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

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2.1 Introduction

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

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

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D.K. Maheshwari (ed.), *Bacteria in Agrobiology: Plant Probiotics*,
DOI 10.1007/978-3-642-27515-9_2,

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

2.2 Plant-Growth-Promoting Rhizobacteria

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

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

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

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

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

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

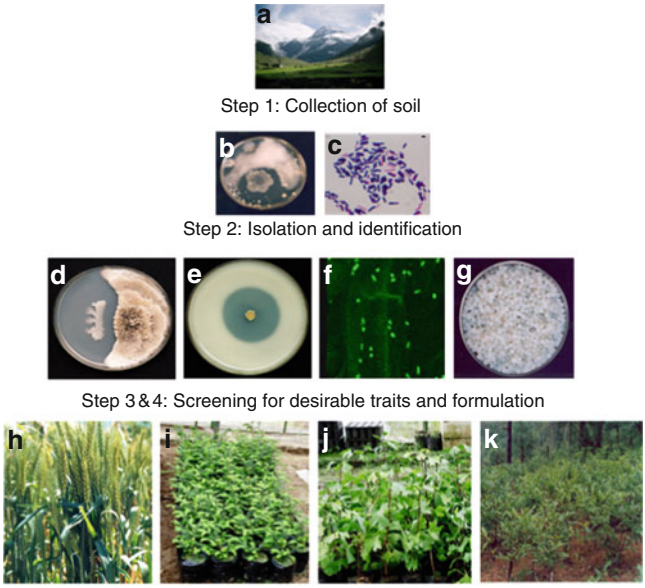

Fig. 2.1 Stepwise schematic representation of steps for the development of microbial inoculants: (**a**) a representative site in IHR for collection of soil samples; (**b**, **c**) isolation and identification of microbes, respectively; (**d**–**f**) screening for desirable traits such as biocontrol,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

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

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

2.3.1 Exploration of Microbial Diversity for the Selection of PGPR

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

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

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

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

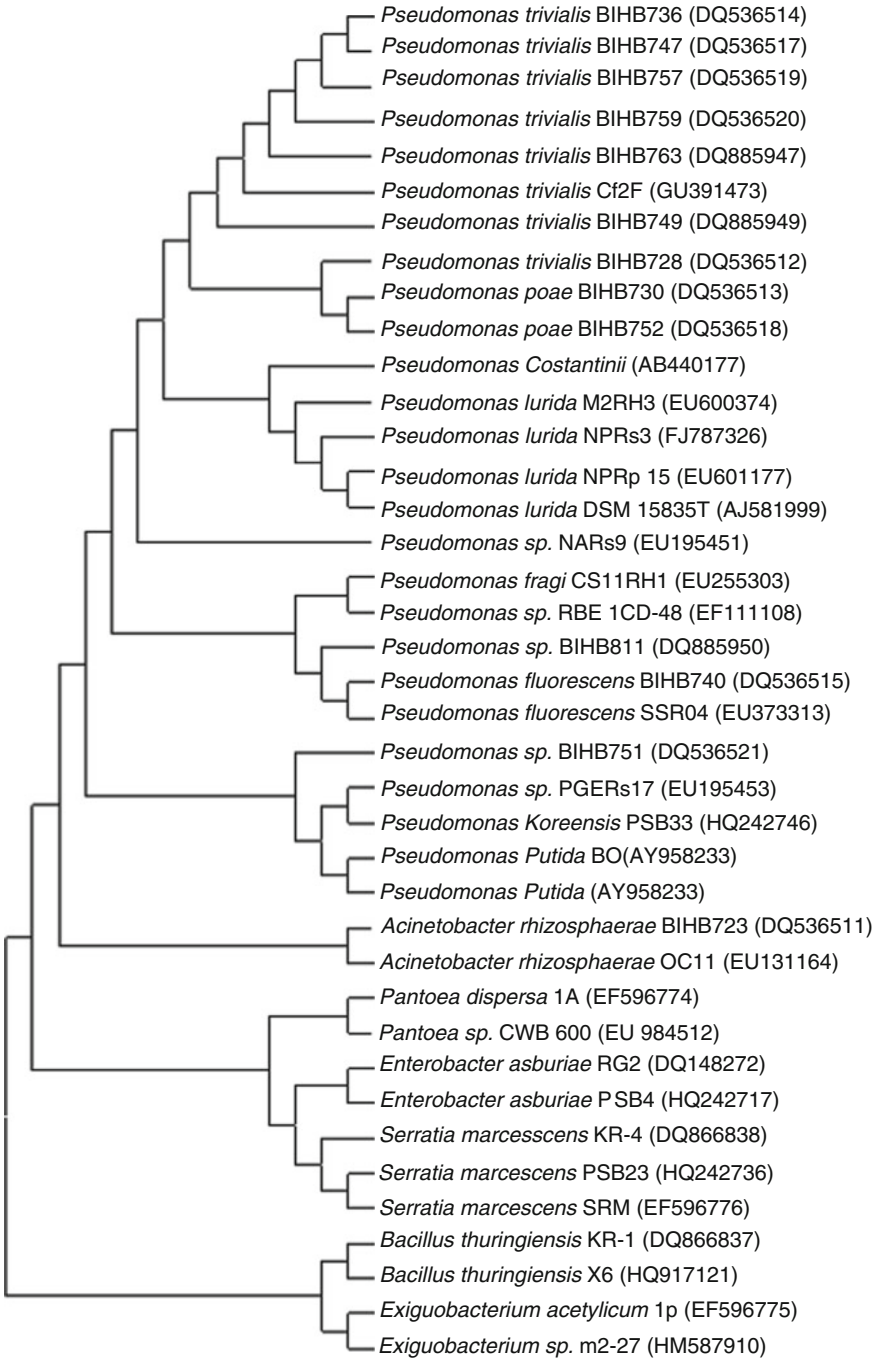

Fig. 2.2 Phylogenetic tree based on 16S rDNA gene sequence of beneficial bacteria isolated from the IHR along with their nearest relatives

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

2.4 Mechanisms of Plant Growth Promotion

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

2.4.1 Fixation, Mobilization and Uptake of Nutrients

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

2.4.1.1 P-Solubilization

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

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

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

2.4.1.2 Siderophore Production

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

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

2.4.1.3 Production of Plant Growth Regulating Substances

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

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

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

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

2.4.2 Biological Control

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

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

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

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

2.4.3 Root Colonization

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

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

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

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

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

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

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

2.5 Role of PGPR in Agriculture

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

2.5.1 Greenhouse Trials

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

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

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

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

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

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

2.5.2 Field-Based Experiments

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

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

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

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

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

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

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

2.5.3 Formulation of Microbial Inoculants

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

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

2.6 New Technologies and Their Potential in PGPR Related Research

2.6.1 Genome Sequencing

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

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

2.6.2 Molecular Analysis of Microbial Communities

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

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

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

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

2.6.3 Reporter Genes and Plant–Microbe Analysis

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

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

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

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

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

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

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

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

2.7 Conclusion

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

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

Acknowledgments Council of Scientific and Industrial Research, the Department of Biotechnology, and the Union Ministry of Environment and Forests, Government of India, New Delhi, and Uttarakhand State Council for Science and Technology, Government of Uttarakhand, Dehradun are thanked for financial support.

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