Chapter 13
Perspectives of PGPR in Agri-Ecosystems

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13.1 Introduction

One of the finest success stories of post independent era is the green revolutions in 1960s, which transformed the country from "begging bowl" to "breadbasket." This has been possible because of the use of chemical fertilizers and hybrid crops. However, in the long run, the use of chemical fertilizers had led to many serious problems which have forced the scientists to explore the other alternatives. One approach in this direction has been the use of biofertilizers, better known as plant growth-promoting rhizobacteria (PGPR). PGPR are those bacteria which are able to colonize plant root systems and promote plant growth (Kloepper and Schroth 1978).

PGPR can affect the plant growth directly by: (1) production or changing the concentration of phytohormones such as IAA (Mordukhova et al. 1991), gibberellic acid (Mahmoud et al. 1984), cytokinins (Tien et al. 1979), and ethylene (Glick et al. 1995); (2) solubilization of mineral phosphates and other nutrients (De Freitas et al. 1997); (3) asymbiotic N_2 fixation (Kennedy et al. 1997), or indirectly by showing antagonism against phytopathogenic microorganisms, e.g., *Fusarium* spp. by production of siderophores (Scher and Baker 1982), β-1,3-glucanase (Fridlender et al. 1993), cyanide (Flaishman et al. 1996), chitinases (Renwick et al. 1991), and antibiotics (Shanahan et al. 1992).

The rhizobacteria may be present (1) in the soil surrounding roots, utilizing the metabolites leaked from roots as the growth nutrients; (2) on the root surface or rhizoplane; (3) in the root tissue, inhabiting spaces between cortical cells; and (4) inside the cells in specialized root structures or nodules. Thus, based on their root proximity and intimacy of association, PGPR are categorized into two different classes: (1) extracellular PGPR (ePGPR), present in rhizosphere; and (2) intracellular

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PGPR (iPGPR), exist inside the cells in specialized nodular structures (Gray and Smith 2005).

The early reports of iPGPR dating back to 1880s, talk about the isolation of rhizobia from the root nodules and demonstrated their ability to convert the atmospheric nitrogen into plant usable forms. Since that time, intensive researches have taken place in this field which has laid the foundation upon which the agricultural use of PGPR is based. To know the PGPRs better, the investigations are going on at genomic, proteomic, cellular, whole plant, and environmental level.

However, the field performance of PGPR has not been very satisfactory and only a few studies have shown consistent performance of these PGPR in the soil. This may be due to various abiotic factors like composition and properties of the soil, availability of soluble organic products and molecular oxygen, etc.; and biotic factors like interactions between PGPR and resident microorganisms, protozoan predation, and bacterial parasitism. The most important factor determining the survival of PGPR is the competition for the limiting resources between the PGPR and natural fauna of the soil. However, PGPR inoculation has been found to be effective in a nutrient deficient or stressed soil, where the development of the resident microorganism is poor (Strigul and Kravchenko 2005).

13.2   Rhizospheric Biodiversity

Biodiversity of soil microbes has been regarded as human and vegetation life resource, especially the one connected with biological and environmental resources because of their vital role in various biogeochemical cycles running through the flora, fauna, and the life of microbes itself. Microbe community composes one of the important components of the soil. A large variety of microbial species reside in the soil. This biodiversity and their activity depend upon the physical and chemical properties of the soil along with the climate and vegetation of that area.

Soil microbes are one of the biota communities, which are very interesting to be studied in order to find out their existence and uses. It is well known that the soil microbes show symbiosis and commensalism with their host, however not every soil microbe is compatible with its host and habitat. Each type of soil microbe fills as a unique niche and plays a different role in nutrient cycling and soil structure.

The microorganisms colonizing in the rhizosphere can be grouped into bacteria, fungi, actinomycetes, algae, and protozoa. Out of these, the microbes that are useful as biofertilizer as the examples cited are mostly Biofertilizers and not Biocontrol agents are *Klebsiella*, *Nitrosomonas*, *Thiobacillus*, *Lactobacillus*, *Azotobacter*, *Azospirillum*, *Rhizobium*, *Bacillus*, *Pseudomonas*, *Enterobacter*, *Arthrobacter*, *Alcaligenes*, *Serratia*, *Streptomyces,* and *Frankia* (Sri Widawati et al. 2004).

Now-a-days, the rhizobacterial community structure can be characterized by the advanced phenotypic and genotypic approaches. Phenotypic methods that rely on the ability to culture microorganisms include standard plating methods on selective

media, community level physiological profiles (CLPP) using the BIOLOG system (Garland 1996), phospholipid fatty acid (PLFA) (Tunlid and White 1992), and fatty acid methyl ester (FAME) profiling (Germida et al. 1998). Culture-independent molecular techniques are based on direct extraction of DNA from soil and 16 S-rRNA gene sequence analysis, bacterial artificial chromosome, or expression cloning systems (Rondon et al. 1999).

An understanding of the mechanisms is imperative to utilize its potential in agri ecosystems plant productivity. The mechanism of action of PGPR falls into two categories – direct and indirect. Direct mechanisms are those that occur inside the plants and directly affect the plants metabolism like nitrogen fixation, phosphate solubilization, production of siderophores, induction of systemic resistance (ISR), etc. whereas, indirect mechanisms include improved nutrient availability to plant, free nitrogen fixation, inhibition of microbes that have a negative growth on plants.

Some of the important mechanisms have been discussed in detail.

13.3   Mechanisms of Plant Growth Promotion

A thorough understanding of the PGPR action mechanisms is fundamental to manipulating the rhizosphere in order to maximize the process within the system that strongly influence the plant productivity in different agri-ecosystems. These includes both direct and indirect mechanisms to support plant growth.

13.3.1   Nitrogen Fixation

The supply of nitrogen to arable plants is about 42 million tons of fertilizer which is being used annually on a global scale for the production of cereals such as wheat, rice, and maize alone as per an estimate. The global demand of fertilizers is estimated to increase by 3.2% reaching to about 175.8 Mt (Heffer and Prud'homme 2008). Of this, only 50% is lost from the soil–plant system through processes such as leaching, volatilization, and denitrification leading to adverse environmental effects such as (1) methemoglobinemia in infants due to NO_3 and NO_2 in water and food; (2) cancer due to secondary amines; (3) respiratory illness due to aerosols of NO_2, NO_3, and HNO_3; (4) eutrophication of surface water; (5) material and ecosystem damage due to HNO_3 in rainwater; (6) plant toxicity due to high levels of NO_2 and NH_4 in soils; (7) excessive plant growth due to more available N; and (8) depletion of stratospheric ozone due to NO and N_2O (Doran et al. 1996). This has led to resurgence in the use of biological preparations containing nitrogen-fixing organisms as fertilizers for assimilation of nitrogen in the nonlegumes, sustaining environmental health and soil productivity.

Rhizobium is the most predominant genus of bacteria which colonize the roots of legumes forming nodules within which they change to a nonreproductive form called bacteroids and start fixing nitrogen. Specificity in this respect is exhibited by both plants and bacteria. Initially it was thought that rhizobia only fixed nitrogen in symbiosis but several investigators reported nitrogenase activity in species of rhizobia grown in pure culture, particularly the slow-growing "cowpea" strains. Since then, symbiotic associations between nonlegumes and nitrogen-fixing bacteria have been reported. These host plants include tropical grasses, rice, sugarcane, and maize. Notable examples of nonsymbiotic diazotrophs include *Azotobacter paspalum* which colonizes under a mucilaginous sheath on the roots of Bahia grass (*Paspalum notatum*) (Dobereiner et al. 1972) and *Spirillum lipoferum* which colonizes the roots of *Digitaria decumbens* and also some kinds of maize (Von Bulow and Dobereiner 1975). Blue-green algae are also agriculturally important for fixation of N (as they are distributed widely on soil surface in all kinds of environments and are tolerant of wide ranges of temperature and oxygen tension). Symbiotic associations between blue-green algae and higher organisms are very effective nitrogen-fixing systems.

In all biological nitrogen fixation systems studied, dinitrogen reduction is catalyzed by a highly conserved enzyme complex called nitrogenase, which consists of two component proteins: the iron (Fe-) protein (MW 64,000) and the molybdenum iron (MoFe-) protein (MW 220,000). Together, the nitrogenase proteins catalyze the ATP-dependent reductions of dinitrogen to ammonia and of protons to hydrogen as well as the reduction of alternative substrates such as acetylene or cyanide. From genetic studies, it is clear that nitrogen fixation needs a whole set of auxiliary proteins and regulatory genes to function inside a cell (Burgess and Lowe 1996).

Regulation of nitrogen fixation at the transcriptional level is affected by factors such as environmental levels of oxygen and ammonium. Nitrogenase components are oxygen labile and the metabolically expensive nitrogenase system is repressed when the cellular level of fixed nitrogen is sufficiently high. Free-living diazotrophs are more sensitive to the cellular ammonium levels (Merrick 1992). Post-translational modes of regulation operate on the nitrogenase to prevent unproductive fixation of nitrogen. In *Rhodospirillum rubrum*, *R. capsulatus* (Purple, nonsulfur photosynthetic bacteria), *Azospirillum brasilense*, *A. lipoferum* (microaerophilic, associative bacteria), and *Chromatium vinosum* (a purple sulfur bacterium), the nitrogenase complex is rapidly, reversibly inactivated by ADP-ribosylation of Fe protein. Optimizing the procedure for screening of efficient diazotropic organisms is essential for obtaining high crop yields. In a study screening for competent diazotrophs, *Azospirillum* associated with the rhizosphere of maize was isolated using semi-solid N-free and solid selective media. The chromosomal DNA of the most promising isolates was extracted and *nifH* gene was amplified and sequenced (Fernando et al. 2006). Nitrogen fixation of the isolates is determined in nitrogen-free medium by acetylene reduction assay and ethylene produced is measured using a gas chromatograph.

Field trials of PGPR that act as nitrogen-fixing and phosphate-solubilizing bacteria have indicated increased yields in the rice crop (Sudha et al. 1999), sugar

beet (Cakmakci et al. 1999), wheat (de Freitas 2000), canola (de Freitas et al. 1997), maize (Pal 1998), and conifer species (Bent et al. 2002). Another PGPR known for nitrogen fixation properties in addition to P solubilization, production of antibiotics, cytokinin, hydrolytic enzymes, colonization of root hair and cortical cells is *Bacillus polymyxa*, now named *Paenibacillus polymyxa* (Timmusk et al. 1999). Field trials of *Bacillus* OSU-142, *Bacillus* M3, and *Microcbacterium* FS01 for determining their competency as PGPR from the year 2002 to 2006 have demonstrated highest N content from combined inoculations of *Bacillus* OSU-142 and *Microcbacterium* FS01 application (Karlidag et al. 2007). N_2-fixing and P-solubilizing abilities of *Bacillus* OSU-142 and *Bacillus* M3 have been reported by many workers.

Studies conducted to study the effects of coinoculation of PGPR with N_2-fixing bacteria such as *Bradyrhizobium* has shown the positive effects of PGPR on nitrogen fixation by an increase in nodule number or mass (Polenko et al. 1987). However, the coinoculation trials conducted by Lucas Garcia et al. (2004) indicated that coinoculation of PGPR with *Sinorhizobium fredii* has no positive effect on nitrogen fixation or nodulation. Furthermore, it is demonstrated that mechanism of biological nitrogen fixation, nodulation, and growth effects on plants is different in gram-positive and gram-negative bacteria.

13.3.2 Phosphate Solubilization

Phosphorus, in spite of being a macro-nutrient essential for energy metabolism, sugar production, regulation of a number of enzymes, membrane physiology as well as structure of the genetic material (Saber et al. 2005), is present only in micromolar or even lesser concentrations in the soil. It has been suggested that 0.3–0.5% of P is required on a dry matter basis for optimal growth of a plant during its vegetative stages whereas the amount of P in soil seldom exceeds 10 μM.

Phosphorus in the soil is of two types: organic, which constitutes 20–80% of the total soil content, and inorganic, which is complexed with cations and converted into insoluble P. (Fixation of phosphorus with calcium is a process observed in calcareous soils and that with iron and aluminum compounds constitutes acidic soils making the soil P unavailable for the plants.)

Limited availability of phosphorus is a global problem. Acidic soils are found in all continents and constitute 30% of the soils worldwide. The deficiency of P is most prominent in acidic soils of the tropical and sub-tropical regions because fixation of P is double the amount added compared to neutral or calcareous soils in acidic soils. Also, in the semiarid tropics bearing sandy soils, deficiency of Pi severely affects crop productivity (Sanchez et al. 1997).

The use of artificial P fertilizers to supplement its unavailability requires farmers to apply four times the quantity of fertilizer required otherwise, because plants are able to fix only 5–25% of the applied fertilizer. This global demand of chemical P fertilizers requires US$ 4 billion per annum (Goldstein et al. 1993) posing an

economic burden on the society. This rate of employing artificial P fertilizers will deplete the nonrenewable P reserves in the coming 60–90 years (Runge-Metzger 1995). The situation is further worsened by the entry of excessive P onto the surface water posing threats of eutrophication and degradation of the environment making the discovery of alternatives for fixation of the unavailable P imperative.

Soil microorganisms generally act as reserves of compounds of labile minerals including phosphorus and their ability to enrich the soil with available P has been reported since the year 1903. The ubiquitously found phosphate solubilizing microorganisms (PSM) increase the yields by enhancing fixation of N by the plants, protecting against pathogens, increasing the availability of trace elements such as iron, zinc, etc., and producing plant growth hormones in addition to making P available to them (Ponmurugan and Gopi 2006) and hence provide lucrative options for use as biofertilizer.

The phosphate solubilization activity of microorganisms has been attributed to production of H^+ ions and organic acids such as acetate, lactate, oxalate, tartarate, succinate, citrate, gluconate, ketogluconate, glycolate, etc. (Lal 2002). The mechanism of action of the organic acids includes acidification of rhizosphere soil, chelation of anions, and ligand exchange reactions (Omar 1998).

A higher concentration of PSM is found in the rhizosphere compared to other sources (Vazquez et al. 2000). Ability of various bacteria and fungi to solubilize insoluble P compounds has been demonstrated. Among bacteria, the most powerful phosphate solubilizers include strains of *Pseudomonas*, *Bacillus*, and *Rhizobium* and the most important fungal genera include *Aspergillus* and *Penicillium* (Motsara et al. 1995). Kucey in 1983 has demonstrated that phosphate-solubilizing bacteria constitute 1–50% while fungi only represent 0.5–0.1% of the total respective population. However, fungi have been reported to be better phosphate solubilizers than bacteria by producing larger quantities of organic acids. In addition to this, bacteria have been demonstrated to lose their phosphate-solubilizing capacity on repeated subculturing unlike the phosphate-solubilizing fungi (Kucey 1983).

In the laboratory, most of the PSMs are isolated depending on their ability to solubilize various compounds of P such as dicalcium phosphate (DCP), tricalcium phosphate (TCP), and hydroxyapatite (HAP), and a smaller number of PSMs have been shown to solubilize ferric and aluminum phosphates (Halder and Chakrabartty 1993) by production of acids. One of the very first screening media devised for checking the efficacy of PSMs isolated from nonrhizosphere and rhizosphere soils, the rhizoplane, and from other soil and marine environments, is the Pikovskaya's medium, on which the PSMs form halos/clear zones surrounding their colonies if phosphate is solubilized. The organisms are cultured by serial dilution/enrichment techniques on a media containing insoluble mineral phosphates such as TCP or HAP. A modified screening assay using bromophenol blue was later devised on which the phosphate solubilizers too yielded yellow halos, leading to generation of clear and visible results (Gupta et al. 1994).

The soluble phosphate concentration in the culture medium used above has been shown to be directly proportional to the titrable acidity and organic acid (principally

gluconic acid) concentration and inversely related to pH. The effects of C and N sources and their concentrations on the screening assays have been marked. Greater solubilization has been observed when ammonium salts are used as the sole source of N compared with use of nitrate salts (Whitelaw 2000); an exception to which was observed by Reyes et al. (1999). In case of C sources, maximum growth yield was observed with sucrose and galactose when Ca–P and Al–P were used as P sources, respectively, in case of the fungus *Aspergillus niger* (Nahas 1996).

All the plate-screening assays are indirect assays, results of which are variable and reliability of which is questionable. Hence, Nautiyal (1999) devised a defined media called the National Botanical Research Institute's Phosphate Growth Medium (NBRIP) which is about threefold more efficient compared to the Pikovskaya's medium in broth assay. Recently, Modified Illmer and Schinner (MIS) media has been devised for isolation of PSB from a typical black wheat growing soil from the North-Western wheat belt of New South Wales (Illmer and Schinner 1992). It is a differential media containing insoluble phosphates and reduced amounts of sugars using root exudates as the inoculums. The results of the same were validated through pot trials (Harris et al. 2006).

Most of the media discussed above have utilized unbuffered media for isolation of PSMs. However, the natural conditions prevailing in the soil are highly buffered leading to their failure when subjected to field trials because of secretion of 10- to 20-folds less organic acids. Therefore, a buffered media was formulated to mimic the alkaline vertisol conditions (Patel et al. 2007).

For the commercialization of the PSM as biofertilizers, inoculants of efficient PSM, also called microphos, need to be developed. The efficiency of various materials as suitable carriers such as peat, pearlite, farm yard manure (FYM), soil, and cow dung cake powder has been evaluated (Kundu and Gaur 1981) for the same. Cultures for mass-supply are packaged in polybags and have a shelf-life of up to 3 months if stored at a temperature of $30 \pm 2°C$. In India, a microbial preparation termed Indian Agricultural Research Institute (IARI) microphos culture (Gaur 1990) has been developed that contains two efficient phosphate-solubilizing bacteria (*Pseudomonas striata* and *B. polymyxa*) and three phosphate-solubilizing fungi (*Aspergillus awamori*, *A. niger*, and *Penicillium digitatum*). Combined inoculation of Rhizobium, a phosphate-solubilizing *B. megaterium* sub sp. *phospaticum* strain-PB and a biocontrol fungus *Trichoderma* spp. produced effects such as increased germination, nutrient uptake, plant height, number of branches, nodulation, pea yield, and total biomass of chickpea compared to either individual inoculations or an uninoculated control (Rudresh et al. 2004). Moreover, it has been demonstrated that plants inoculated with arbuscular mycorrhizal fungi utilize more soluble phosphate from rock phosphate than noninoculated plants (Guissou et al. 2001) and hence they can be used in consortium with other PSM to develop better P biofertilizers.

The use of molecular biology techniques for rapid and easy generation, detection, and characterization of PSM can be highly advantageous and promising (Igual et al. 2001). For improvisation of the solubilization of the organic P reserves in the soil, modification of the genes coding for enzymes such as nonspecific

phosphatases, phytases and phosphonatases, and C–P lyases have been carried out. A large number of important genes have been cloned including acid phosphatase genes from gram-negative bacteria (Rossolini et al. 1998), thermally stable phytase genes (*phy*) from *Bacillus* sp. DS11 (Kim et al. 1998) and *B. subtilis* VTT E-68013 (Kerovuo et al. 1998), acid phosphatase/phytase genes from *Escherichia coli* (*app*A and *app*A2) (Golovan et al. 2000), neutral phytase genes from *B. subtilis* and *B. licheniformis* (Tye et al. 2002), *phy*A gene from the FZB45 strain of *B. amyloliquefaciens*. Table 13.1 enlists few other clone genes involved in solubilization of organic and inorganic phosphate.

13.3.3 Siderophore Production

Iron is present abundantly on earth but is mostly unavailable to the microorganisms. This is due to the aerobic atmosphere of this planet which has converted the surface iron into its oxyhydroxide form having very less solubility. It is found that maximum of 10^{-18} M of free ferric ion is present in solution at biological pH. However iron is one of the essential nutrients required by the microorganism for a variety of functions like reduction of oxygen for synthesis of ATP, reduction of ribotide precursors of DNA, for formation of heme, etc., which has forced the microorganisms to produce certain chemical compounds which can chelate ferric ions from the complexes and make them available for their use.

Siderophores are chemical compounds of low molecular weight (<1,000 molecular weight) which act as ferric ion-specific chelating agents. They scavenge iron, present in the environment as complexes, and make available to the microorganisms (Neilands 1995). More than 500 different siderophores have been described so far. Siderophores are produced by all bacteria and fungi growing under low iron stress condition.

Structurally, siderophores are classified into hydroxymate (e.g., aerobactin, ferrichrome) and catechol [e.g., enterobactin (Fig. 13.1), based on its iron ligation group]. Most of the siderophores have a peptide backbone with several nonprotein amino acid analogs like modified and D-amino acids (Drechsel and Jung 1998). However despite their structural differences, all form an octahedral complex with six binding coordinates for Fe^{3+}.

13.3.3.1 Synthesis

Synthesis of siderophores is similar to that of the antibiotics, i.e., they are assembled by nonribosomal, cytoplasmic peptide synthetases (Wandersman and Delepelaire 2004). In *Ustilago maydis*, a pathogenic fungus causing corn smut disease in plant, the *sid1* which is responsible for initiating siderophore biosynthesis, is present on the genomic DNA (Mei et al. 1993), whereas aerobactin, siderophore first isolated from *Aerobacter aerogenes* (Gibson and Magrath 1969) is a product of

Table 13.1 Cloning of genes involved in solubilization of P reserves

| Organism from which the gene is isolated	Host organism	Gene transferred	Feature	Reference
Erwinia herbicola	*E. coli*	Mps	Produces gluconic acid and solubilizes mineral P in *E. coli* HB101; probably involved in PQQ synthesis	Goldstein and Liu (1987)
Pseudomonas cepacia	*E. coli*	Gab Y	Produces gluconic acid and solubilizes mineral P in *E. coli* JM109; no homology with PQQ genes	Babu-khan et al. (1995)
Enterobacter agglomerans	*E. coli*	pKKY	Solubilizes P in *E. coli* JM109; does not lower the pH	Kim et al. (1997)
Rahnella aquatilis	*E. coli*	pK1M10	Solubilizes P and produces gluconic acid in *E. coli* DH5x; probably related to PQQ synthesis	Kim et al. (1998)
Serratia marcescens	*E. coli*	pKG3791	Produces gluconic acid and solubilizes phosphates	Krishnaraj and Goldstein (2001)
Morganella morganii	*Burkholderia cepacia IS-16*	nAP phosphatase gene	An increase in extracellular phosphatase activity	Fraga et al. (2001)
Morganella morganii	*Azospirillum* spp.	phoC phosphatase gene	Increased phosphatase activity	Fraga et al. (2001)
Aspergillus niger	*Arabidopsis* plants	Phytase gene (phyA)	Improved P nutrition and utilization of inositol phosphates	Richardson et al. (2001)
B. amyloliquefaciens DS11	*B. amyloliquefaciens* strain FZB45	phyA	High extracellular phosphatase activity	Iddris et al. (2002)
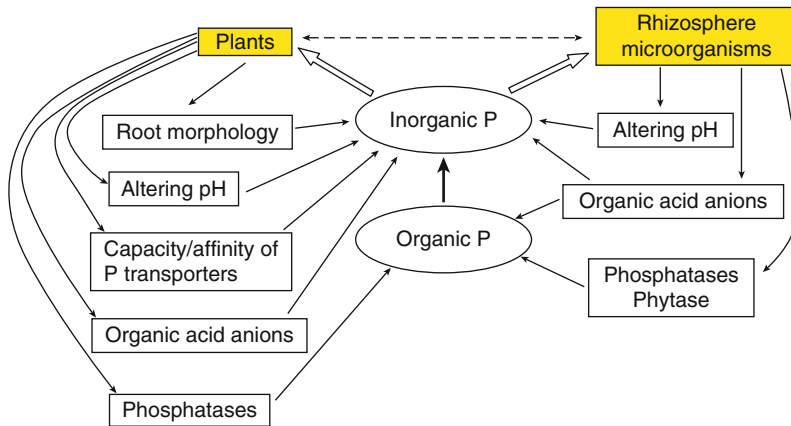
Fig. 13.1 Plant–microbe mechanism to increase phosphorus availability in rhizosphere (Richardson 2007)

plasmid pColV-K30. Thus the gene encoding siderophore may be present on the genomic DNA or on the plasmid varying on the organism to organism.

Iron present in excess amount is also harmful for the bacterial cell. Therefore, the gene encoding the biosynthetic enzyme are iron regulated, i.e., they are expressed only during iron stress conditions and once the intracellular iron concentration rises, the genes are repressed. In *Salmonella typhimurium*, the gene *fur* (ferric uptake regulation) controls the expression of siderophores (Ernst et al. 1978).

The genes responsible for synthesis of siderophores are often clustered with genes involved in its uptake. For uptake of siderophores, receptors are present on the cell membrane – specific membrane anchored binding proteins in case of gram-positive cell and specific outer membrane receptors for gram-negative cells (Wandersman and Delepelaire 2004). But these receptors may also act as entry sites for large number of lethal agents such as bacteriophage, bacteriocin, and antibiotics.

13.3.3.2 Uptake

Siderophores have high affinity for Fe (III) (calculated affinity constant $>10^{30}$ M^{-1}), but at the same time, have weak complexing ability for Fe (II). Thus they take up Fe (III) from the environment and release them inside the cell by reduction by free intracellular electron donors or by extracellular reductases (Schroder et al. 2003). The release of Fe (II) inside the cell may follow two different mechanisms:

1. Reduction and release of Fe (III) from siderophores intracellular electron donors by intracellular and siderophore recycling
2. Cleavage of iron and release of siderophores. In this case, siderophores are used only once and cannot be recycled

Since iron is an essential growth element for all organisms and is present in a complexed form, there exists a furious competition for it among the microorganisms present in the soil habitat and on the plant surfaces. By the production of siderophores, PGPR competitively take up the iron from the soil and thus are able to survive and colonize on the rhizoplane. Some PGPR are even able to secrete siderophores which draws iron from heterologous siderophores produced by cohabiting microorganisms (Whipps 2001). The production of siderophores by PGPR indirectly increases the plant growth by depriving the pathogenic bacteria and fungi of iron and thus inhibiting their growth.

13.3.4 Phytohormone Production

One of the major forms of host plant–microbial interactions is synthesis of phytohormones by plant growth-promoting bacteria which is for plant growth, development, and productivity. Use of bioinoculants-producing phytohormones is gaining importance around the globe as a means of sustainable crop production (Narula et al. 2006). Phytohormones or plant growth regulators are organic substances that influence the physiological processes of plants at very low concentrations. Soil microorganisms produce a variety of phytohormones such as auxins, gibberellins, cytokinins, ethylene, and abscisic acid (ABA).

13.3.4.1 Auxin

Auxins influence many cellular functions, orientation of root and shoot growth in response to light and gravity, differentiation of vascular tissue, apical dominance, initiation of lateral and adventitious roots (Malamy and Benfey 1997), stimulation of cell division, and elongation of stems and roots (Kende and Zeevaart 1997). Some of the auxin-producing organisms are phytopathogens while others are involved in promoting growth. Vandeputte et al. (2005) demonstrated auxin production by the actinomycete *Rhodococcus fascians* which brings about a number of malformations in monocotyledonous and dicotyledonous host plants.

13.3.4.2 Gibberellin

Gibberellins (GA) are another group of phytohormones that are diterpinoid acids. GA is involved in all phases of plant growth and development from germination to senescence. However, the most prominent physiological effect of GA is in shoot elongation. Some other plant growth-related functions of GA include overcoming dormancy and dwarfism in plants, inducing flowering in some photoperiodically sensitive and other low-temperature-dependent plants and contributing to fruit ripening. Several soil microbes are known to produce gibberellins and

gibberellin-like substances (Steenhoudt and Vanderleyden 2000). The common bacterial genera are *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Rhizobium*, *Bacillus*, *Brevibacterium,* and *Flavobacterium*, *Actinomyces* and *Nocardia* are the important actinomycetes and *Fusarium*, *Gibberella*, *Alternaria*, *Aspergillus*, *Penicillium,* and *Rhizopus* are known fungi.

13.3.4.3 Cytokinin

Cytokinins, N^6-substituted aminopurines, regulate cell division and differentiation in certain plant tissues. They play an important role in nodule development and formation. Symbiotic N_2-fixing bacteria, *Rhizobium*, free-living N_2-fixing bacteria *Azospirillum* and *Azotobacter*, and mycorrhizal fungus, *Rhizopogon roseulus*, are known to produce cytokinins in the rhizosphere along with other growth-promoting substances. Other bacteria that produce cytokinins or cytokinin-like substances include *Agrobacterium*, *Bacillus*, *Paenibacillus*, and *Pseudomonas*.

13.3.4.4 Ethylene

Ethylene (C_2H_4) is considered to be a promoter of senescence and an inhibitor of growth and elongation. It can also promote flowering, fruit ripening, and cell elongation in certain plants. Bacterial species of *Aeromonas*, *Citrobacter*, *Arthrobacter*, *Erwinia*, *Serratia*, *Klebsiella*, *Streptomyces*, and fungal species of *Acremonium*, *Alternaria*, *Mucor*, *Fusarium*, *Pythium*, *Neurospora*, and *Candida* are capable of producing ethylene (Subba Rao 1999). However, a sustained high level of ethylene in the plants inhibits root elongation which in turn causes improper germination of the seeds. Glick (1995) has shown that certain free-living bacteria in the rhizosphere release an enzyme called ACC deaminase. ACC (1-aminocyclopropane-1-carboxylate), which is the precursor for ethylene, is broken down by ACC deaminase and thus the concentration of ethylene goes down in a stressed plant and this restores the proper growth in the plant.

13.3.4.5 Abscisic Acid

ABA is generally involved in deceleration or cessation of plant growth. ABA production in two bacterial species *A. brasilense* and *Rhizobium* spp. and several phytopathogenic fungi such as *Cercospora*, *Fusarium*, *Cladosporium*, *Monilia*, *Pestatoria,* and *Verticillium* has been demonstrated. Barea et al. (1976) demonstrated that phosphate solubilizers and nonsolubilizers produced plant growth regulators. Phosphate solubilization bacteria would probably be more effective as inoculants if they produced several growth regulators.

Plant growth regulators are effective at very low concentrations; even less than 1 μM. Barea et al. (1976) reported concentrations of plant growth regulators

produced by microorganisms. These ranged in µg/ml, from 0.01 to 0.09 of IAA, 0.008 to 0.2 of GA, and 0.01 to 0.1 of kinetin.

13.4 Metabolites

PGPR are effectively used in different ecological niches to control phytopathogens by production of secondary metabolites. These may include production of inhibitory allelochemicals, ISR in host plants, and other metabolites in response to biotic and abiotic stresses. It is well known that some soils are naturally suppressive to some soil-borne plant pathogens. This may be due to both the physicochemical properties and microbial composition of the soil. Two types of suppressiveness are known. General suppression is due to the activity of the total microbial population of the soil and is not transferable between soils. Specific suppression is due to the activity of specific individual or groups of microorganisms and is transferable. Bacterial protection of plants depends upon three properties: (1) ecological fitness to maintain an effective population size *in situ*; (2) rapid root colonization; and (3) stable production of secondary metabolites, e.g., siderophores, β-1,3-glucanase, chitinase, cyanide, antibiotics, etc., under variable growth conditions which antagonize to phytopathogens. Secondary metabolite production by PGPR as a basis for bioinoculants has become increasingly popular over the last two decades (Sessitsch et al. 2005). In the last few years, relatively few studies concentrate on the use of PGPR for controlling bacterial diseases that affect plants. One example is the use of nonpathogenic strains of *Streptomyces* to control scab of potato (*Solanum tuberosum* L.) caused by *Streptomyces scabies*. The volume of literature on the use of PGPR for control of fungal diseases and their interactions with fungal pathogens continues to increase. Although, a variable number of bacterial genera and species have been used for such studies, a large number of papers involve the use of *Pseudomonas* species. The features that make this genus so effective and the choice of so many workers are (1) fast growth, (2) ease in culture and genetic manipulation in the laboratory, (3) utilization of a range of organic compounds, and (4) adaptation to rhizosphere.

13.4.1 Antibiotic Production and Regulation

The antibiotics can be classified as polyketides, heterocyclic nitrogenous compounds, cyclic lipopeptides, lipopeptides, aminopolyols, aldehydes, alcohols, ketones, etc. Several factors are involved in modulation of production and efficacy of antibiotics such as type of carbon source, pH, temperature, and other environmental stimuli, growth phase, and cell density. *P. fluorescens* HV37a produces different antibiotics in the presence and absence of glucose (Gutterson and James 1986). It is important to note that many strains produce a variety of different secondary metabolites under different environmental conditions. This bestows

upon the strain a degree of flexibility when confronted with changing environmental conditions. Furthermore, plant growth, development, and genotype may influence antibiotic production by the antagonistic. DAPG production is induced by exudates of older plants which results in selective pressure against other rhizosphere organisms.

Regulation of antibiotic synthesis involves GacA/GacS (global antibiotics and cyanide production) or GrrA/GrrS, RpoD and RpoS, and *N*-acyl-homoserine lactone derivatives. The GacA/GacS, a two-component regulatory system, is found in many plant-associated *Pseudomonads*. The two-component regulatory system and *N*-acyl-homoserine lactone (AHL)-mediated regulatory system function dependent and independent of each other. At a threshold concentration of AHL, which is reached only when a certain density of bacterial cells is present, the AHL will sufficiently bind to and activate a transcriptional regulator which then stimulates gene expression. Microbial metabolites also play a role in the regulation of antibiotic synthesis. Salicylate, fusaric acid, and pyoluteorin have negative effect on DAPG production. Salicylate interacts with repressor *Phl*F and stabilizes its interaction with *phl*A promoter (Abbas et al. 2002). Pyoluteorin, an aromatic polyketide antibiotic, is produced by several *Pseudomonas* species that suppress plant diseases caused by phytopathogenic fungi (Sharma 2008).

13.4.2   Cyanide Production

Cyanide is another secondary metabolite produced from glycine by HCN synthase (Castric 1994). Production of HCN occurs among widely divergent organisms, in many species of fungi but only a few species of bacteria of the genera *Chromobacterium* and *Pseudomonas*. However, action of HCN is ambiguous as it is known to inhibit or enhance plant establishment or inhibit development of plant disease.

13.4.3   Antifungal Activity

Production of antifungal metabolites by PGPR is well documented. A large number of such compounds have been identified including 2,4-di-acetylphloroglucinol (DAPG), hydrogen cyanide, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, tropolone, viscosinamide, oligomycin A, zwittermycin A, xanthobaccin, and several others that are as yet uncharacterized.

Antagonism may also operate by parasitism, which involves the production of several hydrolytic enzymes that degrade cell walls of pathogenic fungi. Chitinases and β-1,3-glucanases are considered key hydrolytic enzymes in the lysis of cell walls of higher fungi. Bacteria of the genera, *Aeromonas*, *Serratia* and *Enterobacter,* and fungi from the genera *Gliocladium* and *Trichoderma* are known to produce chitinolytic enzymes. These enzymes cause the release of elicitors which in turn

elicit various resistance reactions in the plant. Chitinase and cellulase produced by *Pseudomonas* strains showed mycelial growth inhibition of different fungal pathogen on PDA medium (Sindhu and Dadarwal 2001). Production of extracellular β-1,3-glucanases, chitinases, and proteinase increases significantly when *Pseudomonas* spp. are grown in media supplemented with either autoclaved mycelium or isolated purified host fungal cell walls (Viswanathan and Samiyappan 2000). Such induction of chitinases and their antifungal activity together with the fact that chitin, β-1,3-glucan, and protein are the main structural components of most fungal cell walls suggested that hydrolytic enzymes produced by some fluorescent pseudomonads play an important role in the destruction of plant pathogens. Chitinase and β-1,3-glucanase are the key enzymes associated with the decomposition of the fungal hyphal wall. Chitinase (E.C. 3.2.1.39) and β-1,3-glucanase (E.C. 3.2.1.39) have been involved in degradation of fungal cell walls since chitin and β-1,3-glucan are the major components of most fungal cell walls. It has also been demonstrated that extracellular chitinase and laminarinase synthesized by *P. stutzeri* digest and lyse mycelium of *Fusarium solani* (Kumar et al. 2010). Saraf et al. (2008) studied that *Pseudomonas* spp. (M1P3) showed maximum chitinase activity (40 μg/ml *N*-acetyl glucosamine production) and also induced maximum cellulase production. Arora et al. (2008) studied fluorescent *Pseudomonas* (PGC 1 and PGC 2) for their antifungal potential against *R. solani* and *P. capsici*. The results of this study indicated the role of chitinase and β-1,3-glucanase in the inhibition of *R. solani*, however antifungal metabolites of nonenzymatic nature were responsible for inhibition of *P. capsici*.

13.4.4 Elicitors

Elicitors are defined as substances of biotic origin that has the ability to trigger hypersensitive response in a plant. Elicitors may be protein and peptides, carbohydrates, β-glucans (especially heptaglucan), xylans, oligogalacturans (especially 10–15-mers), chitosan (>hexamers), fatty acids, and glycosyl lipids, while most frequently encountered elicitors are polysaccharides, small proteins, or lipids associated with the fungal or bacterial cell wall. Even the pectic fragments resulting from microbial damage to the plant's own cell walls may also act as elicitors. The elicitors interact with the plasma membrane of undamaged cells and trigger activation of genes involved in the defense response.

The elicitors play a major role in the antifungal activity incorporated in the plant by PGPR. When pathogenic fungi attack plant, the PGPR secrete enzymes like chitosanase and chitinase which are capable of hydrolyzing β-1,4-linkages between *N*-acetyl-D-glucosamine and D-glucosamine residues in a partially acetylated fungal cell wall polymer. This hydrolytic action is exploited by many plants as a component of larger post attack defense (Agrios 1997). The fungal cell wall fragments released after hydrolysis with chitinase and chitosanase act as elicitors of plant defense responses such as stomatal closure (Lee et al. 1999), lignifications (Vander

et al. 1998) and PR gene transduction (Jabs et al. 1997). It has been reported by Vander et al. (1998) that the responses elicited by these molecules depend upon the length and acetylation of the fragments released. More specifically, shorter the fragments, more heightened will be the reaction. Long fragments or intact fungal cell wall will cause little or no reaction.

13.4.5 Biosurfactants

Microorganisms produce a wide variety of secondary metabolites that have a diverse spectrum of activity in environmental remediation and biocontrol. Rhamnolipids are examples of bioactive molecules which have found application against phytopathogenic fungi. A variety of biosurfactants like Alasan (*Acinetobacter radioresistens*), Arthofactin (*Arthobacter* sps), Rhamnolipid (*Pseudomonas aeruginosa*), Surfactin (*B. subtilis*), etc., have a broad range of applications including enhanced oil recovery, bioremediation of water insoluble pollutants, emulsification, phase separation, viscosity reduction, etc. (Sharma 2008). Rhamnolipids are effective against three genera of zoosporic plant pathogens *Pythium aphanidermatum*, *P. capsici*, and *Plasmopara lactunae*. These mono- and di-rhamnolipids were produced by *P. aeruginosa* and caused cessation of motility and lysis of culture in less than 1 min (Sharma 2008).

13.5 Exopolysaccharide

The outer membrane proteins, lipopolysaccaharides (LPS) of several bacteria, have been demonstrated to stimulate plant growth. De Weger et al. (1987) characterized the chemical composition of LPS of three plant-associated *Pseudomonads* and found it to be different. LPS production may facilitate efficient colonization of the plant-associated bacteria. The location of individual rhizobacteria on the root can be monitored by using confocal laser scanning microscopy with the help of molecular markers such as green florescent protein or florescent antibodies (Bloemberg and Lugtenberg 2001). This approach in combination with an rRNA targeting probe, that monitors the metabolic activity of a rhizobacterial strain in the rhizosphere, has been used to find out that the bacteria located on the root tips are the most active (Sorensen et al. 2001).

13.6 Production of Insecticide

Jousset et al. (2008) have demonstrated that production of secondary metabolites in *P. fluorescens* CHA0 is crucial for competition for resources and predation control. Keel et al. (2008) have identified a novel genomic locus encoding an insect toxin Fit

(*P. fluorescens* insecticidal toxin) that is related to the insect toxin Mcf (Makes Caterpillars Floppy) of the entomopathogen *Photorhabdus luminescens*. This emphasizes anti-insect properties of plant-associated bacteria which remain to be explored. However, antibiosis as a biocontrol mechanism is challenged by low production of metabolites in natural environment, broad spectrum activity, and production of different metabolites under different conditions.

13.7 Plant Responses to PGPR

Plant roots offer a niche for the proliferation of soil bacteria that thrive on root exudates and lysates. Population of rhizospheric microflora is much higher in the rhizosphere than elsewhere in the soil. These bacteria utilize the nutrients and secrete the metabolites which help in growth promotion of plants. The best studied example of signal exchange is the rhizobium–legume symbiosis. Plant microbe interaction in the rhizosphere lead to increased growth (biofertilizer) and also are deleterious to the pathogenic microflora found in the root system (biocontrol) and leading to induced systemic resistance (van Loon 2007). *P. aurantiaca* SR1 produced compounds that could stimulate the growth of both wheat and alfa-alfa cultivars (Rovera et al. 2008). On the other hand, Tank and Saraf (2009) have reported enhancement of plant growth and decontamination of nickel spiked soil using *P. stutzeri*.

While Glick et al. (2007) concluded that ACC-deaminase containing rhizobacteria can increase root growth by lowering endogenous ACC levels, Remans et al. (2007) have studied the effect of four PGPR on symbiotic interactions between *Rhizobium* and *Phaseolus vulgaris* under deficient versus sufficient phosphorus supply and reported that IAA production and ACC deaminase activity play an important role under low P conditions. The use of PGPR has been reported to ameliorate salinity stress (Tank and Saraf 2010). Whereas, the role of PGPR as biocontrol agents was reported by Singh et al. (2008) in controlling the root rot caused by *Macrophomina phaseolina* using *B. subtilis*.

13.8 Challenges and Future Prospects

A lot has been known regarding the beneficial aspects of the use of PGPR as biofertilizers, and its commercialization can reach much greater heights reaping large benefits to the ecology, environment, and economically. However, there is a lack of efficient screening methods for selection of PGPR and methods to validate their success in field trials. It is important to consider the host plant specificity or adaptation to a particular soil, climatic condition, or pathogen in selecting the isolation considered and screening assays. The traits of PGPR-like ACC deaminase activity, root colonization; antibiotic, and siderophore production can be used for

Table 13.2 List of few siderophores and the genes encoding them

Name of siderophore	Organism	Type	Gene encoding	Reference
Aerobactin	*E. coli*, *Enterobacter aerogenes*	Hydroxamate	pColV-K30, pSMN1 (plasmid)	Loper et al. (1993)
Ferrichrome	*Aspergilllus*, *Ustilago*, *Penicillium*	Hydroxamate	*fso* 1	Welzel et al. (2005)
Yersiniabactin	*Yersinia pestis*	Catechol	*irp1*, *irp2*, *irp3*, *irp4*, *irp5* (13 kb chromosomal DNA)	Pelludat et al. (1998)
Alanguibactin	*Vibrio anguillarum*	Catechol	*Ang R* (plasmid encoded)	Chen et al. (1996)
Mycobactin	*Mycobacterium*	Hydroxamate	*mbt E*	LaMarca et al. (2004)
Enterobactin	*Salmonella typhimurium*	Catechol	*ent F*	Gehring et al. (1997)
Bacillibactin	*Bacillus subtilis*	Catechol	*dhb*ABCD	Raza et al. (2008)
Vibriobactin	*Vibrio cholerae*	Catechol	*Vib F* (chromosomal)	Butterton et al. (2000)
Pseudobactin	*Pseudomonas* B 10	Hydroxamate	*psb A* (monocistronic)	Ambrosi et al. (2000)
Erythrobactin	*Saccharopolyspora erythraea*	Hydroxamate	*nrps 3* and *nrps 5*	Oliveira et al. (2006)
Pyochelin	*Pseudomonas aeruginosa*	Hydroxamate	*pchDCBA* and *pchEF*	Reimmann et al. (2001)
Pyoverdin	*P. aeroginosa*	Mixed	*pvd* (gene cluster of 103 kb of chromosomal DNA)	Tsuda et al. (1995)
Ornibactin	*Burkholderia cepacia*	Hydroxamate	*pvd A* and *ecf 1/Orb S*	Agnoli et al. (2006)
Francobactin	*Frankia*	Hydroxmate		Chincholkar (2000)
Parabactin	*Paracoccus*	Catecholate		Chincholkar (2000)

the selection of PGPR strains. The failure of biofertilizers on the field can be attributed to competition for resources among the rhizobacteria, complex abiotic and biotic factors, and constantly varying environmental conditions (Table 13.2).

Successful commercialization is hindered mainly due to lack of awareness regarding its use in the developing countries and stringent policies governing its use in the developed countries such as Canada and USA. Prior to registration and commercialization, the PGPR products must overcome few hurdles like scale up and production of the organism under commercial fermentation conditions while maintaining quality, stability, and efficacy of the product. Health and safety testing may be required to address the effects on other organisms including toxigenicity, allergenicity, and pathogenicity, persistence in the environment, and potential for horizontal gene transfer. In the process of commercialization, capitalization costs, and potential markets must be considered.

Avenues that still need to be explored for the development of biocontrol strains include screening of microorganisms other than *Pseudomonads* for traits that characterize a PGPR. Moreover, efforts need to be directed toward screening of PGPR for traits, as yet unexplored, such as production of insecticidal toxins, heavy metal resistance, and phytoremediation. Genetic manipulation of the PGPR strains for the transfer of one or more traits for plant growth promotion and that of the host crops for root-associated traits to enhance establishment and proliferation of the beneficial microorganisms may lead to the formation of more effective and stable PGPR strains. The use of multistrain inocula of PGPR with known functions is a

topic of interest for today's scientists working in the field of biofertilizer. Construction of competent strains of PGPR using molecular biology tools is still in its infancy; focused efforts in this direction will lead to huge success stories.

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