased the grain and straw yield besides grain protein content suggesting a strong synergism between the AM fungus and PS bacterium. The impact of co-culture of either *G. intraradices* or *G. Mosseae* with PSB in the presence of 75 % P_2O_5 on crop productivity, nutrient content, nutrient uptake, and soil fertility was at par with sole application of 100 % P_2O_5, but the root colonization and root weight were maximum at flowering stage indicating that *Glycine–Glomus*–PSB interactions in combination with 75 % P_2O_5 dose based on STCR precision model could lead to the reduction in fertilizer application by about one-fourth without disturbing the soybean productivity and soil fertility in a Himalayan acidic alfisol region.

Guiñazú et al. (2010) in a similar study evaluated the effect of single or mixed cultures of nodule bacterium (*S. meliloti* B399) and PS bacterium (*Bacillus* sp. and *Pseudomonas* sp.) on N-fixing efficiency of alfalfa plants and observed that the sole culture of *Pseudomonas* sp. FM7d significantly enhanced the dry matter accumulation in roots and shoots, length of plants and surface area of roots, and symbiotic attributes of alfalfa plants. On the contrary, the mixture of *S. meliloti* B399 and *Bacillus* sp. M7c further increased the measured parameters suggesting a synergistic/additive effect of the two phenotypically different bacterial genera. Likewise, the tripartite combination of *Rhizobium*, PGPR, and PSB has been found to significantly increase nodulation and grain yield relative to uninoculated mung bean

Plate 8.2 Sole and composite impact of microbial inoculation on chickpea grown in soils treated with phosphate fertilizer where T1 indicates control; T2, urea (30 kg/ha); T3, DAP (80 kg/ha); T4, *B. pumilus*; T5, *M. ciceri*; T6, urea with *B. pumilus*; T7, DAP with *M. ciceri*; T8, *B. pumilus* with *M. ciceri*; and T9, urea with DAP

plants. While comparing the impact of all treatments, the mixture of *Rhizobium*, PGPR, and PSB had maximum positive effect and profoundly enhanced the symbiotic properties and yield of mung bean grains (Bansal 2009). In a follow-up study, Dutta and Bandyopadhyay (2009), while conducting a field experiment during the winter seasons, observed that P and biofertilizers, phosphobacterin (*P. striata*) and

co-inoculation of *Rhizobium* with phosphobacterin, when applied together enhanced the early vegetative growth, symbiotic properties like nodule production and excessive synthesis of leg hemoglobin in nodules, nitrogenase activity (NA), and yield components such as seed yields, harvest index (HI), and P uptake by chickpea cultivar Mahamaya-2 plants grown in entisol (laterite soil) under rainfed conditions. Of the various combination treatments, seed inoculation of phosphobacterin with *Rhizobium* was significantly better than other treatments. When P (26.2 kg/ha) was also added to the mixture of *Rhizobium* and phosphobacterin, the biological and chemical properties of chickpeas were further improved relative to other levels of P used with biofertilizer. In yet other study, Messele and Pant (2012) conducted a field experiment to assess the inoculation effects of *Sinorhizobium ciceri* and PS bacteria on the performance of chickpea in Shoa Robit area, Ethiopia, using three levels of NP fertilizer and four levels of inoculants. The sole application of *S. ciceri* increased dry matter yield (DMY) by 156.58 and nodule numbers (NN) by 117.96 % relative to control. In the presence of 18/20 kg N (urea)/P (DCB) ha^{-1}, *S. ciceri* enhanced the DMY, NN, and nodule dry weight (NDW) by 149.6, 143.6, and 200 %, respectively, over uninoculated control suggesting the role of P in nodule tissue development. Similarly, *Pseudomonas* sp. in the presence of 18/20 kg NP ha^{-1} increased NDW, NN, nodule volume (NV), and seed yield (SY) by 240, 188.52, 151.81, and 142.95 %, respectively, compared to control indicating the P-solubilizing efficacy of bacteria in the presence of DCB. In contrast, the co-inoculation of *S. ciceri* and *Pseudomonas* sp. with 18/20 kg NP ha^{-1} dramatically enhanced the NN, NDW,NV, and DMY by 208.8, 220, 221.24, and 172.09 % over uninoculated control at mid-flowering stage of chickpea.

Apart from forming relationship with PGPR, PSM has also been found to establish associative/symbiotic association with arbuscular mycorrhizal (AM)-fungi (Wang et al. 2011) and facilitate plant growth (Osorio and Habte 2013) including legumes (Souchie et al. 2010). Mycorrhizal interactions are ubiquitous and improve plant fitness and soil quality by (1) increasing the nutrient uptake from soil; (2) facilitating uptake of relatively immobile trace elements such as, Zn, Cu, and Fe; (3) increasing protection against biotic and abiotic stresses; and (4) forming soil aggregate (Turnau et al. 2006; Lingua et al. 2008; Garg and Chandel 2010). Conclusively, the interaction of PS organisms with AM fungi is likely to have larger impact on plant health than the sole application of any organism in agricultural practices. The interactive effect of PS organisms with AM fungi on legume development and yield is discussed briefly in the following section.

Souchie et al. (2010) in a study evaluated the synergism between several PS fungi and AM fungi to improve clover growth in the presence of Araxá apatite. The combination of *A. niger* and PSF 21 in the presence of AM fungi showed greatest clover growth; however, *A. niger*, PSF 7, and PSF 21 were found most effective fungal cultures in increasing clover growth when used with AM fungi. Due to greater mycorrhizal colonization, there was a maximum increase in clover plants inoculated with PSF isolates. Of these, isolate PSF 7 was found as the best-performing fungal culture in terms of mycorrhizal establishment and rhizobia symbiosis. Toro et al. (2008) in an experiment tested the efficacy of mixed

microbial cultures: a wild-type (WT) *R. meliloti* strain, its genetically modified (GM) derivative, the AM fungus *G. mosseae* (Nicol and Gerd) Gerd and Trappe, and a PSB *Enterobacter* sp. and rock phosphate (RP), on N and P accumulation in alfalfa plants. Microbial cultures, in general, survived well inside root tissues and colonized alfalfa rhizosphere and did not show any antagonism toward each other. The population of PSB is stimulated due to both AM colonization, RP application, and GM *Rhizobium* inoculation leading to a substantial increase in N and P acquisition by alfalfa plants. Even though the *Enterobacter* application showed no observable effects on N or P accumulation in soil treated with RP, it had an obvious effect on N and P in the non-RP-amended control. In addition, ^{15}N:^{14}N ratio in plant shoots indicated enhanced N_2 fixation rates in *Rhizobium*-inoculated AM plants, compared to those obtained by the same *Rhizobium* strain in non-mycorrhizal plants. Regardless of the *Rhizobium* strain and of whether or not RP was added, AM-inoculated plants showed a lower specific activity (^{32}P:^{31}P) than did their comparable non-mycorrhizal controls suggesting that the plant was using otherwise unavailable P sources. The P-solubilizing, AM-associated, microbiota could in fact release P ions, either from the added RP or from the indigenous "less-available" P. Additionally, the proportion of plant P derived either from the labeled soil P (labile P pool) or from RP was similar for AM-inoculated and non-mycorrhizal controls (without *Enterobacter* inoculation) for each *Rhizobium* strain, but the total P uptake, regardless of the P source, was far higher in AM plants which could probably be due to P mobilization by AM fungi. In other investigation, Mehdi et al. (2006) assessed the responses of lentil to co-culture of P-solubilizing rhizobia and AM fungi in a calcareous soil with high-pH and low available P and N. The effects of AM fungi (*Glomus mosseae* and *G. intraradices*), rhizobial strain (*R. leguminosarum* bv. Viciae) and mixture of rhizobial inoculant and PS *M. ciceri*, and P fertilizers (superphosphate and RP) were highly significant and enhanced the dry matter accumulation in shoots, seed yield, P and N contents, and AM colonization. The PS rhizobial strain had a more pronounced favorable effect on lentil growth and nutrient uptake than the strain without this ability. The P-uptake efficiency was increased when P fertilizers were applied along with AM fungi and/or P-solubilizing rhizobial strains.

8.4.2 Cereal Production and Its Nutritive Value

Cereal crops are grown largely for the edible components of its grain which contains the endosperm, germ, and bran. Cereal is grown in many parts of the world (Table 8.5) and serves as a major energy source worldwide than any other type of crops and hence are called staple crops. They also provide some useful and valuable food nutrients even in their natural form as in the form of whole grain and have been found as a rich source of vitamins, minerals, carbohydrates, fats, oils, and protein (Table 8.6). In some developing countries, rice, wheat, millet, or maize form an important component of dietary system and a sound means of daily

Table 8.5 Top staple foods produced worldwide

	Worldwide production (millions (10^6) of metric tons)		
Grain	2012	2011	2010
Maize (corn)	87	888	85
	2		1
Wheat	671	699	650
Rice	720	725	703
Barley	133	133	124
Sorghum	57	58	60
Millet	30	27	33
Oats	21	22	20
Rye	15	13	12
Triticale	14	13	14
Buckwheat	2.3	2.3	1.4
Fonio	0.59	0.59	0.57
Quinoa	0.08	0.08	0.08

sustenance, while in developed nations, cereal consumption is moderate and varied but still substantial.

8.4.2.1 Impact of Sole/Composite Application of PS Microbes on Cereal Crops

The use of PSB in agricultural practices dates back to the 1950s when some Russian and European scientists applied *Megatierium viphosphateum*, which later on was identified as *Bacillus megatierium* var. *phosphaticum*. The preparation of this bacterium was subsequently called as phosphobacterin (Cooper 1959; Menkina 1963) and, when this was used, increased crop yields from 0 to 70 % in Soviet soils. However, similar experiments conducted in the United States failed to produce any significant effect (Smith et al. 1961). Despite conflicting reports on the performance of PSB in variable agroecosystem against a multitude of crops (Yarzábal 2010) including cereals (Yazdani et al. 2009; Abdel-Ghany et al. 2010; Deshwal et al. 2011; Khalimi et al. 2012; Nico et al. 2012), they have since been applied and have shown promising results in some parts of the world (Chesti and Ali 2007; Baig et al. 2011). For example, Saxena et al. (2014) investigated the synergistic effect of an AM fungus, *Glomus etunicatum,* and an indigenous PSB strain, *Burkholderia cepacia* BAM-6, on wheat plants grown in pots containing soil with low available P in order to find a comparable paring of microbial cultures for enhancing wheat production in semiarid regions.

The dual inoculation of AM fungus and PSB augmented growth and yield parameters relative to the sole application of each culture. Wheat yield and N concentration were enhanced by greater than 50 and 90 %, respectively, following the dual inoculation. Percent root colonization by AM fungi and rhizosphere population of PSB was also increased with time in soil. From this study, it was

Table 8.6 Nutritional value of some major staple foods

	Cereal crops		
Nutritional value per 100 g	Maize	Rice	Wheat
Energy (KJ)	1,528	1,528	1,369
Carbohydrates (g)	74	80	71
Sugars (g)	0.64	0.12	0.41
Dietary fiber (g)	7.3	1.3	12.2
Fat (g)	4.74	0.66	1.54
Protein (g)	9.4	7.1	12.6
Water (g)	10	12	13
Vitamin A equiv. (μg)	214	0	9
Thiamine (vit. B_1) (mg)	0.39	0.07	0.30
Riboflavin (vit. B_2) (mg)	0.20	0.05	0.12
Niacin (vit. B_3) (mg)	3.63	1.6	5.46
Pantothenic acid (B_5) (mg)	0.42	1.01	0.95
Vitamin B_6 (mg)	0.62	0.16	0.3
Folate (vit. B_9) (μg)	19	8	38
Vitamin B_{12} (μg)	–	–	–
Vitamin C (mg)	0	0	0
Vitamin E (mg)	0.49	0.11	1.01
Vitamin K (μg)	0.3	0.1	1.9
Calcium (mg)	7	28	29
Iron (mg)	2.71	0.8	3.19
Magnesium (mg)	127	25	126
Phosphorus (mg)	210	115	288
Potassium (mg)	287	115	363
Sodium (mg)	35	5	2
Zinc (mg)	2.21	1.09	2.65
Manganese (mg)	0.49	1.09	3.99

Source: USDA Nutrient Database (http://en.wikipedia.org)

inferred that *B. cepacia* interacted synergistically well with AM fungus leading eventually to increase in growth and nutrient uptake of wheat plants. Therefore, this study shows a great promise for using biofertilizer for wheat crop grown in arid to semiarid regions. Similarly, Yousefi et al. (2011) investigated the interactive effects of PS bacteria and AM fungi on wheat production, changes in biological population, and inorganic P fractions. In this study, the combined application of PS bacteria and AM fungi increased dry matter accumulation in shoots, seed grain spike number, and grain yields by 52, 19, and 26 %, respectively, compared to the control plants. Moreover, P application increased Olsen–P, Ca_2–P, and Ca_8–P% while biological fertilizers reduced the amount of Ca_2–P and Ca_8–P%. Taking into consideration the success achieved at small scales, Akhtar et al. (2013) conducted a field experiment to investigate the effect of *Rhizobium* and *Bacillus*, alone and in combination, on the yield of wheat (var. Sehar 2006). A uniform rate of N (160 kg ha^{-1}) and K (60 kg ha^{-1}) and two levels of P (57 and 114 kg ha^{-1}) were applied as urea, SOP, and SSP, respectively. Number of tillers, spike length (13.50 cm), number of grains, grain yield (6,171 kg ha^{-1}), biomass (17 t ha^{-1}),

grain protein (11.84 %), and 1,000 grain weight (62 g) were higher for wheat plants co-inoculated with *Rhizobium* and *Bacillus*. The increase in grain yield due to co-inoculation of *Rhizobium* and *Bacillus* was calculated as 17.5 % increase over control. In contrast, the single inoculation of *Bacillus* increased grain yield by 7.7 %. Phosphorus uptake by grains (25.29 kg ha^{-1}) was maximum following dual-culture application which was followed by sole application of *Bacillus* inoculation. Available P in soil after wheat harvest was 16.27 mg kg^{-1} which was significantly higher than all other treatments. This field trial clearly demonstrated a dramatic increase in the availability of P following dual application of *Rhizobium* and *Bacillus* sp. which in effect exerted a strong positive effect on the growth and yield of wheat plants. Similarly, in a trial conducted under both pot and field environments, the biomass and total P of winter wheat was significantly increased following sole application of *Phosphobacterium* strain 9320-SD. However, there was no significant difference in height of the test plants (Chen et al. 2006). Similarly, PSB (*Serratia marcescens*) isolated from cold temperature region, capable of synthesizing IAA, HCN, and siderophore, profoundly enhanced the plant biomass and nutrient uptake of wheat seedlings when grown in cold environment (Selvakumar et al. 2008). In a follow-up study, wheat plants inoculated with ACC deaminase-positive *P. fluorescens* and *P. fluorescens* biotype F had higher growth, yield, and nutrient use efficiency, when grown in soil treated simultaneously with varying levels of three major nutrients like N, P, and K (at 0, 25, 50, 75, and 100 % of recommended doses). However, the overall growth of inoculated wheat plants decreased both under pot and field trials with increasing concentration of synthetic fertilizers. Hence, in most of the cases, significant negative linear correlations were recorded between percentage increases in growth and yield parameters of even inoculated wheat plants. The decline in growth and yield of bacterized wheat plants when grown with increasing chemical fertilizers, however, raised certain questions. For example, do the rates of fertilizers greater than the recommended ones have any direct impact on composition and functional activities of bacteria or do excessive rates have any inhibitory effect on plant metabolism? In this context, it is speculated that low fertilizer application causes reduction in the ACC deaminase activity of PS strains and thereby leads to reduction in the synthesis of stress (nutrient)-induced inhibitory levels of ethylene in the roots through ACC hydrolysis into NH_3 and α-ketobutyrate. Based on this finding, it was suggested that pseudomonads could be used in combination with appropriate doses of fertilizers for better plant growth and savings of fertilizers (Shaharoona et al. 2008) as also observed by Kumari et al. (2009) and Maheshwari et al. (2011). Such increase in cereal production following PSB such as *P. fluorescens* 153, *P. fluorescens* 169, *P. putida* 4, and *P. putida* 108 application has been attributed to both PSA of PSB and their ability to synthesize growth-promoting substances (such as ACC deaminase and IAA-like products) in natural soil ecosystem (Zabihi et al. 2011). Interestingly, *P. putida* 108 among the bacterial cultures displayed enhanced P uptake (96 and 80 %) and grain yield (58 and 37 %) in wheat under greenhouse and field conditions, respectively. Even though this finding suggested that *Pseudomonas* sp. could serve as an alternative to expensive P

application in wheat production system, better results can be achieved when a compatible bioinoculant is added as mixture with 50 % (25 kg/ha P_2O_5) P fertilization. In a recent follow-up study, Abbasi et al. (2011) isolated eight PGPR strains and assessed their morphological and cultural characteristics, PSA, and their ability to secrete IAA. Invariably all strains produced IAA (ranging from 5.5 to 31.0 mg/ml), while only four of them showed P-solubilizing traits. Subsequently, strains WPR-32, WPR-42, and WPR-51 grouped under PGPR category were used both as single and co-culture along with two levels (50 and 100 kg N/ha) of N to evaluate their effect against wheat under greenhouse conditions. As expected, application of PGPR resulted in significant increase in plant height (25 %), shoot fresh weight (45 %), and shoot dry weight (86 %), while it was 27, 102, and 76 %, increase in root length, root fresh, and dry weight, respectively, over uninoculated plants. In addition, the number of tillers per plant, 1,000-grain weight, and grain yield were enhanced by 23, 48, and 59 %, respectively, over control. The nutrient (N and P) uptake by plant organs like shoot was increased threefolds, while K uptake was increased by 58 % following PGPR application. However, the growth, yields, and nutrient uptake were increased even further when bacterial cultures were used together with varying levels of N. Apart from the direct effect of PGPR on wheat plants, the concentration of NO^{-3}, N, and available P in soil also increased with PGPR application. Moreover, of the varying treatments, mixed bacterial cultures showed better efficiency than the individual ones suggesting that there is no reason to doubt why application of PGPR with N fertilizer cannot increase N contents and N uptake by plants. Also, the application of PGPR even with low fertilizer rates could be a more viable option for achieving optimum benefits while reducing the dependence on chemical inputs (Kumar et al. 2009). An interactive and positive effect of PSB, N_2 fixer, and AM fungi on plant vigor, nutrient uptake, and yield in wheat plants was observed following composite application of *P. striata* + *A. chroococcum* + *G. fasciculatum*. The available P contents in soil enhanced significantly due to triple inoculation of *A. chroococcum*, *P. striata*, and *G. fasciculatum*. The residual N content of soil, however, did not change appreciably even among the treatments. The density of *A. chroococcum*, PSB, percentage root infection, and spore density of the AM fungus in inoculated treatments increased at 80 days of wheat growth (Zaidi and Khan 2005).

Like the impact of biofertilizers on wheat production, there have also been reports suggesting similar effects on other cereal crops such as rice and maize. For example, Ebrahimi et al. (2014) observed maximum number of tillers, the highest percentage of fertile tillers, the longest panicles (26.29 cm), and the largest number of seeds per panicle when rice seeds were simultaneously inoculated with bacterial species. With respect to seed yield, it was found that the use of a single bacterial species (*P. putida P. fluorescens*) caused a greater increase in seed yield. The two cultures when used together in the presence of 83 kg of mineral P showed the largest positive effect and resulted in the highest seed yield. This was accompanied by the maximum %age of fertile tillers (94.24 %), long panicles (26.09 cm), and a considerable 1,000-seed weight (22.8 g). Vahed et al. (2012) in a similar study assessed the beneficial effects of PS bacteria on rice crop productivity in a field

experiment at the Rice Research Institute of Iran. Results indicated that PSB and PCF had a significant influence on grain yield, biological yield, and grain P uptake, while there was no effect on the straw P uptake and plant heights. While comparing the overall response, biofertilizer application enhanced the grain yield by 1–11 %, while P uptakes by grains were increased by 6–8 % than control. It was concluded from this study that the application of biofertilizer could stimulate growth and consequently increased the grain yield of rice. Similarly, the inoculation of *Burkholderia vietnamiensis* to rice cultivars in two pot and four field trials at different locations in Vietnam showed an enhancement of 33, 57, 30, and 13 % in shoot weight, root weight, leaf area, and number of tillers/hill, respectively, compared to non-inoculated plants. In other study, strain of *Rhodobacter capsulatus* significantly increased the plant dry weight, number of productive tillers, and grain and straw yields of rice var. Giza 176, grown in pot treated with different levels of N fertilizer compared to non-inoculated plants (Elbadry et al. 1999). The results of this study concluded that N fertilizer could be saved up to 50 % while applying bacterial fertilizers. Similarly, an increase of 41, 12, 11.2–20, and 18.7 % in root weight, straw yield, grain yield, and total biomass, respectively, due to PGPR inoculation over non-inoculated rice is reported (Mehnaz et al. 1998; Sherchand 2000). The liquid culture (for pot experiments) or carrier-based preparation (for field trials) of three bacterial species, such as *B. megaterium*, *B. subtilis,* and *P. corrugata*, isolated from temperate locations in the Indian Himalayan region and exhibiting phosphate-solubilizing activity (PSA) in the order *P. corrugata* > *B. megaterium* > *B. subtilis*, when tested, caused a dramatic increase in overall performance of rice. While comparing the effect of three cultures, *B. subtilis* had the most promising effect and increased the grain yield by 1.7- and 1.6-fold in pot and field trials, respectively (Trivedi et al. 2007). In a recent study, Rajapaksha et al. (2011) conducted experiments under both pot and field environments to assess the substitutability of triple superphosphate (TSP) by a P fertilizer mixture (PFM) involving TSP, RP, and PSB inoculants for wetland rice. For these studies, 6 single and 2 dual inoculants were formulated with *Enterobacter gergoviae* and 5 *Bacillus* species. In pot trials, the mixture of *E. gergoviae* and *B. mycoides* and the sole application of *B. subtilis* enhanced yields by 32 and 25 %, respectively, relative to single application of TSP. The results observed in pot trials were validated under field environment where dual culture of *E. gergoviae* with *B. subtilis* and *E. gergoviae* with *B. pumilus* augmented grain yield by 22–27 % compared to TSP application alone (574 g^{-2}). Overall, it was suggested that about 50 % of TSP could be saved when RP is applied with *E. gergoviae*, *B. pumilus*, and *B. subtilis*, as seed inoculant for raising the productivity of rice both under pot and field conditions.

Similar variable effects of PSB on other cereals used either alone or in combination with other chemical fertilizers have been reported (Panhwar et al. 2011; Yazdani et al. 2011). For example, like wheat and cereals, there has also been a substantial increase in the biomass of maize plants inoculated with fluorescent *Pseudomonas* (Vyas and Gulati 2009) and *S. marcescens* (EB 67) and *Pseudomonas* sp. (CDB 35) (Hameeda et al. 2008). In this experiment, strain EB 67 enhanced

the dry matter accumulation by 99 %, while it was 94 % by strain CDB 35. Grain yields of inoculated maize increased by 85 % and 64 %, following EB 67 and CDB 35 application, respectively. When applied as mixture with arbuscular mycorrhizal (AM) fungi *G. intraradices*, the PSB *Pseudomonas fluorescens* had a positive impact on plant growth, nutrient uptake, grain yield, and yield components in maize plants. Composite inoculation of the two cultures significantly increased grain yield, yield components, harvest index, grain N and P, soil available P, and root colonization percentage under water stress conditions. However, some of the assayed characteristics under well-watered conditions were nonsignificantly higher in chemical fertilizer treatment compared to those observed for dual inoculation treatments. However, the effect of sole application of *P. fluorescens* (Pf) was poor relative to the composite application of AM fungus with PSB or single application of AM fungi. The measured parameters of inoculated plants were in general higher than uninoculated plants under water-deficit stress conditions. In addition, the characteristics determined for co-inoculated plants grown under severe water-stressed conditions were significantly lower than co-inoculated plants grown under well-watered and moderate-stressed conditions. This finding suggested that PSB can interact positively with other organism like AM fungi as observed in this study and can be used to facilitate plant growth and P uptake by maize plants, leading to plant tolerance improving under water-deficit stress conditions (Ehteshami et al. 2007). In another study, Yazdani et al. (2009) investigated the effect of PS bacteria such as *A. coroocoocum*, *A. brasilense*, *P. putida*, and *B. lentus* on yield and growth components of *Zea mays* where they observed an increase in row number, ear weight, grain number/year, grain yield, biological yield, and harvest index relative to control.

8.5 Conclusion

Nitrogen and phosphorus are the two essential nutrients for plant growth and development. The extensive use of chemical fertilizers to provide these nutrients in agriculture is currently under debate due to environmental concern, and questions are raised regarding the consumer's health. Recent advancements in the field of biofertilizers offer an opportunity to environmentally friendly and sustainable agricultural practices to reduce dependence on chemical fertilizers and thereby decrease adverse environmental effects. Phosphate-solubilizing bacteria in association with N_2 fixers and AM fungi can lead to increased legume growth through a range of mechanisms which could be of great practical value in sustainable, low-input agricultural cropping systems that rely on biological processes to maintain soil fertility and plant health. Although there are numerous reports highlighting interactions among P-solubilizers, N_2 fixers, and mycorrhizal fungi, the underlying mechanisms behind these associations are in general not conclusive. Moreover, the development of effective microbial inoculants for raising the productivity of legumes remains a major scientific challenge. And hence, functional properties of

interacting microbes together with the development of suitable microbial pairing still require further experimental confirmation in order to achieve optimum benefits of such natural resources. Future research should therefore strive hard toward an improved understanding of the functional mechanisms behind such microbial interactions, so that compatible organisms could be identified and applied as effective inoculants within sustainable legume production systems.

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Chapter 9
Phosphate-Solubilizing Bacteria Improves Nutrient Uptake in Aerobic Rice

Radziah Othman and Qurban Ali Panhwar

Abstract Phosphate-solubilizing bacteria (PSB) are frequently used in agriculture as plant growth promoters because they provide soluble P to growing plants by solubilizing complex soil inorganic phosphates like Al-P, Fe-P, and Ca-P. Several PSB strains isolated from local aerobic rice are able to solubilize P from insoluble P through production of organic acids, for example, oxalic, malic, succinic, and propionic acids. Hence, the application of PSB plays a vital role in supplying P to growing plants. The application of PSB strains in this study solubilized higher P from the soil and significantly enhanced plant uptake in aerobic rice. Besides possessing P-solubilizing activity, PSB has greater potential to produce phytohormones, for example, indoleacetic acid, and enzymes like phosphatase and phytases. The continuous supply of soluble P to soil P pool and phytohormones in the root environment have resulted in the increased P uptake and consequently improved the growth of aerobic rice. The impact of PSB on aerobic rice is highlighted in this chapter.

Keywords Phosphate-solubilizing bacteria • Aerobic rice • Phosphatase enzymes • Organic acids • Phytohormones • P solubilization

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9.1 Introduction

Rice (*Oryza sativa*) is mostly cultivated under flooded conditions and consumes up to 43 % of the world's irrigation resources (Bouman et al. 2007). Water scarcity is becoming a major problem for agriculture, and it is expected that by 2025, 15–20 million ha of irrigated rice will suffer some degree of water scarcity (Tuong and Bouman 2003). The aerobic rice is a water-saving rice system in which potentially high yielding and fertilizer-responsive adapted rice varieties are grown without standing water. Aerobic rice requires the same amount of nutrients as flooded rice, but there is a problem of phosphorus (P) availability, due to its rapid fixation/immobilization with other soil elements (Goldstein 1986). And hence, P becomes unavailable to plants, and it is estimated that about 70–90 % of externally applied phosphatic fertilizers become fixed in soil (Holford 1997). To obviate this, microorganisms, especially phosphate-solubilizing bacteria (PSB) and arbuscular mycorrhizal (AM) fungi, have been found to have the ability to solubilize P in soil and could reduce fertilizers inputs (Khan et al. 2007). As a result, PSB increases P availability to plants and fulfills the metabolic demands of plant P (Panhwar et al. 2011; Tao et al. 2008). A number of mechanisms involving organic acids (solubilization) and enzyme production (mineralization), the release of H^+, chelation, and respiratory H_2CO_3 production are the documented evidence for P transformation in soils (Khan et al. 2010). Of these, production of organic acids is the main mechanism used to mineralize inorganic P (Khan et al. 2009; Rodríguez et al. 2004). The organic forms of P in contrast are mineralized into inorganic P by some of the enzymes, for example, phosphatase and phytase. Different levels of microbial phosphatase activity have been observed in various types of soils (Kucharski et al. 1996). In addition, enzymes perform a vital role in P release and simultaneously improve crop yields (Wyszkowska and Wyszkowski 2010). Phytases are known to hydrolyze phytates to a series of lower phosphate esters of myoinositol and phosphate which in turn contributes hugely to plant nutrient cycle.

In tropical soils, poor availability of soluble P is a common problem because most of the soil P remains as a fraction of Fe or Al-P. The cheap source of phosphate rock (PR) is relatively less soluble; nevertheless, it has been observed that the bioavailability of PR can be increased by applying PSB (Zapata and Axmann 1995). Here, PSB plays a significant role in solubilizing fixed soil P leading to greater availability of P to plants and, concomitantly, the larger increase in crop yields (Gull et al. 2004). Release of P by PSB from insoluble and fixed/adsorbed forms is, therefore, an important aspect of P availability in soils. Hence, the use of PSB as inoculants simultaneously increases P uptake by the plant and crop yields.

9.2 Aerobic Rice

Aerobic rice requires the same amount of nutrients as flooded rice, but in the former case, there is a problem of P availability, due to its fixation with other elements (Goldstein 1986). In acidic soils (pH < 6.0), most crops suffer from P deficiency due to the ability of soil P to form complexes with Al and Fe and become insoluble under aerobic conditions (Sanchez and Uehara 1980). There is insufficient information available on the cultivation of aerobic rice especially in terms of its nutrient management. On the other hand, there are several reports where potential PSB has been used for the management of P-deficient soils (Panhwar et al. 2012; Vazquez et al. 2000). The use of these beneficial bacteria enhances the sustainable crop production and reduces the dependency on costly imported phosphatic fertilizers. Although several findings are available on the beneficial effects of PSB in many upland crops, there is still inadequate information available on the use of PSB as vital agents for P fertilizer management (especially phosphate rock) in aerobic rice. Hence, the present study was focused on identifying and selecting some biologically efficient PSB strains for improving the uptake of P and simultaneously enhancing the growth of aerobic rice.

9.2.1 Water Requirement of Aerobic Rice

Aerobic rice reduces total water use by 27–51 % and improves water productivity by 32–88 % and can decrease water loss due to seepage, percolation, and evaporation (Bouman et al. 2005). Previous studies in the Philippines and northern China have shown that aerobic rice required 30–50 % less water as compared to flooded systems with 20–30 % lower yields (4.7–5.3 tons ha^{-1}) than Wetland rice (Bouman et al. 2006). Due to this, the cultivation of aerobic rice is increasing very rapidly in both temperate and tropical regions in China (Wang et al. 2002).

9.2.2 Nutrient Management in Aerobic Rice

Aerobic rice can be grown in non-flooding soils and can tolerate flooding, thus it is ideally suited for both flood-prone and drought-prone areas. In aerobic rice, the focus of researchers is more on water use and yields (Bouman et al. 2006); however, little attention is paid on fertilizer response and nutrient use efficiency. The transfer from flooding to non-flooding (aerobic) soil conditions may alter soil water status, soil aeration, and nutrient availability (Timsina and Connor 2001). In addition, the research findings suggest that N and P deficiency in aerobic crops is quite common (Fageria and Breseghello 2001). For availability of P, the mechanism is different in aerobic condition compared to anaerobic rice cultivation system, and P deficiency

has been recognized as one of the main limiting factors for upland rice production in many parts of the world (Sahrawat et al. 2001). This situation requires an extensive research in order to manage the fertilizer application in aerobic rice, especially phosphorus nutrient management.

9.2.2.1 Phosphorus

Phosphorus indeed is a primary essential nutrient for rice production. It stimulates various physiological activities such as root and shoot growth, promotes vigorous seedling growth, advances crop maturity, and plays a vital role in plant metabolism such as cell division, breakdown of sugar, nutrient transport within the plant, regulation of metabolic pathways, and other biochemical characteristics (Theodorou and Plaxton 1993; Khan et al. 2009).

Phosphorus deficiency is a common problem in many soils; globally, about 5.7 billion ha of land has been found to contain too little available P for sustaining optimal crop production. Phosphorus ion concentration in most of the soils ranges from 0.1 to 10 μM, while P in soil should range from 1 to 5 μM for the optimal growth of grasses and 5–60 μM for high-demand crops such as tomato and pea (Hinsinger 2001). Malaysian soils are generally poor in P, and a substantial amount of Malaysia's income (RM 55 billion) was expected to be derived from agricultural activities in 2010. Demand of phosphate fertilizer in the world has increased from 40.6 million tons in 2011 to 41.5 million tons in 2012, at a growth rate of 2.4 %. It is expected to reach 45 million tons in 2016 at a growth rate of 2 % per year. Of the overall increase in demand for a total of 3.5 million tons P_2O_5 between 2012 and 2016, 58 % would be in Asia, 24 % in America, 11 % in Europe, 4 % in Africa, and 3 % in Oceania. Among the Asian countries, about 25 % of growth in world demand of P is expected in India, 14 % in China, 4 % in Pakistan, and 3 % in Indonesia, Malaysia, and Bangladesh. West Asia accounts for 5 % of the increase in consumption, of which Turkey, Iran, and Syria have the bulk of the share (FAO 2012).

9.3 Phosphate-Solubilizing Bacteria

Soil microorganisms have ability to convert insoluble phosphatic compounds into soluble P form for uptake by the crops (Panhwar et al. 2011). There are many rhizosphere microorganisms, which are able to dissolve insoluble P (Henri et al. 2008; Hameeda et al. 2008). Microorganisms play an important role in agriculture by supplying nutrients to the plants and reduce the demand of chemical fertilizers (Çakmakçı et al. 2006). It has been found that the poorly soluble P is usually dissolved by microorganisms, which can then be converted into soluble forms by the process of acidification, chelation, and exchange reactions (Chung et al. 2005). The quantity of PSB, involved in solubilization process, is more abundant in the rhizosphere than non-rhizosphere soil and is metabolically more

dynamic than from other sources (Vazquez et al. 2000). The PSB are ubiquitous, varying in forms and composition. The population of PSB is affected by physical and chemical properties of soils, soil organic matter content, P content, and cultural activities (Kim et al. 1998). Higher populations of PSB are found in agricultural and range land soils (Yahya and Azawi 1998). The PSB also plays a vital role in combination with chemical fertilizers, for example, single super phosphate (SSP) and PR, and application of microbial phosphatic fertilizers has been found to reduce the synthetic P levels by 25–50 % in agricultural practices (Sundara et al. 2002). Direct application of PR is mostly not effective for annual crops (Goenadi et al. 2000), the availability of which however can be enhanced by applying some acid-producing microorganisms: able to solubilize PR (Gyaneshwar et al. 2002). Rodríguez and Fraga (1999) in a study suggested that certain PSB strains were able to solubilize P; examples included were those of *Pseudomonas putida* (51 %), *P. fluorescens* (29 %), and *P. fluorescens* (62 %) (Ghaderi et al. 2008). *Pseudomonas striata* and *Bacillus polymyxa* solubilized 156 and 116 mg P l^{-1}, respectively; *Pseudomonas fluorescens* solubilized 100 mg P l^{-1} containing Ca_3 $(PO_4)_2$, 92 mg P l^{-1} containing $AlPO_4$, and 51 mg P l^{-1} containing $FePO_4$ (Henri et al. 2008).

9.3.1 Production of Organic Acids by Phosphate-Solubilizing Bacteria

There are different mechanisms by which PSB can transform insoluble P into soluble P forms. These include acidification, enzymatic dissolution of phosphates, and ammonium assimilation. Generally, P solubilization is correlated with the production of organic acids through oxidation process that happens on the outer face of the cytoplasmic membrane and is related with the drop in pH of the medium (Maliha et al. 2004; Pradhan and Sukla 2005). Bacteria and fungi can produce organic acids and acidify their surroundings with the release of P ions from mineral P by the substitution of H^+ for Ca^{2+} (Goldstein 1994). The findings by Asea et al. (1988) revealed that fixed P in acidic soils accumulated Fe or Al ions, and there was no correlation found between pH and P solubilization. Hence, there might be other alternative possibilities than organic acids for the insoluble inorganic P solubilization, such as the release of H^+ and production of chelating substances and inorganic acids (Khan et al. 2007). Illmer and Schinner (1995) reported that the production of organic acids by the bacterial cells is not the only reason for P solubilization; therefore, acidification is not the only mechanism of P solubilization, as the capability to decrease the pH in some cases did not correlate with the solubilized mineral P. The chelating ability of the organic acids is also significant (Kucey 1988). Altomare et al. (1999) has examined the ability of plant growth-promoting and biocontrol fungus *Trichoderma harzianum* T-22 to solubilize P in in vitro conditions including PR, whereas organic acids were not detected in culture

Table 9.1 Production of organic acids by PSB grown in broth culture

PSB strains	Oxalic acid (mg l^{-1})	Malic acid (mg l^{-1})	Succinic acid (mg l^{-1})	Propionic acid (mg l^{-1})
PSB1	0.052	0.042	0.150	0.031
PSB6	0.020	0.043	0.125	0.019
PSB9	0.025	0.070	0.245	0.011
PSB10	0.010	0.062	0.175	0.009
PSB14	0.008	0.025	0.205	0.008
PSB15	0.008	0.026	0.172	0.020
PSB16	0.011	0.050	0.250	0.028

Modified from Panhwar et al. (2012)
PSB phosphate-solubilizing bacteria

filtrates, which revealed that the insoluble P might be solubilized by mechanisms other than process of acidification. Soil inorganic P is mostly solubilized by production of organic acids. Low-molecular-weight organic acids play multiple roles in the soil processes, such as root nutrient acquisition, mineral weathering, microbial chemotaxis, and metal detoxification (Jones et al. 2003). Phosphate-solubilizing bacteria can release numerous organic acids including oxalic, citric, butyric, malonic, lactic, succinic, malic, gluconic, acetic, glyconic, fumaric, adipic, and 2-ketogluconic acid (Leyval and Berthelin 1989). Among the organic acids, oxalic and malic acid amounts are more common than the others (Zeng et al. 2008). Phosphate-solubilizing bacterial strains belonging to the genera *Pseudomonas*, *Bacillus*, and *Rhizobium* are among the most powerful P solubilizers (Sharma et al. 2013; Rodriguez et al. 1999). Some of the organic acids released by PSB strains are listed in Table 9.1.

9.3.2 *Phosphate Solubilization by Phosphate-Solubilizing Bacteria*

9.3.2.1 Solubilization of Al and Fe-bound Soil P

The solubilization of Fe and Al takes place through the release of protons by PSB, reducing the adsorbing surface charge to make possible the sorption of negatively charged P ions. Phosphate sorption also might be decreased with the release of protons by acidification that increases $H_2PO_4^-$ in comparison to HPO_4^{2-} having higher similarity to reactive soil surfaces (Whitelaw 2000). The different forms of P, like Al-P and Fe-P, are mostly solubilized by carboxylic acids (Khan et al. 2007; Henri et al. 2008) by the mineral P dissolution as an effect of anion exchange of PO_4^{3-} or by chelation of Al and Fe ions associated with P (Omar 1998). This is due to high affinity of iron uptake system by root-colonizing pseudomonas which depends on the release of Fe^{3+}-chelating molecules like siderophores (Altomare et al. 1999). Furthermore, P is replaced by carboxylic anions through ligand exchange from sorption complexes

(Whitelaw 2000) and chelates Fe and Al ions with phosphate, and after transformation, phosphates become available for plant uptake. Different carboxylic anion lowers the P desorption potential with decrease in the stability constants of Fe- or Al-organic acid complexes (log KAl or log KFe) in the order: citrate > oxalate > malonate/malate > tartrate > lactate > gluconate > acetate > formiate (Ryan et al. 2001).

9.3.2.2 Solubilization of Ca-Bound Soil P by Phosphate-Solubilizing Bacteria

At high pH, soil P forms a complex with Ca and remains unavailable to plants. In the alkaline soil conditions, phosphatic fertilizers and its metabolites are fixed as calcium phosphates. Rock phosphate in soil is insoluble and can become soluble following the release of inorganic P to maintain plant growth (Goldstein 2000). Calcium phosphate solubilization occurs through the secretion of organic acids by microbes (Deubel and Merbach 2005), and lowering of rhizosphere pH (He and Zhu 1988), that break down the bound forms of P like $Ca_3\ (PO_4)_2$; however, the buffering capacity of the medium decreases the effectiveness of PSB in releasing P from tricalcium phosphates (Stephen and Jisha 2009). Thus, any microorganism that acidifies its external medium will result in some level of PS activity. In majority of the soils, proton substitution reactions are determined by microbial production of organic acids, which is shown by the following equation:

$$\left(Ca^{2+}\right)_m\left(PO_4{}^{3-}\right)_n + (HA) = \left(H^+\right)\left(PO_4{}^{3-}\right) + \left(Ca^{2+}\right)\left(A^-\right)$$

There is no stoichiometry in the equation above because of the complexity of Ca-P chemistry and the multiplicity of microbially produced organic acids with differing numbers of dissociable protons (Goldstein 1986).

9.3.2.3 Phosphate Solubilization from Phosphate Rock

Various bacterial species have been reported to solubilize insoluble inorganic P compounds, like TCP, DCP, hydroxyapatite, and PR (Goldstein 1986). *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aerobacter*, *Flavobacterium*, and *Erwinia* are the most common genera which have the ability to solubilize P. For inorganic P solubilization by PSB, there is a relationship between bacterial growth, supernatant acidification, and P solubilization from $Ca_3\ (PO_4)_2$. In vitro study conducted to determine the P solubilization by PSB isolates using different levels of organic acids demonstrated a significant variation in the organic acid secretion by microbes. Of the organic acids, oxalic acid at 20 mM was significantly better than others (Table 9.2).

Table 9.2 Effect of application of organic acids on P solubilization (mg kg^{-1}) in soil

	P solubilization in PSB non-inoculated treatments		P solubilization in PSB inoculated treatments	
Dose of organic acids applied (mM)	Oxalic acid	Malic acid	Oxalic acid	Malic acid
0	18.80	18.80	22.55	22.55
10	21.50	19.12	27.23	22.77
20	24.57	20.35	28.37	21.57
30	23.55	19.70	26.15	19.55

Source: modified from Panhwar et al. (2013)
PSB phosphate-solubilizing bacteria

Significant and constant quantities of organic acids might be detected in the soil solution (Shen et al. 1996). However, the amount of organic acids was found to be very low in the soil solution, usually from 1 to 50 μM (Strobel 2001). In other investigation, low amounts of residual organic acids were found in the soil. However, organic acid concentration in soil is not stable and it can vary with the passage of time (Table 9.3). Similar findings of Jones (1998) and Strobel (2001) showed that the organic acid concentration in soil solution would be different at different space and time.

9.3.2.4 Ammonium Assimilation

Plant supply of nitrogen might have a computable consequence on pH change because of the H^+ release from plant roots through ammonium assimilation (NH_4^+). In the plants that rely on NH_4^+ rather than NO_3^- to reduce pH, the cation uptake ratio will be increased as compared to the anions (Gahoonia et al. 1992). Microbial excretion of H^+ is also similar to plants under the assimilation of cations, mainly associated to N source. It is well understood that microbes H^+ are exerted in exchange for NH_4^+ (Asea et al. 1988), and it is reported that high P is solubilized due to the NH^+_4 rather than NO_3^- nitrogen (Whitelaw et al. 1999). Furthermore, among N sources, ammonium sulfate is known to support high P solubilization for different bacterial species, *Bacillus circulans*, *B. brevis*, and *B. coagulans* (Vora and Shelat 1998).

9.3.2.5 Organic Phosphate Solubilization

In most soils, the organic forms of P are 30–50 %, while in other soils they may be as low as 5 % and as high as 95 % (Paul and Clark 1989). Organic P in the soil is mostly present in the form of inositol phosphate (soil phytate). An organic form is the most stable form in the soil and accounts for up to 50 % of the total organic P. It is synthesized mostly in soil by microorganisms and by plants (Harley and Smith 1983). Phosphomonoesters, phosphodiesters including phospholipids and nucleic acids, and phosphotriesters are the other organic P compounds in soil. Organic P

Table 9.3 Organic acid recoveries after 40 days of application in aerobic rice soil

	Recovery (%) in PSB non-inoculated treatments		Recovery (%) in PSB inoculated treatments	
Dose of organic acids applied (mM)	Oxalic acid	Malic acid	Oxalic acid	Malic acid
0	0	0	0	0
10	6.0	2.2	14.4	4.5
20	3.8	2.4	7.7	3.8
30	3.7	1.6	5.7	2.7

Modified from Panhwar et al. (2013)
PSB phosphate-solubilizing bacteria, *mM* millimole

compounds degradability mostly depends on the physicochemical and biochemical properties of their molecules, e.g., nucleic acids, phospholipids, and sugar phosphates are easy to break down, but phytic acid, polyphosphates, and phosphonates decay more slowly (McGrath et al. 1998). Alkaline and acid phosphatases use organic phosphate as a substrate to change it into inorganic form (Beech et al. 2001). The major mechanism for mineralization of soil organic P is the production of acid phosphatases. Phosphate can be released from organic compounds in soil by three different enzymes. Acid phosphatases and phytases perform major role for the P solubilization due to the presence of their substrates in soil.

9.3.2.6 Enzymatic Dissolution of Phosphates

Organic P compounds in soil are mineralized by phosphatases, phytases, phosphonatases, and C-P lyases. The microbial-released phosphatases are an extensively distributed exoenzyme and play vital roles in mineralization and dissolution of organic P compounds in the rhizosphere (Rodriguez et al. 2006). Phosphate-solubilizing bacterial strains have the ability to produce phytohormones and phosphatase enzymes and make it available to plants (Ponmurugan and Gopi 2006; Relwani et al. 2008; Aseri et al. 2009). While plants do not take up P in the insoluble form, it can become soluble by acid and alkaline phosphatase. Phosphatase is an enzyme which eliminates P from its substrate by hydrolyzing phosphoric acid monoesters into a P ion and a molecule with a free hydroxyl group. The root phosphatase activities can be helpful to the plants for their greater consumption of soil organic P (Asmar et al. 1995). Different PSB, namely, *Sinorhizobium* sp. AS017 and *Sinorhizobium* sp. AS016, have shown maximum activity of acid phosphatase. A corn experiment by Pantujit and Pongsilp (2010) concluded that PSE008 had maximum alkaline phosphatase activity. Besides this, PSB increased plant dry weight and P content as compared to control in corn crop. The majority of the microorganisms in soil contain phytase (myoinositol hexaphosphate phosphohydrolase) that can hydrolyze sodium phytate, resulting in inorganic P (Greaves et al. 1963). There are many bacterial strains such as *Escherichia coli*, *B. subtilis*, *B. amyloliquefaciens*, and *Klebsiella* spp.; yeasts like, *Schwanniomyces castellii*, *S. occidentalis*, *Hansenula polymorpha*, and *Rhodotorula gracilis*; and

Table 9.4 Enzyme activity by phosphate-solubilizing bacteria

PSB strains	Phosphatase activity (EU ml^{-1})	Phytase activity (EU ml^{-1})
PSB1	200	82
PSB6	356	86
PSB9	245	106
PSB10	180	125
PSB14	150	78
PSB15	280	120
PSB16	417	142

Modified from Panhwar et al. (2012)
EU enzyme unit

fungi such as *Aspergillus niger* and *A. ficuum* species being used for the production of microbial phytases (Pandey et al. 2001). Some PSB strains capable of producing phosphatase and phytase are listed in Table 9.4.

Enzyme activities increase due to the inoculation of PSB. There were two enzymes, such as phosphatase and phytase, which were found in the roots of aerobic rice. The PSB inoculation produced higher values of both enzymes as compared to non-inoculated plants. The highest phosphatase (10.68 μg p-NP g^{-1} root dry wt ha^{-1}) activity (Fig. 9.1) and phytase (25.71 U mg^{-1}) activity (Fig. 9.2) were found following the application of mixtures of PSB, PR, and oxalic acid. In this study, it was found that the inoculated plants had significantly higher amounts of phosphatase enzyme. It is known that this enzyme excreted from roots can hydrolyze a wide range of organic P compounds in soil and release Pi for plant uptake (George et al. 2002). Therefore, increased Pi pool confirmed the role of PSB in solubilizing P from organic substances.

9.4 Effect of PSB on Nutrient Uptake and Growth of Aerobic Rice

9.4.1 Phosphorus Uptake by Plants Inoculated with PSB

A number of bacterial species including PSB associated with the plants' rhizosphere are able to exert a beneficial effect on plants growth. Therefore, their use as biofertilizers or as biocontrol agents in agricultural practices has been the focus of numerous researchers (Glick 1995). This group of bacteria has often been termed as "plant growth-promoting rhizobacteria" (PGPR), and among them are strains belonging to the genera *Pseudomonas*, *Azospirillum*, *Burkholderia*, *Bacillus*, *Enterobacter*, *Rhizobium*, *Erwinia*, *Serratia*, *Alcaligenes*, *Arthrobacter*, *Acinetobacter*, and *Flavobacterium* (Sharma et al. 2013; Panhwar et al. 2012) which facilitate the growth of plants. Inoculation of PSB and the application of oxalic acid increased P uptake in aerobic rice (Panhwar et al. 2013). The lower

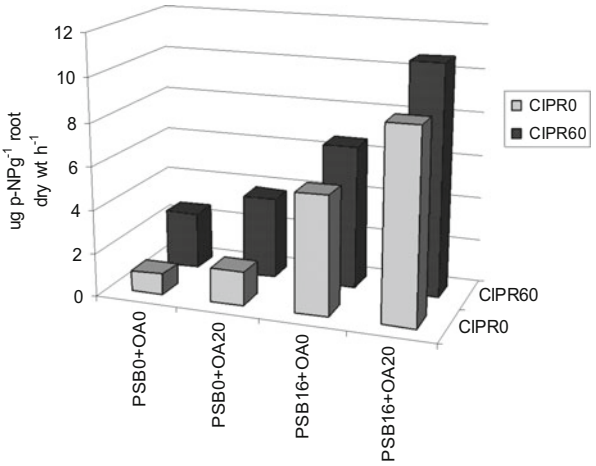

Fig. 9.1 Effect of PSB and PR with OA on phosphatase activity in rice roots. PSB16 = *Bacillus* sp., PSB 0 = non-inoculated, CIPR = phosphate rock, OA = oxalic acid

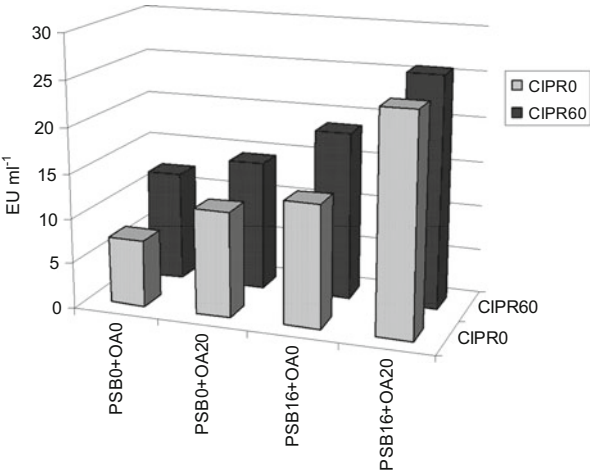

Fig. 9.2 Effect of PSB and PR with OA on phytase activity in rice roots. PSB16 = *Bacillus* sp., PSB 0 = non-inoculated, CIPR = phosphate rock, OA = oxalic acid

specific activity (^{32}P) in the aerobic rice tissue showed a positive effect of PSB inoculation or OA application to make the bioavailable P from PR and native soil sources (Table 9.5). This result is in concurrence with the earlier findings of Bolan (1991), who found lower values of ^{32}P in the inoculated treatments. The PSB treatments showed effectiveness at releasing ^{31}P from sparingly soluble sources and the total amount of P derived either from the available (labeled) soil fraction (Pdfl) or from the added PR (PdfCIPR) in plants. In fact, PSB released P from the low-available P sources. The inoculation of PSB with CIPR (expand) and OA showed higher values in plant P uptake and the amount of P derived from the unavailable sources. Our results are in agreement with the findings of Toro et al. (1997), who also found that plant total P and ^{32}P activity were lowered due

Table 9.5 Specific activity of ^{32}P and concentration of P in plant tissues of aerobic rice

	Specific activity ^{32}P		Pdfl (%)		PdfPR (%)		P uptake (mg P pot^{-1})		PUE (%)	
Treatments	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$
$PSB_0 + OA_0$	152.9a	84.8b	55.4d	91.6a	8.3g	44.5d	0.34f	0.84d	0.0	0.43f
$PSB_0 + OA_{20}$	83.4b	48.0dc	38.4e	78.5b	21.4f	61.5c	0.66d	0.99c	0.16g	0.77d
$PSB_{16} + OA_0$	54.1c	32.1de	30.9f	73.3b	26.6f	69.0b	0.75e	1.09b	0.56e	1.04b
$PSB_{16} + OA_{20}$	35.3d	12.5e	17.2g	67.1c	32.8e	82.7a	0.95c	1.20a	0.89c	1.12a

CIPR = phosphate rock, PSB16 = (*Bacillus* sp.). Means within the same column followed by the same letters are not significantly (NS) different at $P < 0.05$ [Adapted from Panhwar et al. (2013)]

OA oxalic acid, *PUE* phosphate use efficiency

to the dilution effects from the P solubilized by the PSB. Thus, this decreased the ^{32}P activity, as compared to the control, where no P was solubilized from the added PR.

9.4.2 *Effect of PSB on Grain Yield*

A synergistic effect on aerobic rice in this study was observed which enhanced the P uptake and plant biomass production. In a similar study, Barea et al. (2003) also found an identical trend in plants P uptake following inoculation of PSB while *P. putida* inoculation enhanced the growth of canola (Lifshitz et al. 1987). Since the production of IAA by PSB has a positive effect on root architecture (Naher et al. 2009), therefore, an extensive root system improves the nutrient uptake from the surroundings leading to the higher plant biomass production. The IAA exudation can further increase the carbon fixation through increased nutrient uptake. Moreover, inoculation with PSB, for example, *Azospirillum lipoferum* 34H, has reported to improve rice seeds P ion content and consequently improved root length and fresh and dry shoot biomass (Murty and Ladha 1988). Concurrently, increases in P uptake and crop yields have been observed after PSB inoculation with *Bacillus* sp. (Panhwar et al. 2013), *Pseudomonads*, and *Bacilli* (Sharma et al. 2013). Inoculation of PSB enhanced P uptake and simultaneously increased the yield of aerobic rice. Plant P uptake, P use efficiency (PUE %), total biomass, and total protein content increased with the inoculation of PSB with CIPR and OA. In addition, PSB inoculation and the application of PR and OA significantly increased the grain yield and plant biomass of aerobic rice (Table 9.6). The highest grain yield and plant biomass were determined when PSB was inoculated with CIPR and OA and was statistically at par with those of PSB and PR application (Panhwar et al. 2013).

9.5 Conclusion

The phosphate-solubilizing bacteria have generally been found effective for enhancing the bioavailable P by several mechanisms, such as production of organic acids, ammonium assimilation, or enzyme production. In the acidic soil, P is mostly bound with Fe/Al and in alkaline soil with Ca. Moreover, phosphate-solubilizing bacteria are widely distributed in different soil-plant ecosystem and perform a major role in soil organic and inorganic P solubilization. The inoculation of PSB solubilization/mineralization endowed with phytohormone-producing ability is likely to have a synergistic and productive effect, which might increase P uptake, growth and yield of various crops including aerobic rice.

Table 9.6 Effects of PSB, CIPR, and OA on different yield parameters of aerobic rice at harvest

	Number of panicle plant^{-1}		Number of unfilled grains (%)		1,000 grain weight (g)		Wt of grains pot^{-1} (g)		Increment (%) of grain yield over control		Plant biomass (g pot^{-1})		Increment (%) of biomass over control	
Treatments	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$
$PSB_0 + OA_0$	1.31d	2.30bc	25.9a	22.97b	15.65e	17.53c	12.21e	15.03cd	–	23.10c	7.48f	12.87e	–	72.10d
$PSB_0 + OA_{20}$	1.87cd	3.37ab	24.94ab	19.18dc	16.46de	17.81b	14.89de	15.74b	21.98cd	28.91b	10.26e	15.91c	37.20e	112.08c
$PSB_{16} + OA_0$	1.94bcd	3.55ab	20.18c	16.84de	16.68cd	18.43ab	15.39b	16.76a	26.04b	34.81a	15.54d	17.82a	107.90c	138.30a
$PSB_{16} + OA_{20}$	3.58a	4.04a	18.71dc	16.30e	18.09ab	19.81a	15.92b	16.83a	32.05b	37.87a	16.39b	18.36a	119.30b	145.60a
PSB_{16}	***		***		***		***		–		***		–	
OA	***		**		**		***		–		***		–	
PR	NS		*		*		***		–		***		–	
$PSB_{16} \times OA$	**		**		NS		***		–		*		–	
$PSB_{16} \times PR$	***		*		***		*		–		NS		–	
$OA \times PR$	***		**		**		**		–		**		–	
$PSB_{16} \times OA \times PR$	***		*		***		**		–		***		–	

Means within the same column followed by the same letters are not significantly (NS) different at $P < 0.05$ [Adapted from Panhwar et al. (2013)]
PR phosphate rock, *PSB* PSB16 (*Bacillus* sp.), *OA* oxalic acid
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

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Chapter 10
Role of Phosphate-Solubilizing Microbes in the Management of Plant Diseases

Almas Zaidi, Ees Ahmad, and Md. Saghir Khan

Abstract Soilborne phytopathogens are one of the major problems in sustainable crop production world over. To alleviate the damaging impact of pathogens on crop yields, huge quantities of toxic chemicals especially pesticides are used in modern agronomic practices, which, however, are extremely destructive to the environment. The non-desirability of applying huge quantities of pesticides to soil due in part to residue problems, emergence of resistance among soil phytopathogens, and lack of pathogen-resistant crop varieties has forced researchers to find solutions to the increasing pesticides problems. To this end, biological control measures consisting of microbial preparations are considered a promising option to the use of expensive and environment disruptive pesticides. Microorganisms including plant growth-promoting rhizobacteria (PGPR) in general have been found to synthesize a wide array of metabolites with significant fungicidal and bactericidal capabilities. The use of phosphate-solubilizing (PS) microorganisms among PGPR has produced both direct and indirect effects on growth and development of plants. The PS microbes endowed with biocontrol activity manage the pathogens by one or simultaneous mechanisms of antibiosis, lysis, competition, and mycoparasitism and prevent the yield losses. Even though the literature on the physiological role of PS microorganisms in crop enhancement via P supply is adequately available, the information on the ability of such organisms in the control of phytopathogens is scarce. Here, different mechanisms utilized by PS organisms for plant disease suppression are discussed. It is envisioned that the PS bacteria in the near future are expected to reduce, if not completely eliminate, the use of pesticides in insect-pests management strategies.

Keywords PSM • Phytopathogens • Disease management • Antibiosis • Lytic enzymes

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10.1 Introduction

Among various crop declining factors, the phytopathogens capable of destructing plant vitality are major and chronic threat to the sustainability and food production worldwide. On global basis, plant diseases caused by more than 60 pathogens account for about 30 % loss of crop yields amounting to the tune of about 416 million US dollars (Nehl et al. 1996). Plant diseases, therefore, need to be controlled to preserve the quality and abundance of food, feed, and fiber produced by growers around the world. In order to mitigate the damage caused by phytopathogens and, hence, to achieve optimum crop yields, various approaches are adopted. For example, apart from good agronomic and horticultural practices, growers very frequently and excessively use agrochemicals in high input modern agriculture systems to manage phytopathogens. The long-term use and misuse of synthetic chemicals have, however, resulted in severe deleterious impacts on ecological balance of soil, associated beneficial soil microflora, soil fertility, crop production, and emergence of resistance among pathogens (De Weger et al. 1995; Gerhardson 2002). In addition, the ever-increasing cost of pesticides has also been an acutely worrying factor among progressive crop producers world over. Considering such challenges, scientists indeed are desperate to find environmentally friendly alternatives to the extensive use of toxic pesticides, for combating crop diseases. To this end, the use of valuable microbes especially those capable of suppressing/inhibiting the pathogen populations, often called "biocontrol agents" or biological control agents (BCA), is considered one of the most suitable choices for a meaningful and secure crop-management practices (Welbaum et al. 2004; Fravel 2005; Ongena and Jacques 2008; Hyakumachi 2013). The terms "biological control" and its abbreviated synonym "biocontrol" have been used in different fields of biology, most notably among entomology and plant pathology. In entomology, it involves the use of live predatory insects, entomopathogenic nematodes, or microbial pathogens to suppress populations of different pest insects. In plant pathology, the term applies to the use of microbial antagonists to suppress diseases and the use of host-specific pathogens to control weed populations. In both fields, the organism that suppresses the pest or pathogen is referred to as the *biological control agent (BCA)*. Also, the term biological control involves the use of natural products extracted or fermented from various sources (Pal and Gardener 2006). Undeniably, many soilborne microorganisms have been found useful and are perpetually included purposely into differing production systems as a part of integrated pest and productivity management practices (Avis et al. 2008; Singh et al. 2010; Tallapragada and Gudimi 2011; Guiñazú et al. 2013). Among the variously distributed microbial communities, the PGPR (Kloepper and Schroth 1978; Bashan and Holguin 1998) facilitate the growth of plants (Bhattacharya and Jha 2012) both directly by way of supplying N (nitrogen fixation) and P (phosphate solubilization) to plants (Khan et al. 2009) or by providing other growth promoting substances like IAA (Ahmad et al. 2013; Oves et al. 2013) and trace elements and indirectly by diminishing the activity of plant pathogens (biocontrol) (Mamaghani et al. 2009;

Ingle and Deshmukh 2010; Yaqub and Shahazad 2011; Parikh and Jha 2012; Geethapriya and Krishnaveni 2012) by virtue of synthesizing siderophores (Ahmad et al. 2013; Walia et al. 2013), antibiotics (Compant et al. 2005), lytic enzymes (Postma et al. 2010; Kumar et al. 2012) or cyanogenic compounds (Hallman et al. 1997; Sturz et al. 2000; Welbaum et al. 2004; Ahmad et al. 2013), etc. In general, the symbiotic PGPR, for example, rhizobia (Khan et al. 2002), free-living PGPR (Khan et al. 2006), and endophytic PGPR, have been reported to continuously promote plant growth by restricting/inhibiting the populations of disease-causing phytopathogens (Sturz et al. 2000; Lodewyckx et al. 2002; Dobbelaere et al. 2003). Experimental evidence that is consistent with the involvement of PS biocontrol bacteria in the suppression of fungal pathogen causing plant disease comes from several different studies. For example, P-solubilizing fluorescent pseudomonads demonstrated a profound antifungal activity against *R. solani* and effectively protected the pepper plants against damping off under in vivo conditions through the release of disease inhibiting lytic enzymes (chitinase and β-1,3-glucanase), siderophores, and HCN (Rajkumar et al. 2008). On the other hand, one study observed that ACC deaminase-producing P-solubilizing strain BPR7 of *Bacillus* sp. recovered from Indian Himalayan region was most efficient at protecting plants against fungal pathogens like *M. phaseolina*, *Fusarium oxysporum*, *F. solani*, *Sclerotinia sclerotiorum*, *R. solani*, and *Colletotrichum* sp. (Kumar et al. 2013).

10.2 Examples of Plant Pathogenic Bacteria, Fungi, and Nematode Causing Plant Diseases

Plant diseases are reported to cause economical loss of billions of dollars by reducing crop yields and result in poorer quality produce. When pesticides are used to control phytopathogens, it contaminates food grains which later on become nonconsumable for humans (Guo et al. 2013). Taking into account the magnitude of yield losses by the phytopathogens, a survey was conducted to identify the most prominent fungi, bacteria, and nematodes able to cause diseases onto plants, and the results of this finding were published in *Molecular Plant Pathology* Journal. The top 10 fungi (Ralph et al. 2012), bacteria (Mansfiels et al. 2012), and nematodes (Jones et al. (2013) inflicting heavy losses to crops are listed in Table 10.1. Bacteria which were very close to top ten listed bacteria but did not find place in top ten club included *Clavibacter michiganensis* (*michiganensis* and *sepedonicus*), *Pseudomonas savastanoi*, and *Candidatus liberibacter asiaticus*.

Table 10.1 Top 10 fungal, bacterial, and nematode plant pathogens

	Plant pathogens				
Rank	Fungal[a]	Author of description	Bacterial[b]	Author of description	Nematode[c]
1	*Magnaporthe oryzae*	Ralph Dean	*Pseudomonas syringae* pathovars	John Mansfield	Root-knot nematodes *Meloidogyne* spp.
2	*Botrytis cinerea*	Jan A. L. van Kan	*Ralstonia solanacearum*	Stéphane Genin	Cyst nematodes (*Heterodera* and *Globodera* spp.)
3	*Puccinia* spp.	Zacharias A. Pretorius	*Agrobacterium tumefaciens*	Shimpei Magori, Vitaly Citovsky	Root lesion nematodes (*Pratylenchus* spp.)
4	*Fusarium graminearum*	Kim Hammond-Kosack	*Xanthomonas oryzae* pv. *oryzae*	Pamela Ronald	The burrowing nematode *Radopholus similis*
5	*F. oxysporum*	Antonio Di Pietro	*Xanthomonas campestris* pathovars	Malinee Sriariyanum, Max Dow	*Ditylenchus dipsaci*
6	*Blumeria graminis*	Pietro Spanu	*Xanthomonas axonopodis* pv. *manihotis*	Valérie Verdier	The pine wilt nematode *Bursaphelenchus xylophilus*
7	*Mycosphaerella graminicola*	Jason J. Rudd	*Erwinia amylovora*	Steven V. Beer	The reniform nematode *Rotylenchulus reniformis*
8	*Colletotrichum* spp.	Marty Dickman	*Xylella fastidiosa*	Marcos A. Machado	*Xiphinema index*
9	*Ustilago maydis*	Regine Kahmann	*Dickeya* (*dadantii* and *solani*)	Ian Toth	*Nacobbus aberrans*
10	*Melampsora lini*	Jeff Ellis	*Pectobacterium carotovorum* (and *P. atrosepticum*)	George Salmond	*Aphelenchoides besseyi*

[a,b,c]Adapted from Dean et al. (2005) and Jones et al. (2013), respectively

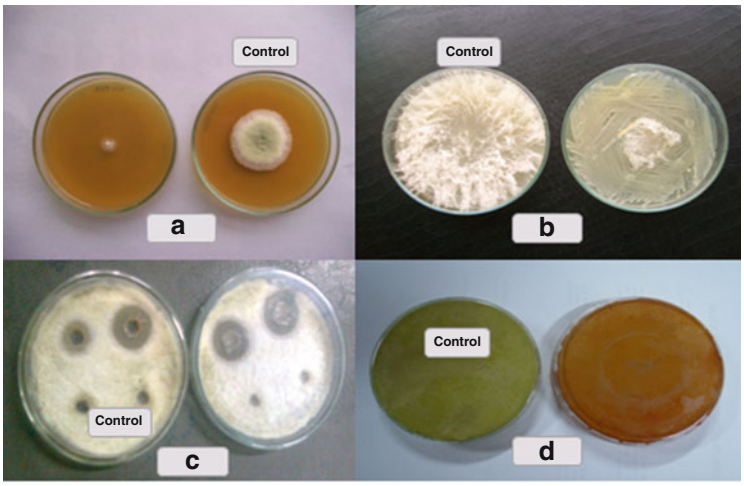

Fig. 10.1 Antifungal activity expression of certain unidentified PS organisms: (**a**) The volatile compounds inhibiting the growth of phytopathogenic fungi in a dual plate assay, (**b**) the metabolite produced by bacteria inhibiting the growth of phytopathogenic fungi, (**c**) the antifungal metabolite that inhibit the growth of fungi in a well-diffusion method, and (**d**) cyanogenic compound (HCN) production by PS bacterial strain

10.3 Importance of PSM in the Management of Plant Diseases: An Overview

The production and release of certain secondary metabolites by PSM in the rhizosphere (Khan et al. 2013) deleteriously affects the soilborne phytopathogens (Vassilev et al. 2006; Postma et al. 2010) (Fig. 10.1), and some of the pathogen-suppressing metabolites, for example, siderophores (Ahmad et al. 2013), antibiotics (de Werra et al. 2009), and lytic enzymes (Kumar et al. 2013), produced by PSM in vitro are listed in Table 10.2.

In a most recent study, Son et al. (2014) evaluated the effects of PS bacteria, identified by 16S rDNA sequence analysis as *Kluyvera cryocrescens* KUDC1771 and *Brevibacterium iodinum* KUDC1716, on growth promotion of pepper and antagonistic activity against a gray leaf spot disease pathogen, *Stemphylium lycopersici*. The selected PS bacteria enhanced the growth of *K. cryocrescens* KUDC1771 inoculated plants. Of these, *B. iodinum* KUDC1716 significantly decreased the severity of gray leaf spot disease and concurrently enhanced the plant health. Also, KUDC1716 strain of *B. iodinum* considerably increased the expression of pathogenesis-related (PR) protein genes including CaPR4 and CaChi2 in the absence of pathogen suggesting that *B. iodinum* could induce defense response against *S. lycopersici* and, hence, it may be used as a potential biological control agent. Similarly, PS bacteria, *Janibacter*, *Pseudomonas*, and *Bacillus* isolated from Uruguay, Chile, and Argentina produced siderophores and exopolysaccharides (EPS), hydrolyzed starch, and demonstrated biological control activity

Table 10.2 P-solubilizing bacteria with biocontrol activity

Bacterial strains	PGPR activity	Metabolites released	Fungal phytopathogens	References
Brevibacterium iodinum	Siderophore, IAA	ISR	*Stemphylium lycopersici*	Son et al. (2014)
Bacillus pumilus	Siderophore, IAA	ISR	*Stemphylium lycopersici*	Son et al. (2014)
Kluyvera cryocrescens	Siderophore, IAA	ISR	*Stemphylium lycopersici*	Son et al. (2014)
Enterobacter ludwigii	Siderophore, IAA	ISR	*Stemphylium lycopersici*	Son et al. (2014)
Pseudomonas putida	Siderophore, IAA	ISR	*Stemphylium lycopersici*	Son et al. (2014)
P. putida PSE3 and *R. leguminosarum* RP2	Siderophore, IAA, EPS, Ammonia, HCN, ACC deaminase		*Rhizoctonia*, *Penicillium*, *Alternaria*	Ahmad et al. (2013)
Burkholderia cepacia MPC-7		Gluconic acid, α, 2-ketogluconic acid, benzoic acid, phenylacetic acid	*Phytophthora capsici*	Mao et al. (2013)
Pseudomonas plecoglossicida	IAA, siderophore, HCN		*M. phaseolina*	Gopalakrishnan et al. (2011)
Brevibacterium antiquum	IAA, siderophore		*M. phaseolina*	Gopalakrishnan et al. (2011)
Bacillus altitudinis	IAA, siderophore, HCN		*M. phaseolina*	Gopalakrishnan et al. (2011)
Enterobacter ludwigii	IAA, siderophore, HCN		*M. phaseolina*	Gopalakrishnan et al. (2011)
Acinetobacter tandoii	Siderophore		*M. phaseolina*	Gopalakrishnan et al. (2011)
Bacillus spp.	Siderophore, HCN, IAA, ACC deaminase	β-1,3-Glucanase, chitinase, β-1,4-glucanase	*Macrophomina phaseolina*, *F. oxysporum*, *F. solani*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, and *Colletotrichum* sp.	Kumar et al. (2013)
Pseudomonas chlororaphis	ND	Chitinase activity	*Pythium*, *Fusarium*	Postma et al. (2010)
Paenibacillus polymyxa	ND	Chitinase activity	*Pythium*, *Fusarium*	Postma et al. (2010)

Burkholderia sp.	ND	Chitinase activity	*Pythium*, *Fusarium*	Postma et al. (2010)
Serratia plymuthica	ND	Chitinase activity	*Pythium*, *Fusarium*	Postma et al. (2010)
Bacillus, *Paenibacillus*	Siderophore, HCN, IAA	Chitinase	*Rhizoctonia bataticola*, *Macrophomina phaseolina*, *Fusarium udum*, and *Sclerotium rolfsii*	Senthilkumar et al. (2009)
B. subtilis PSRB1	IAA		*F. oxysporum*, *A. solani*	Hariprasad and Niranjana (2009)
P. putida PSRB6	IAA, siderophore		*F. oxysporum*, *A. solani*	Hariprasad and Niranjana (2009)
Azotobacter sp. PSRB7	IAA		*F. oxysporum*, *A. solani*	Hariprasad and Niranjana (2009)
P. fluorescens PSRB27	Siderophore	Chitinase	*F. oxysporum*, *A. solani*	Hariprasad and Niranjana (2009)
B. megaterium PSRB38	IAA	Glucanase	*F. oxysporum*, *A. solani*	Hariprasad and Niranjana (2009)
P. aeruginosa (BFPB9)	IAA, siderophore, HCN	Protease, cellulase	*Cylindrocladium floridanum*, *Sarocladium oryzae*, *Rhizoctonia solani*, *Botrytis cinerea*	Jha et al. (2009)
P. plecoglossicida (FP12)	IAA, siderophore	Protease	*Cylindrocladium floridanum*, *Sarocladium oryzae*, *Rhizoctonia solani*, *Botrytis cinerea*,	Jha et al. (2009)
P. mosselii (FP13)	IAA, siderophore	Protease	*Cylindrocladium floridanum*, *Sarocladium oryzae*, *Rhizoctonia solani*, *Botrytis cinerea*	Jha et al. (2009)
Bacillus subtilis		Amylase, cellulase	*Fusarium oxysporum*, *Botryodiplodia theobromae*	Swain and Ray (2009)

(continued)

Table 10.2 (continued)

Bacterial strains	PGPR activity	Metabolites released	Fungal phytopathogens	References
Pseudomonas fluorescens CHA0	ND	2,4-Diacetylphloroglucinol (DAPG) and pyoluteorin (PLT)	*Gaeumannomyces graminis* var. *Tritici*	de Werra et al. (2009)
Pseudomonas spp	Siderophore, HCN, IAA	β-1,3-Glucanase, chitinase	*Rhizoctonia solani*	Rajkumar et al. (2008)
Achromobacter xylosoxidans, *Bacillus pumilus*	ND	Jasmonates (JAs) and abscisic acid (ABA)	*Verticillium orense* and *Sclerotinia sclerotiorum*, *Alternaria* sp.	Forchetti et al. (2007)
Pseudomonas corrugate	IAA, HCN	Protease	*B. cinerea*, *A. solani*, *F. oxysporum*, *M. laxa*	Guo et al. (2007)

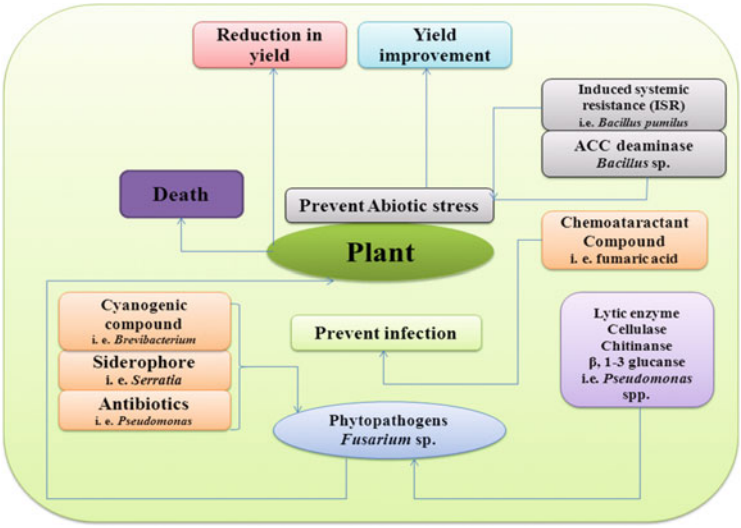

Fig. 10.2 Possible mode of disease management using P-solubilizing microorganism

inhibiting the growth of *Macrophomina phaseolina* and *Rhizoctonia* spp. (Guiñazú et al. 2013).

10.3.1 Mechanism of Biocontrol by P-Solubilizing Bacteria

Some of the common modes of disease suppression adopted by the PGPR include (1) competition for an ecological niche or a substrate, (2) the production of inhibitory allelochemicals, and (3) induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens (Haas et al. 2000, 2002; Lugtenberg et al. 2001; Ryu et al. 2004a). A possible mode of disease management using P-solubilizing microorganism is presented in Fig. 10.2.

10.3.1.1 Rhizosphere Competence and Colonization

The variable response of PS microbes as a biocontrol agent in plant protection strategies against phytopathogens under filed trials has largely contributed to the failure of developing microbial biocontrol agents at commercial levels. The commonly differing performance of such microbes has been ascribed mainly to the poor rhizosphere competence (Van Wees et al. 1997; Schroth and Hancock 1981; de Weert and Bloemberg 2006) among other factors (McLean et al. 2005; Ghirardi et al. 2012). Rhizosphere competence of any microbial cell is a prerequisite for the expression of their beneficial effects on plant growth and health. The rhizosphere

competence of biocontrol agents involves the efficient root colonization coupled with the ability to survive and proliferate all along the growing plant roots and in the presence of soil microflora over a considerable time period (Weller 1988; Whipps 1997; Lugtenberg and Dekkers 1999; Compant et al. 2005). Since rhizosphere competence plays a central role in deciding the fate of success of biocontrol agents under field soil, it is equally important to better understand the impact of some soil variables on such biocontrol agents also. In the rhizospheres, the plant exudates (photosynthates) discharged by many plant genotypes are the primary source of nutrients and, therefore, distinctly attract a huge number of diverse microflora including both PS organisms (acting as biocontrol agents) and phytopathogens (Van Overbeek and Van Elsas 1995). However, due to restricted availability of nutrients in and around the extremely vital rhizosphere, a furious competition occurs among soil microbiota resulting either in colonization or in exclusion (weak colonizers) of microbial populations from the rhizospheres. Such competitive factors which drives the microbial populations in soils could be the flagella of the organisms (which facilitates motility) and the chemotactic substances (Turnbull et al. 2001; Uroz et al. 2003; Dennis et al. 2010) like organic acids, amino acids, and specific sugars, exuded from the growing plants (Badri and Vivanco 2009). As an example, root exudates have been found to positively mediate the interaction of plants, for example, cucumber root and rhizosphere bacteria such as *Bacillus amyloliquefaciens* SQR9, and ultimately enhanced its root colonization (Liu et al. 2014). Some of these chemicals may also have the antimicrobial activity and, hence, give an edge to the secreting organisms over other soil dwellers. The quantity and composition of chemoattractants and antimicrobials, however, differ from plants to plants and are influenced largely by environmental factors (Bais et al. 2004). Among the various cellular components, the bacterial lipopolysaccharide (LPS), for example, is reported to influence the root colonization (Duijff et al. 2008). It has also recently been shown that the rapid bacterial growth rate and their ability to synthesize vitamin B1 and to discharge NADH dehydrogenases contribute immensely to plant colonization by rhizobacteria (Simons et al. 1996; Camacho et al. 2002). Of the total 43 isolates of PS bacteria recovered from 37 rhizospheric soils of tomato, growing in the Karnataka regions of India, only 33 isolates were found P solubilizer and colonized the roots of tomato, increased the biological and chemical properties of plants, and improved the quality of seeds under laboratory conditions relative to control. Some of these PS bacteria also protected plants from fusarium wilt infection, but none of them had any antagonistic activity against early blight (Hariprasad and Niranjana 2009).

10.3.1.2 Allelochemicals Mediated Biocontrol Activity

The allelochemicals including iron-chelating siderophores, antibiotics, biocidal volatiles, lytic enzymes, and detoxification enzymes (Glick 2012; Saraf et al. 2014) synthesized by the P-solubilizers and PGPR and used in the management of plant diseases are listed in Table 10.3. A few of these metabolites released

Table 10.3 Types and mode of action of allelochemicals in plant disease management

Types of allelochemicals	PGPR/PSB strain	Diseases/phytopathogens controlled/prevention	References
Antibiotics, 2,4-diacetylphloroglucinol (2,4 DAPG)	*Pseudomonas* fluorescens	Effective against *Sclerotium rolfsii* (up to 75 % inhibition)	Asadhi et al. (2013)
Lytic enzymes: chitinase, chitosanase, β-1,3-glucanase as well as cellulases, proteases	*Bacillus alvei* NRC 14	Root-rot of tomato plants; *F. oxysporum* inhibition in vitro and in vivo conditions	Abdel-Aziz et al. (2013)
Lytic enzymes: chitinase, chitosanase, β-1,3-glucanase as well as cellulases, proteases	*Bacillus alvei* NRC 14	Reduced the incidence and pathogenicity of the root-knot nematode, *Meloidogyne javanica*	Abdel-Aziz et al. (2013)
Siderophores	*P. aeruginosa* JAS-25	*F. oxysporum*, *F. udum*, *Aspergillus niger*	Sulochana et al. (2013)
Siderophore, phytase, organic acid, ACC deaminase, cyanogens, lytic enzymes, oxalate oxidase	*Bacillus* sp. BPR7	Strongly inhibited the growth of several phytopathogens: *M. phaseolina*, *F. oxysporum*, *F. solani*, *S. sclerotiorum*, *R. solani*, and *Colletotrichum* sp.	Kumar et al. (2012)
Fengycin, iturin, and surfactin lipopeptides but strong antifungal activity was found associated only with fengycin lipopeptides	*Bacillus subtilis* CPA-8	Peach brown rot; *strong antifungal activity against Monilinia laxa* and *Monilinia fructicola*	
Chitinase	*Bacillus alvei* NRC 14	Wide range of fungal pathogens	Abdel-Aziz et al. (2012)
ACC deaminase activity, antifungal activity	*Bacillus* sp	Wide range of antifungal activities	Minaxi et al. (2012)
Siderophores	*Alcaligenes* sp. STC1 and *Acinetobacter*	*A. niger*, *A. flavus*, *F. oxysporum*, *A. alternata*, *C. arachichola*, *M. anisophilia*, and *P. solanacearum*	Sayyed and Patel (2011)
Pyrrolnitrin	*P. chlororaphis* O6	*Rhizoctonia solani* and *Fusarium graminearum*	Park et al. (2011)
Extracellular hydrolytic enzyme	Bacillus subtilis	Wilting of cotton seedlings and inhibited *F. oxysporum*	Gajbhiye et al. (2010)
Iturin A	*Bacillus subtilis* RP24	Many fungal growth inhibitions and confirmed antifungal gene by PCR	Grover et al. (2010)

(continued)

Table 10.3 (continued)

Types of allelochemicals	PGPR/PSB strain	Diseases/phytopathogens controlled/prevention	References
Iturin A	*Bacillus amyloliquefaciens*	Postharvest diseases, citrus postharvest pathogens; *Alternaria citri*, *Botryosphaeria* sp. *Colletotrichum gloeosporioides etc.*	Arrebola et al. (2010)
Siderophore	*Alcaligenes faecalis*	Growth inhibitions of *A. niger*, *A. flavus*, *F. oxysporum*, and *A. alternate*	Sayyed and Chincholkar (2009)
Lytic enzymes: chitinase, β-1,3-glucanase, protease, etc.	Pseudomonas PGC2	*R. solani* and *P. capsici* growth inhibition	Arora et al. (2008)
Volatile metabolites	*P. fluorescens*, *P. corrugata*, *P. chlororaphis*	Inhibition of mycelial growth and spore germination	Fernando et al. (2005)

Modified from Saraf et al. (2014)

by PS bacteria and their consequential impact on plant pathogens are discussed briefly in the following section.

Role of Siderophores in Disease Suppression

Iron is one of the most important nutrients for both eukaryotes (Sayyed et al. 2007a, b) and nearly all prokaryotes (Kaplan and Kaplan 2009) which play fundamental roles in both iron metabolism and virulence of most fungi. Some of the crucial functions of iron in different metabolism include (1) transport, storage, and activation of molecular oxygen and amino acid syntheses, (2) respiration, (3) DNA biosynthesis, (4) nitrogen fixation, (5) reduction of ribonucleotides and dinitrogen, and (6) activation and decomposition of peroxides and electron transport (Duhan et al. 1998; Faraldo-Gomez and Sansom 2003; Katiyar and Goel 2004; Miethke and Marahiel 2007; Sandy and Butler 2009). However, when iron concentration in the environment is reduced, the growth of organisms requiring such element is inhibited. Under iron-starved conditions, numerous prokaryotes including bacteria such as *Chryseobacterium* sp. (Radzki et al. 2013), *Pseudomonas* sp. (Babana et al. 2013), *Pantoea agglomerans* and *Burkholderia anthina* (Walpola and Yoon 2013), and *P. fluorescens* (Parani and Saha 2012); fungi such as *A. fumigatus* and *A. nidulans* (Gründlinger et al. 2013); and actinomycetes *Streptomycetes* spp. (Das et al. 2007), however, synthesize a wide range of siderophores (a Greek phrase for "iron bearer"), a relatively low molecular weight (below 2 kDa), ferric ion-specific chelating agents (Neilands 1995; Budzikiewicz et al. 2010) in order to solubilize, capture, and transport inorganic iron to the cell (Carrillo-Castaneda et al. 2005; Sandy and Butler 2009). Some of the common siderophores produced by many fungi include ferrichromes by *Aspergillus* spp. (Charlang et al. 1981), *Suillus variegatus* (Wallander and Wickman 1999), and *Microsporum* spp. (Bentley et al. 2008); coprogens by *Fusarium dimerum* (Van der Helm and Winkelmann 1994) and *Epicoccum purpurascens* (Frederick et al. 1981); and fusigen by *Fusarium* spp. (Van der Helm and Winkelmann 1994) and *Histoplasma capsulatum* (Burt 1982). Siderophores produced by many organisms play some vital roles, for example, it functions as plant growth promoters (Yadav et al. 2011; Gamit and Tank 2014), biocontrol agents (Arora et al. 2001; Schenk et al. 2012), and bioremediation agents (Wang et al. 2011; Ishimaru et al. 2012), in addition to their valuable role in soil mineral weathering (Reichard et al. 2005; Buss et al. 2007; Shirvani and Nourbakhsh 2010). Realizing these properties, siderophore-positive strains have been exploited in the management of plant diseases (Kloepper et al. 1980; Wong et al. 1996; Sindhu et al. 1997; Sritharan 2000; Verma et al. 2011). The siderophoregenic rhizobacteria inhabiting soil/rhizospheres protect the plants from damage by preventing the iron acquisition by phytopathogens (Lemanceau and Albouvette 1993; Estrella and Chet 1998; Bloemberg and Lugtenberg 2001; Johri et al. 2003). Apart from iron, siderophores also form complex with heavy metals such as Cd, Pb, Ni, As (III, V), Al, Zn, Cu, Co, and Sr (Nair et al. 2006; Sayyed and Chincholkar 2010). Therefore, iron-deficient

situation in turn leads to reduction in the proliferation and root colonization by phytopathogens. The siderophore-mediated mechanism of biocontrol is considered extremely effective because PS bacteria/PGPR-produced siderophores have a much greater affinity for iron than do the fungal pathogens (Schippers et al. 1987). In the presence of siderophores, the fungal pathogens, therefore, become unable to proliferate in the root rhizospheres of the host plants due largely to the unavailability of iron (O'Sullivan and O'Gara 1992). While employing this strategy of biocontrol, the siderophore-secreting PS strains successfully out-compete fungal pathogens for available iron. In contrast, the growth of plants is generally not affected even by the reduction in rhizosphere iron concentration which results from the siderophores released by the biocontrol agents because plants in general can grow at much lower iron concentrations than most microorganisms. Also, many plants can bind, take up, and then utilize the iron siderophore complex (Bar-Ness et al. 1991; Wang et al. 1993). There are several scientific evidences which confirm the role of siderophores in the prevention of pathogen infestation (Vandenbergh and Gonzalez 1984; Sulochana et al. 2013). For example, of the 41 bacterial isolates collected from rhizosphere soil, 12 exhibited a maximum antagonistic activity in dual culture assay. These 12 bacterial cultures were further screened for disease suppression on red pepper plants in both natural and greenhouse conditions. All the antagonists showed varying levels of antagonism, whereas the isolates R33 and R13 exhibited the maximum (86.8 % and 71 %) ability to reduce the disease severity under in vivo conditions. Based on the 16S rDNA sequencing, the most effective isolate was identified as *Bacillus subtilis*. Further, the bacteria inoculated red pepper plants had longer and thicker roots and shoots, while there was great reduction in the severity of diseases which was possibly due to the release of siderophores, cyanogenic compounds, and hydrolytic enzymes secreted by the test bacteria (Lee et al. 2008). In a follow-up study, *B. cepacia* XXVI when used as antagonist in Petri-dish bioassay test inhibited massively the population of *Colletotrichum gloeosporioides*. The halo formation on CAS agar plates indicating growth inhibition of the pathogen was due to hydroxamate siderophore (deferoxamine mesylate salt-equivalent) production by strain XXVI. Interestingly, even the lowest concentration (0.64 μg ml^{-1}) of siderophore resulted in more than 91 % inhibition of the pathogens and the biocontrol activity of the test bacterium against *C. gloeosporioides* ATCC MYA 456 correlated directly with the siderophore production by *B. cepacia* XXVI. The growth of other five strains of *C. gloeosporioides*, isolated from mango "Ataulfo" orchards located in the municipality of Chahuites, State of Oaxaca in Mexico, was also inhibited when tested against *B. cepacia* XXVI. This finding, therefore, suggested that *B. cepacia* with siderophore-producing ability could be utilized as prospective microbial agent controlling the *C. gloeosporioides* infection. The use of such biocontrol materials is likely to reduce the environmental hazards which otherwise could be caused by the current practices of applying pesticides to control such diseases (Santos-Villalobos et al. 2012). Similarly, both siderophore rich-culture broth and cell-free supernatant of *Alcaligenes* sp. and *Pseudomonas aeruginosa* RZS3 in other investigation have shown growth inhibition of phytopathogenic fungi, namely,

A. niger, *A. flavus*, *F. oxysporum*, *A. alternata*, *C. arachichola*, *M. anisopliae*, and *P. solanacearum* (Sayyed and Patel 2011). However, the control preparation (free of any siderophore activity) did not inhibit the growth of any of the test fungal species consolidating the role of siderophores in disease suppression. Rhizobacteria capable of synthesizing and releasing siderophores are also known to be involved in inducing systemic resistance (ISRs) to the plants (Wees et al. 2000; Pieterse et al. 2001) and suppressiveness to the soil (Mazzola 2002). Summarily, the siderophore-based biological control agents must be popularized among field practitioners for reasons as they (1) are inexpensive and nondestructive (safer) to the environment, (2) are self-replicating in the environment and hence do not require repeated application, (3) do not lead to biomagnification, and (4) have no emergence of resistance among target organisms (Sayyed et al. 2005).

Antibiosis

Antibiotics are bioactive microbial metabolites that at low concentrations inhibit the growth or metabolic activities of other organisms (Thomashow and Weller 1995). Microbial communities able to produce antibiotics are common in natural environment. Historically, the natural antibiotics are reported to contribute to (1) microbial defense, (2) fitness, (3) interference, and (4) competitiveness (Wiseman et al. 1996; Haas and Defago 2005; Mavrodi et al. 2006; Fajardo and Martinez 2008; Little et al. 2008). The antibiotic-mediated inhibition of plant pathogens by rhizosphere-inhabiting biocontrol microorganisms is well documented (Raaijmakers et al. 2002; Haas and Keel 2003; Haas and Defago 2005; Raaijmakers and Mazzola 2012). Among all the PGPR strains, *Bacillus* and *Pseudomonas* are the two most common genera widely used in the disease management practices through antibiotics production. Perhaps, a well-known example is the suppression of take-all disease in wheat by 2,4-diacetylphloroglucinol, produced by *P. fluorescens* in the rhizosphere (Weller et al. 2007). However, like many other bacterial species, the antibiotics production by PS organisms is also one of the important traits by which the PS bacteria prevent the proliferation of plant pathogens (Sunish et al. 2005; Naik et al. 2008; Mazurier et al. 2009). Since then, a variety of antibiotics have been identified, including compounds such as amphisin, 2,4-diacetylphloroglucinol (DAPG), oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, tropolone, and cyclic lipopeptides produced by pseudomonads (Defago 1993; Nielsen and Sørensen 2003; Raaijmakers and Mazzola 2012; Zhou et al. 2012; Saraf et al. 2014) and oligomycin A, kanosamine, zwittermicin A, and xanthobaccin produced by *Bacillus*, *Streptomyces*, and *Stenotrophomonas* spp. (Milner et al. 1995, 1996; Hashidoko et al. 1999; Nakayama et al. 1999; Mavrodi et al. 2012). In a study, 2,4-diacetylphloroglucinol (DAPG) and pyoluteorin (PLT) producing *P. fluorescens* CHA0 promoted the growth of various plants and protected them against root diseases caused by pathogenic fungi. Among the organic acids, gluconic acid was the principal acid produced by *Pseudomonas* spp. and the mutant strain (genes encoding glucose dehydrogenase (*gcd*) and

gluconate dehydrogenase (*gad*) were deleted) acidified the environment and solubilized mineral P. Furthermore, the formation of gluconic acid by CHA0 completely inhibited the production of PLT and partially hampered the synthesis of DAPG. In the Δ*gcd* mutant, which did not produce gluconic acid, the enhanced production of antifungal compounds was associated with improved biocontrol activity against take-all disease of wheat, caused by *Gaeumannomyces graminis* var. *tritici*. This study provided a new evidence for a close association of gluconic acid metabolism with antifungal compound production and biocontrol activity in *P. fluorescens* CHA0 (de Werra et al. 2009). *Pseudomonas fluorescens* strain Q8r1-96, an aggressive colonizer of the wheat rhizosphere, in a study was found to produce 1,850 μg ml^{-1} 2,4-DAPG after 48 h of growth in King's B Medium, while strain Q2-87V1 could produce only 19.4 μg ml^{-1} metabolites under the identical conditions. Rhizoplane levels of 2,4-DAPG after 4 days of Q8r1-96 colonization were 1,946, 1,650, and 2,767 ng g^{-1} for Buchanan, Finley, and Tara wheat cultivars, respectively. Metabolite levels obtained for Q2-87V1 colonization were 1,468, 366, and 80 ng g^{-1} on the respective cultivars. Thus, strain Q8r1-96 produced significantly more 2,4-DAPG than Q2-87V1 on Tara and Finley roots, whereas both strains produced similar amounts of the metabolites on Buchanan roots. In greenhouse experiments, take-all damage was reduced only on Tara roots inoculated with Q8r1-96. Moreover, in a recent study, bacterial strain, *Pseudomonas brassicacearum* J12, isolated from the rhizosphere soil of tomato plants strongly inhibited the growth of phytopathogenic bacteria *Ralstonia solanacearum*. J12 could produce 2,4-diacetylphloroglucinol (2,4-DAPG), HCN, siderophore(s), and protease. The maximum growth and antagonistic activity were recorded at 30 °C and pH 8. Strain J12 significantly suppressed tomato bacteria wilt by 45.5 % in the greenhouse experiment. The main antimicrobial compound of J12 was identified as 2,4-diacetylphloroglucinol (2,4-DAPG) by HPLC–ESI-MS analysis (Zhou et al. 2012). One problem with depending too much on antibiotic-producing bacteria as biocontrol agents is, however, that with the increased use of these bacterial strains, some phytopathogens may also develop resistance to specific antibiotics in a manner similar to those exhibited for chemically synthesized antibacterial drugs. To obviate this, it is suggested that the HCN positive biocontrol agents should be utilized along with antibiotics producing bacterial strains in order to suppress the pathogens and to avoid emergence of antibiotic resistance among bacterial species. This approach seems more credible because while HCN may not have a strong biocontrol activity by itself, it may act synergistically with bacterially encoded antibiotics.

Lytic Enzyme

Lytic enzymes such as chitinase, pectinases, and cellulases secreted by a variety of microorganisms including PS organisms disrupt the functionality of pathogens by hydrolyzing chitin, pectins, and cellulose, respectively, and thus play a pivotal role

in direct suppression of plant pathogens (Chernin and Chet 2002; Kamensky et al. 2003; Ovadis et al. 2004; Kim et al. 2008). The extracellular secretion of hydrolytic enzymes, for example, chitinase, is considered distinctly crucial in disease management strategies. For instance, *S. plymuthica* C48 through the production of chitinase has been found to inhibit spore germination and germ-tube elongation in fungal pathogen, *Botrytis cinerea* (Frankowski et al. 2001). Likewise, the PS bacterium *Serratia marcescens* (Wani et al. 2005) has shown antagonist activity against *Sclerotium rolfsii* (Ordentlich et al. 1988), while *Paenibacillus* sp. strain 300 and actinomycetes *Streptomyces* sp. strain 385 suppressed *F. oxysporum* f. sp *cucumerinum*. Extracellular chitinase and laminarinase synthesized by *Pseudomonas stutzeri* digested and lysed mycelia of *F. solani* (Lim et al. 1991). In yet other example, secretion of β-1,3-glucanases and chitinases and induction of host resistance by *P. guilliermondii* M8 played a major role in the biocontrol of *P. guilliermondii* M8 against *B. cinerea* (Zhang et al. 2011). The 1,3-glucanase synthesized by *Paenibacillus* sp. strain 300 and *Streptomyces* sp. strain 385 lysed fungal cell walls of *F. oxysporum* fsp. *cucumerinum* (Singh et al. 1999). Similar degradation of cell wall of *R. solani*, *S. rolfsii*, and *Pythium ultimum* by 1,3-glucanase of *B. cepacia* is reported (Fridlender et al. 1993). In other investigation, the PS bacterial strains produced protease and exhibited a broad-spectrum antifungal activity against phytopathogenic fungi. Also, when tested in PCR using the gene-specific primers, PS strain BFPB9 showed the presence of hcnBC genes that encode HCN. On the basis of phenotypic traits, 16S rRNA sequence homology, and subsequent phylogenetic analysis, PS strains BFPB9, FP12, and FP13 were identified as *P. aeruginosa*, *P. plecoglossicida*, and *P. mosselii*, respectively. Due to the inherent ability of protease, cellulase, and HCN production by *P. plecoglossicida* and *P. mosselii* and *P. aeruginosa*, these PS strains were suggested to be developed as biofertilizers and biocontrol agents (Jha et al. 2009).

The extracellular chitinase and an antifungal compound produced by *Chromobacterium* sp. strain C61 were investigated by Kim et al. (2014) to elucidate their biological control activity. They observed that strain C61 had antifungal activities under in vitro conditions and successfully controlled plant diseases in field conditions. The bacterium possessed a locus *chi54* encoding chitinase, while *chi54* mutant did not produce chitinase. The wild-type strain showed significantly increased production of the extracellular enzymes and expression of the *chi54* transcript, when grown in culture medium treated with chitin. Furthermore, the in vitro assays demonstrated that purified chitinase inhibited spore germination of multiple pathogens. However, the in planta biocontrol activity of filtrates of cultures grown in the presence of chitin was lower than that of filtrates grown without chitin indicating that correlation between chitinase and biocontrol activity was missing. The further analysis of C61 culture filtrates revealed an antifungal cyclic lipopeptide, chromobactomycin, whose structure contained a unique nonameric peptide ring. The purified chromobactomycin inhibited the growth of several phytopathogenic fungi in vitro, and plant application significantly reduced disease severity for several pathogens. These data suggest that both the extracellular

chitinase and the antibiotic chromobactomycin can act synergistically to suppress plant disease by *Chromobacterium* sp. strain C61.

ACC Deaminase

Plant growth can also be stimulated by PGPR including PS bacteria that produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which cleaves ACC, the immediate precursor of the plant hormone ethylene, to produce α-ketobutyrate and ammonia (Todorovic and Glick 2008; Ahmad et al. 2013). Ethylene is an important signaling molecule in plants which under pathogen attack or abiotic stress results in plant growth inhibition (Abeles et al. 1992). Following infection by a pathogen, a strong correlation between the timing of ethylene production increase and the development of chlorotic, necrotic, or wilt symptoms is reported (Goto et al. 1980; Elad 1988; Boller 1991). Inoculation of plants with ACC deaminase positive PGPR reduced the stress levels of ethylene and resulted in longer roots and promoted plant growth following environmental- or pathogen-induced stress (Glick et al. 1998, 2007; Farwell et al. 2007). For example, ACC deaminase-producing biocontrol bacteria were more effective in preventing growth inhibition on cucumber plants caused by root pathogen *P. ultimum* and in potato plants by root rot pathogen *Erwinia carotovora* (Wang et al. 2004). In a recent example, the ACC deaminase-producing *Methylobacterium* inoculated with tomato showed significantly reduced disease symptoms caused by *Ralstonia solanacearum* and lowered ethylene emission under greenhouse condition. The ACC and ACO (1-aminocyclopropane-1-carboxylate oxidase) accumulation in tomato leaves was significantly reduced with *Methylobacterium* strain inoculation. While ACC oxidase gene expression was found higher in plants treated with *R. solanacearum* than *Methylobacterium* sp. treatment, PR proteins related to ISR like β-1,3-glucanase, PAL, PO, and PPO were increased in *Methylobacterium* sp. inoculated plants. A significant increase in β-1,3-glucanase and PAL gene expression was found in all the *Methylobacterium* spp. treatments compared to the *R. solanacearum* treatment (Yim et al. 2013). In addition, transgenic tomato plants expressing a bacterial ACC deaminase gene under the transcriptional control of a root-specific promoter, which mimics the effect of adding ACC deaminase-producing plant growth-promoting bacteria to the plant roots, have been reported to significantly protect against damages from *Verticillium* wilt compared to non-transformed tomato plants (Robison et al. 2001). Stearns et al. (2012) also observed the ACC deaminase activity in PS bacterium *P. putida* and used it to evaluate its effect on growth promotion of *Brassica napus* (canola). Transcriptional changes in bacterially treated canola plants were investigated employing an *A. thaliana* oligonucleotide microarray. The results indicated that the transcription of genes involved in plant hormone regulation, secondary metabolism, and stress response was altered in inoculated plants, whereas the upregulation of genes for auxin response factors and the downregulation of stress response genes were observed only in the presence of bacterial ACC deaminase. These results, therefore, support the hypothesis that

there is a direct link between ethylene and the auxin response and that this study provides more evidence for the stress-reducing benefits of ACC deaminase-expressing plant growth-promoting bacteria (Stearns et al. 2012).

10.3.2 Indirect Plant Growth Promotion Through Induced Systemic Resistance

Inoculation of plants with sole or multiple plant growth-promoting activities containing PGPR including PS bacteria is reported to provide systemic resistance against a broad spectrum of plant pathogens, for example, those belonging to fungal, bacterial, and viral groups (Ryu et al. 2004b; Latha et al. 2009; Raj et al. 2012; Alizadeh et al. 2013; Son et al. 2014).

10.3.2.1 Induced Systemic Resistance

Plants have numerous active defense apparatuses that can aggressively be expressed when exposed to biotic stresses such as phytopathogens and parasites or viruses to phytophagous insect. Induced systemic resistance (ISR) is a phenomenon in plants which is triggered principally following inoculation of microbial cultures and is phenotypically similar to the systemic acquired resistance (SAR) that occurs when plants activate their defense systems in response to infection by a pathogenic agent (Pieterse et al. 2009). Induced systemic resistance of plants against pathogens is a widespread phenomenon that has its potential use in the management of phytopathogens, for instance, fungi, bacteria, and viruses. Induced systemic resistance involves jasmonate (JA), ethylene, and salicylic acid (SA) signaling within plants, and these hormones stimulate the host plant's defense responses to a range of pathogens (Verhagen et al. 2004). Besides ethylene, JA, and SA, other bacterial molecules such as the *O*-antigenic side chain of the bacterial outer membrane protein lipopolysaccharide, flagellar proteins, pyoverdine, chitin, β-glucans, and cyclic lipopeptide surfactants have been reported to act as signaling molecules inducing ISR. However, ISR does not require any direct interaction between the resistance-inducing bacteria and the pathogens (Bakker et al. 2007). Strains of PGPR are reported to suppress diseases by antagonism between the bacteria and soilborne pathogens (Khan et al. 2002) and by ISR in plants against pathogens (Beneduzi et al. 2012; Alizadeh et al. 2013). The PGPB-elicited ISR was first observed on carnation (*Dianthus caryophillus*) with reduced susceptibility to wilt caused by *Fusarium* sp. (Van Peer et al. 1991) and on cucumber (*Cucumis sativus*) with reduced susceptibility to foliar disease caused by *Colletotrichum orbiculare* (Wei et al. 1991). Manifestation of ISR is dependent on the combination of both host plants and bacterial strains (Van Loon et al. 1998; Kilic-Ekici and Yuen 2004). The bacterial triggered ISR strengthens plant cell wall and alters host

physiology and metabolic responses, leading to an enhanced synthesis of plant defense chemicals upon challenge by pathogens and/or abiotic stress factors (Ramamoorthy et al. 2001; Nowak and Shulaev 2003; Alizadeh et al. 2013). Interestingly, the rhizobacteria-assisted ISR is identical to that of pathogen-induced SAR in that both types of induced resistance make uninfected plant parts more resistant to a broad spectrum of plant pathogens. The type of bioprimed plant response induced after challenge with a pathogen results in the formation of structural barriers, such as thickened cell wall papillae due to the deposition of callose and the accumulation of phenolic compounds at the site of pathogen attack (Benhamou et al. 1996a, 1998). Similarly, biochemical or physiological changes in plants following inoculation include induced accumulation of pathogenesis-related proteins (PR proteins) such as PR-1, PR-2, chitinases, and some peroxidases (Viswanathan and Samiyappan 1999; Park and Kloepper 2000; Jeun et al. 2004; Latha et al. 2009; Raj et al. 2012; Chowdappa et al. 2013). However, certain PGPB do not induce PR proteins (Hoffland et al. 1995; Pieterse et al. 1996; Van Wees et al. 1997); instead, it increases the accumulation of peroxidase, phenylalanine ammonia lyase, phytoalexins, polyphenol oxidase, and/or chalcone synthase (Van Peer et al. 1991; Ongena et al. 2000; Chen et al. 2000; Ramamoorthy et al. 2001; Chowdappa et al. 2013). In several studies, numerous rhizobacteria have been found to trigger the salicylic acid (SA)-dependent SAR pathway by producing SA at the root surface, whereas some other rhizobacteria triggered different signaling pathway independent of SA. The SA-independent ISR pathway has been observed in *Arabidopsis thaliana*, which is dependent on jasmonic acid (JA) and ethylene signaling. Similar ISR in plants, for example, carnation, cucumber, radish, tobacco, and *Arabidopsis* following *Pseudomonas* inoculation, is reported (Choudhary et al. 2007). In addition to *Pseudomonas* strains, ISR is developed following inoculation of different *Bacillus* species, for example, *B. amyloliquifaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus*, and shown considerable reduction in the incidence or severity of various diseases on a variety of hosts (Choudhary et al. 2007). Similarly, the ISR in one of the study was triggered by *Trichoderma harzianum* Tr6 and *Pseudomonas* sp. Ps14, both isolated from the rhizosphere of cucumber, and had inhibitory activity against *Fusarium oxysporum* fsp. *radicis cucumerinum* (cucumber) and in *A. thaliana* against *Botrytis cinerea* (Alizadeh et al. 2013). In other reports, the ISR was triggered by *P. fluorescens* (Pf1 and Py15) and *B. subtilis* (Bs16) when used either alone or together and as mixture with the most effective plant extract, Zimmu, in both in vitro and in vivo experiments against early blight disease in tomato caused by *A. solani* (Latha et al. 2009), *P. fluorescens* EP1 against red rot caused by *Colletotrichum falcatum* on sugarcane (Viswanathan and Samiyappan 1999), *Burkholderia phytofirmans* PsJN against *Botrytis cinerea* on grapevine (Barka et al. 2000, 2002), *Verticillium dahliae* on tomato, *P. denitrificans* and *P. putida* against *Ceratocystis fagacearum* on oak (Brooks et al. 1994), *P. fluorescens* against *F. oxysporum* fsp. *radicis-lycopersici* on tomato, *B. pumilus* SE34 against *F. oxysporum* fsp. *pisi* on pea roots (Benhamou et al. 1996b), and *F. oxysporum* fsp. *vasinfectum* on cotton roots (Conn et al. 1997). Recently, *B. pumilus* strain

INR-7 effectively induced downy mildew resistance in pearl millet. The histochemical analysis of *B. pumilus* INR-7-mediated systemic resistance indicated that IR was associated with the expression of hypersensitive response (HR), enhanced lignification, callose deposition, and H_2O_2 in addition to the increased expression of the defense enzymes β-1,3-glucanase, chitinase, phenylalanine ammonia lyase (PAL), peroxidase (POX), and polyphenol oxidase (PPO). The HR was rapidly expressed in the resistant pearl millet. The further microscopic investigation of inoculated pearl millet tissues showed the presence of significantly higher levels of lignin, callose, and H_2O_2 in resistant and induced resistant seedlings. Accumulation of various defense enzymes (e.g., β-1,3-glucanase, chitinase, PAL, POX, and PPO) located in vascular bundles was an immediate response to *Sclerospora graminicola* infection and preceded the development of induced resistance elicited by strain INR-7. This study clearly demonstrated that the differences between the responses, susceptibility, INR-7 treated, or resistant pearl millet seedlings exhibited variations in the speed, intensity, and pattern of different histochemical responses to *S. graminicola* infection (Raj et al. 2012).

10.4 Conclusion

Naturally abundant yet functionally diverse rhizosphere microorganisms have immense potential in sustainable crop production and have shown significant increase in crop yields both directly and indirectly under fluctuating field environments. Besides supplying soluble P to plants and increasing crop production directly, phosphate-solubilizing microorganisms also promote plant growth and yields (primary effect) indirectly by suppressing the plant diseases (secondary effect) caused by so many phytopathogens. In some cases, the secondary effect is more obvious and effective than the primary ones. Therefore, the simultaneous biocontrol activity and other plant growth-promoting properties of PS organisms provide one of the better options to replace pesticides and chemical fertilizers in sustainable agriculture practices. From the disease management point of view, more researches aimed at finding quality biocontrol PS organisms with multiple growth-promoting activities are urgently required. Moreover, understanding the precise mode of action and the ecophysiology of the PSM in relation to other soilborne inhabitants is important which may help in developing the appropriate inoculants for their efficient use under different production systems. Further investigations including efficacy test under field conditions are however needed to consolidate the role of PS organisms as proficient biofertilizers. If the field trials show promising results, this could allow further exploiting the full potential of PS organisms as multifaceted beneficial bio-inoculants at commercial scale for increasing the growth and health of plants which in turn is likely to reduce problems associated with the use of toxic chemicals in agriculture practices.

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Chapter 11
Microbial Consortium of Plant Growth-Promoting Rhizobacteria Improves the Performance of Plants Growing in Stressed Soils: An Overview

Meenu Panwar, Rupinder Tewari, and Harsh Nayyar

Abstract Soil and associated stress conditions not only influence dwelling microbial populations and soil ecosystems but also affect growth and yield of plants. Major soil stress includes salinity, drought, and metal contamination. Due to burgeoning populations and expanding food demands, it has become imperative to alleviate the stressful soil conditions so that the crop production is increased and, consequently, the food demands are fulfilled. Different strategies are followed to resolve this problem, and one such approach involves exploiting microbial potential for plant's benefit. The multifunctional microscopic life-forms are already known for their applications in industries, medicine, and agricultural field. One of the major attributes of microbes from agronomic point of view is their ability to solubilize difficultly available forms of soil phosphorus. Phosphate-solubilizing microbes are also known to produce enzymes, siderophores, and growth hormones; embellish plant growth and biocontrol activity; and improve soil properties. Such microorganisms possessing attributes, beneficial for plants are termed as plant growth-promoting rhizobacteria (PGPR). There are plentiful reports on bacterial-mediated plant growth promotion under nonstressed conditions although fewer reports are available on their effects under stressed condition. The bacterial ability to enhance tolerance of plants in stressed soils and the impact of PGPR consortium (mixture) on different crops are highlighted. The major idea here is to consolidate the fact that PGPR consortium can be used directly in stress-affected soil with an aim to refurbish soil conditions to foster crop productivity in stressed soils.

Keywords PGPR • Microbial consortium • Siderophores • Biocontrol • Soil stressors

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11.1 Introduction

Soil inhabits different life-forms including plants, animals, and microorganisms and is a nutrient hoarded treasure, a support system (for plant) furnishing with plentiful crops and yields. Any change in soil conditions eventually affects plant growth. Human activities and ever-increasing populations are continuously exploiting this natural system, consequently affecting the growth and productivity of plants. However, there are certain soil factors which negatively affect plant growth. These are (1) moisture content, (2) salt, (3) nutrient pool of soils, (4) microbial composition and their functional variation, and (5) soil pollution especially deposition of toxicants (heavy metal and pesticides) in soil. When deviated from optimal conditions, these factors cause adverse effects and are specified as stress conditions for soil. The deleterious impacts of these stresses include dwindling productivity, burden on delimited resources, and economic fall. Considering these threats, researchers from different fields are working in unison to avert such problems. One such area involves the exploitation of microbiological resources of soils. Microorganisms are known to be omnipresent and possess multifunctional characteristics even though the full potential of microorganisms is still unrevealed. Most of the chemical reactions occurring in soil leading to nutrient availability are mediated by different microorganisms like N_2 fixers, P solubilizers, or decomposers (Powlson et al. 2001). Considering the available information and application of microorganisms, there has been greater interest in using such organisms to restrain the adverse effects also (Vassilev et al. 2012).

Microorganisms colonizing the rhizospheres are known to have beneficial effects on the nutrient acquisition, mineral solubilization, disease resistance, and stress tolerance and are collectively described as plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978; Vessey 2003). Reports are available in the literature on the effectiveness of rhizospheric microorganisms as plant growth promoters as well as on their potential for imparting stress resistance or improving stress tolerance in plants, presenting PGPR as viable option to cope with these problems (Yang et al. 2009; Zelicourt et al. 2013; Ahemed and Kibret 2014). Another aspect of exploiting microbial potential is to combine the attributes of different microbes to get an outcome encompassing numerous or complementing beneficial effects. Microorganisms are known to have attributes like cooperation/mutualism where they benefit each other or other life-forms to enhance the positive outcomes (Singh et al. 2010). Multiple properties of resistance/tolerance and plant growth promotion, therefore, serve as an appraisal and make PGPR one of the most suitable choices to manage these problems (Bano and Fatima 2009; Egamberdieva and Kucharova 2009; Zelicourt et al. 2013). Judicial application of the stress-tolerant PGPR consortium can be a viable solution and need to be further strengthened through field trials. The present chapter gathers reports on the experimental studies done on PGPR consortium helping plant/crops cope with stressful soil conditions. Also, the focus is given here on soil stress and associated effects including mechanisms of PGPR in stress alleviation.

11.2 Stresses Occurring in Soils

Soil can be defined as upper layer of earth where plant grows and have their roots (Brady 1974). Soil indeed is the habitat for both microscopic (millions of microorganisms) and macroscopic (insects, animals, plants) life (Pelczar et al. 1993; Saika 2013). The plants along with soil inhabiting microbes affect the soil structure, fertility, and porosity; prevent erosion; and serve as source of organic matter; likewise, any alteration in soil influences these life-forms. Soil stress is one of the abiotic factors and can be defined as environmental variables affecting soil, which can induce potentially injurious effects on the growth and yield of plants. Stress in plants is mainly measured in relation to survival, growth, crop yield, biomass, and primary assimilation processes associated with growth (Oliveira et al. 2013). These abiotic stresses also reduce the number, activity, and diversity of soil microflora, which in turn may limit the crop production (Sgroy et al. 2009).

11.2.1 Types of Soil Stresses

Soil stresses involve drought stress (decreased water availability to plants), salt stress (increase salts in soil solution), heavy metal stress (excessive toxic metals in soil), nutrient stress (insufficient nutrients in soil), and temperature stress (extremes of temperature both high and freezing). Of these, drought is one of the most important stresses followed by salinity stress (Kinje 2006; Carmen and Roberto 2011). Extensive areas of land are affected by these two stresses and are reported to have maximum deleterious effects on the agricultural productivities (Oliveira et al. 2013). The effects of drought and salt stress are highly interrelated and influence practically almost every aspect of plant. The effects of stresses on plants involve disrupted photosynthesis leading to leaf senescence, accumulation of excessive reactive oxygen species (ROS), nutrient deficiency, and destruction of cellular organelles and metabolism leading to decreased plant growth. The after-effect includes both physiological and metabolically disturbed homeostasis of plant (Carmen and Roberto 2011; Oliveira et al. 2013). Metal stress is another important soil stress, which is becoming increasingly intensive due to numerous anthropogenic factors (Glick 2010). Unchecked increase in population and industrial revolution is resulting in accumulation of toxic metals and organic wastes in soil making it unsuitable for agricultural practices and also harmful to all life-forms (Glick 2010). Some of the effects of these stresses are briefly outlined in Fig. 11.1.

11.2.1.1 Drought Stress

Water comprises 80–90 % of the plant biomass and plays central role in all major physiological processes of the plants involving nutrient uptake and photosynthesis.

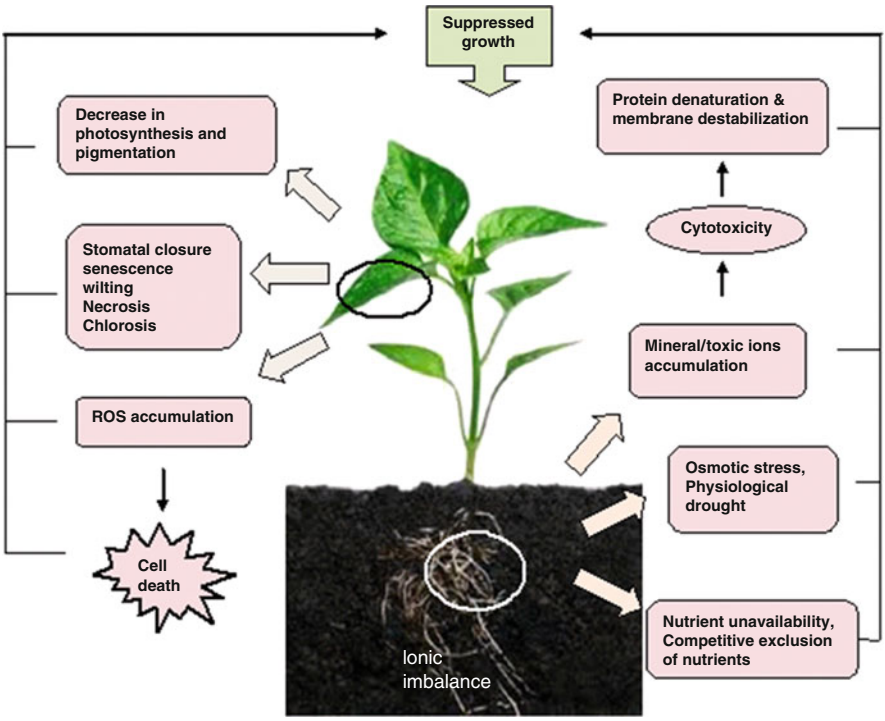

Fig. 11.1 An overview of stress effects on the plant. Effects include a combinatorial picture of salt and metal stress (ionic imbalance) and drought stress (osmotic stress) on physiological and metabolic aspects of plants focusing mainly on leaves and root-associated processes which ultimately lead to inhibition in growth

Drought stress can be defined as low water or moisture content in soil, not enough to fulfill the plant requirements. When the water loss occurs due to metabolic processes and transpiration exceeds the water availability for absorption or when water content of plant gets low enough to interfere with normal plant processes, water deficit/stress is created. It can also result from reduced moisture of soil, due to less rainfall or supplemental irrigation. Water stress has been found as an important factor affecting deleteriously various stages/metabolic processes of plants (Upadhyay and Panda 2013). For example, water stress reduces the water potential of plant cell and thus enhances the solute concentration, which further hinders cell enlargement, stem proliferation, and root elongation, thereby hampering the plant growth (Akinci and Losel 2012). However, when plants are growing under stressed situation, it exhibits visible symptoms. As an example, "wilting" is the condition of plants where the non-wooden parts of the plants become nonrigid due to low turgor pressure and is one of the most common symptoms of water stress (Correia et al. 2001; Cabuslay et al. 2002). Also, water stress may cause stomata closure. Accumulation of plant hormone, for instance, abscisic acid (ABA), is responsible for the stomatal closure (Socias et al. 1997). This further reduces gaseous exchange,

transpiration, and CO_2 assimilation during photosynthesis (Cornic 2000). Also, water stress results in reduced chlorophyll content, inhibits chloroplast activity and disorganizes thylakoid membranes, decreases the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase and other enzymes in carbon reduction cycle (Reddy et al. 2004), impairs electron transport, and increases the concentration of ROS. The imbalance in scavenging and formation of ROS and increased O_2 photoreduction in chloroplast results in ROS accumulation (Robinson and Bunce 2000). The ROS damages photosynthetic apparatus, cell membrane, and macromolecules. DNA nicking, denaturation of structural and functional macromolecules, lipid peroxidation, oxidation of amino acids and proteins, and photosynthetic pigments are some of the effects of ROS accumulation (Lisar et al. 2012). Stomata closure under drought stress is also found to be related to altered nutritional status, xylem sap pH, and hydraulic conductivity as well as declines water content in leaf (Oren et al. 1999). Summarily, drought stress interrupts the enzymatic reactions mainly involved in CO_2 fixation and ATP synthesis and thus affects the plant by altering (1) photosynthesis, (2) transpiration, (3) nutrient uptake, (4) hormone production, (5) homeostasis, and (6) other metabolic processes.

11.2.1.2 Saline Stress

In agricultural terms, salinity can be defined as salt level exceeding the plant requirements (Yadav et al. 2011). In other words, it can also be defined in terms of dissolved mineral salt concentration, i.e., electrolytes of cations and anions where major cations involve Na^+, Ca^{2+}, Mg^{2+}, and K^+ and anions involve Cl^-, SO_4^{2-}, CO_3^{2-}, HCO_3^-, and NO_3^-. According to USDA Salinity Laboratory (Seidahmed et al. 2013), saline soil is defined as soil having electrical conductivity 4 dS m^{-1} or higher. The excessive concentrations of salt change the physicochemical properties of soil and affect the nutrient uptake from soil, making nutrients inaccessible for plants. Some of the effects of salt stress on plants include deteriorated growth, nitrogen content, photosynthetic capacity, and metabolic processes including protein and lipid metabolism (Upadhyay et al. 2011). Salt stress has been reported to drastically affect the growth and yield of several crops (Parida and Das 2005; Ondrasek et al. 2011). Broadly, effects of salt stress can be categorized as (1) unavailability of water causing drought-like conditions; (2) high salt content in plants, i.e., Na^+ and Cl^-, leading to disrupted physiological and biological processes; and (3) high salt content affecting availability of other soil nutrients. One of the most dominant symptoms of salt stress involves stunted growth. Cessation of leaf expansion and reduction in dry weight and fresh weights of stem, roots, and leaves are some other effects of the salt stress (Hernandez et al. 1999; Wang and Nil 2000). Salt stress affects largely the shoot growth compared to root growth and hence influences both vegetative and reproductive stages of plants. It creates osmotic and ionic stress due to less water content and high salt concentration, respectively. The osmolarity of external tissues results in

decreased growth of plant (Munns 2002), whereas the ionic effect leads to ion (mainly Na^+) accumulation mainly in leaf tissues leading to necrosis. "Necrosis" is death or degeneration of tissue, visible as yellowing or dark patches on plant leaves. Due to excessive salt in soil, the required nutrient becomes unavailable for plants. The salt ions (Na^+) intervene the transporters of root plasma membrane and hamper root growth, thus obstructing the nutrient uptake by plants (Yadav et al. 2011). Salt stress causes water deficit, which results in oxidative stress due to formation of ROS, causing membrane dysfunction and cell death (Parida and Das 2005). Lipids also act as a target for oxidative reactions and, being structural constituent of membranes and insulator for internal organs, damage the cellular structure aggravating negative effects of the salt stress (Singh et al. 2002). The high concentration of solutes in root medium interferes with the water absorption by roots and reduces root conductivity. These effects further lead to decreased plant growth and photosynthetic rate. The chlorophyll and carotenoid content in leaves decline under salt stress. Symptoms of chlorosis appear on leaves due to the reduction of photosynthetic pigments. Salt stress affects different physiological processes such as cessation of carbon assimilation in leaves, reduction in permeability due to dehydration, closure of stomata affecting chloroplast activity, senescence, ionic leakage into the cytosol leading to inactivation of photosynthetic and respiratory electron transport (Allakhverdiev et al. 2000; Parvaiz and Satyawati 2008), and altered enzyme activity due to change in cytoplasmic structure.

11.2.1.3 Metal Stress

Heavy metals (HM) can be defined as elements with metallic properties and higher range of molecular weight and include transition elements. The industrial revolution and anthropogenic activities have dramatically raised the metal concentration in soil (Yan-de et al. 2007; Oves et al. 2012). Among these metals, iron (Fe), molybdenum (Mo), and manganese (Mn) are known as essential micronutrients required by the plants, while a few, for example, cadmium (Cd), do not have any biological activity. Other metals like chromium (Cr), copper (Cu), mercury (Hg), lead (Pb), and nickel (Ni) are also common in soil. Based on the requirement, HM can be divided into essential and nonessential elements, although the excessive accumulation of both of these in soils adversely affects the plants (Wani et al. 2012; Morsy et al. 2013) as well as soil microflora (Oliveira and Pampulha 2006; Wani and Khan 2010). The plentiful HM in soil is absorbed and translocated to various organs of plants and impairs plant metabolism and growth (Bingham et al. 1986; Cheng 2003; Ahmad et al. 2012b). The excessive metals in soil also affect soil properties and fertility, making it unsuitable for agricultural activities.

The possible toxic impact of heavy metals on plant includes (1) disintegration of cell organelles and (2) disruption of membranes and physiological processes like (a) photosynthesis, (b) inactivation of protein synthesis, (c) inactivation of respiration and carbohydrate metabolism, and (d) nutrient uptake (Jing et al. 2007; Wani et al. 2007; Wani et al. 2008; Khan et al. 2012). Metal accumulation also results in

reduced microbial population (Wani and Khan 2013) thereby affecting the soil fertility and making it unsuitable for sustainable agriculture (Cheng 2003). Germination rate and root vitality of the plant are also affected by the metal stress (Shu et al. 1997). Heavy metals were also known to affect the cell division by causing inhibition of DNase and RNase activity; damaging nucleolus and disrupting DNA synthesis; and causing chromosomal aberration, coagulation, and fragmentation (Yang and He 1995; Musarrat et al. 2011). Reduced cell division and elongation along with decreased cell membrane integrity are some other effects of membrane toxicity. Some of the visible symptoms include interfoliar chlorosis, wilting, necrosis, crinkling of leaf, reddening, and purpling (Reichman 2002). Lessened chlorophyll content, reduced photosynthetic rate, and augmented carotenoid breakdown are also some of the results of metal toxicity. Accumulated metals are believed to replace Mg ion of the chlorophyll molecule thus affecting photosynthesis (Kupper et al. 1996). Heavy metals are also known to disrupt the photosystems ensuing decreased proton availability, consequently affecting photosynthesis. Reduced ATP synthesis and disrupted activity of chloroplast are some other effects reported for metal toxicity by disruption of enzymatic systems (Teige et al. 1990). Like any other stress, free radical production is increased in plant as a response to metal stress. The concentration of metal plays an important role here as at low concentration the protective antioxidant enzymes balance the effect, but at higher metal toxic condition these accumulated free radicals damage membranes by lipid peroxidation (Yadav 2010) followed by injury to surrounding cells. Free radicals also damage macromolecules like nucleic acids and proteins, thus disrupting normal metabolism and leading to cell death. Leaf senescence is another effect of oxidative damage due to ROS accumulation (Luna et al. 1994). Since growth, yields, and many other physiological functions of plants are affected negatively by toxic metals (Yadav 2010; Selvakumar et al. 2012), remedial measures are urgently required for its cleanup from the contaminated sites (Khan et al. 2011; Zaidi et al. 2012). In this context, scientists around the world have attempted to use molecular tools and breeding programs for exploiting physiological traits of plants, developing new stress-tolerant crop varieties, altering crop calendars, and managing agronomic resources to circumvent stress-related impact on plants. Another well-considered option in this direction is the use of microorganisms for combating stress (Khan et al. 2009). In this regard, reports on the individual/combined use of metal-tolerant/normal microorganisms in growth promotion and other positive effects on plants are available (Selvakumar et al. 2012; Ahmad et al. 2013; Oves et al. 2013).

11.3 Plant Growth-Promoting Rhizobacteria

Soil is inhabited by numerous microorganisms, which can be categorized as beneficial or detrimental based on their effect on the soil, plants, and ultimately plant's yield (Singh et al. 2011a). The diverse microbial population of soil plays a pivotal role in processes determining soil fertility and plant's productivity (Tilak

et al. 2005). Soil microorganisms participate in processes like decomposition, mineralization, and nutrient availability, improve soil structure (soil aggregation by production of polysaccharides), increase the nutrient acquisition efficiency of the plants, and improve plant health through growth hormone production (Hayat et al. 2010; Singh et al. 2011b). Microbial populations having the ability to colonize root surface and imparting beneficial effects to plants are known as plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978; Joshi and Bhatt 2011). Plant growth-promoting rhizobacteria facilitate plant growth both directly and indirectly (Glick 2012). Some of the notable PGPR belong to genera *Arthrobacter* (Banerjee et al. 2010), *Azotobacter* (Ponmurugan et al. 2012), *Azospirillum* (Jacoud et al. 1999), *Bacillus* (Kumar et al. 2011), *Enterobacter* (Shoebitz et al. 2009), *Pseudomonas* (Noori and Saud 2012), and *Serratia* (Zhang et al. 1997). Based on the proximity with the plant roots, PGPR can be divided into (1) extracellular PGPR, existing in rhizosphere, rhizoplane, or spaces between root cortices, and (2) intracellular PGPR, present within roots or nodules of the plant. Also, based on the mode of action, PGPR have been classified as (1) bio-stimulants which promote plant growth via phytohormone production, including auxins IAA and similar compounds like abscisic acid, gibberellic acid, cytokinins (Carmen and Roberto 2011); (2) biofertilizers which enable nutrient availability and acquisition via N_2 fixation (Mohammadi and Sohrabi 2012) and P solubilization (Khan et al. 2007; Zaidi et al. 2009; Khan et al. 2010; Das et al. 2013); and (3) bioprotectants which provide protection to plants against phytopathogens via production of antibiotics (Labuschagne et al. 2011), siderophores (Glick 2012), and induced systemic resistance (Figueiredo et al. 2011).

11.3.1 Direct Mechanisms

11.3.1.1 Production of Plant Growth Regulators

Microorganisms are known to produce plant growth-stimulating substances such as phytohormones, for example, auxins (Spaepen and Vanderleyden 2011), cytokinins (Nieto and Frankenberger 1990), gibberellins and abscisic acid (Singh 2013), etc., as well as certain volatiles (Ryu et al. 2003). The phytohormone-producing microorganisms include *Acetobacter diazotrophicus* (Patil et al. 2011), *Azospirillum brasilense* (Perrig et al. 2007), *Herbaspirillum seropedicae* (Bastian et al. 1998), *Bacillus pumilus* and *B. licheniformis* (Gutirrez-Manero et al. 2001), etc.

11.3.1.2 Nitrogen Uptake

Specialized microorganisms have capability to fix atmospheric N (biological nitrogen fixation; BNF) and maintain the balance of N in soil ecosystem. Nitrogen fixers are categorized into two groups: (a) symbiotic nitrogen fixers and (b) nonsymbiotic

nitrogen fixers. *Rhizobium* and *Frankia* belong to symbiotic N_2 fixers that associate with legumes, whereas nonsymbionts are free-living N_2 fixers which interacts with nonleguminous plants (Ahemed and Kibret 2014). Numerous PGPR are also known to possess this attribute although the mechanism responsible for their growth promotion is not N_2 fixation. Some of these PGPR are *Azotobacter* (Kizilkaya 2009), *Bacillus* (Ding et al. 2005), *Clostridium*, *Klebsiella* (Iniguez et al. 2004), *Alcaligenes*, *and Arthrobacter* (Mohammadi and Sohrabi 2012).

11.3.1.3 Increased Mineral Uptake

Plant growth-promoting rhizobacteria are reported to provide nutrients to plants via mineralization/solubilization of unavailable minerals like P (Khan et al. 2007). Also, the siderophores, secreted by PGPR strains, play important roles in mineral transport (Vessey 2003; Ahmad et al. 2013). Mineralization process involves conversion of organic P into soluble forms through enzymes like phytases and phosphatases (Walpola and Yoon 2012), whereas in solubilization the inorganic P is transformed into soluble forms via organic acid production, acidification of medium (Park et al. 2009; Khan et al. 2010), chelation, and exchange reactions (Walpola and Yoon 2012). Both solubilization and mineralization mechanism can occur in one bacterial species also. Some of the phosphate solubilizing (PS) bacteria include *Acinetobacter* (Rokhbakhsh-Zamin et al. 2011), *Burkholderia* (Gupta et al. 2012), *Enterobacter* (Gupta et al. 2012; Maheshwari and Sudha 2013), *Klebsiella* (Ahemad and Khan 2011), *Pseudomonas* (Rajkumar and Freitas 2008), and *Stenotrophomonas* (Mehnaz et al. 2010). Numerous studies have been conducted globally to analyze the effects of various P solubilizers on growth, yield, and other important parameters of plants (Khan et al. 2009; Ahmad et al. 2012a). Some of the examples supporting the effectiveness of these microorganisms against different crops are listed in Table 11.1.

11.3.2 Indirect Mechanisms

11.3.2.1 Antibiotic Production

Antibiotics are defined as heterogenous low molecular weight organic compounds secreted by microorganism, having destructive/inhibitory effects on the growth and metabolism of other microorganism/s (Duffy 2003; Beneduzi et al. 2012). PGPR are also known to produce antibiotics and other small molecules preventing plants from damage caused by the plant pathogens. These antibiotics are categorized as (A) nonvolatiles including polyketides (e.g., pyoluteorin), heterocyclic nitrogenous compounds such as phenazine derivatives, phenylpyrrole (e.g., pyrrolnitrin), lipopeptides (e.g., bacillomycin), aminopolyols (e.g., zwittermicin A) and (B) volatile antibiotics such as hydrogen cyanide (HCN), aldehydes, sulfide,

Table 11.1 Examples of P-solubilizing microorganism and their effects on the plants

P solubilizer	Plants	Effect	Reference
Bacillus megaterium	Sugarcane (*Saccharum officinarum*)	Enhanced sugarcane and sugar yield, P content in soil	Sundara et al. (2002)
Bacillus sp.	Banana cultivars (*Musa paradisiaca*)	Improved yield and mineral content, fresh biomass (aerial and root), aerial dry mass, diameter, and foliar surface	Jaizme-Vega et al. (2004)
Pseudomonas sp.	Tomato (*Solanum lycopersicum*)	Enhanced growth	El-Tantawy and Mohammed (2009)
Pantoea eucalypti	Slender trefoil (*Lotus tenuis*)	Enhanced growth	Castagno et al. (2011)
Variovorax paradoxus	Pea (*Pisum sativum)*	Increased root-shoot biomass, stomatal conductance, enhanced nutrient availability, and P accumulation	Jiang et al. (2012)
Burkholderia multivorans WS FJ9	Poplar (*Populus euramericana cv.*)	Increased height, root collar diameter, biomass, P content	Li et al. (2013)
B. tropica KS04	Chili (*Capsicum frutescens L. cv. Hua Rua*)	Significant increase in height, fresh weight, root and shoot dry weight, as well as number of flowers	Boonlue et al. (2013)

ketones, and alcoholic compounds (Fernando et al. 2006). Some of the antibiotics like 2,4-diacetylphloroglucinol (Shanahan et al. 1992), phenazine-1-carboxylate (Chin-A-Woeng et al. 2001), pyoluteorin (Howell and Stipanovic 1980), pyrrolnitrin (Thomashow and Weller 1988), and HCN are produced by *Pseudomonas* sp. (Hass and Defago 2005); bacillomycin (Volpon et al. 1999), kanosamine (Milner et al. 1996), and iturin A (Constantinescu 2001) are produced by *Bacillus* sp. (Fernando et al. 2006). Toluene, dimethyl disulfide, and terpenoid compounds like α-pinene and limonene are the other volatiles produced by *Burkholderia* sp. (Tenorio-Salgado et al. 2013).

11.3.2.2 Siderophore Production

Siderophores are low molecular weight peptide molecules with side chains and functional groups acting as ligand for Fe^{3+} (Beneduzi et al. 2012). Siderophores are also known as "iron carriers" and act as biocontrol agents by sequestering iron (Fe), required for phytopathogens. By limiting the iron availability, siderophores inhibit the growth of phytopathogens in immediate vicinity of plant and hence indirectly protect plant from pathogen damage (Glick 2012). Siderophore-producing PGPR, for example, *Pseudomonas* sp. and *Enterobacter* sp. (Gram-negative bacteria) and

Bacillus sp. and *Rhodococcus* sp. (Gram-positive bacteria) (Saharan and Nehra 2011) also deprive native microflora from available iron and thus outnumber the native microbes and exhibit plant growth-promoting effect (Kloepper et al. 1980).

11.3.2.3 Induced Systemic Resistance

Induced systemic resistance (ISR) is another indirect mode of action where PGPR or nonpathogenic rhizobacteria act as stimuli, and in response, plants develop enhanced resistance to pathogens. ISR involves actions of nonpathogenic bacteria and is mainly dependent on jasmonic acid and ethylene signaling in plants (Lugtenberg and Kamilova 2009). Some of the putative mechanisms responsible for enhanced resistance include accumulation of phenolic compounds, increased activity of defense enzymes, enhanced lignifications, etc. Many *Pseudomonas* sp. and *Bacillus* sp. are recognized to act as biocontrol agents and protect plant from pathogens through this mechanism (Kloepper et al. 2004). PGPR-mediated ISR against bacteria, fungi, and viruses has already been reported (Niranjan et al. 2005).

11.4 Microbial Consortium

"Consortium" is a Latin word, which stands for partnership, association, or group, that works for common interest. From the microbiological perspective, consortium constitutes a group of compatible organisms belonging to different species in contact with one another, implicated in different biological processes ranging from sewage treatment to metabolic processes in rumen (Mark 2009). Two or more microorganisms living in symbiosis can be called as consortium. Microbes with different attributes can be used as consortium, which can work synergistically promoting each other's beneficial effects. Some of the PGPR consortium-related studies are summarized in Table 11.2. A study involving N_2 fixing, *R. leguminosarum* bv. *viceae* (LB-4); P solubilizing, *B. megaterium*; and PGPR, LK-786 (*Kurthia* sp.) and LK-884 (*Pseudomonas diminuta*) was carried out to ascertain their effects on lentil (*Lens culinaris*) crop following single and dual culture inoculation (Kumar and Chandra 2008). Maximum increase in dry weight, yield, mineral uptake, and nodule number was reported in case of all microbial combination as compared to dual combinations of *Rhizobium* + *B. megaterium or Rhizobium* + LK-884/LK-786 (*Kurthia* sp.), whereas no positive effects were observed in uninoculated controls. A similar study was carried out using consortium of *Burkholderia gladioli* 10242, *Enterobacter hormaechei* 10240, *Pseudomonas synxantha* 10223, and *Serratia marcescens* 10241, for their effect on the *Aloe vera* plants. The result indicated augmented biomass as well as aloin-A content of the plants (Gupta et al. 2012). An experimental study was conducted on the evaluation of effects of PGPR consortium comprising FCA-8, FCA-56, and FCA-60 of *P. putida* and arbuscular mycorrhizal fungi (AMF) on citrus (*Citrus*

Table 11.2 Examples of PGPR consortium effects on various crops

PGPR	Crop/plant	Effects	Reference
Rhizobium + *B. megaterium or Rhizobium* + LK-884 (*P. diminuta*)/LK-786 (*Kurthia sp.*)	Lentil crop (*Lens culinaris*)	Increased dry weight, yield, mineral uptake, and nodule number	Kumar and Chandra (2008)
A. brasilense strain Az39 and *B. japonicum* strain E109	Soybean (*Glycine max*) and corn/maize (*Zea mays*)	Augmented germination rate, shoot-root length, dry weight, and nodulation	Cassan et al. (2009)
A. lipoferum, *P. fluorescens*, and *P. putida*	Maize (*Zea mays*)	Improved biomass and yield	Adjanohoun et al. (2011)
PGPR strains FCA-8, FCA-56, FCA-60 of *P. putida* and AM-fungi	Citrus (*Citrus volkameria*)	Plant height, stem-base diameter, root length and volume, biomass, and colonization similar to fertilization	Chiquito-Contreras et al. (2012)
Different combinations of PGPR	Artichoke (*Cynara scolymus*)	Increased shoot length, root and shoot weight, vigor, germination percentage, and mean time of germination	Jahanian et al. (2012)
Pantoea cypripedii and *Enterobacter aerogenes*	Chickpea (*Cicer arietinum*)	Increased P uptake by plant	Singh et al. (2013)
Trichoderma viride, *P. fluorescence*, and *A. chroococcum*	Chili (*Capsicum annum* L.)	Improved growth and yield	Sateesh and Sivasakthivelan (2013)

volkameriana) (Chiquito-Contreras et al. 2012). The study involved consortium treatment with 50 % fertilization, whereas control involved no PGPR inoculation with 100 % fertilization. Different parameters studied involved plant height, stem-base diameter, root length and volume, biomass, and colonization; results so obtained were similar to the effects obtained with control, suggesting that their effectiveness is similar to fertilizers.

Besides agricultural crops, PGPR were also found effective in facilitating the growth of flower crops (Kumari et al. 2013). One such study involved the combination of four PGPR (*A. chroococcum*, *A. lipoferum*, *B. megaterium*, and *P. fluorescens*) on rose plants (*Catharanthus roseus*). Mixed inoculation enhanced growth, vigor, nutrient content (P, K, and N by 2.34 %, 2.2 %, and 0.34 %, respectively), and chlorophyll content (Lenin and Jayanthi 2012). Another comparative experiment involving single, double, and consortium inoculation of *A. chroococcum*, *P. fluorescence*, and *T. viride* was carried out for chili crop (*Capsicum annum* L.). Maximum growth and yield were recorded for consortium cultures relative to single and double inoculation (Sateesh and Sivasakthivelan

2013). Phosphate-solubilizing *Pantoea cypripedii* and *Enterobacter aerogenes* used together increased P uptake by 53 % in chickpea crop compared to control (Singh et al. 2013).

11.5 PGPR and Stress Alleviation

Different studies have suggested that such microorganisms can also divulge some degree of tolerance to the plants thus imparting resistance to these plants. Tolerance can be defined as microbe's intrinsic property to encounter stressful conditions, whereas resistance is microorganism's ability to withstand stressful conditions by certain mechanisms. Some of the experimental evidence indicates that microorganisms with tolerance/resistance abilities can help plants to successfully adapt to different stressed situations. Therefore, the organisms endowed with tolerance/resistance abilities can be used effectively as beneficial inoculants for enhancing crop production in stressed/derelict soils (Khan et al. 2011; Milosevic et al. 2012). Some of the mechanisms by which PGPR ameliorate stress situations are discussed in the following section and are illustrated in Fig. 11.2.

11.5.1 Mechanisms and Role of PGPR in Stress Alleviation

11.5.1.1 Exopolysaccharide Secretion

Microorganisms belonging to different functional groups for example rhizobia secrete exopolysaccharides (EPS), which provide resistance to cell against different stressors and thus protect the microorganism from stress. The EPS also improve the soil structure by forming macroaggregates with soil, which further increase the water retention ability of soil (Alami et al. 2000). Macroaggregates uphold equilibrium in aerobic and anaerobic conditions in soil and also ascertain gradual uptake of nutrients from soil. In case of salt stress, these aggregates help by binding cations making them unavailable to plants (Haynes and Swift 1990). The rhizobacteria have the ability to form biofilms by secreting polysaccharides and proteins, the matrix so formed limits the diffusion of compounds like plant growth hormones and nutrients from the plant's vicinity, thus promoting plant growth by alleviating stress conditions (Timmusk et al. 2013).

11.5.1.2 Accommodation: Accumulation and Sequestration of Metals

Plant growth-promoting rhizobacteria produce metal-chelating agents, known as siderophores, an iron-chelating agent, which can make the required iron available to plants and hence prevent plants from becoming chlorotic and indirectly

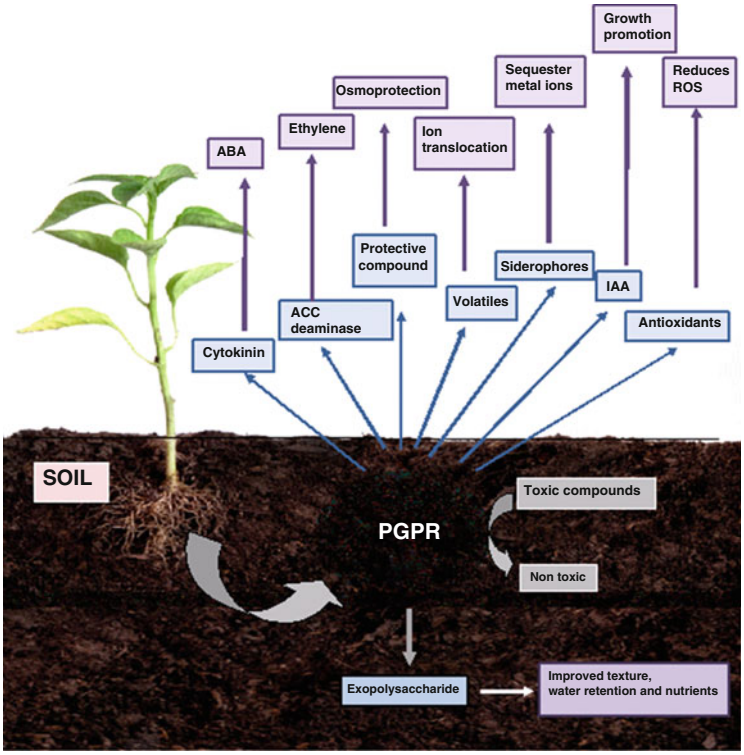

Fig. 11.2 Stress alleviation modes of PGPR (modified from Yang et al. 2009). *ABA* abscisic acid, *IAA* indole acetic acid, *ROS* reactive oxygen species

ameliorating metal stress. The siderophores also bind to other metals like Mg, Mn, and Cr and chelate the solubilized/leached metals (Akhtar et al. 2013). Siderophore-producing PGPR can therefore be used to remove toxicants (metals) from metal polluted soil (Mani et al. 2010). Along with metal stress, siderophore-producing rhizobacteria also inhibit the phytopathogen (Kloepper et al. 1980; Glick 2012) as discussed in Sect. 11.3.2.2.

11.5.1.3 Biotransformation: Conversion of Toxic Forms to Less Toxic Forms

Microorganisms especially PGPR can help in relieving metal toxicity by transforming highly toxic metals to less toxic forms or in forms more readily accessible to plant roots (Khan et al. 2009). The conversion of metals involves mainly a change in the valence state of metals, for example, change of organic selenium to selenate or organo-selenium (Zayed et al. 1998) which can easily be taken up by plants. This feature of PGPR has been well exploited in

phytoremediation technology for enhancing metal removal by plants (Jing et al. 2007). Furthermore, rhizobacteria affect the adsorption/desorption of metals by altering their chemical properties, pH, organic matter content, redox state, etc., consequently affecting their solubility and mobility (Gray et al. 1998). PGPR also improve the efficiency of phytoremediation strategy of metal cleanup by increasing the hyper-accumulating abilities of certain plants through their rapid growth in metal stress (Varsha et al. 2011).

11.5.1.4 P Solubilization

The amount of P available to plants is very less as compared to total soil P pool. One of the important attributes of PGPR is phosphate solubilization and the group of microorganisms capable of converting inorganic P into soluble forms is known as P-solubilizing microorganisms (Khan et al. 2007). Along with P assimilation, these microorganisms release a fair amount of soluble P into soil which can be used as P source by the plants. The most efficient PS bacterial strains are *Pseudomonas* (Das et al. 2003) and *Rhizobium* (Sridevi and Mallaiah 2009), whereas *Penicillium* (Chai et al. 2011) and *Aspergillus* (Singh and Reddy 2011) are the most powerful fungal PS strains (Khan et al. 2010; Brahmaprakash and Sahu 2012).

11.5.1.5 Improves Plant Defense Mechanisms Under Stressed Environment

Modulating Enzyme: 1-Aminocyclopropane-1-Carboxylate Deaminase

Under normal condition, plant maintains its homeostasis by producing a hormone "ethylene" which plays important role in various developmental processes. Under stress conditions, the amount of ethylene produced by plant increases due to which it is also known as "stress ethylene." At higher concentrations, it decreases root and shoot growth and also induces defense responses of plant to mitigate adverse effects. Plant growth-promoting rhizobacteria produce an enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which degrades ACC, the precursor for ethylene, into ammonia and α-ketobutyrate. Bacteria utilize ammonia as N source thereby restricting the ethylene accumulation consequently rescuing the plant growth from the stress (Khan et al. 2009). Also, PGPR synthesize growth hormone IAA from tryptophan produced in plant root exudates which in turn enhances both plant growth and activates enzyme ACC synthase involved in ACC production. ACC so produced is then exuded from the plant roots and acted upon by the bacteria (Selvakumar et al. 2012).

Volatile Production

PGPR are known to produce volatile organic and inorganic compounds which can affect the plant growth and resistance/tolerance against biotic and abiotic stresses via different mechanisms. Some of the volatile compounds produced by different PGPR include 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol by *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a (Ryu et al. 2003) and C-13 hydrocarbon tridecane by *Paenibacillus polymyxa* E681 (Lee et al. 2012). These volatile compounds were found to affect the tissue-specific regulation of high-affinity K^+ transporter 1 (HKT1), which is further involved in the regulation of Na^+ homeostasis in salt stress. The volatiles downregulate hkt1 in roots but upregulate them in the shoot, lowering Na^+ levels and recirculation of Na^+ levels in plant (Yang et al. 2009). Other mechanisms include enhanced iron uptake by upregulating FIT1 (Fe-deficiency-induced transcription factor) during metal stress and production of compatible solutes like betaine under oxidative stress (Farag et al. 2013). These volatiles are also found to have negative effects on the plant growth under certain circumstances (Bailly and Weisskopf 2012). Some of the volatiles involved in stress resistance against biotic factors like pathogens have been already discussed in Sect. 11.3.2.1.

Synthesis of Auxins and Similar Compounds

Microbial auxins can affect the plant's auxins governed developmental processes such as root development including root length, surface area, and number of root tips. This root development further enables nutrient uptake by plants, thereby improving plant health in the presence of inhibitory compounds or under stress conditions (Egamberdieva and Kucharova 2009). Plant exudates contain tryptophan, which is when acquired by rhizobacteria converted to IAA. The microbial IAA along with plant's pooled auxins stimulates plant growth and proliferation (Glick 1995).

Protective Compounds

Microorganisms are known to produce osmo-protectants such as proline, betaine, trehalose, and glutamate which modulate their cytoplasmic osmolarity and hence protect plants from stress conditions (Blanco 1994). Plant also produces protective compounds or compatible osmolytes in response to stress conditions, mainly salt stress. Some of these compounds include amino acids, imino acids, amides, proteins, quaternary ammonium compounds, and polyamines (Carmen and Roberto 2011). Increased production of proline in response to stressors has been reported (Lalelou et al. 2010; Marin et al. 2010) which plays a role in osmo-adaptation in salt stress (Meloni et al. 2001), and as a molecular chaperone it protects and stabilizes

macromolecules like proteins during dehydration and also acts as a scavenger for hydroxyl radical, thus protecting from osmotic stress (Csonka 1989; Upadhyay et al. 2012).

Antioxidative Enzymes

Another mechanism of PGPR to counteract stress involves the production of ROS scavengers. Enhanced production of ROS, such as H_2O_2, hydroxyl radicals, singlet oxygen, and superoxide, ensues oxidative damage to DNA, proteins, and lipids. This response is mainly an outcome of imbalance in production and scavenging of ROS due to stress condition. Major ROS scavengers include catalase, superoxide dismutase, and ascorbate peroxidase. PGPR, for instance, *Serratia* sp., *Rhizobium* sp. (Han and Lee 2005), *Bacillus* sp., *Arthrobacter* sp. (Upadhyay et al. 2012), *Azospirullum* sp., and *Pseudomonas* sp. (Baniaghil et al. 2013), are reported to enhance the production of these antioxidant enzymes responsible for ROS degradation/breakdown, thereby helping plants to ameliorate stress response and also growth promotion (Kohler et al. 2009; Carmen and Roberto 2011).

Induced Systemic Tolerance

Similar to ISR for biotic factors, another term "induced systemic tolerance (IST)" had been proposed for abiotic stress alleviation by PGPR. IST is defined as physical and chemical changes elicited by PGPR in response to abiotic stresses such as salt stress, drought stress, temperature stress, metal stress, or nutrition deficiency (Yang et al. 2009). These microbial communities follow different mechanisms such as production of (1) volatiles to modulate Na^+ homeostasis under salt stress (Farag et al. 2013); (2) abscisic acid causing closure of stomata, thus preventing water loss in drought stress; (3) antioxidant enzymes like superoxide dismutase and catalase, which degrade the reactive oxygen species, bringing down cell damage (Selvakumar et al. 2012); (4) IAA, cytokinins, and other metabolites stimulating root growth, thus helping nutrient acquisition combating nutrient deficiency; etc. (Yang et al. 2009). Some of the PGPR reported for IST include *B. cereus*, *B. subtilis*, *Serratia* sp. (Wang et al. 2012), *Paenibacillus polymyxa* (Timmusk and Wagner 1999), *Achromobacter piechaudii* (Mayak et al. 2004), etc.

11.6 PGPR Consortium Application in Plants Growing in Stressed Soils

11.6.1 Drought Stress

The consortia of *Paenibacillus polymyxa* (DSM 36) and *P. polymyxa* Loutit (L) along with *Rhizobium tropici* (CIAT 899) significantly increased growth, N content, and nodulation of common bean (*Phaseolus vulgaris*) growing under drought stress conditions (Figueiredo et al. 2008) compared to plants inoculated only with *Rhizobium*. However, negative effects of drought stress on the measured parameters were observed suggesting that the mixture of bacteria had a positive mitigating impact on stressor. Single and multiple inoculations with different *Pseudomonas* sp. were carried out to study the effect on Asparagus (*Asparagus officinalis*) cultivars (Guelph millennium and Jersey giant) under both drought and flood stress up to 8 weeks. The results so obtained were significantly convincing in one of the cultivars in case of both single and multiple inoculation (Liddycoat et al. 2009). Five drought-tolerant bacterial strains, namely, *Pseudomonas entomophila* strain BV-P13, *P. monteillii* strain WAPP53, *P. putida* strain GAP-P45, *P. stutzeri* strain GRFHAP-P14, and *P. syringae* strain GRFHYTP52, were used to inoculate maize grown under water-deficit conditions. The PGPR inoculation reduced the drought stress damage and improved plant biomass, leaf water potential, relative water content, aggregation stability, sugars, amino acids, and proline content. The effects also included decreased electrolyte leakage and water loss from leaves (Sandhya et al. 2010). In other experiment, three plant growth-promoting strains—*B. cereus AR*156, *B. subtilis* SM21, and *Serratia* sp. XY21—decreased wilting symptoms and leaf monodehydroascorbate in cucumber (*Cucumis sativus*) plant, while they showed 3.45-fold increase in proline content along with increased SOD activity, supporting the hypothesis of induced systemic tolerance in drought stress (Wang et al. 2012). The combined application of PGPR (*A. brasilense*, *B. lentus*, and *Pseudomonades* sp.) improved antioxidant activity and also indicated better photosynthetic capacity and improved photosynthetic pigments in Basil (*Ocimum basilicum*) (Heidari and Golpayengani 2012), while the combined inoculation of different PGPR strains increased superoxide dismutase and peroxidase activity along with better chlorophyll content and transpiration in runner bean plants (*Phaseolus coccineus* L.) (Stefan et al. 2013).

11.6.2 Salt Stress

Effects of dual inoculation of *Serratia* sp. and *Rhizobium* sp. on the growth and other parameters of lettuce plant grown under salt stress were variable. PGPR negated the effects of salt stress on the antioxidant enzymes and on photosynthesis,

mineral content, and growth (Han and Lee 2005). And hence, the consortia of microbial cultures showed both growth-promoting activity and the stress alleviation activity. Another greenhouse study was carried out on two legumes like common bean and soybean under moderate salt conditions (25 mM) where rhizobial strains *R. tropici* (CIAT899) or *R. etli* (ISP42) and *Ensifer fredii* (*Sinorhizobium*) SMH12 and HH103 along with PGPR *Chryseobacterium balustinum* Aur9 strains were used both individually and in combination to determine their effects on nodulation and growth. The coinoculation significantly increased the nodule primordial formation in common bean and showed better nodulation and shoot-root growth in both crops (Estevezi et al. 2009). In yet other report, the coinoculation of *Pseudomonas* sp. and *Rhizobium* sp. showed maximum increase in growth (dry weight and height), mineral accumulation, ion uptake, chlorophyll content, and proline content in maize (cv. Agaiti 2002 and Av 4001) plants grown under salt stress compared to single inoculations of either culture (Bano and Fatima 2009). The consortia of EPS producing salt-tolerant PGPR strains comprising of *Bacillus* sp., *Burkholderia* sp., *Enterobacter* sp., *Microbacterium* sp., and *Paenibacillus* sp. increased the biomass of wheat (Upadhyay et al. 2012). The mixture of salt-tolerant bacteria such as strains of *Brachybacterium saurashtrense* (JG-06), *Brevibacterium casei* (JG-08), and *Haererohalobacter* (JG-11) augmented the water content, metal ion ratio K^+/Na^+, and mineral and auxin content and decreased the electrolyte leakage and oxidative damage in peanut (*Arachis hypogaea*) plants compared to uninoculated control plants (Shukla et al. 2012). In a similar study, Nadeem et al. (2013) observed a significant increase in germination rate and percentage, growth, yield, and nutritional status of wheat inoculated with consortia of *Enterobacter cloacae*, *Pseudomonas putida*, *P. fluorescens*, and *Serratia ficaria*, when grown under saline-stressed environment. The co-culture of *Pseudomonas syringae* Mk1, *P. fluorescens* Mk20, and *P. fluorescens Biotype* G Mk25 in combination with *R. phaseoli* (M1, M6, and M9) increased the shoot weight, root weight, number of pods, and total dry weight of mung bean plants by 145 %, 173 %, 150 %, and 269 %, respectively, when grown in saline condition. Furthermore, the seedling growth, nodulation, and mineral uptake were significantly enhanced following mixture of PGPR where there was a substantial reduction in salt stress due to microbial application (Ahmad et al. 2012a; Aamir et al. 2013). Two bacterial strains *A. brasilense* and *Pantoea dispersa* showed a significant increase in dry weight and K^+/Na^+ level of salt-sensitive sweet pepper (*Capsicum annuum*) compared to uninoculated controls. The net assimilation rate remained unaffected even at higher salinity level (80 mM) in case of inoculated plants. Inoculated plants were also found to have higher stomatal conductance at higher stress (Amor and Cuadra-Crespo 2012).

11.6.3 Metal Stress

Plant growth-promoting attributes of metal-tolerant *Flavobacterium* sp., *Rhodococcus* sp., and *Variovorax paradoxus* were found to stimulate the root growth of rapeseed both in the presence and the absence of Cd, supporting their role as promoters under metal-stressed situation (Belimov et al. 2005). A study on the effect of metal-tolerant PGPR *Burkholderia* sp. CMBM40 and *Methylobacterium oryzae* CMBM20 inoculation on tomato plants grown in Ni- and Cd-treated soil was carried out. The PGPR were found to decrease the metal uptake by plants and also enhanced the plant growth by producing growth hormones (Madhaiyan et al. 2007). Consortia of *Bradyrhizobium* sp. with metal-tolerant PGPR *Pseudomonas* sp. and *Ochrobactrum cytisi* significantly improved biomass, yield, and N content of metal accumulating *Lupinus luteus* plants but they decreased metal accumulation within plants (Dary et al. 2010). Likewise, the metal-tolerant PGPR consortia significantly increased root length, shoot length, biomass, and chlorophyll content of mung bean by 138 %, 88 %, 256 %, and 54.1 %, respectively, when grown in chromium-treated soils (Singh et al. 2010). Similar enhancement in some cereals, for example, wheat following metal-tolerant PGPR, *B. thuringiensis* and *P. fluorescens* (Shahzadi et al. 2013) and *A. brasilense* and *A. chroococcum* (Janmohammadi et al. 2013), has been reported. The PGPR *Ralstonia eutropha* (B1) and *Chryseobacterium humi* (B2) inoculated sunflower (*Helianthus annuus*) plants when grown in Zn- and Cd-contaminated soil had decreased metal concentration inside plant tissues, suggesting that metal-resistant PGPR might have served as effective stabilizers for plants grown in metal-contaminated soil (Marques et al. 2013).

11.7 Conclusion

Among various abiotic stresses, drought, salinity, and metal pollution are the most stronger and stringent ones, which restrict the overall performance of plants growing in such derelict soils. The sole or composite (consortia) application of PGPR is an emerging area of interest because these microbes have been found to enhance the growth and development of plants both under conventional and stressed environments in different production systems across varying ecological niches. Moreover, microbial inoculation is cost effective, environmentally friendly, and easy option for farm practitioners. However, before they are made commercially available, more field trials are needed to get the full benefit of this strategy in combating stress-related problems caused to agronomically important crops. Considering the available information, it is believed that the practice of PGPR consortium application is likely to grow faster and agricultural practices will slowly be able to shifting its focus from fertilizer to efficacious use of PGPR.

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Chapter 12
Inoculation Impact of Phosphate-Solubilizing Microorganisms on Growth and Development of Vegetable Crops

Asfa Rizvi, Md. Saghir Khan, and Ees Ahmad

Abstract Vegetables are one of the important food components of dietary systems in many countries including Asian regions. It provides some of the essential nutrients such as carbohydrates, proteins, and fats and therefore plays a critical role in the human health. Vegetables while growing in soil require significant amounts of phosphorus for better biological growth and optimum yields. The soluble and available forms of phosphorous in soil are, however, limited and not accessible for uptake by vegetable crops. To this end, apart from chemical phosphatic fertilizers, one strategy to provide phosphorus to vegetable crops is the use of phosphate-solubilizing microorganisms, which are ubiquitous and both inexpensive and safe to the environment. Phosphate-solubilizing microorganisms secrete organic acid which in turn solubilizes the complex forms of phosphorus and makes it available to vegetable plants, besides exhibiting other growth-promoting activities. Here, the impact of phosphate-solubilizing microorganisms onto the growth and yield of vegetables is discussed and considered. This approach of using PS microorganisms in vegetable cultivation is likely to help in reducing, if not completely eliminating, the use of synthetic fertilizers in vegetable production across different regions of the world.

Keywords PSM • Vegetables • Synthetic fertilizers • Brinjal • Potato • Tomato

12.1 Introduction

Vegetables are the source of several important nutrients and form an intricate part of our daily routine diets. For proper development and higher yields, vegetable crops grown in different production systems rely hugely on various plant nutrients

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(Solaiman and Rahbbani 2006) including the major element phosphorous (P). Even though the vegetables require high amounts of P for its luxuriant growth and development, the accessibility of P to such crops is restricted due to its rapid fixation ability (Khan et al. 2007; Bushman et al. 2009). Therefore, the deficiency of soluble P in soil has become one of the most limiting factors in crop production in different agroclimatic regions. The plants obtain their P requirements from the soil pool where it occurs as inorganic P, produced as a result of weathering of parent rock or as organic P derived from decayed plants, animal remains, or microorganisms. Mineral forms of P present in soil are apatite, hydroxyapatite, and oxyapatite, while organic P occurs chiefly in the form of inositol phosphate. Other organic P compounds in soil are in the form of phosphomonoesters, phosphodiesters (including phospholipids, nucleic acids), and phosphotriesters (Paul and Clark 1988). Although P is present in soil in abundance, yet it is the least soluble and majority of it is immobilized and rendered unavailable for plant uptake. Plants acquire P from soil solution as phosphate anions which are extremely reactive and are immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+}, and Al^{3+}. And hence, the soluble fraction of P within the soils is usually very low relative to other mineral nutrients.

Deficiency of P is a common and quite widespread problem among many soils including the Indian soils, because of which, the growth of vegetable suffers heavily. Phosphorous, therefore, needs to be applied frequently and externally in the form of phosphatic fertilizers in order to maintain a lavish crop growth. The chemical fertilizers when used, however, also become rapidly immobilized soon after application and thus remain unavailable to the plants (Sanyal and Datta 1991; Rodriguez and Fraga 1999). In this context, soil microorganisms play an important role in phosphate solubilization by mineralizing the organic P in the soil and thus making it available to the plants. Some microbes, isolated from various rhizospheric soils including those of vegetable rhizospheres, popularly known as the phosphate-solubilizing microorganisms, have this ability of solubilizing insoluble mineral P by various mechanisms (Sung-Man et al. 2010; Varsha et al. 2010; Sagervanshi et al. 2012; Sharma et al. 2012; Alia et al. 2013; Onyia and Anyanwu 2013; Karpagam and Nagalakshmi 2014). Some of the important genera of phosphate-solubilizing bacteria include *Achromobacter*, *Aerobacter*, *Alkaligenes*, *Bacillus*, *Pseudomonas*, *Serratia*, and *Xanthomonas* (Li 1981; Sharma et al. 2005; Chen et al. 2006; Ivanova et al. 2006). Besides providing P, PSM also facilitate the growth of vegetables by other mechanisms (Jeon et al. 2003; Lucy et al. 2004; Egamberdiyeva 2005; Calvo et al. 2010; Kang et al. 2010; Sung-Man et al. 2010; Dastager et al. 2011; Sagervanshi et al. 2012).

12.2 Importance of Vegetables in Human Dietary System

Vegetables are considered a cheap source of energy as compared to other foods (Alertor et al. 2002; Hussain et al. 2009) but serve as a rich source of essential nutrients such as carbohydrates, carotene, protein, vitamins, calcium, iron, ascorbic acid, dietary fiber, and concentration of trace minerals (Salunkhe and Kadam 1995). Major nutritional components of some of the widely consumed vegetables are listed in Table 12.1. Indeed, vegetables are one of the important food components of human diets world over and have numerous health benefits. Cruciferous vegetables, for instance, contain protein, carbohydrate, and vitamins (ascorbic acid, folic acid, tocopherols, and provitamin A). The cruciferous vegetables contain both major essential mineral elements such as Ca, K, P, and Na, Mg, etc. (Singh et al. 2001), while Fe, Se, Cu, Mn, and Zn are micronutrients found in these vegetables. Among the root vegetables, carrot is rich in carotenoids and dietary fibers with high levels of several other functional components that aid in improving human health. Spinach, on the other hand, has a high nutritional value and is extremely rich in antioxidants and has vitamin A, vitamin C, vitamin E, vitamin K, Mg, Mn, folate, betaine, Fe, vitamin B_2, Ca, K, vitamin B_6, Cu, protein, P, Zn, niacin, Se, omega-3 fatty acids, and folic acid. Spinach also has a high Ca content. Potato contains several vitamins and minerals along with carbohydrate (starch) (≈26 g/medium-sized potato). The starch of potato has the similar physiological effects and health benefits as fiber and offers protection against colon cancer, improves glucose tolerance and insulin sensitivity, lowers plasma cholesterol and triglyceride concentrations, and reduces even fat storage. Tomatoes on the contrary are versatile vegetable in daily dietary practice and contain lycopene, one of the most powerful natural antioxidants. Lycopene has also been shown to protect against oxidative damage in many epidemiological and experimental studies. In addition to its antioxidant activity, other metabolic effects of lycopene have also been demonstrated. Tomato consumption has been associated with decreased risk of breast, head, and neck cancers and might be strongly protective against neurodegenerative diseases. In general, diets rich in these foods are associated with a lower risk of the chronic disease of cancer (Hennekens 1986) and heart diseases (Vanpoppel et al. 1994). Apart from human health benefits, vegetables in general improve the quality of the soil where they are growing (Hussain et al. 2010).

12.3 Importance of P to Some Vegetable Crops

Worldwide crop production remains limited due to low phytoavailability of P (Abd El-Salam et al. 2005; Khan et al. 2010). Therefore, it is required at regular basis to overcome the P deficiency to crop plants in P-deficient soils. Among different vegetables, potato, for example, has a relatively high P requirement, but it uses soil P inefficiently due to the limited accessibility of P. However, from the primary

Table 12.1 Nutritional value of some common vegetables

	Vegetables			
Nutrient components (g/100 g)	Brinjal	Cabbage	Tomato	Potato
Energy (kcal)	25	25	18	77
Carbohydrate	5.88	5.80	3.9	17.47
Protein	0.98	1.28	0.9	2.00
Fat	0.18	0.10	0.2	0.10
Dietary fibre	3.00	2.50	1.2	2.20
Sugars	3.53	3.20	2.6	15.44

Source: USDA Nutrient Database

growth until the maturity stage, an adequate supply of P is required by the plant (Grant et al. 2001). When sufficient concentration of P is taken up by potato, it promotes rapid canopy development, root cell division, tuber set, and starch synthesis in potato. An ample amount of P is therefore essential for optimizing the tuber yield, nutritional quality, and resistance of potato to some diseases also. Other studies have also demonstrated a significant increase in yield, number of tubers, and tuber size distribution due to fertilizer P application (Jenkins and Ali 1999; Maier et al. 2002; Sanderson et al. 2003). Even though an inverse relationship between tuber number and tuber size is reported (Knowles and Knowles 2006), an increase in tuber number with P fertilization has shown both an increase and decrease in tuber size (Freeman et al. 1998; Jenkins and Ali 1999). Also, P is an important constituent of nucleoproteins and nucleic acids of other vegetables such as brinjal (Parihar and Tripathi 2003). Onion (*Allium cepa* L.) is yet another most important commercial bulbous vegetable which requires sufficient amount of P among other macro- and micronutrients, from very early stages of growth for optimum production (Grant et al. 2001).

12.3.1 Relevance of Phosphate-Solubilizing Bacteria to Vegetable Crops

Vegetable crops require highest quantity of N, P, and K, while other nutrients, including Fe, Cu, Mn, and Zn, are needed in much smaller amounts. Apart from N and P, majority of these nutrients are most likely available in the soil in adequate or even excessive amounts. When nutrients are not needed by vegetables yet they are added to soil, may lead to deficiencies of other nutrients and can result into nutrient imbalance within soils. For example, when P is applied, but not needed, it can kill off the symbiotic mycorrhizal-forming fungi required by the plant and reduce the ability of vegetables to absorb iron and other micronutrients. Similarly, excess soil P also shuts down the plant's ability to produce phytochelates, organic molecules produced by roots to increase its iron uptake. Considering the cost and some side effects of excessive application in vegetable cultivation, there is an urgent need to

protect the vegetable crops from deleterious impact of chemical fertilizers. In this regard, bacteria possessing the capability to solubilize/mineralize insoluble/organic forms of P, known as phosphate-solubilizing bacteria (PSB), have provided some solutions to the expensive synthetic P fertilizers (Khan et al. 2009, 2010; Calvo et al. 2010; Madgaonkar and Lakshman 2013). Phosphate-solubilizing bacteria isolated from different ecological habitats have been used for improving crop production including vegetables (Han and Lee 2005; Turan et al. 2007; El-Tantawy and Mohamed 2009) since 1903 (Khan and Joergensen 2009). The PSB are ubiquitous with variation in forms and population in different soils. These, PS bacteria are being used as biofertilizer since the 1950s (Kudashev 1956; Krasilinikov 1957) to supply soluble P to vegetable crops in an environment friendly and sustainable manner (Khan et al. 2007) by production of organic acids (solubilization) or by catalyzing organic P by enzymes (mineralization) (Khan et al. 2009, 2013).

12.4 Examples of Effects of PS Bacteria on a Few Notable Vegetable Crops

12.4.1 Brinjal

Brinjal (*Solanum melongena* L.) is one of the most popular and widely grown vegetables in the world. Generally, solanaceous vegetables require larger quantities of major nutrients like N, P, and K for optimum yields. In this context, PSB strain has been used to provide P to plants (Han and Lee 2005; Turan et al. 2007). The PS bacterium *Bacillus megaterium*, for example, when used as microbial inoculant against brinjal plants grown in nutrient-deficient soils, resulted in a higher P availability in the soil, and consequently there was more uptake of P by brinjal plants leading eventually to enhanced growth. Furthermore, the shoot and root dry weight of eggplants were increased substantially by 30 and 27 %, respectively, due to sole application of PSB or inoculation combined with RP after 30 days of planting. A significant increase in plant height, dry weight, and rate of photosynthesis was also observed following PSB application. Also, photosynthetic rates were enhanced by 12 % under the influence of PSB inoculation. From this study, it was suggested that *Bacillus megaterium* could be used as a biofertilizer to enhance various growth parameters and yield of eggplant in P-limited soils (Han and Lee 2005). Single and composite inoculation effects of some other PSB on two varieties of brinjal, viz., "Muktajhuri" and "VNR60," were found to be greatly variable. There was a significant positive effect of PSB on the vegetative growth of brinjal plants, and hence, an increase in fruit yield was observed when compared with uninoculated control. The PSB isolates when used either alone or in combination had a pronounced impact on growth and yields. However, the tripartite combinations of all the three P solubilizers resulted in highest crop yield compared to single

inoculation of PSB. In summary, there was an overall improvement in average plant height, plant canopy, and other measured yield parameters when the brinjal cultivars were inoculated with the PSB strains, indicating a clear-cut role of PS bacteria in the development of eggplants (Roy and Sengupta 2008).

12.4.2 Potato and Tomato

Globally, approximately 40 % of world's land has low crop production efficiency especially for potato because its roots have limited access to P in the soil (Igual et al. 2001). Moreover, potato needs high amounts of P because of its high biomass producing ability. In order to circumvent this P deficiency, chemical fertilizers are used, but due to rapid fixation ability, P is not available for consumption by potato plants. PSM here play an important role and supply P to potato by secreting certain organic acids (Rashid et al. 2004; Uma and Sathiyavani 2012). Three PS bacterial strains, namely, *Pantoea agglomerans*, *Microbacterium laevaniformans*, and *Pseudomonas putida*, when used singly or in combination against potato (*Solanum tuberosum*), demonstrated a positive response under three sets of experiments, i.e., laboratory, greenhouse, and fields. The combinations of either *P. agglomerans* or *M. laevaniformans* strains with *P. putida* led to higher biomass and potato tuber growth in greenhouse and in field trials. This increase was attributed to the fact that mixture of an acid- and a phosphatase-producing bacterium might have allowed the simultaneous utilization of both inorganic and organic P compounds by potato plants. On the contrary, the Pi levels of soil or application of chemical Pi fertilizer, however, did not cause much difference in potato yields. Of all the three PSB, *P. agglomerans* significantly increased the growth and yield of potato plants by about 20–25 % (Malboobi et al. 2009). Likewise, the dry weight of creole potato roots, and the soil available N, showed better results with the inoculation of 50 % of the inoculum consisting of PSB (*Pseudomonas cepacia*, *Xanthomonas maltophilia*, *Enterobacter cloacae*, and *Acidovorans delafieldii*, formerly called *P. delafieldii*) and four strains of *Azotobacter chroococcum* plus 50 % of chemical fertilizer. A dual inocula of PSB and *A. chroococcum* resulted in significant production of "criolla" potato, Yema de Huevo variety (*Solanum phureja*), at a level matching that of crops grown solely with 100 % NPK fertilizer. Furthermore, approximately 7.4 % reduction in costs of production was observed following microbial inoculation (Faccini et al. 2007). According to Naderi et al. (2012) in a follow-up study, the tuber number per plant, stem number per plant, and plant height of potato were not affected, but the PSB application had significant effects on tuber formation (yield) and tuber mean weight. Leaf area index (LAI), crop growth rate (CGR), and relative growth rate (RGR) were all higher in the first stage of growth due to PSB application which further increased at later stages of plant growth. Among all treatments, spraying PSB on the soil treated with 100 kg/ha P chemical fertilizer displayed the best production of potato,

suggesting that this combination of fertilizer and PSB could serve as a sound strategy for sustainable production of potato in any conducive environment.

Tomato (*Lycopersicon esculentum* Mill) is the other important vegetable crop, which contains some important minerals and vitamins. Tomatoes, eaten freely throughout the world, are believed to benefit the heart among other things. Lycopene is one of the most powerful antioxidants found in tomato, and, when cooked, tomatoes have been found beneficial in preventing prostate cancer. The NPK are the most important nutrients supporting its growth, while deficiency of any one of these nutrients limits growth and yield. To increase the availability of P for plants, large amounts of phosphatic fertilizer are used on a regular basis. However, due to reasons explained in the other section (Sect. 12.3.1), PS microorganisms are considered to supply P to tomato plants in a more economical and hazard-free manner. For example, the PS bacteria isolated from tomato rhizosphere efficiently promoted the growth of tomato plants under laboratory conditions. Moreover, shoot length, root length, fresh weight, dry weight, and P content of the plants were increased following PSB application over control. The concentration of available P in rhizospheric soil collected after 30 days growth of tomato plants was higher in rhizospheric soil samples of plants bacterized with PSB over control. The inoculated tomato plants accumulated more P than control plants. Subsequently, the PSB-inoculated plants were healthier and were protected well from diseases like *Fusarium* wilt and early blight, and hence, the overall disease incidence was significantly decreased in the inoculated plants (Hariprasad and Niranjana 2009). Also, two PS bacterial isolates (*Pantoea agglomerans* and *Burkholderia anthina*) in a pot experiment under greenhouse conditions remarkably enhanced plant height, root length, shoot and root dry weight, P uptake of tomato plants, and available P content of soil compared to the control. The enhancement was more pronounced in co-inoculation of PSB strains with TCP. It was, therefore, concluded that the PSB strains possessed greater potential to be developed as biofertilizers for enhancing soil fertility and concurrently the health of tomato plants (Walpola and Min-Ho 2013) Also, the impact of *Bacillus* application along with fertilizer treatment on growth and phosphorous content of tomato was studied. Similar increase in tomato growth and yields following *Pseudomonas* (El-Tantawy and Mohamed 2009) or other PSB inoculation is reported (Awasthi et al. 2011).

12.4.3 Cucumber and Pepper

The impact of a P solubilizer *Bacillus megaterium* var. *phosphaticum* on cucumber and pepper in nutrient-deficient soils was variable, but this strain enhanced nutrient P uptake from the soil and promoted the growth of plants. The availability of P increased further for plants inoculated with PSB when applied with RP (Han and Supanjani 2006). Plant growth-promoting rhizobacteria (*Pseudomonas* sp.), PS biofertilizer prepared from *Pseudomonas putida* strain P13 and *P. agglomerans* strain P5, and chemical fertilizers were used in a separate experiment to evaluate

their effect on yield and yield components of cucumbers under field environment. The results clearly showed that the mixture of PGPR (*Pseudomonas* sp.), strains of *P. putida* and *P. agglomerans*, and chemical fertilizers demonstrated a profound increase in length, fresh and dry weight of roots and shoots, and yield of cucumber plants (Isfahani and Besharati 2012). Bacterial cultures, for instance, *Pseudomonas* sp., exhibiting high PS ability isolated from the rhizosphere soil and root cuttings of bush black pepper (*Piper nigrum* L.) when used in combination with N_2 fixing *Azospirillum sp.* and VAM showed additive effect on black pepper under greenhouse trials and reflected their potentiality as efficient P solubilizer for black pepper growing in soils (Ramachandran et al. 2007). In a similar way, a Gram-positive, rod-shaped potential PSB *Bacillus* strain which shared highest sequence similarity to *Bacillus tequilensis* NRRL B-41771T (99.5 %) produced good amount of IAA and was positive for siderophore production. The seed inoculation with this strain (NII-0943) resulted in significantly higher root initiation in black pepper cuttings grown under pot experiments. The soil N and P and P and N uptake by inoculated plants were also enhanced significantly following bacterial inoculation (Dastager et al. 2011).

12.5 Conclusion and Future Prospects

Even though phosphorus is an essential nutrient required for proper growth and development of vegetable plants, it is generally unavailable due to its rapid fixation ability with soil constituents. No doubt, PSM in this context can act as a better and viable substitute and may supply an ample quantity of P to vegetable crops in an inexpensive way. Therefore, researchers need to identify more and more potentially sound PSM so that they could be developed as microbial P inoculants for raising the production of vegetables in eco-friendly way under different agroecological regions. Broadly, the use of PSB in vegetable cultivation has genuinely provided an exciting and meaningful option for enhancing its production and simultaneously preserving the inherent characteristics of diverse agroecosystems from the unpleasant shock of synthetic fertilizers. The success of PSB application, however, depends on selection and delivery of quality PSB inoculants, which requires considerable attention of the scientists to overcome such challenges.

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