# Chapter 19
# Plant Growth-Promoting Rhizobacteria of Medicinal Plants in NW Himalayas: Current Status and Future Prospects

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## 19.1 Introduction

India is a natural, invaluable storehouse of medicinal plant diversity of great importance for mankind. The Himalayas are one of the largest and youngest mountain ranges of the world and cover about 10 % of India's land area. Extending across much of the northern and north-eastern borders of the country, the Himalayan massif regulates climate for a broad portion of Asia and provides ecosystem services especially perennial water streams to much of the heavily populated plains of India. In addition, due to its unique location as the meeting place of three biogeographic realms (the Palaearctic, Indo-Malayan and Mediterranean), the species diversity and endemism in the region are unique. At the same time, the region is extremely fragile as a complex result of tectonic activities and anthropogenic influences. On account of its unique and diverse ecosystems and high levels of threat, the Himalayas have recently been designated as a global biodiversity hotspot by Conservation International (Joshi et al. 2010). Some of the important medicinal plants are known to grow only in their indigenous niches, and it is very difficult to increase their population. Overexploitation of natural resources due to the increase in population may lead to the extinction of important medicinal plants. Therefore, medicinal plants need to be protected in their natural habitat through careful management so as to achieve a sustainable balance through systematic agrotechnique.

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Over the last decades, world agriculture has experienced high increase in crop yields, which is being achieved through massive use of inorganic fertilizers and pesticides and mechanization driven by fossil fuel. The global necessity to increase agricultural production from a steadily decreasing and degrading land resource base has placed a considerable strain on agroecosystem health (Tilak et al. 2005; Rao 2013). Especially in developing countries including India, the demand of chemical fertilizers for crop production has increased tremendously due to the release of several high-yielding and nutrient-demanding varieties of crop plants. The excessive and imbalanced use of chemical fertilizers has resulted not only in the deterioration of soil health but also leads to some major environmental problems. This has evinced a great interest in the implementation of environmental friendly sustainable agricultural practices. A progressive reduction in the application of agrochemicals in farming practices without compromising on the yield or quality of the crops and advancement of new generation technologies can be the only possible sustainable alternative. During the last couple of decades, the recent biotechnological advancements in agriculture have unlocked new avenues for the augmentation of productivity in a sustainable manner and have made possible exploitation of soil microorganisms for improving the crop health (Hayat et al. 2010; Lugtenberg and Kamilova 2009) and mitigating environmental stresses (Rao and Sharma 1995; Tank and Saraf 2010).

## 19.2 Plant Growth-Promoting Rhizobacteria

The concept of rhizosphere was first given by Hiltner (1904) to describe the microbial population in the rhizosphere that colonizes the roots of plants, is beneficial and enhances crop productivity and protects the environment. Root colonization comprises the ability of introduced bacteria to survive and establish on or in the plant root, propagate and disperse along the growing root in the presence of indigenous microflora (Kloepper and Schroth 1978). Numerous microorganisms such as algae, bacteria, protozoa and fungi coexist in the rhizospheric region, but bacteria are the most predominant. Plants preferentially select those bacteria contributing to the plants by releasing sugars, amino acids, organic acids, vitamins, enzymes and organic or inorganic ions through root exudates which contribute to creating a rich environment for microbial proliferation. Plant growth-promoting rhizobacteria are soil bacteria inhabiting around/on the root surface and are directly or indirectly involved in promoting plant growth and development via production and secretion of various regulatory chemicals in the vicinity of rhizosphere. They stimulate plant growth through mobilizing nutrients in soils, producing numerous plant growth regulators, protecting plants from phytopathogens by controlling or inhibiting them, improving soil structure and bioremediating the polluted soils by sequestering toxic heavy metal species and degrading xenobiotic compounds (Ahemad and Malik 2011). Indeed, the bacteria lodging around/in the plant roots (rhizobacteria) is more versatile in transforming,

mobilizing and solubilizing the nutrients compared to those from bulk soils (Hayat et al. 2010). Therefore, the rhizobacteria are the dominant driving forces in recycling the soil nutrients, and, consequently, they are crucial for soil fertility (Glick 2012). Strains with PGPR activity, belonging to genera *Azoarcus*, *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Gluconacetobacter*, *Pseudomonas* and *Serratia*, have been reported by many workers. 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. PGPRs promote plant growth by direct and indirect mechanisms and act as biofertilizers as well as biopesticides (Das et al. 2013). With recent upsurge in the interest in organic farming, several biodynamic preparations based on cow dung fermentations are used all of which contain plant growth-promoting bacteria. *Bacillus safensis*, *Bacillus cereus*, *Bacillus subtilis*, *Lysinibacillus xylanilyticus* and *Bacillus licheniformis* were reported recently from cow dung ferments (Radha and Rao 2014). Of these, *L. xylanilyticus* and *B. licheniformis* were reported for the first time in biodynamic preparations.

### *19.2.1 Relationship Between PGPR and Plant Host*

For PGPR to have impact on plant growth, there is an obvious need for an intimate association with the host plant. However, the degree of intimacy can vary depending on where and how the PGPR colonizes the host plant. Relationships between PGPR and their hosts can be categorized into two levels of complexity: (1) rhizospheric and (2) endophytic.

#### 19.2.1.1 Rhizospheric

The rhizosphere can be defined as any volume of soil specifically influenced by plant roots and/or in association with roots, hairs and plant-produced material. This space includes soil bound by plant roots, often extending a few mm from the root surface (Bringhurst et al. 2001) and can include the plant root epidermal layer (Mahafee and Kloepper 1997). Plant exudates in the rhizosphere, such as amino acids and sugars, provide a rich source of energy and nutrients for bacteria, resulting in bacterial populations greater in this area than outside the rhizosphere. Extracellular PGPR (ePGPR) existing in the rhizosphere increases plant growth through a variety of mechanisms; they include genera such as *Bacillus*, *Pseudomonas*, *Chromobacterium*, *Agrobacterium* and free-living nitrogen-fixing bacteria such as *Azotobacter* and *Azospirillum*. Most rhizosphere organisms occur within 50 mm of root surface, and their populations within 10 mm of root surface may reach $1.2 \times 10^8$ cells $kg^{-1}$ soil. Despite large numbers of bacteria in rhizosphere,

only 7–15 % of the total root surface is generally occupied by microbial cells (Gray and Smith 2005).

#### 19.2.1.2 Endophytic

Rhizobacteria that establish inside plant roots, forming more intimate associations, are called endophytes. To aid in this conceptualization, simple terms have been adopted; intracellular PGPR (iPGPR) refers to bacteria residing inside plant cells, producing nodules and being localized inside those specialized structures. These include a wide range of soil bacteria forming less formal associations than the rhizobia–legume symbiosis; endophytes may stimulate plant growth, directly or indirectly, and include the rhizobia. Soil bacteria in the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Azorhizobium*, belonging to the family Rhizobiaceae, invade plant root systems and form root nodules (Wang and Martinez-Romero 2000). Collectively, they are often referred to as rhizobia. These PGPRs are mostly Gram-negative and rod-shaped, with a lower proportion being Gram-positive rods, cocci and pleomorphic forms.

## 19.3 Mechanism of Plant Growth Promotion

Plant growth-promoting rhizobacteria (PGPR) colonizes plant roots and stimulates plant growth. PGPRs control the damage to plants from phytopathogens and promote the plant growth by a number of different mechanisms. According to Glick (1995), the general mechanisms of plant growth promotion by PGPR include associative nitrogen fixation, lowering of ethylene levels, production of siderophores and phytohormones, induction of pathogen resistance, solubilization of nutrients, promotion of mycorrhizal functioning and decreasing pollutant toxicity. The PGPR strains can thus promote plant growth and development either directly or indirectly or both.

### *19.3.1 Direct*

There are several ways in which different PGPRs may directly facilitate the proliferation of their plant hosts. They may (1) solubilize minerals such as phosphorus, (2) fix atmospheric nitrogen and supply it to the plants and (3) synthesize various phytohormones, including auxins and cytokinins (Chen et al. 2006).

### *19.3.2 Indirect*

The indirect mechanism of plant growth occurs when PGPR lessens or prevents the deleterious effects of plant pathogens on plants by the production of inhibitory substances or by increasing the natural resistance of the host (Nehl et al. 1997). PGPRs provide different mechanisms for suppressing plant pathogens. These include competition for nutrients and space (Elad and Chet 1987); antibiosis by producing antibiotics, viz., pyrrolnitrin, pyocyanin and 2,4-diacetylphloroglucinol (Pierson and Thomashow 1992) and production of siderophores (fluorescent yellow pigment), viz., pseudobactin, which limits the availability of iron necessary for the growth of pathogens (Lemanceau 1992). Other important mechanisms include production of lytic enzymes such as chitinases and β-1,3-glucanases which degrade chitin and glucan present in the cell wall of fungi (Frindlender et al. 1993), HCN production and degradation of toxin produced by pathogen (Duffy and Defago 1997). PGPRs have attracted much attention for their role in reducing plant diseases. Although the full potential has not been reached yet, the work to date is very promising and may offer organic growers effective control of serious plant diseases.

## 19.4 PGPR Associated With Medicinal Plants

Ecosystems in the Indian Himalayas encompass one of the largest altitudinal gradients in the world and range from the subtropical forests of the Siwaliks to alpine meadows and scrub in the higher peaks of the Great Himalayas. Some of the richer assemblages of wild and medicinal plants are found in this region. It has been estimated that the region supports over 4,500 species of vascular plants (Western Himalaya Ecoregional BSAP 2002). Ancient Indian literature incorporates a remarkably broad definition of medicinal plants and considers all plants as potential sources of medicinal substances. However, this plant wealth is eroding at a fast pace due to habitat loss, land fragmentation, overexploitation, invasion of exotics, pollution and climate change. The population explosion and economic development and urbanization the world over have been basic and fundamental reasons for the depletion of natural resources. The biosphere has lost some valuable species, and many more are threatened. According to some estimates, tropical forests alone are losing one species per day. The erosion of species richness is going to erode the valuable genes, genomes, ecosystem balance, ecosystem stability and a host of other characteristics which are hard to retrieve back. The anthropogenic interferences have deflected the natural directions, posing threat to these pristine ecosystems. To protect these herbal medicinal plants in their natural habitat, a systematic agro-technique needs to be developed (Malleswari and Bagyanarayana 2013).

Plant growth-promoting microbes found in the rhizosphere of various medicinal plants grown in different soils and climatic conditions can provide a wide spectrum

of benefits to plants (Mayak et al. 2004). Arbuscular mycorrhizal fungi (AMF) are also known to increase the growth of many plant species, including medicinal and aromatic plants (Selvaraj et al. 2008). Various PGPR strains have also proven to be able to increase nutrient availability in the rhizosphere (Cakmakci et al. 2007). The occurrence of *Azospirillum*, *Azotobacter*, *Pseudomonas* and *Bacillus* in the rhizosphere of *Withania somnifera* has been reported by Thosar et al. (2005). Species such as *Azospirillum*, *Azotobacter* and *Pseudomonas* have been found in the rhizosphere of *Catharanthus roseus*, *Coleus forskohlii*, *Ocimum sanctum* and *Aloe vera* (Karthikeyan et al. 2008). Turrini et al. (2010) reported the occurrence of AMF species such as *Glomus coronatum*, *G. mosseae*, *G. etunicatum*, *G. geosporum*, *G. viscosum* and *G. rubiforme* in the rhizosphere of *Smilax aspera* and *Helichrysum litoreum*. Species belonging to the genus *Bacillus* has been registered as the dominant rhizobacteria associated with medicinal plants, *Valeriana jatamansi*, *Podophyllum hexandrum* and *Picrorhiza kurroa* grown in their natural habitat of Northwestern Himalayas (AINP on Biofertilizer Solan Centre, UHF, Nauni).

In the rhizosphere, a synergism between various bacterial genera such as *Bacillus*, *Pseudomonas*, *Arthrobacter* and *Rhizobium* has been shown to promote plant growth of various plants such as peanut (*Arachis hypogaea* L.) (Dey et al. 2004), maize (*Zea mays* L.), soybean (*Glycine max L.*) (Cassan et al. 2009), fodder galega (*Galega orientalis* L.) (Egamberdieva et al. 2011) and sweet basil (*Ocimum basilicum* L.) (Hemavathi et al. 2006). Compared to single inoculation, co-inoculation has improved the absorption of nitrogen (N), phosphorus (P) and mineral nutrients by plants (Bashan and Holguin 1997). Such PGPR activity has been reported in species belonging to *Azospirillum*, *Azotobacter*, *Pseudomonas*, *Bacillus*, *Burkholderia*, *Bradyrhizobium*, *Sinorhizobium* and *Trichoderma* (Sudhakar et al. 2000; Hemavathi et al. 2006; Rajasekar and Elango 2011).

An intensive practice to obtain high yield from cultivated plants requires the extensive use of chemical fertilizers, fungicides and pesticides, which may create environmental problems. Nowadays, the use of biofertilizers in production plays an important role as a supplement to improve the growth and yield of several agricultural, horticultural and medicinal plants (Rao 2008; Lugtenberg and Kamilova 2009). There are several reports that PGPRs have promoted the growth of cereals, ornamentals, vegetables and MAPs (Vessey 2003; Lugtenberg and Kamilova 2009; Egamberdieva 2011; Radha and Rao 2014). Since some medicinal plants are on the verge of extinction, therefore their domestic cultivation is thought to be a viable alternative (Sekar and Kandavel 2010). But, certain drawbacks exist including variability in yield and difference in phytochemical profile over those growing in the wild habitat (Kala et al. 2006). Limited studies have been undertaken on rhizobacteria associated with medicinal plants. The present effort is an exercise to review the efforts on isolation, screening and characterization of PGPR with multiple traits associated with medicinal plants, with an emphasis on methods, and more importantly dwell on the nature of future investigations needed in the field.

## 19.5 Isolation, Enumeration and Characterization of Culturable Rhizobacteria and Endorhizobacteria by Replica Plating Technique

### *19.5.1 Isolation*

Root systems of medicinal plants are exposed carefully by manual excavation and shaken vigorously to remove the rhizospheric soil adhering to the roots. Isolation of bacteria is done by diluting the soil suspension in tenfold dilution series (Fig. 19.1). For endorhizobacteria, the root samples are surface sterilized in 0.2 % mercuric chloride ($HgCl_2$) for 3 min followed by washing in sterilized distilled water.

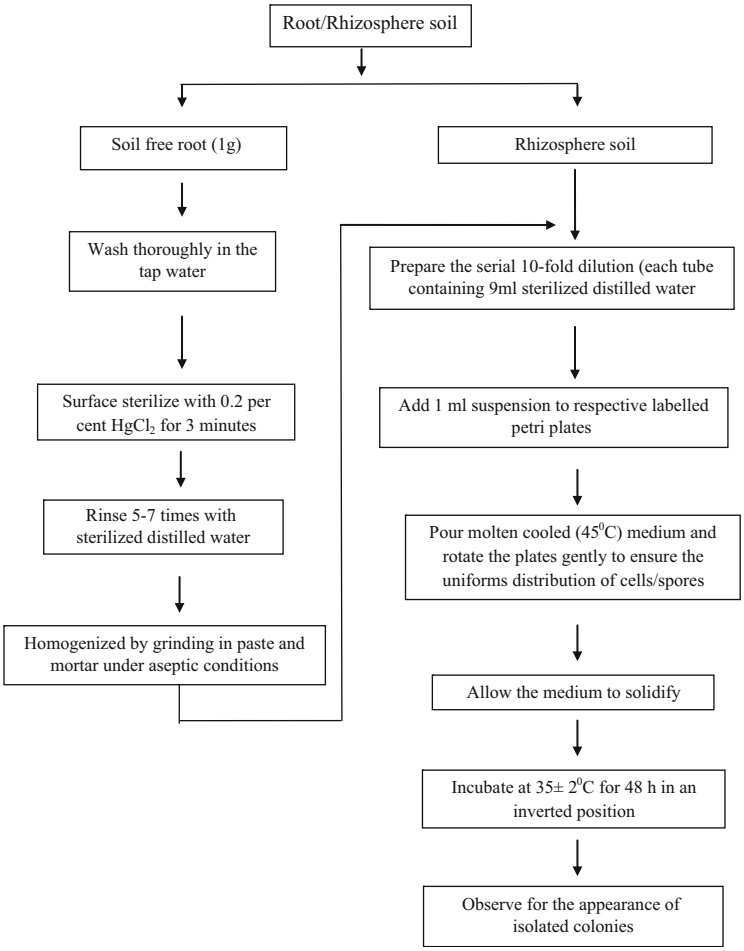


**Fig. 19.1** Flow sheet for the isolation of rhizospheric and endophytic rhizobacteria

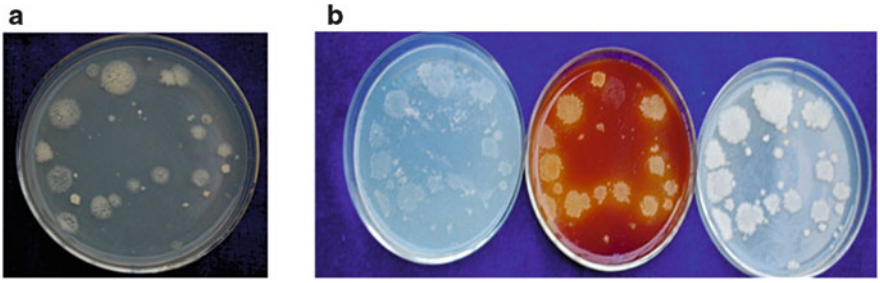


**Fig. 19.2** Master plate for isolation of PGPR of *Picrorhiza kurroa* (**a**); replica plating on different media (**b**): NA, PVK, N-free medium

The surface sterility of roots needs to be cross-checked by incubating the surface-sterilized roots in sterilized nutrient broth overnight. For isolation, one gram of surface-sterilized root sample is placed in 9 ml of sterilized distilled water and then grounded to produce slurry using pestle and mortar under aseptic conditions, and finally plating of soil/root sample is done by pour plate technique on nutrient agar (master plate) under aseptic conditions as per procedure depicted in Fig. 19.2. Plant growth-promoting bacterial isolates from *Picrorhiza kurroa* and other medicinal plants were also isolated by modified replica plating technique developed by AINP on Biofertilizer laboratory, Solan Centre, UHF, Nauni. Populations are expressed as colony-forming unit (CFU) per gram of dry soil weight and per gram of the root weight.

The representative bacterial isolates of the total plated population from the rhizosphere soil and rhizome/roots of the *Picrorhiza kurroa* from two locations of Chamba district isolated by modified replica plating are presented in Fig. 19.2. Replica plating technique was originally developed to isolate auxotrophic mutants, but it can also be used for the quick isolation and screening of PGPRs for plant growth-promoting traits (AINP on Biofertilizers Laboratory Solan, Shirkot and Vohra 2007; Mehta et al. 2013). Rhizospheric and endorhizospheric bacterial populations obtained on nutrient agar (master plate) are replica plated in the same position as the master plate with the help of a wooden block, covered with sterilized velveteen cloth onto the selective media: CAS medium (Schwyn and Neilands 1987) for siderophore-producing ability, nitrogen-free medium for nitrogen-fixing ability and Pikovskaya medium for phosphate-solubilizing ability. At the end of the incubation period (72 h), the location of the colonies appearing on the replica plates is compared to the master plate (Mehta et al. 2010).

All the bacterial isolates were able to grow on nutrient agar, Pikovskaya's medium, nitrogen-free media and CAS media and were selected for screening PGP traits. Four efficient P-solubilizing bacterial isolates exhibited very good chitinase activity on agar plates with a zone size ranging from 30 to 45 mm. Maximum IAA production (30.0 µg/ml) was exhibited by two isolates, and seven isolates were found antagonistic against common fungal pathogens: *Alternaria solani*, *Fusarium oxysporum*, *Pythium aphanidermatum*, *Sclerotium rolfsii and*

*Dematophora necatrix*, and maximum siderophore unit (27.2 %) was observed by one isolate (unpublished).

### 19.5.2 Enumeration

Pearson correlation analysis for total culturable rhizosphere soil and root endophytic bacterial population among six *Valeriana jatamansi* growing sites, viz., Bharmour, Salooni, Padri, Naingra, Holi and Hadsar of Chamba district of Himachal Pradesh, was done (unpublished data from research work underway at AINP on Biofertilizer laboratory, Solan Centre, UHF, Nauni, Table 19.1). There was a positive and significant correlation ($r = 0.67$) between the bacterial population in the rhizosphere and that inside the plants. The sampling sites differed in soil physicochemical properties and environmental conditions. Significant variation in the population of both indigenous rhizosphere soil bacteria and *V. jatamansi* root endophytes was attributed to plant source, time of sampling and environmental conditions, thus suggesting a close association between bacterial population and medicinal plants.

### 19.5.3 Characterization

The in vitro screening of bacterial isolates for important PGPR attributes is depicted in Fig.19.3. PGPR may use more than one mechanism (direct and indirect) to enhance plant growth, as experimental evidence suggests that plant growth stimulation is the net result of multiple mechanisms that may be activated simultaneously. Recent investigations on PGPR revealed that it can promote plant growth mainly by the following means: (1) producing 1-aminocyclopropane-1-

**Table 19.1** Enumeration of total culturable rhizosphere and endophytic bacterial populations of *Valeriana jatamansi* seedlings

| Location | Sites | Rhizosphere soil bacterial population[a] ($\times 10^6$ cfu $g^{-1}$ soil) | Root endophytic bacterial population[a] ($\times 10^3$ cfu $g^{-1}$ root) |
|---|---|---|---|
| Chamba | Bharmour | 29.3 | 26.2 |
| | Salooni | 20.2 | 16.2 |
| | Padri | 28.4 | 20.0 |
| | Naingra | 27.8 | 23.2 |
| | Holi | 25.0 | 21.2 |
| | Hadsar | 27.4 | 17.3 |
| LSD | | 9.0 | 10.8 |
| Correlation coefficient | | $r = 0.67$ | |

[a]Average of five samples

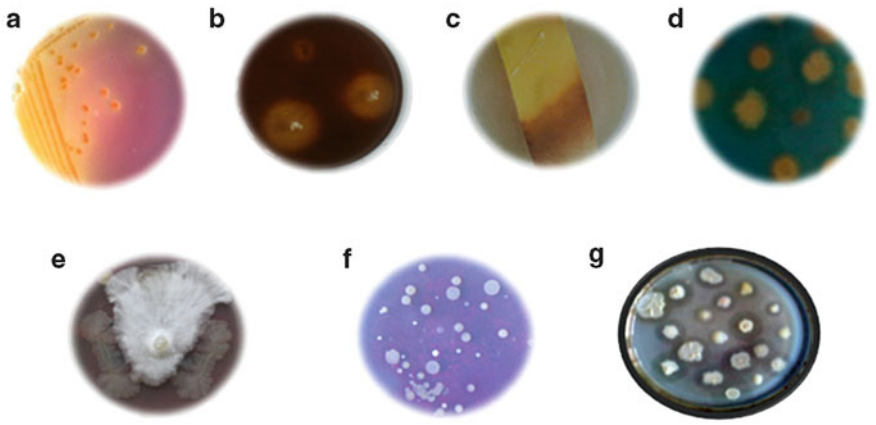


**Fig. 19.3** Multifarious plant growth-promoting traits of P-solubilizing bacterial isolates: P-solubilization (**a**), chitinase activity (**b**), HCN production (**c**), siderophore production (**d**), antifungal activity against *Dematophora necatrix* (**e**), growth on nitrogen-free medium (f) and proteolytic activity (g)

carboxylic acid (ACC) deaminase to reduce the level of ethylene in the roots of developing plants (Dey et al. 2004); (2) producing plant growth regulators like indole acetic acid (IAA) (Mishra et al. 2010), gibberellic acid, cytokinins (Sánchez-Castro et al. 2012) and ethylene (Saleem et al. 2007); (3) asymbiotic nitrogen fixation (Ardakani et al. 2010); (4) exhibition of antagonistic activity against phytopathogenic microorganisms by producing siderophores, β-1,3-glucanase, chitinases, antibiotics, fluorescent pigment and cyanide; and (5) solubilization of mineral phosphates and other nutrients (Hayat et al. 2010). Recently, biochemical and molecular approaches are providing new insight into the genetic basis of these biosynthetic pathways, their regulation and their importance in biological control.

In AINP on Biofertilizers laboratory, Solan Centre, UHF, Nauni, work has been carried out on the plant growth-promoting potential of PGPRs isolated from *Podophyllum hexandrum* (*unpublished data*). Forty-one bacterial isolates were isolated by modified replica plating technique, and representatives of the total plated population from the rhizosphere and rhizome/roots of the *P. hexandrum* were selected. All the bacterial isolates were able to grow on nutrient agar, Pikovskaya's, nitrogen-free media and CAS media and selected for further screening for various plant growth-promoting traits. Proportion of PGPR exhibiting phosphate solubilization and siderophore production is depicted in Fig. 19.4. Percentage of bacteria exhibiting phosphate solubilization activity is arranged in the order of S3(76.3 %) > S2(72.1 %) > S4(71.4 %) > S1(50.0) > S5 (40.0 %). In particular, 100.0 % of the bacteria isolated from the endo-rhizosphere (ER) of site S4 could solubilize phosphorus, even though only 16.7 % of isolates from rhizosphere soil (RS) of site S5 samples could display this activity. The bacterial isolates showing siderophore production were in the order of S2 (66.7 %) > S4 (64.3 %) > S3(61.5 %) > S1(46.2 %) > S5(40.0 %). The highest siderophore

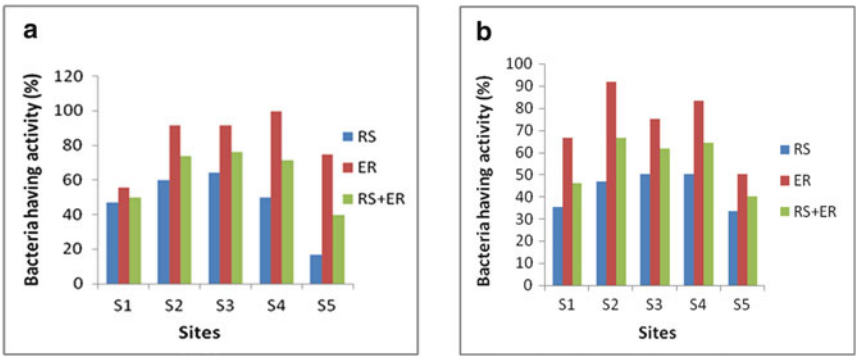


**Fig. 19.4** Graphical representation of percentages of rhizosphere and endophytic bacterial isolates of five sites for PGP traits: (**a**) phosphate solubilization and (**b**) siderophore production (AINP on Biofertilizer laboratory, Solan Centre, UHF, Nauni)

producers were recorded in samples collected from ER of site S2 (91.7 %), and lowest percentages were recorded in RS of site S1 (35.3 %).

## 19.6 Plant Growth-Promoting Attributes of PGPR

### *19.6.1 Biological Nitrogen Fixation*

Nitrogen is an essential element for all forms of life and a basic requisite for synthesizing nucleic acids, proteins and other organic nitrogenous compounds. The ability to reduce and derive such appreciable amounts of nitrogen from the atmospheric reservoir and enrich the soil is confined to bacteria and archaea (Young 1992). Biological nitrogen fixation includes symbiotic nitrogen fixation in the case of *Rhizobium*, the obligate symbionts in leguminous plants, and *Frankia* in nonleguminous trees, while non-symbiotic nitrogen-fixing forms (free-living, associative or endophytic) include *Azotobacter*, *Azospirillum*, *Azoarcus*, *Acetobacter diazotrophicus* and cyanobacteria.

Diazotrophs represent a physiologically and phylogenetically highly diverse functional group, and consequently the functional gene *nifH* (nitrogenase reductase) is the prevailing marker gene for the detection and identification of potential diazotrophs in environmental samples. However, for simple initial screenings to test the efficacy of the rhizobacteria as nitrogen fixer, a loopful of 24-h-old culture of each isolate is streaked on nitrogen-free medium (Jansen et al. 2002) and incubated for 72 h, and the colonies that are able to grow are selected as putative nitrogen fixers.

#### 19.6.1.1 Nitrogenase Activity (Husen 2003)

The ability of the bacteria to fix dinitrogen can be measured by standard protocol of acetylene reduction assay given by Hardy et al. (1968). 50 µl of bacterial culture is inoculated in 1 ml of Burk's nitrogen-free medium (Subba Rao 1999) in 6 ml vacutainer sealed with cotton plugs and incubated for 48 h at room temperature. The cotton plug is then replaced with a rubber stopper, and 0.5 $cm^3$ of the atmosphere (10 %) in the vacutainer is replaced with acetylene and then incubated for 20–24 h. Gas sample (1 ml) was removed from the vacutainer using 1 ml syringe, and the ethylene gas concentration is measured by gas chromatography.

### *19.6.2 P-Solubilization*

Phosphorous is one of the major nutrients required for the growth and development of plants and microorganisms. Microorganisms offer a biological means of solubilizing the insoluble inorganic P of soil and make it available to the plants as orthophosphate. The phosphate-solubilizing bacteria are a promising source of plant growth-promoting agents in agriculture that help sustain agriculture. Most efficient phosphate-solubilizing microorganisms (PSMs) belong to genera *Bacillus* and *Pseudomonas* (Illmer and Schinner 1995; Richardson 2001) and among fungi, *Aspergillus* and *Penicillium*. Certain strains of *Rhizobium* can also solubilize both organic and inorganic phosphate (Alikhani et al. 2006).

#### 19.6.2.1 Mechanism of Phosphate Solubilization

There are two components of P in soil: organic and inorganic phosphates. Inorganic P occurs in soil, mostly in the form of insoluble mineral complexes; some of these appearing after the application of chemical fertilizers. Organic matter, on the other hand, is an important reservoir of immobilized P that accounts for 20–80 % of soil P (Richardson 1994). Organic phosphate solubilization is also called mineralization of organic phosphorus, and it occurs in soil at the expense of plant and animal remains, which contain a large amount of organic phosphorus compounds. The degradability of organic phosphorous compounds depend mainly on the physico-chemical and biochemical properties of their molecules, e.g. nucleic acids, phospholipids and sugar phosphates are easily broken down, but phytic acid, polyphosphates and phosphonates are decomposed more slowly (McGrath et al. 1995).

Several reports have suggested the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite and rock phosphate (Goldstein 1986; Mehta et al. 2010; Walia et al. 2013). In two thirds of all arable soils, the pH is above 7.0,

so that most mineral P is in the form of poorly soluble calcium phosphates (CaPs). Microorganisms must assimilate P via membrane transport, so dissolution of CaPs to Pi ($H_2PO_4$) is considered essential to the global P cycle. Evaluation of samples from soils throughout the world has shown that, in general, the direct oxidation pathway provides the biochemical basis for highly efficacious phosphate solubilization in Gram-negative bacteria via diffusion of the strong organic acids produced in the periplasm to the adjacent environment.

#### 19.6.2.2 Qualitative Estimation on Agar Plates

For the qualitative estimation of phosphorus, positive bacterial isolates obtained after isolation by replica plating are streaked on the PVK agar plates containing known amount of tricalcium phosphate [$Ca_3(PO_4)_2$] and incubated at 37 °C for 48 h. The bacterial solubilization of phosphorus exhibited with yellow-coloured zones produced around the isolated bacterial colony can be calculated by subtracting colony size from total size. Phosphate solubilization index (PSI) is measured using the formula given by Edi-Premono et al. (1996) (Fig. 19.5).

#### 19.6.2.3 Quantitative Estimation in Liquid Broth

Fifty millilitre of PVK broth is dispensed in 250 ml of Erlenmeyer flask containing 0.5 % tricalcium phosphate (TCP) and autoclaved at 15 psi for 20 min, inoculated with 10 % of the bacterial suspension (OD 1.0 at 540 nm) and incubated at 35±2°C under shake conditions for 72 h along with two controls of PVK broth, one with TCP plus inoculum and the other one with inoculum, and no TCP. The culture supernatant is used for determination of the soluble phosphate as described by Bray and Kurtz (1945). An aliquot (0.1–1.0 ml) from the culture supernatant is made to final volume of 5 ml with distilled water and 5 ml ammonium molybdate. The mixture is then thoroughly shaken. The contents of the flasks are finally diluted to 20 ml. Then add 1.0 ml of chlorostannous acid, and make its volume to 25 ml in the volumetric flask. The contents are mixed thoroughly, and the blue-coloured intensity is measured after 10 min at 660 nm. An appropriate blank is kept in which all

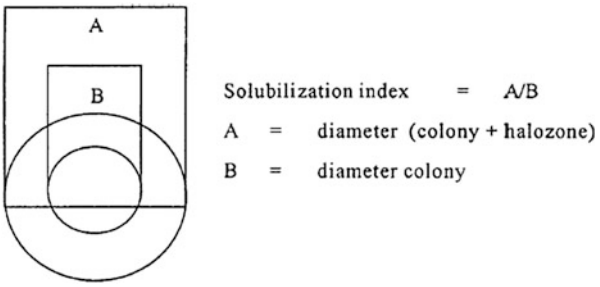


**Fig. 19.5** Figure showing formula for calculating phosphate solubilization index (PSI)

reagents were added except the culture. The results were extrapolated by standard curve drawn using potassium dihydrogen phosphate.

$$P \text{ solubilization} = T - C$$

where

$T =$ PVK with TCP, inoculated
$C =$ PVK with TCP, uninoculated

In a study conducted in AINP on Biofertilizer laboratory, Solan Centre, an isolate *Bacillus subtilis* $CB_8A$ from apple rhizosphere was found to produce phosphate metabolite even without the addition of insoluble phosphate source to the Pikovskaya's broth and also possess five plant growth-promoting attributes (IAA production, siderophore synthesis, chitinase activity, ability to fix atmospheric nitrogen and antifungal activity against *Dematophora necatrix*) at wide range of temperatures (30–45 °C), pH 7 to 9 and salt concentration (0–5 %). The presence of *gdh* gene in *Bacillus subtilis* $CB_8A$ isolate along with organic acid production has been detected which is considered as a possible mechanism responsible for phosphate solubilization (Mehta et al. 2013).

Similarly, in the case of medicinal plants, efficient PGPRs were isolated and screened for P-solubilization and other PGP traits. Almost all the isolates from all the three medicinal plants, viz., *Valeriana jatamansi*, *Picrorhiza kurroa* and *Podophyllum hexandrum*, screened were P-solubilizers and showed high P-solubilization under in vitro conditions. Thirty P-solubilizing strains were isolated from *V. jatamansi*, and among them *Aneurinibacillus aneurinilyticus* strain CKMV1 showed maximum P-solubilization of 257.0 mg/l; 40 strains were from *P. kurroa*, *Bacillus subtilis* strain PkR(7a) exhibited high TCP solubilization of 320.0 mg/l, and 45 P-solubilizing isolates were from *P. hexandrum*, while the maximum P-solubilization was observed with *B. subtilis* strain $4a_1$ (320.0 mg/l).

### *19.6.3 Phytohormone Production*

Phytohormones are organic compounds which are effective at low concentration but play important role as regulators of growth and development of plants. They are the chemical messengers that effect plant's ability to respond to its environment. There are five groups of phytohormones: auxins, gibberellins, cytokinins, ethylene, and abscisic acid. The root is one of the plant's organs that is most sensitive to fluctuations in IAA, and its response to increasing amounts of exogenous IAA extends from elongation of the primary root, formation of lateral and adventitious roots, to growth cessation; hence, IAA is considered as the most important native auxin (Ashrafuzzaman et al. 2009).

IAA is secreted by 80 % of microorganisms and especially secreted by rhizobacteria and interferes with the many plant developmental processes because

the endogenous pool of plant IAA may be altered by the acquisition of IAA (Glick 2012; Spaepen et al. 2007a, b). IAA acts as a reciprocal signalling molecule and affects the gene expression in several microorganisms, and therefore it is considered to play a very important role in rhizobacteria–plant interactions (Costacurta and Vanderleyden 1995). Tryptophan (Trp) is generally considered as the IAA precursor, because its addition to IAA-producing bacterial cultures promotes an increase in IAA synthesis since it requires Trp-dependent pathways (Costacurta and Vanderleyden 1995).

IAA affects plant cell division, extension and differentiation; stimulates seed and tuber germination; increases the rate of xylem and root development; controls the processes of vegetative growth; initiates lateral and adventitious root formation; mediates responses to light, gravity and florescence; and affects photosynthesis, pigment formation, biosynthesis of various metabolites and resistance to stressful conditions. Moreover, bacterial IAA increases root surface area and length and thereby provides the plant greater access to soil nutrients. Also, the rhizobacterial IAA loosens plant cell walls and as a result facilitates an increasing amount of root exudation that provides additional nutrients to support the growth of rhizosphere bacteria (Glick 2012). The downregulation of IAA as signalling is associated with the plant defence mechanisms against a number of phytopathogenic bacteria as evidenced in enhanced susceptibility of plants to the bacterial pathogen by exogenous application of IAA or IAA produced by the pathogen.

#### 19.6.3.1 Quantitative Estimation of Indole-3-Acetic Acid (Auxins)

Quantitative measurement of auxin is done by colorimetric method (Gorden and Paleg 1957) with slight modification. 2–3 drops of orthophosphoric acid are added to 2 ml supernatant along with 4 ml of Salkowski reagent (2 ml of 0.5 M $FeCl_3$ in 98 ml of 35% $HClO_4$). This mixture is then incubated at room temperature in dark for 25 min. Absorbance is measured at 535 nm for the development of pink colour. Concentration of indole-3-acetic acid is estimated by preparing calibration curve using indole-3-acetic acid.

### *19.6.4 Siderophore Production*

Iron is one of the bulk minerals present in plentiful amounts on earth, yet it is unavailable in the soil for the plants. This is because $Fe^{3+}$ (ferric ion) is a common form of iron found in nature and is meagrely soluble. To overcome this problem, PGPR secretes siderophores which are iron-binding protein of low molecular mass and high binding affinity with ferric ion. Siderophores are small molecular weight compounds that bind to iron in the soil and make it unavailable to some of the disease-causing microflora and thus starving them of the iron they otherwise need to survive. Lankford coined the term siderophore in 1973 to describe low molecular

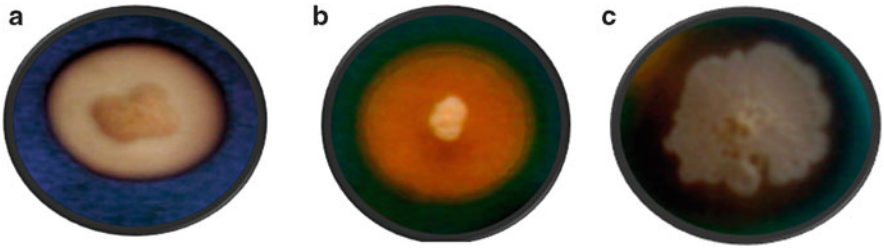


**Fig. 19.6** Plate assay for detection of type of siderophore: (**a**) catecholate, (**b**) hydroxamate and (**c**) carboxylate

weight molecules that bind ferric iron with an extremely high affinity (Lankford 1973). Siderophores are of three types, (a) catecholate, (b) hydroxamate and (c) carboxylate (Fig. 19.6), and have molecular weight ranging from approximately 600 to 1,500 Da, and because passive diffusion does not occur for molecules greater than 600 Da, siderophores must be actively transported. Once actively transported into the periplasm, the iron siderophore complex is bound to a periplasmic binding protein (Braun and Braun 2002).

Siderophores secreted by PGPRs improve plant growth and development by increasing the accessibility of iron in the soil surrounding the roots. Plants such as oats, sorghum, cotton, peanut, sunflower and cucumber demonstrate the ability to use microbial siderophores as sole source of iron than their own siderophores (phytosiderophores). Microbial siderophores are also reported to increase the chlorophyll content and plant biomass in cucumber plants (Das et al. 2013). Nakouti and Hobbs (2012) isolated organisms on the basis of their survival in an iron-limited environment. The survivors of this treatment were largely actinomycetes, and the most prolific producers as assessed and characterized by the chrome azurol sulfonate assay were found to belong to the genus *Streptomyces*.

#### 19.6.4.1 Estimation of Siderophores by Chrome-Azurol-S (CAS) Assay (Schwyn and Neilands 1987)

Siderophore production is detected by chrome-azurol-S (CAS) plate assay and assayed by procedure of Schwyn and Neilands 1987. Sterilized CAS blue agar is prepared by mixing CAS (60.5 mg/50 ml distilled water) with 5 ml iron solution (1 mM $FeCl_3 \cdot 6H_2O$) and 5 ml of 10 mM HCl. This solution is slowly added to hexadecyltrimethylammonium bromide (HDTMA) (72.9 mg/40 ml distilled water). Then the CAS dye is poured into nutrient agar, and plates are poured for spotting of 24-h-old test bacterial culture. Formation of a bright zone with a yellowish (hydroxamate), pinkish (catecholate) and whitish (carboxylate) colour in the dark blue medium indicated the production of siderophore after incubating for 72 h at 37 °C. In the case of liquid assay, the absorbance is recorded at 630 nm, and the

minimal medium is used as a blank with reference (r) cell free extract of culture supernatant. The siderophore units can be calculated using the formula:

$$\text{Percent siderophore unit} = \frac{A_r - A_s}{A_r} \times 100$$

where

$A_r$ is defined as absorbance at 630 nm of reference
$A_s$ is the absorbance at 630 nm of the test bacteria

## *19.6.5 HCN Production*

A secondary metabolite produced commonly by rhizosphere microorganisms is hydrogen cyanide (HCN), a gas known to negatively affect root metabolism and growth (Schippers et al. 1990). Cyanide production is one of the possible ways by which rhizobacteria may suppress plant growth in soil. Although cyanide acts as a general metabolic inhibitor, it is synthesized, excreted and metabolized by hundreds of organisms, including bacteria, algae, fungi, plants and insects, as a means to avoid predation or competition. It affects sensitive organisms by inhibiting the synthesis of ATP-mediated cytochrome oxidase and is a potential environmentally compatible way for biological control of weeds.

### 19.6.5.1 HCN Production Method (Baker and Schippers 1987)

The bacterial cultures are streaked on King's medium B amended with 1.4 g/l glycine agar plates, and the Whatman No. 1 filter paper strips soaked in 0.5 % picric acid in 2 % sodium carbonate are placed inside the top lid of petri plates. Then the petri plates are sealed with parafilm, inverted and incubated at $28 \pm 2°C$ for 1–4 days. Uninoculated plates are kept as a control for comparison. The results are observed for change of colour of filter paper from yellow to orange brown to dark brown.

## *19.6.6 Biocontrol Ability*

The term "biological control" and its abbreviated synonym "biocontrol" have been used in different fields of biology, but in plant pathology, this term is applied for the use of microbial antagonists (the biological control agent or BCA) to suppress diseases. Most narrowly, biological control refers to the suppression of a single pathogen (or pest) by a single antagonist in a single cropping system.

Soil-borne fungal diseases pose serious constraints on agro-productivity. Biological control is a non-hazardous strategy to control plant pathogens and improve crop productivity. The use of indigenous endophytic bacteria is considered as an environmentally friendly and ecologically efficient strategy. Further, it appears inevitable that fewer pesticides will be used in the future and that greater reliance will be laid on biological and biotechnological applications including the use of microorganisms as antagonists. Therefore, the interest in biological control has been increased in the past few years partly due to the 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 (Chen et al. 2006).

A total of 31 endophytic bacteria belonging to different genera, viz., *Pseudomonas*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Acetobacter, Burkholderia*, *Rhizobium* and *Xanthomonas*, were isolated from soybean (*Glycine max* (L) Merril) and were screened in vitro for the antagonistic activity against soil-borne fungal pathogens of soybean, viz., *Rhizoctonia solani*, *Fusarium oxysporum*, *Sclerotium rolfsii*, *Colletotrichum truncatum*, *Macrophomina phaseolina* and *Alternaria alternata* (Dalal and Kulkarni 2013). *Pseudomonas* sp. and *Bacillus* sp. are the major constituents of rhizobacteria, encourage the plant growth through their diverse mechanisms and act as biocontrol agents for various agriculture plants and medicinal plants (Noori and Saud 2012; Shehata et al. 2012; Shanmugam et al. 2011; Zhang et al. 2011; Chauhan et al. 2014).

#### 19.6.6.1 Assay for Antagonists by Agar Streak Method (Vincent 1947)

The rhizobacterial antagonists are screened by streaking a loopful of 48-h-old culture of test isolates a little below the centre of the pre-poured petri plates of malt yeast extract agar and then kept for overnight incubation at 37 °C to check for contamination. Mycelial disc of 4-day-old culture of the test fungal pathogen is placed simultaneously on one side of the streak. A check inoculated with the test pathogen only is kept for comparison. The plates are incubated at 24 ± 1 °C and per cent growth inhibition is calculated according to Vincent (1947).

$$I = \frac{C - T}{C} \times 100$$

where

$I$ = per cent growth inhibition
$C$ = growth of fungus in control
$T$ = growth of fungus in treatment

#### 19.6.6.2 Production of Antibiotic in Liquid Culture

The inhibitory effect of the culture filtrate of test organisms and their consortium is studied using standard method of agar dilution technique. Seventy-two-hour-old culture is first centrifuged at 15,000 rpm for 20 min at 4 °C and then filter sterilized using millipore filter (pore size = 0.22 μm). Different concentrations like 10 and 20 % of the filtrate are poured in malt yeast extract agar (MEA), and plates are incorporated with fungal bits of the test pathogens. The plates are incubated at temperature 24 ± 1 °C for 7 days when the control plate is filled completely with fungal growth, and then colony diameter is measured.

### *19.6.7 Lytic Enzymes Production*

Various kinds of enzymes are produced by microorganisms. The antagonistic activity against different type of microbes may also be attributed to the production of lytic enzymes that are produced by microorganisms. An enzyme chitinase and chitobiase produced by some bacteria and fungi like *Mucor*, *Trichoderma* and *Pseudomonas* species possessed a lytic effect which was related to antagonistic behaviour (Pedraza Reyes and Lopez Romero 1991; Ulhoa and Peberdy 1991). Chitinases are particularly useful in agriculture as biocontrol agents against fungal phytopathogens because of their ability to hydrolyse the chitinous fungal cell wall (Suresh et al. 2010; Wahyudi et al. 2011). Different *Paenibacillus* strains are inhibitory to bacteria and/or fungi (Kajimura and Kaneda 1997) due to the production of antimicrobial substances and cell wall-degrading enzymes (β-1,3-glucanases, cellulases, chitinases and proteases) (Budi et al. 2000). Increased induction of the pathogenesis-related chitinase isoform in *Pseudomonas*-treated rice in response to *R. solani* infection indicated that the induced chitinase has a definite role in suppressing disease development (Radjacommare et al. 2004).

#### 19.6.7.1 Chitinase Assay (Robert and Selitrennikoff 1988)

Preparation of colloidal chitin (Berger and Reynolds 1958)

1. Powdered chitin is digested overnight with concentrated hydrochloric acid at 4 °C.
2. After digestion step, distilled water is added carefully and mixed thoroughly.
3. Centrifuge and remove the supernatant carefully (the first two–three washes are highly acidic).
4. Continued washing with distilled water until the pH of solution reaches around 4.0.
5. The pH of the colloidal chitin solution is adjusted by using 2N NaOH (a pH around 6–6.5).

6. The liquid (10 ml of chitin in 100 ml media) is added directly or the chitin suspension in water and is centrifuged, and the pellet is collected, dried and used at 0.3 % in minimal salt media.

The bacterial culture is spotted on prepared minimal agar plates amended with 0.3 % colloidal chitin and incubated at 30 °C for 7 days. Development of halo zone around the colony after addition of iodine was considered as positive for chitinase enzyme production. In a study conducted in AINP on Biofertilizers laboratory, Solan Centre, the chitinase activity was found in 11 isolates of medicinal plant *Picrorhiza kurroa* (91.7 %) out of selected 12 endophytes and only in 21 rhizosphere soil (75 %) isolates out of total 28 isolates selected. The highest chitinase activity was observed in the case of four isolates with a zone size ranging between 30 and 45 mm.

#### 19.6.7.2 Proteolytic Activity by Plate Assay (Fleming et al. 1975)

Screening for proteolytic activity in bacterial isolates is done by spot inoculation of bacterial culture (72-h-old) on skim milk agar (nutrient agar 100 ml supplemented separately with sterilized skim milk) and incubation at 28 °C for 28–48 h. Clear zone (diameter, mm) formation around the bacterial spot is taken as positive test for proteolysis.

#### 19.6.7.3 Amylolytic Activity by Plate Assay (Shaw et al. 1995)

Spot inoculation of 24-h-old bacterial culture is done on starch agar plate and incubated at 37 °C for 24–48 h. After incubation, the petri plates are flooded with iodine solution. Agar plates are observed for starch hydrolysis which is indicated by the formation of clear zone (diameter, mm) around the bacterial spot.

## 19.7 Induced Systemic Resistance

Several rhizobacterial strains have been shown to act as plant growth-promoting bacteria through both stimulation of growth and induced systemic resistance (ISR), but it is not clear how far both the mechanisms are connected. Induced resistance is manifested as a reduction of the number of diseased plants or in disease severity upon subsequent infection by a pathogen. Such reduced disease susceptibility can be local or systemic, result from developmental or environmental factors and depend on multiple mechanisms. The spectrum of diseases to which PGPR elicited ISR confers enhanced resistance overlaps partly with that of pathogen-induced systemic acquired resistance (SAR). Both ISR and SAR represent a state of enhanced basal resistance of the plant that depends on the signalling compounds,

jasmonic acid and salicylic acid, respectively. Pathogens are differentially sensitive to the resistance activated by each of these signalling pathways. ISR-eliciting rhizobacteria can induce typical early defence-related responses in cell suspensions; in plants, they do not necessarily activate defence-related gene expression. Instead, they appear to act through priming of effective resistance mechanisms, as reflected by earlier and stronger defence reactions once infection occurs (Van Loon 2007).

## 19.8 Identification and Characterization of PGPR

Plant growth-promoting rhizobacteria (PGPRs) establish positive interactions with plant roots and play a key role in agricultural environments and are being currently exploited commercially for agricultural uses. Their identification involves a polyphasic approach based on cultural, physiological and biochemical tests followed by sequencing the 16S rDNA gene. Amplified ribosomal DNA restriction analysis as well as RAPD patterns revealed a high level of intraspecific genetic diversity.

In a study conducted by Chauhan et al. (2014), a bacterial collection of approximately thirty native strains from rhizosphere soil associated with the seedlings of *Valeriana jatamansi* grown in moist temperate forest located in and around Chamba district of Himachal Pradesh were characterized. Four strains were selected and analyzed for plant growth-promoting traits under in vitro (Fig. 19.7). Strain CKMV1 of the total four selected strains identified as *Aneurinibacillus aneurinilyticus* on the basis of morphological, biochemical and 16S rDNA analysis showed maximum phosphate solubilization (257.0 mg $l^{-1}$), indole acetic acid (6.5 μ g $ml^{-1}$) and siderophore production (53.4 %) at $35 \pm 2$ °C (Table 19.2). Besides, the strain also exhibited growth on nitrogen-free medium, hydrogen cyanide production and antifungal activity against different fungal pathogens. Significant growth inhibition of fungal pathogens occurred in the order *Sclerotium rolfsii* > *Rhizoctonia solani* > *Dematophora necatrix* > *Phytophthora* spp. > *Alternaria* spp. > *Fusarium oxysporum*. The results suggested that the rhizosphere of native *V. jatamansi* growing in their natural habitat of Himachal Pradesh is a rich source of PGPRs which have a potential to be used in the future as PGP inoculants to improve crop productivity.

The identification and analysis of genetic polymorphisms of strains isolated from medicinal plants can be carried out by a combination of molecular, PCR-based techniques like analysis of the restriction patterns produced by amplified DNA coding for 16S rDNA. An analysis of RAPD patterns by the analysis of molecular variance method revealed a high level of intraspecific genetic diversity in this *Burkholderia cepacia* population (Cello et al. 1997). Whole-cell fatty acid methyl ester (FAME) profile and 16S rDNA sequence analysis were employed to isolate and identify the bacterial groups that actively solubilized phosphates in vitro from rhizosphere soil of *Valeriana jatamansi* and other important medicinal plants (unpublished data from AINP on Biofertilizer laboratory, Solan Centre).

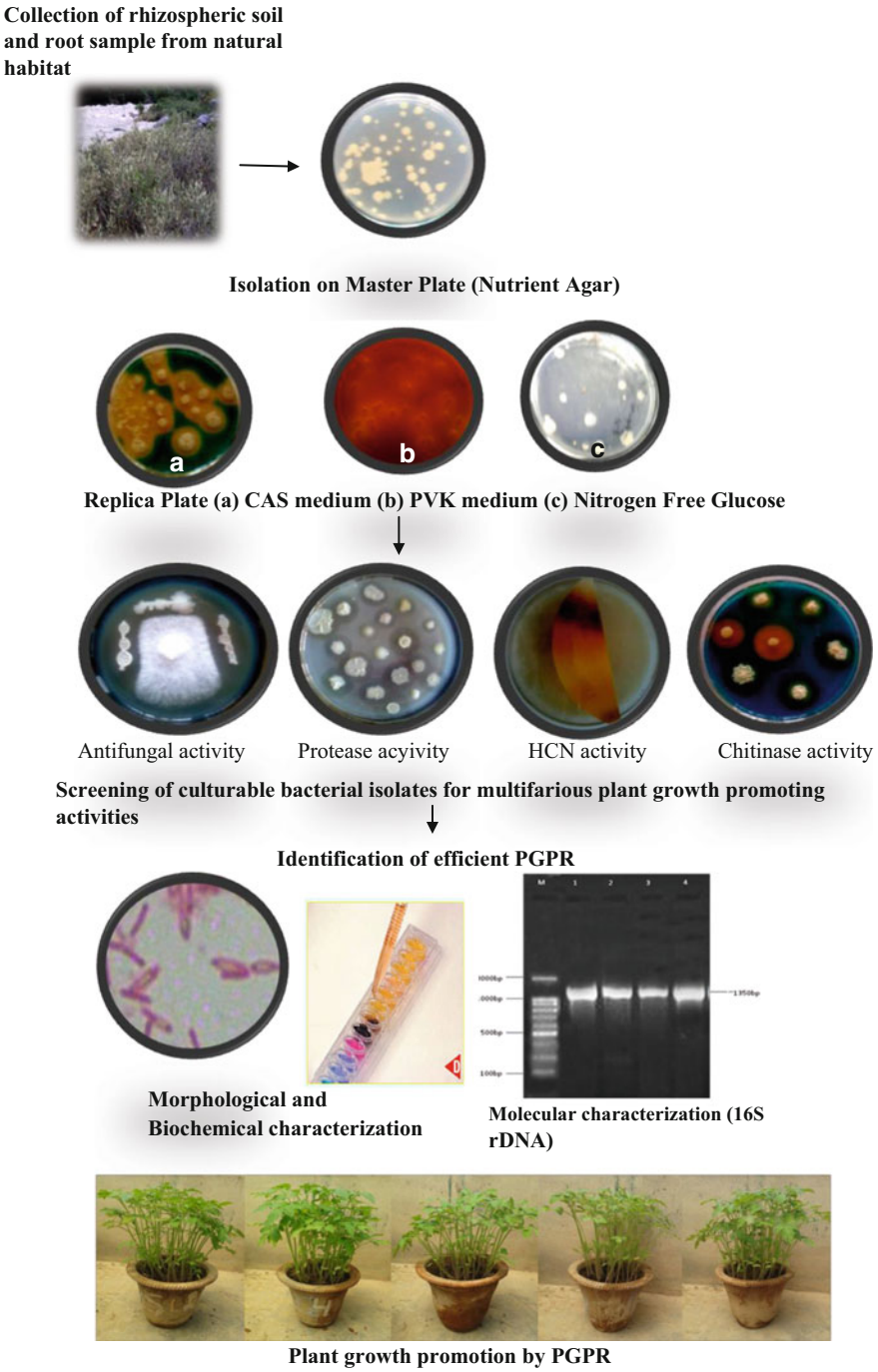


**Fig. 19.7** Stepwise schematic representation of steps for the isolation, identification and characterization of plant growth-promoting rhizobacteria

**Table 19.2** Characterization of selected P-solubilizing bacterial isolates for quantitative estimation of plant growth promoting traits (Unpublished data from AINP on biofertlizer, Solan centre)

| | Plant growth promoting traits | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Quantitative assay | | | % Growth inhibition against fungal pathogens[a] | | | | | |
| Isolates | P-solubilization (mg/l)[b] | Siderophore unit (%)[c] | IAA production (μg/ml)[d] | *F. oxysporum* | *R. Solani* | *S. Rolfsii* | *Phytophthora* spp. | *Alternaria* spp. | *D. necatrix* |
| CKMV1 | 250.00 | 53.43 | 6.5 | 64.30 | 91.58 | 93.58 | 71.37 | 71.08 | 75.73 |
| CKMV2[e] | 120.00 | 40.21 | 3.21 | 67.89 | 51.11 | 57.78 | 64.45 | 62.24 | 60.00 |
| CKMV3[e] | 89.0 | 37.08 | 2.1 | 66.67 | 55.56 | 68.89 | 55.56 | 55.55 | 45.0 |
| CKMV4 | 119.00 | 38.75 | 3.98 | 68.89 | 68.89 | 66.67 | 60.00 | 68.89 | 53.0 |
| LSD | 7.02 | 3.0 | 0.23 | 2.50 | 3.02 | 2.50 | 3.02 | 2.52 | 3.02 |

[a] $I = \frac{C-T}{C} \times 100$

[b] **T** − C; Where, T = Inoculated PVK with TCP, C (uninoculated PVK with TCP)

[c] % Siderophore unit $= \frac{Ar-As}{As} \times 100$, $A_r$ = Absorbance at 630 nm of reference; $A_s$ = Absorbance at 630 nm of test sample

[d] $A_r$ = Absorbance at 630 nm of reference; $A_s$ = Absorbance at 630 nm of test sample

[e] Endophyte

Where, I = Per cent growth inhibition, C = Growth of fungus in control, T = Growth of fungus in treatment

## 19.9 Application of PGPR

The application of plant growth-promoting rhizobacteria (PGPR) as crop inoculants for biofertilization, phytostimulation and biocontrol is an attractive alternative to reduce the use of chemical fertilizers which are costly inputs and also affect the environment. Potential indigenous isolates from medicinal plants can be used as biofertilizer/biostimulant/bioprotectant for protection of the endangered herbal medicinal plants in their natural habitat by a systematic agro-technique. Inoculation with efficient PGPR isolates has produced significantly positive effects on germination and growth (shoot, root length and biomass) of the plants.

The techniques for isolation (rhizobacteria and endorhizobacteria), screening for PGP traits (P-solubilization, siderophore production, nitrogen fixation, hydrolytic enzyme activity) and characterization (morphological, biochemical, physiological and molecular) of PGPR of endangered medicinal plants of NW Himalayas are depicted in Fig. 19.6. There is a further need to explore the varied agro-ecological niches/habitat for the presence of native and new beneficial microflora associated with medicinal plants. It is important to screen an ecoregion-specific PGPR strain which can be used as potential plant growth promoter and bioprotectant. In studies conducted on medicinal plants of trans-Himalayas under AINP on Biofertilizers laboratory, Solan Centre, it has been found that the rhizosphere of *Picrorhiza kurroa*, *Podophyllum hexandrum* and *Valeriana jatamansi* is a rich source of potential PGPR strains with multifarious plant growth-promoting attributes. These potential strains can be further explored for increasing growth parameters/biomass/nutrient uptake under field conditions not only for parent host plant but also for other agricultural crops because microflora associated with medicinal plants possessed maximum number of PGP traits. The presence of specific and limited population of PGPRs associated with medicinal plants unequivocally suggests the hypothesis that natural medicinal plant genotypic variants of a single species can select specific microbial consortia as a result of their unique root exudates profile which exerts selective influence in microbial colonization.

Our results revealed that the native strains rhizosphere of *Valeriana*, *Podophyllum* and *Picrorhiza* possessed a maximum number of PGP traits (Fig. 19.3) like IAA production, phosphorus solubilization, in vitro antagonism to plant pathogens, siderophore production and HCN production. These strains when further screened to show their effect on growth promotion of tomato in terms of increase in growth and biomass registered an increase of 22.6 % root length and 13.8 % of increase in shoot length over control. In another study conducted under net house conditions for plant growth-promoting attributes of the bacterial isolates of seabuckthorn growing in trans-Himalayas (depicted in Fig. 19.7, AINP, Solan Centre), a significant increase in germination was observed from 87.5 % to 100 % when the seeds were treated with $SH3^{5}$ and $T2^{R}$ (out of six PGPR isolates evaluated) as compared to control and seedlings treated with other isolates, thus clearly indicating the possible direct effect on seed germination in soil. However, for growth parameters, $T7^{6*}$ showed maximum per cent increase in shoot length (13.8 %), shoot dry weight

(29.5 %), root length (25.1 %) and root dry weight (33.3 %). All the three $SH3^5$, $T2^R$ and $T7^{6*}$ belonged to genus *Bacillus* and were isolated from stress environments and can therefore be further explored as biofertilizer/bioprotectant for sustainable agricultural practice under conditions of stressful environments. The increased growth and biomass in seedlings raised from seeds treated with P-solubilizing isolates may be attributed to the cumulative effect of phosphate solubilization, nitrogen fixation and production of plant growth regulators.

A similar study was done elsewhere by Mundra et al. (2011), where a phosphate-solubilizing yeast strain PS4 identified as *Rhodotorula* sp. was isolated from the rhizosphere of seabuckthorn (*Hippophae rhamnoides* L.) growing in the Indian trans-Himalayas. The strain solubilizes $Ca_3(PO_4)_2$ to a greater extent than $FePO_4$ and $AlPO_4$. The solubilization of insoluble phosphate was associated with a drop in pH of the culture media. Inoculation of tomato seedling with the strain increased the root and shoots length and fruit yield. Therefore, *Rhodotorula* sp. PS4 with phosphate-solubilizing ability under stress conditions appears to be attractive for exploring the plant growth-promoting ability for deployment as a microbial inoculant in stressed regions.

In another study conducted by Ghodsalavi et al. (2013) on *Valeriana officinalis*, 40 bacterial isolates showed different plant growth-promoting traits like production of siderophores, indole acetic acid (IAA), hydrogen cyanide (HCN), lipase and protease under in vitro conditions and growth promotion study under greenhouse conditions. Rajasekar and Elango (2011) conducted field trials with microbial consortium of *Azospirillum*, *Azotobacter*, *Pseudomonas* and *Bacillus* in combination or single inoculant application on *Withania somnifera* for two consecutive years and recorded a significant increase in plant height, root length and alkaloid content when compared to uninoculated control.

Malleswari and Bagyanarayana (2013) isolated 219 bacterial strains from the rhizosphere sample from different locations of Andhra Pradesh and screened for PGP activity like ammonia production, IAA production, phosphate solubilization, HCN production and antifungal activity. They reported a significant increase with inoculation of *Pantoea* sp., *Bacillus* sp. and *Pseudomonas* sp. on growth promotion (germination and root/shoot length) of sorghum, maize and green gram.

We characterized 510 bacterial isolates from the rhizosphere of soybean, chickpea and wheat and from fresh vermicompost and vermicasts in central India. Twelve bacterial isolates P2 (*Bacillus amyloliquefaciens*), P3 (*Bacillus megaterium*), P4 (*Bacillus subtilis*), P6 (*Bacillus subtilis*), P10 (*Bacillus subtilis*), P17 (*Staphylococcus succinus*), P25 (*Lysinibacillus fusiformis*), P26 (*Dyella marensis*), P53 (*Bacillus subtilis*), P33 (*Bacillus amyloliquefaciens*), P41 (*Bacillus megaterium*) and P48 (*Bacillus licheniformis*) showed multiple PGPR activities in vitro and enhanced plant growth in vivo. 60 isolates shortlisted from above were characterized for in vitro plant growth-promoting attributes. 70 % of the isolates grew in N-free medium and 45 % solubilized phosphate. 76 % of isolates produced IAA production, and none of them showed ACC deaminase activity. 83 % of the isolates produced siderophores and 76 % of the isolates produced ammonia. Only 7 % isolates were HCN positive, and all of them were from wheat rhizosphere

(AINP on Soil Biodiversity-Biofertilizers, IISS, Bhopal). In field studies, P3, P10 and P25 consistently performed well on soybean, chickpea and wheat. It will be interesting to see how these strains from tropical vertisols perform in a different rhizosphere (medicinal plants) in temperate climates.

In another study conducted for plant growth-promoting effect of PGP bacterial isolates of *Podophyllum hexandrum* on tomato seedlings under growth chamber conditions (AINP on Biofertilizer laboratory, Solan Centre), inoculation registered a significant increase in root/shoot parameters. The effect of seed treatment by *Bacillus subtilis* $2a_1$ improved the root length (90 %), shoot length (86.7 %), shoot dry weight (334.5 %) and plant biomass (240.3 %) which was statistically significant as compared to other isolates. *Bacillus subtilis* strain $2a_1$ possessed maximum PGP traits (IAA productivity, siderophore synthesis, chitinase activity, protease activity, amylase activity and antifungal activity against *Alternaria solani*, *Dematophora necatrix*, *Sclerotium rolfsii* and *Phytophthora* sp.)

Plant–microbe ecology is a complex system with all members interrelated. Plants are always subjected to biotic and abiotic factors in their environment which influence their growth and development. This is important from economical point of view in most medicinal plants as these factors greatly affect the root development and production. It is well known that rhizosphere and soil microorganisms (PGPR) play an important role in maintaining crop and soil health through versatile mechanisms: nutrient cycling and uptake, suppression of plant pathogens, induction of resistance in plant host and direct stimulation of plant growth (Kloepper et al. 2004). Maintaining biodiversity of PGPR in soil is thus an important component of environment-friendly sustainable agriculture strategies. Some studies have demonstrated that agricultural practices affected the diversity and function of rhizosphere and soil microorganisms. Therefore, the continued use of growth-promoting rhizobacteria (PGPR) as inoculants is a promising solution for environmentally friendly agriculture including the cultivation of medicinal plants.

## 19.10 Conclusions

Soil–plant–microbe interactions have been much studied in recent decades. Plant species are considered to be one of the most important factors in shaping rhizobacterial communities, but specific plant–microbe interactions in the rhizosphere require further studies to fully understand them. Plant-associated beneficial microorganisms or plant growth-promoting rhizobacteria (PGPRs) fulfil important functions in promoting plant growth and sustaining plant health (Walia et al. 2013). Direct plant growth promotion by microbes is based on improved nutrient acquisition and hormonal stimulation (Walia et al. 2014). Diverse mechanisms are involved in the suppression of plant pathogens which are often indirectly connected with plant growth. Beneficial plant–microbe interactions have led to development of microbial inoculants for use in agricultural biotechnology (Berg 2009). These

rhizospheric microorganisms are being exploited for their innumerable properties and active metabolites (Tamilarasi et al. 2008).

This chapter provides an insight for the exploitation of beneficial plant–microbe interactions and use of beneficial microorganisms occurring in their natural habitat as biofertilizer. This offers an environmentally friendly strategy and is considered as a potential tool for sustainable agriculture for enhanced production of medicinally important plants without creating any side effects. Such strategies will be useful in reducing the use of chemical loads on plant production and a step forward in the development of chemical-free herbals. However, the interactions among PGPR and plants are still not well understood, especially in field applications and different environments (Niranjan et al. 2005). Therefore, there is a need for attention on the following aspects:

1. Many types of microorganisms are known to inhabit soil, especially rhizobacteria which play an important role in plant growth and development due to a number of plant growth-promoting traits. More studies are needed on plant–microbe interactions and their activities in different regions and ecologies, including stressed ones. This will throw light on the exact mechanisms involved in stimulation of plant growth in vivo through biologically active compounds, potential competition between PGPR strains and indigenous soil microflora in the rhizosphere of plants including medicinal plants. Availability of more information will enable the development and widespread acceptance of new inoculants and inoculation strategies that can improve soil ecology, plant development and resistance against diseases and pests.
2. Screening and application of root-colonizing rhizobacteria with enhanced colonizing potential is essential for developing sound strategies to manage the rhizosphere in such a way that it becomes more difficult for pathogens to colonize the rhizosphere; thus, these beneficial bacteria can engineer positive interactions in the rhizosphere, control plant diseases and stimulate plant growth.
3. The question of whether medicinal plants grown ex situ in a different soil and climatic zone and with applied fertilizers and organic manures in an integrated way would have the same activity profile of the medicinally active ingredients as those plants growing in the wild needs to be studied. If not, whether inoculation of PGPR isolated from their native environments and inoculated on these ex situ grown plants would help restore the activity profile needs to be assessed.
4. In their native wild, pristine habitat in the Himalayas growing in the adapted soils and climatic zone, how would these plants respond to inoculation with PGPR isolated from their own rhizosphere in situ? In case they respond in terms of better growth, would there still be an improvement in the profile of active ingredients? This would help to achieve the full potential of medicinal plants even in their own habitats.
5. Is there a species endemism in PGPR like in rhizobia? How would medicinal plants in the Himalayas respond to inoculation with PGPR from tropical crop rhizosphere? Would they influence the profile of active ingredients in a similar

way as PGPR isolated from temperate soils from the rhizosphere of medicinal/cultivated plants?

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## References

Ahemad M, Malik A (2011) Bioaccumulation of heavy metals by zinc resistant bacteria isolated from agricultural soils irrigated with wastewater. Bacteriol J 2:12–21

Alikhani HA, Saleh-Rastin N, Antoun H (2006) Phosphate solubilization activity of rhizobia native to Iranian soils. Plant Soil 287:35–41

Ardakani SS, Heydari A, Tayebi L, Mohammadi M (2010) Promotion of cotton seedlings growth characteristics by development and use of new bioformulations. Int J Bot 6:95–100

Ashrafuzzaman M, Hossen FA, Ismail MR, Hoque MA, Islam MZ, Shahidullah SM, Meon S (2009) Efficiency of plant growth promoting Rhizobacteria (PGPR) for the enhancement of rice growth. Afr J Biotechnol 8:1247–1252

Baker PD, Schippers MN (1987) Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* sp. mediated plant growth stimulation. Soil Biol Biochem 19:451–457

Bashan Y, Holguin G (1997) *Azospirillum*-plant relationships: environmental and physiological advances (1990–1996). Can J Microbiol 43:103–121

Berg G (2009) Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Appl Microbiol Biotechnol 84:11–18

Berger LR, Reynolds DM (1958) The chitinase system of a strain of S*treptomyces griseus*. Biochim Biophys Acta 29:522–534

Braun V, Braun M (2002) Active transport of iron and siderophore antibiotics. Curr Opin Microbiol 5:194–201

Bray RH, Kurtz LT (1945) Determination of total organic and available forms of phosphorus in soils. Soil Sci 59:39–46

Bringhurst RM, Cardon ZG, Gage DJ (2001) Galactosides in the rhizosphere: utilization by *Sinorhizobium meliloti* and development of a biosensor. Proc Natl Acad Sci USA 98: 4540–4545

Budi SW, van Tuinen D, Arnould C, Dumas-Gaudut E, Gianinazzi-Pearson V, Gianinazzi S (2000) Hydrolytic enzyme activity of *Paenibacillus* sp. strain B2 and effect of antagonistic bacterium on cell wall integrity of two soil-borne pathogenic fungi. Appl Soil Ecol 15:191–199

Cakmakci RM, Erat UG, Donmez MF (2007) The influence of PGPR on growth parameters, antioxidant and pentose phosphate oxidative cycle enzymes in wheat and spinach plants. J Plant Nutr Soil Sci 170:288–295

Cassan F, Perrig D, Sgroy V, Masciarelli O, Penna C, Luna V (2009) *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109, inoculated singly or in combination, promote seed germination and early seedling growth in corn (*Zea mays* L.) and soybean (*Glycine max* L.). Eur J Soil Biol 45:28–35

Cello FDI, Bevivino A, Chiarini L, Fani R, Paffetti D, Tabacchioni S, Dalmastri C (1997) Biodiversity of a *Burkholderia cepacia* population isolated from the maize rhizosphere at different plant growth stages. Appl Environ Microbiol 63:4485–4493

Chauhan A, Balgir PP, Shirkot CK (2014) Characterization of *Aneurinibacillus aneurinilyticus* strain CKMV1 as a plant growth promoting rhizobacteria. Int J Agric Environ Biotechnol 7: 37–45

Chen YP, Rekha PD, Arun AB, Shen FT, Lai WA, Young CC (2006) Phosphate solubilizing bacteria from subtropical soil and their tri-calcium phosphate solubilizing abilities. Appl Soil Ecol 34:33–41

Costacurta A, Vanderleyden J (1995) Synthesis of phytohormones by plant-associated bacteria. Crit Rev Microbiol 21:1–18

Dalal J, Kulkarni N (2013) Antagonistic and plant growth promoting potentials of indigenous endophytic bacteria of soybean (*Glycine max* (L) Merril). Curr Res Microbiol Biotechnol 1: 62–69

Das AJ, Kumar M, Kumar R (2013) Plant growth promoting rhizobacteria (PGPR): an alternative of chemical fertilizer for sustainable, environment friendly agriculture. Res J Agric Forest Sci 1:21–23

Dey R, Pal KK, Bhatt DM, Chauhan SM (2004) Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) by application of plant growth-promoting rhizobacteria. Microbiol Res 159:371–394

Duffy BK, Defago G (1997) Zinc improves biocontrol of Fusarium Crown and root rot of tomato by *Pseudomonas fluorescens* and represses the production of pathogen metabolites inhibitory to bacterial antibiotic biosynthesis. Phytopathology 87:1250–1257

Edi-Premono M, Moawad AM, Vlek PLG (1996) Effect of phosphate-solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. Indones J Crop Sci 11:13–23

Egamberdieva D (2011) Survival of *Pseudomonas extremorientalis* TSAU20 and *P. chlororaphis* TSAU13 in the rhizosphere of common bean (*Phaseolus vulgaris*) under saline conditions. Plant Soil Environ 57:122–127

Egamberdieva D, Kucharova Z, Davranov K, Berg G, Makarova N, Azarova T, Chebotar V, Tikhonovich I, Kamilova F, Validov S, Lugtenberg B (2011) Bacteria able to control foot and root rot and to promote growth of cucumber in salinated soils. Biol Fertil Soils 47:197–205

Elad Y, Chet I (1987) Possible role of competition for nutrition in biocontrol of *Pythium*-damping-off by bacteria. Phytopathology 77:190–195

Fleming HP, Etchells JL, Costilus RH (1975) Microbial inhibition by an isolate of *Pediococcus* from cucumber brines. Appl Microbiol 30:1040–1042

Frindlender M, Inbar J, Chet I (1993) Biological control of soil-borne plant pathogens by a ß-1, 3-glucanase producing *Pseudomonas cepacia*. Soil Biol Biochem 25:1211–1221

Ghodsalavi B, Ahmadzaeh M, Soleimani M, Madloo PB, Taghizad-Farid R (2013) Isolation and characterization of rhizobacteria and their effects on root extracts of *Valeriana officinalis*. Aust J Crop Sci 7:338–344

Glick BR (1995) The enhancement of plant growth by free living bacteria. Can J Microbiol 41: 109–117

Glick BR (2012) Plant growth-promoting bacteria: mechanisms and applications. Scientifica 1–15

Goldstein AH (1986) Bacterial solubilization of mineral phosphates: historical perspective and future prospects. Am J Altern Agric 1:51–57

Gorden SA, Paleg LG (1957) Observations on the quantitative determination of indoleacetic acid. Physiol Plantarum 10:37–48

Gray EJ, Smith DL (2005) Intracellular and extracellular PGPR: commonalities and distinctions in the plant-bacterium signalling processes. Soil Biol Biochem 37:395–412

Hardy RWF, Holsten RD, Jackson EK, Burns RC (1968) The acetylene-ethylene assay for N2 fixation: Laboratory and Field Evaluation. Plant Physiol 43:1185–1207

Hayat R, Ali S, Amara U, Khalid R, Ahmed I (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. Ann Microbiol 60:579–598

Hemavathi VN, Sivakumar BS, Suresh CK, Earanna N (2006) Effect of *Glomus fasciculatum* and plant growth promoting rhizobacteria on growth and yield of *Ocimum basilicum*. Karnataka J Agric Sci 19:17–20

Hiltner L (1904) Uber neuere Erfahrungen und Probleme auf dem Gebiet der Bodenbaksteriologie und unter besonderer Berücksichtigung der Gründüngung und Brache. Arb Dtsch Landwirt Ges 98:59–78

Husen E (2003) Screening of soil bacteria for plant growth promoting activities in vitro. Indones J Agric Sci 4:27–31

Illmer P, Schinner F (1995) Solubilization of inorganic calcium phosphates-solubilization mechanisms. Soil Biol Biochem 27:257–263

Jansen PH, Yates PS, Grinton BE, Taylor PM, Sait M (2002) Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia*. Appl Environ Microbiol 68:2391–2396

Joshi R, Nailwal TK, Tewari LM, Shukla A (2010) Exploring biotechnology for conserving Himalayan biodiversity. Life Sci J 7:20–28

Kajimura Y, Kaneda M (1997) Fusaricidins B, C and D, new depsipeptide antibiotics produced by *Bacillus polymyxa* KT-8. Isolation, structure elucidation and biological activity. J Antibiot 50: 220–228

Kala CP, Dhyani PP, Sajwan BS (2006) Developing the medicinal plants sector in northern India: challenges and opportunities. J Ethnobiol Ethnomed 2:32

Karthikeyan B, Jaleel CA, Lakshmannan G, Deiveekasundaram M (2008) Studies on the microbial bio diversity of some commercially important medicinal plants. Colloids Surf B 62:143–145

Kloepper JW, Schroth MN (1978) Plant growth-promoting rhizobacteria on radishes. In: Proceedings of the 4th international conference on plant pathogenic bacteria, angers, France, pp 879–882

Kloepper JW, Ryu CM, Zhang S (2004) Induced systemic resistance and promotion of plant growth by *Bacillus* sp. Phytopathology 94:1259–1266

Lankford CE (1973) Bacterial assimilation of iron. Crit Rev Microbiol 2:273–331

Lemanceau P (1992) Effets bénéfiques de rhizobactéries sur les plantes: exemple des *Pseudomonas* spp. fluorescents. Agronomie 12:413–437

Lugtenberg BJ, Kamilova F (2009) Plant-growth-promoting rhizobacteria. Annu Rev Microbiol 63:541–556

Mahafee WF, Kloepper JW (1997) Temporal changes in the bacterial communities of soil, rhizosphere and endorhiza associated with field-grown cucumber (*Cucumis sativus* L.). Microbiol Ecol 34:210–223

Malleswari D, Bagyanarayana G (2013) *In vitro* screening of rhizobacteria isolated from the rhizosphere of medicinal and aromatic plants for multiple plant growth promoting activities. J Microbiol Biotech Res 3:84–91

Mayak S, Tirosh T, Glick BR (2004) Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers. Plant Sci 166:525–530

McGrath JW, Wisdom GB, McMullan G, Lrakin MJ, Quinn JP (1995) The purification and properties of phosphonoacetate hydrolase, a novel carbon-phosphorus bond-cleaving enzyme from *Pseudomonas fluorescens* 23F. Eur J Biochem 234:225–30

Mehta P, Chauhan A, Mahajan R, Mahajan PK, Shirkot CK (2010) Strain of *Bacillus circulans* isolated from apple rhizosphere showing plant growth promoting potential. Curr Sci 98: 538–542

Mehta P, Walia A, Chauhan A, Shirkot CK (2013) Plant growth promoting traits of phosphate-solubilizing rhizobacteria isolated from apple trees in trans Himalayan region of Himachal Pradesh. Arch Microbiol 195:357–369

Mishra RK, Prakash O, Alam M, Dikshit A (2010) Influence of plant growth promoting rhizobacteria (PGPR) on the productivity of *Pelargonium Graveolens* l. herit. Recent Res Sci Technol 2:53–57

Mundra S, Arora R, Stobdan T (2011) Solubilization of insoluble inorganic phosphates by a novel temperature-, pH-, and salt-tolerant yeast, *Rhodotorula* sp. PS4, isolated from seabuckthorn rhizosphere, growing in cold desert of Ladakh, India. World J Microbiol Biotechnol 27: 2387–2396

Nakouti I, Hobbs G (2012) Characterization of five siderophore producing actinomycetes from soil samples and the use of antibiotic resistance to differentiate the isolates. Int J Agr Sci 4:202–206

Nehl DB, Allen SJ, Brown JF (1997) Deleterious rhizosphere bacteria: an integrating perspective. Appl Soil Ecol 5:1–20

Niranjan RS, Shetty HS, Reddy MS (2005) Plant growth-promoting rhizobacteria: potential green alternative for plant productivity. In: Siddiqui ZA (ed) PGPR: biocontrol and biofertilization. Springer, Dordrecht, pp 197–216

Noori MSS, Saud HM (2012) Potential plant growth-promoting activity of *pseudomonas* sp. isolated from paddy soil in Malaysia as biocontrol agent. J Plant Pathol Microbiol 3: 2157–7471

Pedraza Reyes M, Lopez Romero E (1991) Detection of nine chitinase species in germinating cells of *Mucor rouxii*. Curr Microbiol 22:43–46

Pierson LS, Thomashow LS (1992) Cloning and heterologous expression of phenazine biosynthetic locus from Pseudomonas aureofaciens. Mol Plant Microbe Interact 5:330–339

Radha TK, Rao DLN (2014) Plant growth promoting bacteria from cow dung based biodynamic preparations. Indian J Microbiol. doi:10.1007/s12088-014-0468-6

Radjacommare R, Kandan A, Nanda KR, Samiyappan R (2004) Association of the hydrolytic enzyme chitinase against *Rhizoctonia solani* in rhizobacteria treated rice plants. J Phytopathol 152:365–370

Rajasekar S, Elango R (2011) Effect of microbial consortium on plant growth and improvement of alkaloid content in *Withania somnifera* (Ashwagandha). Curr Bot 2:27–30

Rao DLN (2008) Biofertilizer research progress (2004-07). All India Network Project on Biofertilizers, IISS, Bhopal, p 105

Rao DLN (2013) Soil biological health and its management. In: Tandon HLS (ed) Soil health management: productivity-sustainability-resource management. FDCO, New Delhi, pp 55–83

Rao DLN, Sharma PC (1995) Alleviation of salinity stress in chickpea by *Rhizobium* inoculation and/or nitrate supply. Biol Plantarum 37:405–410

Richardson AE (1994) Soil microorganisms and phosphorous availability. In: Pankhurst CE, Doube BM, Gupta VVSR (eds) Soil biota: management in sustainable farming systems. CSIRO, Victoria, Australia

Richardson AE (2001) Prospects for using soil microorganisms to improve the acquisition of phosphorous by plants. Aust J Plant Physiol 28:897–906

Robert WK, Selitrennikoff CP (1988) Plant and bacterial chitinase differ in antifungal activity. J Gen Microbiol 134:169–176

Saleem M, Arshad M, Hussain S, Bhatti AS (2007) Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. J Ind Microbiol Biotechnol 34:635–648

Sánchez-Castro I, Ferrol N, Barea JM (2012) Analyzing the community composition of arbuscular mycorrhizal fungi colonizing the roots of representative shrubland species in a Mediterranean ecosystems. J Arid Environ 80:1–9

Schippers B, Bakker A, Bakker P, van Peer R (1990) Beneficial and deleterious effects of HCN-producing Pseudomonads on rhizosphere interactions. Plant Soil 129:75–83

Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56

Sekar S, Kandavel D (2010) Interaction of plant growth promoting rhizobacteria (pgpr)and endophytes with medicinal plants – new avenues for phytochemicals. J Phytol 2:91–100

Selvaraj T, Rajeshkumar S, Nisha MC, Wondimu L, Tesso M (2008) Effect of *Glomus mosseae* and plant growth promoting rhizomicroorganisms (PGPR's) on growth, nutrients and content of secondary metabolites in *Begonia malabarica* Lam. Mj Int J Sci Tech 2:516–525

Shanmugam V, Kanoujia N, Singh M, Singh S, Prasad R (2011) Biocontrol of vascular wilt and corm rot of gladiolus caused by *Fusarium oxysporum* f. sp. gladioli using plant growth promoting rhizobacterial mixture. Crop Prot 30:807–813

Shaw FJ, Lin PF, Chen CS, Chen CH (1995) Purification and characterization of an extracellular α-amylase from thermos species. Bot Bull Acad Sin 36:195–200

Shehata HS, Hamed ER, Eleiwa ME (2012) Potential for improving healthy and productivity of Soybean by plant growth promoting rhizobacteria. Aust J Basic Appl Sci 6:98–107

Shirkot CK, Vohra I (2007) Characterization of novel carbendazim tolerant Bacillus subtilis with multiple plant growth promoting activities. In: Proceedings of XVI International plant protection congress, vol. 1. BCPC, SECC, Glasgow, Scotland, UK, pp 272–277

Spaepen S, Vanderleyden J, Remans R (2007a) Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiol Rev 31:425–448

Spaepen S, Versees W, Gocke D, Pohl M, Steyaert J, Vanderleyden J (2007b) Characterization of phenylpyruvate decarboxylase, involved in auxin production of *Azospirillum brasilense*. J Bacteriol 189:7626–7633

Subba Rao NS (1999) Soil microbiology (fourth edition of soil microorganisms and plant growth). Science Publishers, Inc., New York, NY

Sudhakar PG, Chattopadhyay N, Gangwar SK, Ghosh JK (2000) Effect of foliar application of *Azotobacter*, *Azospirillum* and *Beijerinckia* on leaf yield and quality of mulberry (*Morus alba*). J Agric Sci 134:227–234

Suresh A, Pallavi P, Srinivas P, Praveen Kumar V, Jeevan Chandra S, Ram Reedy S (2010) Plant growth promoting activities of fluorescent Pseudomonads associated with some crop plants. Afr J Microbiol Res 4:1491–1494

Tamilarasi S, Nanthakumar K, Karthikeyan K, Lakshmanaperumalsamy P (2008) Diversity of root associated microorganisms of selected medicinal plants and influence of rhizomicroorganisms on the antimicrobial property of *Coriandrum sativum*. J Environ Biol 29:127–134

Tank N, Saraf M (2010) Salinity-resistant plant growth promoting rhizobacteria ameliorates sodium chloride stress on tomato plants. J Plant Int 5:51–58

Thosar NJ, Ingle VG, Jadhar JC (2005) Effect of FYM and Biofertilizers on dry root and seed yield of Ashwagandha (*Withania somnifera*). Crop Prod 1:27–28

Tilak KVBR, Ranganayahi N, Pal KN, De R, Saxena AK, Nautiyal CS, Mital S, Tripathi AK, Joshi BN (2005) Diversity of plant growth and soil health supporting bacteria. Curr Sci 89:136–150

Turrini A, Sbrana C, Strani P, Pezzarossa B, Risaliti R, Giovannetti M (2010) Arbuscular mycorrhizal fungi of a Mediterranean island (Pianosa), within a UNESCO Biosphere Reserve. Biol Fertil Soils 46:511–520

Ulhoa CJ, Peberdy JF (1991) Purification and characterization of an extracellular chitobiase from *Trichoderma harzianum*. Curr Microbiol 23:285–289

Van Loon LC (2007) Plant responses to plant growth-promoting rhizobacteria. Eur J Plant Pathol 119:243–254

Vessey JK (2003) Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255:571–586

Vincent JM (1947) Distortion of fungal hyphae in the presence of certain inhibitors. Nature 159: 850

Wahyudi AT, Astuti RP, Widyawati A, Meryandini A, Nawangsih A (2011) Characterization of *Bacillus* sp. strains isolated from rhizosphere of soybean plants for their use as potential plant growth for promoting rhizobacteria. J Microbiol Antimicrob 3:34–40

Walia A, Mehta P, Chauhan A, Shirkot CK (2013) Effect of *Bacillus subtilis* strain CKT1 as inoculum on growth of tomato seedlings under net house conditions. Proc Natl Acad Sci Ind B 84:145–155

Walia A, Mehta P, Chauhan A, Shirkot CK (2014) Antagonistic Activity of plant growth promoting rhizobacteria isolated from tomato rhizosphere against soil borne fungal plant pathogens. Int J Agric Environ Biotechnol 6:571–580

Wang ET, Martinez-Romero E (2000) *Sesbania herbacea-Rhizobium huautlense* nodulation in flooded soils and comparative characterization of *S. Herbacea*-Nodulating Rhizobia in different environments. Microb Ecol 41:25–32

Western Himalaya Ecoregional BSAP (2002) Full citation. In: Singh SP (ed) 2002. Himalaya ecoregional biodiversity: strategy and action plan. Prepared under the NBSAP-India

Young JPW (1992) Phylogenetic classification of nitrogen-fixing organisms. In: Stacey G, Burris RH, Evans HJ (eds) Biological nitrogen fixation. Chapman and Hall, New York, pp 43–86

Zhang N, Wu K, He X, Li S, Zhang ZH, Shen B, Yang X, Zhang R, Huang Q, Shen Q (2011) A new bioorganic fertilizer can effectively control banana wilt by strong colonization with *Bacillus subtilis* N11. Plant Soil 344:87–97