Chapter 2
Bacillus as PGPR in Crop Ecosystem

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2.1 Introduction

Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria which have the ability to colonize the roots and either promote plant growth through direct action or via biological control of plant diseases (Kloepper and Schroth 1978). They are associated with many plant species and are commonly present in varied environments. Strains with PGPR activity, belonging to genera *Azoarcus*, *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Gluconacetobacter*, *Pseudomonas*, and *Serratia,* have been reported (Hurek and Reinhold-Hurek 2003). Among these, species of *Pseudomonas* and *Bacillus* are the most extensively studied. These bacteria competitively colonize the roots of plant and can act as biofertilizers and/or antagonists (biopesticides) or simultaneously both.

Diversified populations of aerobic endospore forming bacteria (AEFB), viz., species of *Bacillus*, occur in agricultural fields and contribute to crop productivity directly or indirectly. Physiological traits, such as multilayered cell wall, stress resistant endospore formation, and secretion of peptide antibiotics, peptide signal molecules, and extracellular enzymes, are ubiquitous to these bacilli and contribute to their survival under adverse environmental conditions for extended periods of time. Multiple species of *Bacillus* and *Paenibacillus* are known to promote plant growth. The principal mechanisms of growth promotion include production of growth stimulating phytohormones, solubilization and mobilization of phosphate, siderophore production, antibiosis, i.e., production of antibiotics, inhibition of plant ethylene synthesis, and induction of plant systemic resistance to pathogens (Richardson et al. 2009; Idris et al. 2007; Gutierrez-Manero et al. 2001;

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Whipps 2001). It is very likely that plant growth promotion by rhizosphere bacilli may be a result of combined action of two or more of these mechanisms (Fig. 2.1).

Pathogenic microorganisms affecting plant health are a major threat to food production, and traditional methods, viz., crop rotation, breeding for resistant plant cultivars, and application of chemical pesticides, seem to be insufficient to control root diseases of important crop plants (Johri et al. 2003). Further, it appears inevitable that fewer pesticides will be used in future and that greater reliance will be laid on biotechnological applications including use of microorganisms as antagonists. Therefore, interest in biological control has been increased in the past few years partly due to change in the public concern over the use of chemicals and the need to find alternatives of chemicals used for disease control. Both *Bacillus* and *Paenibacillus* species express antagonistic activities by suppressing the pathogens and numerous reports covering this aspect both under *in vitro* and *in vivo* conditions are available (Arrebola et al. 2010; Chen et al. 2009; Joshi and McSpadden Gardener 2006).

Enhancement of plant growth by root-colonizing species of *Bacillus* and *Paenibacillus* is well documented and PGPR members of the genus *Bacillus* can provide a solution to the formulation problem encountered during the development of BCAs to be used as commercial products, due in part to their ability to form heat- and desiccation-resistant spores (Kloepper et al. 2004; Emmert and Handelsman 1999). In the past few years, research has been directed more toward the induced systemic resistance (ISR), a process by which PGPR stimulate the defense

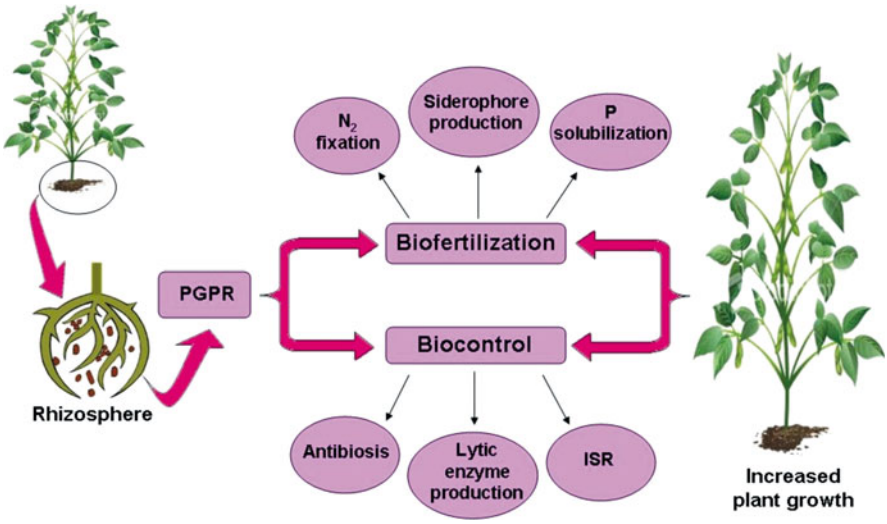

Fig. 2.1 Schematic illustration of important mechanisms known for plant growth promotion by PGPR. Different mechanisms can be broadly studied under (1) Biofertilization, and (2) Biocontrol of pathogens. Biofertilization encompasses: (a) N_2 Fixation, (b) Siderophore production, (c) $P_{inorganic}$ solubilization by rhizobacteria. Biocontrol involves: (a) Antibiosis, (b) Secretion of lytic enzymes, and (c) Induction of Systemic Resistance (ISR) of host plant by PGPR

mechanisms of host plants without causing apparent harm to the host. More recently, Choudhary and Johri (2008) have reviewed ISR by *Bacillus* spp. in relation to crop plants and emphasized on the mechanisms and possible applications of ISR in the biological control of pathogenic microbes. Various strains of species *B. amyloliquefaciens, B. subtilis*, *B. pasteurii, B. cereus, B. pumilus, B. mycoides*, and *B. sphaericus* are known as potential elicitors of ISR and exhibit significant reduction in the incidence or severity of various diseases on diverse hosts (Choudhary and Johri 2008; Kloepper et al. 2004). It is believed that plants have the ability to acquire enhanced level of resistance to pathogens after getting exposed to biotic stimuli provided by many PGPRs and this is known as rhizobacteria-mediated ISR (Choudhary et al. 2007).

The aim of this chapter is to perpetuate the ecological perspectives and role of *Bacillus* species studied in the past few years, pertaining to its plant growth promotory activities with emphasis on the biocontrol mechanisms and possible implications in crop ecosystem. Published and some previously unpublished work have been summarized in this chapter, showing that strains of *Bacillus* and *Paenibacillus* species, including *B. subtilis, B. cereus*, *B. amyloliquefaciens, B. pumilus, B. pasteurii, B. mycoides, B. sphaericus, P. polymyxa*, *P. azotofixans,* and some other newly discovered species (*B. endophyticus*), influence the growth, development, and yield of crops under controlled and varied natural conditions either directly or indirectly following various mechanisms.

2.2 Ecology of *Bacillus* and *Paenibacillus* Species

Most species of *Bacillus* and *Paenibacillus* are distributed globally and the widespread occurrence of subspecies of *B. subtilis* and *B. cereus* with their ability to suppress the plant pathogens has been widely recognized.

2.2.1 Distribution, Diversity, and Population Dynamics

Plant growth promoting strains of *Bacillus* and *Paenibacillus* have been widely studied for enhancement of plant growth (Choudhary and Johri 2008; Kloepper et al. 2004). Cultivation-dependent approaches have revealed the occurrence of multiple isolates of phylogenetically and phenotypically similar species of *B. subtilis* and *B. cereus* ranging from log 3 to log 6 counts (CFU) per gram fresh weight (Vargas-Ayala et al. 2000). While culture-independent studies of soil confirmed the uncultured diversity of both *Bacillus* and *Paenibacillus* rRNA lineages, there are contradictions about the relative abundance of culturable and unculturable representatives of these genera in different soils (McSpadden Gardener 2004; Smalla et al. 2001). Though multiple species of *Bacillus* and *Paenibacillus* are frequently found in the soil and rhizosphere, only limited

information is available about the most commonly isolated species of this genus. In some cases, *B. megaterium* has been found as the most abundant species, but it is improbable that a single species will dominate numerically in most soils (Liu and Sinclair 1992). Species of *B. polymyxa* group, recently renamed *Paenibacillus*, are autotrophs, commonly associated with rotting plant materials, composts, and the rhizosphere. Some of them are able to fix nitrogen and thus contribute significantly to the acquisition of nitrogen by crops such as Canadian wheat (Priest 1993). Members of *B. brevis* group, renamed *Brevibacillus,* are found in both soil and water habitats. The species *B. sphaericus* is most noted as an insect pathogen and is found in the sediments of pools, lakes, and drainage ditches where insect larvae thrive.

Limited attempts have been made to study the diversity of bacterial populations in and around the rhizosphere, probably due to lack of appropriate techniques required to isolate sufficient number of strains belonging to the same species. Due in part to the unavailability of suitable methods to explore the community dynamics, our understanding of the variation in microbial community dynamics in response to soil type, plant type, or stage of plant development is limited as yet (McSpadden Gardener 2004; Duineveld et al. 1998). 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 (Wieland et al. 2001 and references therein). To come to an improved understanding of factors affecting the ability of bacteria to colonize the rhizosphere, the plant has to be taken into account.

Rhizospheric competence is a necessary prerequisite for PGPR. It comprises of effective root colonization combined with the ability to survive and proliferate along the growing plant roots in the presence of indigenous microbiota over a period of time. Given the importance of rhizospheric competence as a prerequisite, understanding the plant–microbe communication as affected by genetic and environmental factors in the context of their ecological niche can contribute significantly toward understanding the mechanisms of action (Bais et al. 2004; Whipps 2001). *Bacillus* species are believed to be less rhizosphere competent than *Pseudomonas* species. As a consequence, most research even today is aimed at the development of BCAs based on *Pseudomonas* species (Weller 1988). However, studies on the genetic diversity of *Bacillus* from soil as well as from the wheat rhizosphere implied that rhizosphere competence is a characteristic of the strain (genotype) not exclusive to the genus or species. Based on studies of wheat rhizosphere colonization by *Bacillus* species, it seems that rhizosphere competent genotypes occur in this bacterium (Milus and Rothrock 1993; Maplestone and Campbell 1989).

Experiments with different wheat varieties conducted by Juhnke et al. (1987) and Milus and Rothrock (1993) have revealed that seeds bacterized 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 the top few centimeters of root below the seed could not be confirmed. However, in another

study, high populations of *B. mycoides* and *B. pumilus* in the rhizosphere of wheat at a depth of 20–30 cm below the site of bacterial inoculation (200 μl/seed) at the time of planting have been reported; the bacterial population was believed to be carried downward either in conjunction with water infiltration or along with elongating tips of growing roots (Maplestone and Campbell 1989).

2.2.2 Spatiotemporal Aspects

Variations are known to exist in the genetic microdiversity within the species of *Bacillus* and *Paenibacillus* (McSpadden Gardener 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 temperature gradient gel electrophoresis (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. McSpadden Gardener (2004) studied the population structure of these two groups by terminal restriction fragment length polymorphism (TRFLP) 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. In addition, the diversity of *Bacillus* spp. decreased from nine species at 45 days to just one species at 133 days, shortly before harvesting. *B. pumilus* was reported as the most prominent species from soybean phylloplane at all sampling times till the end of cropping season, followed by *B. subtilis* as second most abundant species from 15 to 108 days after sowing. Several other *Bacillus* spp., such as *B. subtilis*, *B. brevis*, *B. firmus,* and *B. circulans,* were found as regular or as dominant microflora at an early stage of plant growth, but were no longer detected

after 85 days from the phylloplane of trifoliate leaves. The cause of apparent reduction in *Bacillus* spp. populations at the end of soybean cropping season, however, remained unclear.

The genus *Paenibacillus* encompasses several species described as nitrogen-fixing bacilli, including *P. polymyxa, P. azotofixans*, and *P. macerans* (Ash et al. 1993). In contrast to other species of this genus, strains belonging to *P. azotofixans* are efficient nitrogen fixers and are prevalent in the rhizosphere of maize, sorghum, sugarcane, wheat, banana, and forage grasses (Rosado et al. 1998a; Seldin 1992). Rosado et al. (1998b) showed that bacterial diversity of *P. azotofixans* was high in bulk soil compared to the rhizosphere. Seldin et al. (1998) determined the diversity of *P. azotofixans* strains isolated from the rhizoplane, rhizosphere, and nonroot associated soil of maize grown in two different field soils of Brazil (Cerrado and Varzea). On the basis of phenotypic traits, 60 strains from Varzea soil and 46 strains from Cerrado were identified as *P. azotofixans* and they could be categorized into six groups for each soil. Fifteen different hybridization patterns were obtained in 60 *P. azotofixans* strains from Varzea while only two patterns were obtained from 46 strains of Cerrado when specific plasmids for nifH genes were used as probes. Data from the phenotypic and hybridization studies were used to construct a dendrogram; all strains could be distributed into 29 groups. Strains isolated from Varzea soil were more heterogeneous than those obtained from Cerrado soil. This heterogeneity is believed to be a result of difference in soil type but it remained unclear whether the difference in soil type could account for differences demonstrated by the heterogeneity between Varzea and Cerrado soil populations. These observations were in agreement with the findings of Berge et al (1991) who also reported variations in the population structure of *B. circulans* from the rhizosphere of maize with the soil type.

2.2.3 Rhizospheric Effect and Host Specificity

It is not certain if plants actively select beneficial soil microbial communities in their rhizosphere through rhizodeposition, though earlier studies showed that plants select for taxonomic functional groups in the rhizosphere (Grayston et al. 2001; 1998). 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; Smalla et al. 2001). The root exudation is believed to be plant specific and this specificity may reflect the evolution or specific physiological adaptation to conditions of a particular soil habitat (Crowley and Rengel 1999). The type of root exudates is crucial for the ecosystem distribution and niche specificity of certain plants. Composition of root exudates was shown to vary with plant species and stage of plant growth (Jaeger et al. 1999). Concomitantly, the plant is supposed to influence the population structure of indigenous rhizobacteria as well as the population dynamics of introduced BCAs. Under certain conditions, many compounds present in the root

exudates (sugar, amino acids, or organic acids) stimulate a positive chemotactic response in 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. Oger et al. (1997) demonstrated that genetically engineered plants producing opines have an altered rhizosphere community. In fact due to high diversity of chemical influences in the rhizosphere of different plants, roots drive specific selections of microbes out of indefinite pool of soil microbial diversity.

Nevertheless, the cultivation practices being followed have also been recognized as an important determinant of rhizospheric microbiota (Mittal and Johri 2007). Agriculture management strategies can induce clear shifts in the structures of plant-associated microbial communities (Garbeva et al. 2004). For example, plant genotype can exert strong effects on the bacterial communities associated with the plants (Gu and Mazzola 2003; Adams and Kloepper 2002). Growth stage of plant is another important factor that provides shape to the rhizobacterial community structure and as reported in case of potato rhizosphere it could be the strongest one affecting the bacterial communities (van Overbeek and van Elsas 2008). Besides, land use, soil history, cultivation practices, and plant growth stage are some of the other factors which govern the structure of plant-associated microbial communities (van Overbeek and van Elsas 2008 and references therein, Mittal and Johri 2007).

Among the existing practices, use of biofertilizer is of utmost importance in crop ecosystem pertaining to agriculture production. A study was carried out to evaluate the effect of cultivation practices (traditional and modern), on the community structure of culturable bacteria antagonistic toward soilborne pathogenic fungus *Sclerotinia sclerotiorum*, associated with the soybean (*Glycine max* L.) rhizoplane and rhizosphere/endorhizosphere and bulk soil (Kumar et al. 2009). The cultivation parameters for both kinds of practices were otherwise similar except that the traditional system of cultivation involved use of farmyard manure (FYM) as fertilizer input while modern cultivation system was based on application of commercially available inorganic chemical fertilizers. The community structure of bacterial antagonists isolated following traditional system of cultivation was structurally more diverse than modern system. Further, traditional system of cultivation was found to support higher population density of the antagonists. The bacterial diversity was found to increase with the stages of plant growth gradually from seedling up to maturation stage and then eventually followed a decline with only transient changes. Little variation was observed in bulk soil for community structure, implying that the bulk soil was highly stable while the gradual shifts observed in bacterial diversity may be a consequence of change in composition of root exudates excreted from the plant roots which are known to change the chemistry and biology of root microenvironments (Hartmann et al. 2009). The nature of organic amendments used in traditional system of cultivation may account for the occurrence of high bacterial diversity of antagonists in the traditional system. As a matter of fact, these organic substrates can act as ideal source of nutrients for the antagonists in soils and offer an opportunity to introduce

and establish specific BCAs into soils, which in turn leads to sustainable disease control based on activities of microbial communities.

Smalla et al. (2001) demonstrated for the first time that roots of each model plant species are colonized by its own bacterial communities using cultivation-independent methods on three phylogenetically different and economically important crops – strawberry (*Fragaria ananassa* Duch.), potato (*Solanum tuberosum* L.), and oilseed rape (*Brassica napus* L.). It was possible to differentiate the plant species on the basis of the rhizosphere communities using DGGE in a randomized field trial (Smalla et al. 2001). The DGGE fingerprints showed plant-dependent shifts in the relative abundance of bacterial populations in the rhizosphere. All rhizobacteria showed some bands in common, and also specific bands intriguingly, e.g., *Nocardia* populations were identified as strawberry-specific bands.

2.2.4 Endophytic Colonization and Plant Growth Promotion

Bacteria residing in the rhizosphere of plants may gain access into the root interior and establish endophytic populations. Several bacteria can transcend the endodermis barrier, reach the vascular system by crossing through the root cortex, and subsequently thrive as endophytes in plant tissues, viz., stem, leaves, tubers, etc. (Compant et al. 2005). The endophytic colonization of host plant by bacteria reflects on their ability to selectively adapt themselves to these specific ecological niches resulting in an intimate association without any apparent harm to the plant (Compant et al. 2005 and references therein). Bacterial endophytic communities are presumed to be a product of colonization process initiated in the root zone but they may originate from other sources, viz., phyllosphere, anthosphere, or spermosphere (Sturz et al. 2000).

Species of *Bacillus* are common inhabitants among the resident microflora of inner tissues of various species of plants, including cotton, grape, peas, spruce, and sweet corn, where they play an important role in plant protection and growth promotion (Berg et al. 2005; Shishido et al. 1999; Bell et al. 1995). Almost all the endophytic, aerobic, spore forming bacteria described so far belong to the species generally recognized as free-living soil organisms, such as *B. cereus*, *B. insolitus, B. megaterium, B. pumilus, B. subtilis,* and *P. polymyxa*, though in some cases the bacteria have not been identified beyond the genus level (Shishido et al. 1999; Benhamou et al. 1996; Sturz et al. 1997; Bell et al. 1995).

Reva et al. (2002) studied the diversity of endophytic AEFB in the inner tissues of healthy cotton plants (*Gossypium* sp. Dushanbe, Tajikistan). A total of 76 strains were characterized phenotypically and majority of them were identified as *B. amyloliquefaciens*, *B. licheniformis*, *B. megaterium*, *B. pumilus,* and *B. subtilis*; four strains could not be assigned to any known species. Among the isolates, *B. subtilis* was most abundant (43 strains) followed by *B. licheniformis* (15 strains), *B. megaterium* (eight strains), and *B. pumilus* (six strains). Phenotypically all the four unusual strains appeared similar and showed some resemblance to *B. insolitus*, another well-known colonizer of plants but differed from the latter in some

physiological properties (Sturz et al. 1997; Bell et al. 1995). Molecular typing of these four strains revealed similar RAPD patterns that were different from those of the reference strains of common plant-associated species such as *B. licheniformis, B. megaterium, B. pumilus*, and *B. subtilis*. Based on similarity level of RAPD profiling, the four strains were grouped into single distinct taxon but two different amplification profiles were obtained when the hypervariable spacer regions between 16S and 23S rRNA genes were targeted, suggesting that these four bacteria encompass two lineages within the same taxon. Complete 16S rRNA sequencing of the two representatives unravelled the distinction between them; one of these was characterized as a new species, *B. endophyticus*.

2.3 Phtyostimulation and Biofertilization Effects

The physiology of plant and signaling are affected by bacterial hormones in different ways depending upon the physiological role played by hormone or recalcitrance of the plant tissues to change in hormonal level and the concentration of the hormone being produced. Biofertilizing PGPR, in particular, refers to the rhizobacteria that are able to promote plant growth by increasing nutrient uptake by plants.

2.3.1 Phytostimulation

Enhancement of plant growth by root colonizing species of *Bacillus* and *Paenibacillus* is well known (Idris et al. 2007; Kloepper et al. 2004). It is also very likely that growth promoting effects of various PGPRs are due to bacterial production of plant growth regulators such as indole-3-acetic acid (IAA), gibberellins, and cytokinins (Bottini et al. 2004; Bloemberg and Lugtenberg 2001). A large proportion (80%) of bacteria colonizing the rhizosphere have been reported positive for IAA production, but reports depicting IAA production by Gram-positive soil-living bacteria are only few (Loper and Schroth 1986). However, Idris et al. (2004) showed production of substances with auxin (IAA)-like bioactivity from strains of *B. subtilis*/*B. amyloliquefaciens* including strain FZB42. Further, gibberellin production was confirmed from *B. pumilus* and *B. licheniformis* (Gutierrez-Manero et al. 2001). Tryptophan has been identified as main precursor molecule for biosynthesis of IAA in bacteria. IAA controls a diverse array of functions in plant growth and development and acts as a key component in shaping plant root architecture such as root vascular tissue differentiation, regulation of lateral root initiation, polar root hair positioning, and root gravitropism (Aloni et al. 2006).

Idris et al. (2007) first demonstrated the production of reasonable quantities of IAA from Gram-positive bacterium *B. amyloliquefaciens* FZB42 and IAA production was enhanced when the bacterium was fed with tryptophan. Production of IAA was dramatically reduced in the mutants deficient in *trp* gene responsible for biosynthesis

of IAA, suggesting that main route of IAA biosynthesis in this bacterium was dependent on tryptophan. Spaepen et al. (2007) reviewed different pathways involved in the biosynthesis of IAA based on the chemical nature of intermediate molecules produced using tryptophan as precursor. The plant beneficial Gram-negative bacteria synthesize IAA following different pathways that involves indole-3-pyruvic acid (IPA), indole-3-acetamide (IAM), or indole-3-acetonitrile (IAN) as important intermediates (Patten and Glick 1996; Kobayashi et al. 1995). However, in Gram-positive bacteria the main route for biosynthesis of IAA involves IPA (Vandeputte et al. 2005).

Plant hormones affect the spatial and temporal expression of various phenotypes such as cell elongation, division, and differentiation. Besides they are believed to play an important role in plant's response to biotic and abiotic stresses. Many bacteria are capable of producing more than one type of plant hormone; however, some of them can produce and degrade the same hormone, produce one, and degrade the precursor of another, thus affecting the physiology of plant in several ways (Boiero et al. 2007; Leveau and Lindow 2005). Further, bacterial production of IAA may be beneficial or detrimental to the plant health. For example, IAA production by *P. putida* GR12-2 has been found to improve the root proliferation of *Azospirillum brasilense* resulting in increased root surface area which helps in augmentation of nutrient and water uptake from soil (Patten and Glick 2002). On the other hand, in some reports IAA production has been found necessary for pathogenesis (Yang et al. 2007; Vandeputte et al. 2005). There is a growing body of literature showing that IAA can act as a signal molecule, indicating that use of hormones as signaling molecules is not confined only to the plants but also takes part in communication between bacteria and other microorganisms (Spaepen et al. 2007).

2.3.2 *Biofertilization*

PGPR stimulate the plant growth directly through increase in nutrition acquisition, such as phosphate solubilization, or more generally by rendering the inaccessible nutrients available to the plants (Persello-Cartieaux et al. 2003). After nitrogen, perhaps the essential mineral element that most frequently limits the growth of plants is P, which is taken up from soil solution as phosphate (Pi, $H_2PO_4^-$). Although soils generally contain a large amount of total P but only a small proportion is available for uptake by the plants. On an average, most of mineral nutrients in soil are present in millimolar amounts but P is present in micromolar or even lesser quantities (Khan et al. 2006). However, plants are well adapted to uptake of P from low concentration soil solution (Jungk 2001). Therefore, it is presumed that the supply and availability of P to the root surface is influenced by the root and microbial processes.

Phosphate-solubilizing microorganisms (PSM) include a wide range of symbiotic and nonsymbiotic organisms, such as *Pseudomonas, Bacillus,* and *Rhizobium*

species; actinomycetes; and various fungi-like *Aspergillus* and *Penicillium* species (Richardson et al. 2009 and references therein). Phosphate-solubilizing bacteria have already been applied in the agronomic practices as potential bioinoculants to increase the productivity. For example, in Soviet Union, a biofertilizer product under the trade name "phosphobacterin" was prepared and commercialized for agricultural applications. Phosphobacterin contained *Bacillus megaterium* var. *phosphaticum* and later on it was also introduced to other countries, like Eastern Europe and India (Khan et al. 2006). Similarly, in India, a consortium, termed as Indian Agricultural Research Institute (IARI) microphos culture, has been developed containing two very efficient phosphate-solubilizing bacteria (*Pseudomonas striata* and *Bacillus polymyxa*) and three phosphate-solubilizing fungi (*Aspergillus awamori*, *A. niger,* and *Penicillium digitatum*) (Gaur 1990).

Application of phosphate solubilizers alone or in combination with nitrogen fixers has been found beneficial for cotton and wheat fields (Zaidi and Khan 2005; Kundu and Gaur 1980). A study had been carried out under green house conditions to explore the effects of combined inoculation of *Rhizobium* and phosphate-solubilizing *P. striata* or *B. polymyxa* with or without added fertilizers on chickpea yield and nutritional contents (Algawadi and Gaur 1988). Whereas, inoculation with *Rhizobium* alone was found to increase nodulation, addition of phosphate solubilizers increased the phosphorus content of the soil. Combined inoculation increased the nodulation and available phosphorus of the soil coupled with improved grain yield and phosphorus and nitrogen uptake by the plants. Natarajan and Subramainan (1995) suggested that following a combined inoculation of *Rhizobium* (strain Tt 9) with *B. megaterium* var. *phosphaticum* could meet with about 50% of the phosphatic fertilizer requirement of the groundnut. This consortium was found very effective for groundnut, resulting in increased nodulation, increased root and shoot length, as well as increased pod yield.

Tomar et al. (1993) reported that inoculation with the phosphate-solubilizing bacterium *B. firmus* resulted in significant increase in seed yield in field trials on lentil (*Lens esculentus)* and black gram (*Vigna mungo*). Similarly, Bethlenfalvay (1994) demonstrated the impact of a consortium comprising *Glomus mosseae*, *Bacillus* sp., and *Rhizobium* sp. on plant growth and soil aggregation upon *Pisum sativum* cultivation and observed a dramatic increase in plant growth and soil aggregation. While in case of *P. sativum*, inoculation of *Rhizobium, B. polymyxa,* and *Glomus faciculatum* resulted in enhanced dry matter production and PO_4^{3-} uptake, no significant response of soybean to dual inoculation was observed (Kloepper et al. 1980).

2.4 Biological Control: Gram-Positive Perspectives

Biological control, using microorganisms to suppress plant disease, offers a powerful alternative to the use of synthetic chemicals. The rich diversity of the microbial world provides a seemingly endless resource for this purpose. While a diverse array

of microorganisms contribute toward the biological control of plant pathogens, most research has utilized species of *Bacillus*, *Trichoderma,* and *Pseudomonas* (McSpadden Gardener and Driks 2004). There are eight species of microorganisms registered by U.S. Environmental Protection Agency for commercial use against soilborne plant pathogens in the United States (Cook et al. 1996). These include two fungi (*Gliocladium virens* G-21 and *Trichoderma harzianum* KRL-AG2), three Gram-negative bacteria (*Agrobacterium radiobacter* K84, *Pseudomonas fluorescens* EG1053, and *Burkholderia cepacia* type Wisconsin), and three Gram-positive bacteria (*Bacillus subtilis* GB03, *B. subtilis* MBI 600, and *Streptomyces griseoviridis* K61). Other than *A. radiobacter* K84, all others are used to control damping-off diseases and improve stand establishment and seedling vigour.

There is a growing body of literature which describes different mechanisms for biocontrol ability of *Bacillus,* viz., siderophore production, secretion of hydrolytic enzymes, antibiosis, ISR, etc. However, discussion on all these aspects of biocontrol is beyond the scope of this chapter, hence antibiosis, quorum quenching (QQ), and ISR, the mechanisms of major importance being emphasized in current scenario involved in biocontrol, will be discussed in detail. Moreover, numerous reports on *in vitro* antimicrobial activity of *Bacillus* species are available, but here we emphasize on the selective studies that combine the successful *in situ* demonstration of antagonism in addition to *in vitro* studies, i.e., success stories of *Bacillus* species used as BCAs in the field.

2.4.1 Success Stories of Bacillus Species *as Biocontrol Agents*

Extensive research including the field testing of different *Bacillus* strains has led to the development of a number of products widely used as commercial BCAs (McSpadden Gardener and Fravel 2002). There is a list of biopesticides (available online: http://www.oardc.ohio-state.edu/apsbcc) registered for pests and disease control in the United States, approved by the U.S. Environmental Protection Agency (EPA), wherein the commercial formulations of different *Bacillus* strains used as BCAs are specified. The products are available as different formulations, viz., liquid or suspension in a liquid, wettable powder, or dry cakes depending upon the compatibility of the biocontrol strain with the carrier molecule.

Products like Companion, Kodiak, Serenade, Subtilex, and Taegro are based on exploitation of different strains of *B. subtilis* as BCAs. Although Companion and Kodiak manufactured by Growth Products Ltd, NY, and Gustafson Inc., TX, of the United Staes, respectively, use the same strain *B. subtilis* GB03, the formulations used differ; the former is used as liquid while the latter as dry flakes. While Kodiak is labeled for the control of root pathogens of cotton and legumes (soybean) such as *Rhizoctonia solani*, *Fusarium* spp., *Aspergillus* spp., and *Alternaria* spp., Companion is known to control the diseases caused by species of *Rhizoctonia*, *Phytophthora*, *Pythium,* and *Fusarium*. The principal component of Subtilex (Becker Underwood, Ames, IA) is *B. subtilis* MBI600 and is marketed for control of root- and

seed-borne infections of ornamental and vegetable crops, such as root rot of soybean and *Botrytis* species, infection of vines, strawberry and cucumber, and brown rust of cereals. Likewise, Serenade (AgraQuest, Davis, CA, USA) containing *B. subtiis* strain QST713 has been proposed to mitigate the downy mildew, *Cercospora* leaf spot, and early blight and late blight diseases associated with various crop plants. However, until today the genetic basis of biocontrol ability of *B. subtilis* strains is not clearly understood and much has been emphasized on the antibiotic production (Joshi and McSpadden Gardener 2006).

2.4.1.1 Antibiosis

Bais et al. (2004) demonstrated the protective action of surfactin produced by *B. subtilis* against infection caused by *Pseudomonas syringae* in *Arabidopsis thaliana* and suggested that surfactin was necessary not only for root colonization but also provided protection against the pathogen. The disease suppression was correlated with inhibitory concentrations of surfactin produced by the organism on roots. Moyne et al (2001) identified *B. subtilis* strain AU195 capable of producing antifungal peptides showing similarity with bacillomycin (group iturin A). The strain AU195 exhibited strong antagonistic activity against *Aspergillus flavus* and a broad range of other plant pathogenic fungi. In another study, *B. amyloliquefaciens* strain A_1Z isolated from soybean rhizosphere was found to produce iturin-like compounds, which successfully inhibited three taxonomically diverse fungal pathogens, *Sclerotinia sclerotiorum*, *Macrophomina phaseolina,* and *Fusarium oxysporum*, the causal agents of sclerotinia stem rot, charcoal rot, and fusarial wilt of soybean plants, under controlled conditions. Chromatographic analysis and mass spectrometric studies showed that the principal antifungal components show similarity with iturin-like compounds (Kumar et al. unpublished). However, the efficacy of antifungal compounds has not been evaluated in the field as yet.

Romero et al. (2007) showed the involvement of iturin and fengycin antibiotics from four *B. subtilis* strains UMAF6614, UMAF6616, UMAF6639, and UMAF8561 in the suppression of powdery mildew of cucurbits caused by *Podosphaera fusca*. The culture supernatant could successively inhibit the powdery mildew at levels previously reported for vegetative cells (Romero et al. 2004). The chemical analysis of culture filtrate together with the recovery of inhibitory components (surfactin, fengycin, and iturin A or bacillomycin) from the melon leaves treated with two strains (UMAF6614 and UMAF6639) strongly supported the evidence of *in situ* production of these antimicrobials.

2.4.1.2 Quorum Quenching and Biological Control

Bacteria sense their population density and coordinate the expression of target genes, including the virulence factors in Gram-negative bacteria, by N-acylhomoserine lactones (AHLs) dependent mechanism known as quorum sensing (QS). While

AHLs and other substituted γ-butyrolactones are synthesized by Gram-negative bacteria, certain oligopeptides and substituted γ-butyrolactones are the primary signal molecules found in Gram-positive bacteria (Faure et al. 2009). The most widely studied signal molecules involved in quorum sensing are the AHLs (Whitehead et al. 2001). In Gram-positive bacteria, QS signaling molecules are generally peptides, except for the universal pheromone LuxS found in both Gram-positive and Gram-negative bacteria (Schauder et al. 2001). QS is believed to play a crucial role in bacterial physiology including regulation of rhizospheric competence factors such as antibiotic production, horizontal gene transfer, and control of those functions that are directly or indirectly related to plant–microbe interactions (Whitehead et al. 2001). However, several soil bacteria are able to interfere with the QS by enzymatic degradation of AHLs, a process known as QQ. AHL inactivation has been reported in α-proteobacteria (e.g., *Agrobacterium*, *Bosea,* and *Ochrobactrum*), β-proteobacteria (e.g., *Variovorax*, *Ralstonia*, *Comamomonas,* and *Delftia*), and γ-proteobacteria (e.g., *Pseudomonas* and *Acinetobacter*) (Faure et al. 2009). In case of Gram-positive bacteria, AHL degradation occurs in both low G + C% strains, i.e., Firmicutes, such as *Bacillus*, and in high G + C% strains or actinobacteria, such as *Rhodococcus* and *Arthrobacter*.

Acylhomoserine lactonase activity (AiiA) that hydrolyzes the lactone ring of AHLs was first observed in a *Bacillus* isolate from soil (Dong et al. 2001, 2000). Until now, two types of enzymes that inactivate AHLs have been identified in several species/genera of bacteria: the AHL lactonases that cause lactonolysis (opening of the gamma-butyrolactone ring) resulting in acyl-homoserine with reduced biological activity and AHL acylases that break the amide linkage of AHLs to produce homoserine lactone and fatty acids with no biological activity (Uroz et al. 2008; Zhang and Dong 2004).

QQ covers various phenomena that lead to perturbation of expression of QS-regulated functions. Dong et al. (2007) evaluated the mechanisms and functions of QQ *in vivo* and threw light on the possible applications of this phenomenon in control of plant diseases and promotion of plant health. It has been suggested by many researchers to take advantage of QQ to develop novel biocontrol strategies for plant pathogens (Dong et al. 2007). For example, Park et al. (2008) identified a potential AHL-degrading enzyme, AiiA, from *B. thuringiensis* which could effectively attenuate the virulence of Gram-negative bacterium *Erwinia carotovora* in the root system of pepper plant by QQ. Recent studies on *B. thuringiensis* show that many subspecies of this organism produce AiiA homolog enzymes to degrade AHLs (Dong et al. 2004, 2000). In another case, genetically modified plants which expressed AHL lactonase, AiiA of *Bacillus*, were found to be more resistant to *Pectobacterium carotovorum* infection than their parental, wild-type plants (Dong et al. 2001). Moreover, studies carried out by Molina et al. (2003) clearly demonstrated the role of AHL-lactonase enzyme in biocontrol of phytopathogens. A significant reduction was observed in the severity of soft rot of potato caused by *P. carotovorum* and crown gall of tomato caused by *A. tumefaciens* when applied with soil bacterium *Bacillus* sp. A24 or *P. fluorescens* P3 modified with lactonase gene AiiA, suggesting that disease inhibition was a result of QQ.

Thus, QQ, in a way, can be used under antivirulence/antidisease strategies to develop novel medical/animal therapies or novel biological control strategies for phytopathogens (Dong et al. 2007). These studies elegantly suggest that QQ can be used as a potential weapon for biological control of pathogenic microorganisms targeting the QS pathway, however, little is known toward ecological aspects of QQ enzymes under *in situ* conditions. All QQ strategies have so far been developed under *in vitro* or under the green house conditions and their efficacy under field conditions remains to be evaluated. Assessment of interconnections in the signal molecules is a future challenge that needs the help of advanced analytical tools and techniques including transcriptomics, proteomics, and metabolomics to account for the intra- and inter-species communications in the rhizosphere and their ecological impact on the rhizospheric microbiota.

2.4.1.3 Induced Systemic Resistance: Ecological Significance and Applicability

Induced resistance may be defined as a physiological "state of enhanced defensive capacity" elicited in response to specific environmental stimuli and consequently the plant's innate defenses are potentiated against subsequent biotic challenges (van Loon 2000). In addition, there is another defined form of induced resistance, popularly known as systemic acquired resistance (SAR) which is different from ISR in context to the nature of elicitor and regulatory pathways involved. While ISR relies on pathways regulated by jasmonic acid (JA) and ethylene (ET), SAR involves accumulation of salicylic acid (SA) and pathogenesis related (PR) proteins – chitinase and cellulase. PGPRs are among the various groups of plant-associated microorganisms that can elicit the plant defense systems resulting in reduction of disease severity or incidence of diseases caused by pathogens which are spatially different from the inducing agent (van Loon and Glick 2004).

Recently, Choudhary and Johri (2008) explicated the mechanisms and role of *Bacillus* species as inducers of systemic resistance in relation to plant–microbe interactions and demarketed the pathways involved in their regulation. Available reports suggest that specific strains of the species *B. amyloliquefaciens*, *B. subtilis, B. pasteurii*, *B. cereus*, *B. pumilus, B. mycoides*, and *B. sphaericus* elicit significant reductions in the incidence or severity of various diseases on a diversity of hosts including greenhouse studies or field trials on tomato, bell pepper, muskmelon, watermelon, sugarbeet, tobacco, *Arabidopsis* species, cucumber, loblolly pine, and tropical crops (Kloepper et al. 2004).

2.4.1.4 Greenhouse Studies on Induction of Plant Resistance Systems

A greenhouse test was performed for ISR study of *B. mycoides* strain Bac J isolated from sugarbeet leaves infected with *Cercospora beticola*, the causal agent of *Cercospora* leaf spot on sugarbeet. The strain was sprayed (1.0 log 8 CFU/ml)

onto one leaf of test plant and bagged. After 3 days of treatment with Bac J, plants were challenge inoculated with the spore suspension of pathogen. There was a significant reduction in disease severity in plants treated with Bac J on a highly susceptible and a moderately susceptible variety of sugarbeet (Bargabus et al. 2002). In another study performed by Krause et al. (2003), bacterial strains isolated from compost were screened for their capacity to elicit systemic protection against *Xanthomonas campestris* py. *armoraciae*. A total of eleven isolates were found to elicit significant reduction in the disease severity in two of the three repeated experiments: four of the top performing strains were characterized as members of *Bacillus* species.

A comparative study of the results obtained by microtiter-based bioassays to assess elicitation of ISR and pot experiments was conducted in greenhouse against blue mold of tobacco caused by *Peronospora tabacina* Adam (Zhang et al. 2002). The disease incidence was significantly reduced in terms of mean percentage of leaf area under infection from *P. tabacina* Adam when strains of *B. pasteurii* C-9 and *B. pumilus* SE34 and T4 were applied as soil drenches on three tobacco cultivars. Also, the sporulation of the pathogen was significantly decreased when compared with the treated strains and nonbacterized control. To explore the relationship between elicitation of plant growth promotion and ISR, the three strains were further evaluated and applied separately as seed treatment. Tobacco growth was significantly increased by strains SE34 and C-9 but not by T4. It was found to be induced by C-9, not by SE34 and T4. However, application of bacteria by seed treatment following soil drenches resulted in elicitation of ISR by all three strains, in addition to the enhancement of plant growth.

In another study, *B. subtilis* strain AF1, isolated from soils suppressive to pigeon pea (*Cajanus cajan*) wilt caused by *Fusarium udum*, was presumed to induce resistance against *Aspergillus niger* on peanut (*Arachis hypogea*) (Podile and Dube 1988). Further, it was found that strain AF1 stimulated production of phenylalanine ammonia lyase (PAL) and peroxidase activity, indicating that AF1 elicited ISR (Podile et al. 1995). Strong experimental evidence that AF1 elicited ISR came from the findings of Sailaja et al. (1997) who reported a notable reduction in the incidence of crown rot of peanut caused by *A. niger* corresponding to increase in lipoxygenase activity, a phenomenon associated with ISR.

2.4.1.5 Field Experiments for Protection Against Systemic Disease

It is not surprising that many biological control agents showing promising results under the controlled environmental conditions of greenhouse fail to exhibit same results in the field under natural environments where competition is more severe. Therefore, shifting from greenhouse to field trials is an important step to evaluate the efficacy of PGPR eliciting ISR and *Bacillus*. Species were found effective in reduction of disease incidence or plant growth promotion have been examined under field conditions.

In a field trial conducted on sugarbeet for six consecutive growing seasons, the disease severity due to *Cercospora* leaf spot was reduced significantly when sprayed with *B. mycoides* strain Bac J (log 7.0 CFU/ml). About 38–91% reduction in disease severity was found in comparison to the nontreated control. However, in 2 of the 6 years, reduction in disease severity achieved by treatment with Bac J was not significantly different from that attained by using triphenyltin hydroxide, the most commonly used fungicide for *Cercospora* leaf spot. It has been suggested previously that ISR was presumed to be the mechanism of disease control in greenhouse test that provided spatial separation of pathogen and PGPR but spatial separation was not maintained in the field experiments (Bargabus et al. 2002).

In addition to the bacterial and fungal diseases, reduction in the incidence or severity of viral diseases has also been studied in the field employing selected strains of ISR-eliciting *Bacillus* species. Zehnder et al. (2000) assessed three strains, *B. subtilis* IN937b, *B. pumilus* SE34, and *B. amyloliquefaciens* IN937a for ISR activity against CMV on tomato plants under field conditions for two consecutive cropping seasons. The PGPR strains were applied as seed treatments at the time of transplanting to the pots prior to their transplantation in the field, while CMV inoculation was done on plants 1 week before transplantation to the field. Treatment with all three *Bacillus* strains resulted in significant reduction of disease compared to the nonbacterized control.

Resistance-inducing rhizobacteria offer an attractive alternative, providing a natural, safe, effective, persistent, and durable type of protection. But protection based on biological agents is not always trustworthy and is seldom as effective as chemical treatments. However, different treatments may be combined and combinations of BCAs that suppress diseases by complementary mechanisms may further reduce the incidence or severity of disease. Rhizobacteria-mediated ISR thus may be a valuable addition to the alternatives available for environmentally friendly plant disease control.

2.5 Conclusions

Considerable efforts toward understanding the ecology and management of PGPR have been directed, yet their development as inoculants remains a considerable challenge. The rhizospheric community is highly complex, comprises of a myriad of organisms interacting in various ways, acting upon each other and reacting to the external environment. Several isolates of *Bacillus* spp. have been developed as BCAs of plant pests and pathogens. However, to be used as successful BCAs a greater understanding of their ecology is desired. In this context, greater knowledge of the diversity, distribution, and physiology of Gram-positive species will be helpful for identification of new strains compatible with the cropping systems. Paramount to success of PGPR is a need to better understand the ecology of rhizobacteria either indigenous or introduced within the rhizosphere. Exploration and identification of traits involved in the ability of certain bacteria to establish

themselves into the rhizosphere at levels sufficient to exert effects on plant growth, effectively compete with the indigenous microflora, cooperatively interact with other beneficial members of rhizospheric biota, and understand the mechanisms (signaling, growth promotory actions, disease suppression etc.) that occur between plants and bacteria are also required.

Clearly, the taxonomic and physiological diversity of *Bacillus* spp. appears capable of reducing the disease incidence or severity but also indicates that much remains to be done on the mechanisms by which these bacteria promote plant growth. The molecular mechanisms involved in the root colonization are under study nowadays and advancement in the molecular and genomic tools offers new possibilities for improving the selection, characterization, and management of biological control. Development of proteomics and functional genomics will be helpful to determine and follow expression of crucial genes of BCAs during mass production, formulation, and application. Transformation of BCAs by inserting genes that improve the tolerance of antagonists to abiotic stresses, such as increased tolerance or resistance to cold, heat, drought, high salinity, heavy metal rich soils, or acidic soils, etc., could be another exciting and challenging task and may provide with better opportunities to implement the concept of biocontrol in the field under the dynamic natural environments.

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