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Plant-Growth-Promoting Rhizobacteria (PGPR) and Medicinal Plants

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Foreword

The editors of the volume "PGPR and Medicinal Plants" asked me to write the Foreword to this book. I have gone through the volume contents and some chapters, which prompted me to write the Foreword. Microorganisms are abundantly distributed in the soil, ranging from bacteria, actinomycetes, fungi, algae, cyanobacteria, and protozoa. These microbes contribute many beneficial elements, like carbon, sulfur, phosphorus, and nitrogen, to the soil by taking part in the nutrient cycle. The zone of contact between the root and soil is the rhizosphere. This region has intense activity and concentration of microbes and is considered vital for plant vigor and full development to maturity. Plant growth and development promoting rhizobacteria (PGPR) are present in the vicinity of the root system and at times adhering to the root. Such bacteria have been applied to a wide range of agriculturally important crops for the purpose of plant growth promotion, including emergence seed germination and value addition.

The influence of root exudates on the proliferation of soil microorganisms around and inside roots as well as interactions between soil microorganisms, rhizosphere colonies, and plant hosts have been widely studied. Studies based on molecular techniques have estimated about 4,000 microbial species per gram of soil samples. Powerful methods of estimation provide only the crudest measure of its magnitude. Nonetheless, many such estimates exist, suggesting that a single gram of soil may contain over 10 billion microbial cells and more than 1,800 bacterial species (Zhang et al. 2008).

PGPRs are well established to colonize plant roots and stimulate plant growth. They serve the purpose of being used as biofertilizers, plant growth regulators, and biotic elicitors and promote plant growth by several mechanisms such as phosphorus solubilization, production of volatile organic compounds, induction of systemic disease resistance, nitrogen fixation, maintenance of soil fertility and nutrient uptake, and resistance of water stress. How to classify the newly found diverse microorganisms remains an open question. As molecular methods, such as whole genome sequencing, are more widely applied to characterize bacterial diversity, our ability to make taxonomic sense of what we learn is severely challenged.

From a structural, functional, and taxonomical perspective, soil bacteria are an impressively diverse group. It is well known that they vary from free-living bacteria to single fungi capable of extending their growth over a large distance of multiple square kilometers. Still, we know little about soil bacteria because of the difficulties associated with their cultivation (Witzany 2011). Only a very limited number of species have been classified because less than 1 % grow easily on nutrient agar plates. Consequently, scientists depend on indirect analytical methodologies, mainly biochemical markers, as well as on measures of the metabolic activity in either entire soil microbial communities or selected segments of such communities. Research has underscored the essential functions that microorganisms play in soil quality. This is particularly evident in the important areas of the cycling of essential nutrients, the decomposition of organic materials, the regulation of essential nutrients, the decomposition of organic materials, and the regulation of the productive capacity of plant life, in the dynamics of soil microbial community considered holistically.

Restoration-related research into the roles of microbes has branched off in two principal directions: investigation that describes conditions and target locations in the ecosystem and research that focuses on system manipulation. This second direction stresses the creative manipulation of system components to facilitate more rapid arrival at desired systematic states through overcoming challenges posed by the paucity of mutualists and other positive components or by the presence of invasive plants and other negative influences.

Plant–PGPR associations are mediated through an exchange of chemical metabolites. Root exudates provide energy-rich organic acids, sugars, and amino acids that are metabolized within a short time by soil microorganisms, while specialized microorganisms generate an array of biologically active compounds that elicit plant growth promotion.

Fuqua et al. (1996, 2002) defined the term “quorum sensing” (QS) as the bacterial regulatory process that couples gene expression to cell density. This process is mediated by low-molecular-weight signal molecules that are synthesized by bacterial population and accumulate in the environment. The presence of molecules is sensed by bacteria and induces either the expression or repression of QS-regulated gene(s). Earlier it was thought that bacteria are unable to communicate. Investigations on QS have drastically changed this view that biologists had on bacteria. Indeed, bacteria not only communicate but they do so in multiple languages using QS signals.

Considering that several plant pathogenic bacteria also rely upon QS molecules to regulate virulence or virulence-related functions, the same evolutionary reading provides potential explanation for the plant capacity to detect the presence of the bacterial signal molecules. The general occurrence of functions capable of inducing QS signal degradation in fungi and bacteria, including noncultivable ones, strongly suggests that these functions might play a significant biological role. The QS strategies have been developed and present a multifaceted value. They may be developed to prevent or limit biofilm functions on several structures, or the impact of bacterial diseases in plants.

The moot questions remain: how do microbes perceive environmental and metabolic signals and how do they integrate this information to modulate gene expression? Genome-wide comparisons indicate that the ability to accurately sense the environment and to change gene expression accordingly is critical to the bacteria that spend at least a part of their life cycle in the complex and uncertain environments of soil. Quorum sensing (QS) is one of the mechanisms by which microbes change global patterns of gene expression in response to increases in their population densities within a diffusion-limited environment.

The time is ripe to commercialize the products by establishing strong linkages between academic and industries. The volume "PGPR and Medicinal Plants" edited by eminent microbiologists Drs. Dilfuza Egamberdieva, Smriti Srivastava, and Ajit Varma presents innovative ideas and thoughts . I congratulate them. This volume should be useful for active researchers, teachers, and scientists. It is published as part of the Soil Biology Series by Springer, Heidelberg, Germany.

Secunderabad, India K.V.V. Sairam

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Preface

This book was conceptualized during finalizing the Soil Biology volume "Root engineering: Basic concepts and Applications" edited by Asuncion Morte and Ajit Varma (2014). Soon it was realized that the basic functions of roots are heavily regulated by the microorganisms around them and thus a new volume "PGPR and Medicinal Plants" was depicted. The prime aim and objective of this volume is to highlight various aspects of action, effect, and application of PGPRs in medicinal plants to lend a hand to scientists throughout the world working in this field.

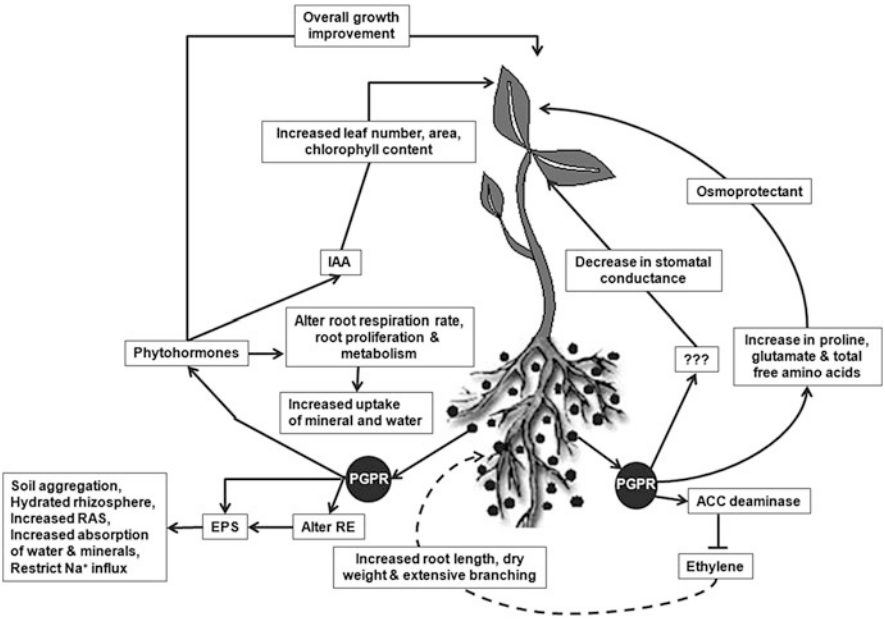
The rhizosphere concept was first introduced by Hiltner (1904) to describe the narrow zone of soil surrounding the roots where microbial populations are stimulated by root activities. The term "plant growth-promoting rhizobacteria (PGPR)" was first used by Joseph W. Kloepper in the late 1970s and has become commonly used in scientific literature. A large number of microorganisms such as bacteria, fungi, protozoa, and algae coexist in the rhizosphere; however, the most abundant organism is bacteria. Plants select those bacteria contributing most to their fitness by releasing organic compounds through exudates creating a very selective environment where diversity is low. Since bacteria are the most abundant microorganisms in the rhizosphere, it is highly probable that they influence the plants' physiology to a greater extent, especially considering their competitiveness in root colonization, hence, referred as plant growth-promoting rhizobacteria (PGPR). PGPRs are the group of microorganisms which colonize and have symbiotic relationship with the plant roots and promote plant growth via various plant growth-promoting substances and also act as biofertilizers.

The world today comes up with a new ailment after every short span of time and thus our requirement of medicines and drugs continues to amplify. Natural compounds are most preferred over synthetic drugs for curing diseases and these natural compounds are variedly obtained from medicinal plants. All we need is to enhance quality and quantity of plant secondary metabolites, which can be skillfully used for drug production. Numerous plant growth-promoting rhizobacteria are well known to exhibit beneficial effects on plenty of medicinal plants.

PGPRs have different relationships with different species of host plants, mainly rhizospheric and endophytic. Rhizospheric relationships consist of the PGPRs that colonize the surface of the root, or superficial intercellular spaces of the host plant, often forming root nodules. The dominant species found in the rhizosphere is a microbe from the genus *Azospirillum*. Endophytic relationships involve the PGPRs residing and growing within the host plant in the apoplastic space. It is well established that only 1–2 % of bacteria promote plant growth in the rhizosphere while acting as PGPR. PGPRs have been known to be present within many different bacterial taxa, among which most commercially industrial PGPRs are species of *Bacillus* which form endospores that confer population stability during formulation and storage of products. The main groups of PGPR can be found along with the phyla *Cyanobacteria, Actinobacteria, Bacteroidetes, Firmicutes,* and *Proteobacteria.* Fluorescent pseudomonads are identified to suppress soilborne fungal pathogens by producing antifungal metabolites and by sequestering iron in the rhizosphere through the release of iron-chelating siderophores, rendering it unavailable to other organisms.

PGPRs have several applications like increasing the availability of nutrients in the rhizosphere, increased root volume which is related to more nutrient absorption, to stimulate plant growth, e.g., through the production of plant hormones, to control or inhibit the activity of plant pathogens, to improve soil structure, and mineralization of organic pollutants, i.e., bioremediation of polluted soils, and are also used as biofertilizers and also known for phytohormone production, phosphate solubilization, siderophore production, biocontrol agents, and biological fungicides, etc. PGPRs are a healthier choice to improve the crop efficiency as well as quality. PGPRs improve the chemical and microbial property of soil and enhance the amount of plant enzymes for better defense mechanism in plant.

During the past couple of decades, the use of PGPRs for sustainable agriculture has increased tremendously in various parts of the world. Significant increases in growth and yield of agronomically important crops in response to inoculation with PGPR have been repeatedly reported. Recent reports have identified several volatile organic compounds produced by a variety of bacteria that promote plant growth and induce systemic resistance in *Arabidopsis thaliana*. Beneficial effects of PGPRs have also been attributed to shifts in the microbial ecology of the rhizosphere. Previous research has shown the practicality of introducing PGPR into commercial peat-based substrates for vegetable production in order to increase plant vigor, control root diseases, and increase yields. Results of tomato (*Lycopersicon esculentum*) and pepper (*Capsicum annuum*) trials in Florida included significant increases in tomato and pepper transplant growth during greenhouse production in response to various formulations of PGPR tested. As a result of increased growth, the time required to produce a standard sized transplant was reduced as were greenhouse applications of fertilizer. Also, transplant vigor and survival in the field were improved by PGPR treatments in both tomato and pepper. An overall view on the salient functions of PGPRs is depicted in the diagram below.



Morphological and physiological changes in plants by application of PGPR leading to abiotic stress tolerance [Adopted from Dutta and Khurana (2015)]

This volume is composed of 20 chapters, divided into 5 parts, encompassing various aspects of effect of PGPRs on medicinal plants. The first chapter provides an overview on PGPR and medicinal plants and their state of the art. The first section of this book focuses on plant improvement and is composed of 5 chapters. Chapter 2 provides a wide and comprehensive account on interaction of rhizosphere microbes with medicinal plants. Chapter 3 covers the handsome story toward enhancement of efficiency of medicinal and aromatic plants on interaction with PGPRs, and Chap. 4 deliberates the usefulness of vermicompost and associated microorganisms in enhancing soil health and agriculture productivity. Following this Chap. 5 describes the effect of Arbuscular mycorrhizae fungus and plant growth-promoting rhizobacteria of potential bioinoculants on growth, yield, and forskolin content of *Coleus forskohlii*, and Chap. 6 beautifully describes emergence and future facets of plant growth-promoting rhizobacteria upon interaction with medicinal plants. The second part with Chaps. 7, 8, and 9 relates to alleviation of plant stress tolerance with the help of PGPRs. The third section of this book focuses on biological control activity of PGPRs. Chapters 10, 11, and 12 highlight the ecological manifestation of rhizobacteria for curbing medicinal plant diseases, mechanism and control of plant associated diseases, and role of PGPRs in increasing soil fertility and plant health, respectively. The fourth part of the book brilliantly highlights some mechanisms of actions of PGPRs. It includes Chaps. 13–16 and highlights systemic induction of secondary metabolites, new frontiers for

phytochemicals, and rhizosphere microflora in advocacy of heavy metal tolerance in plants. The last part, composed of Chaps. 17–20, evidently describes diversity and characterization of PGPRs and also focuses locations like North West Himalayas and Argentina.

It has been a pleasure to edit this book, primarily due to the stimulating cooperation of the contributors. We wish to thank Hanna Hensler-Fritton and Jutta Lindenborn at Springer, Heidelberg, for their generous assistance and patience in finalizing the volume. Finally we give special thanks to our families—immediate and extended—for their kind support and their incentive to put everything together.

Ajit Varma and Smriti Shrivastava are particularly very thankful to Dr. Ashok K. Chauhan, Founder President of Ritanand Balved Education Foundation (an umbrella organization of Amity Institution), New Delhi, for his kind support and constant encouragement.

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New Delhi, India — Smriti Shrivastava
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Contents

Part V PGPR: Diversity and Characterization

Chapter 1
Plant Growth-Promoting Rhizobacteria (PGPR) and Medicinal Plants: The State of the Art

Smriti Shrivastava, Dilfuza Egamberdieva, and Ajit Varma

1.1 Introduction

Plant growth-promoting rhizobacteria (PGPR) are bacteria colonizing rhizospheres of plant that enhance plant growth through various mechanisms like nitrogen fixation, solubilization of phosphate, quorum sensing, etc. (Bhattacharya and Jha 2012). PGPR offer various ways to replace chemical fertilizers, pesticides, etc., and thus this quality has significantly led to their increased demand.

Before we start with the current applications and state of the art related to PGPR and medicinal plants, it will really be interesting to know the basic and history behind this wonderful science. Basis of application of plant growth-promoting bacteria may be said to be led days back when Theophrastus (372–287 B.C.) suggested mixing of different soil samples to remove defects of one and add life to soil (Tisdale and Nelson 1975). Certainly the technical approach behind the same only became clear after microscopy came into play. Establishment of legumes on cultivable land was recorded for the first time by Virgil (Chew 2002). Investigation of rhizosphere root colonization in grasses and confirmation of the fact that soil bacteria could convert atmospheric nitrogen into plant-usable forms were reported by Hellriegel and Wilfarth (1888). The term "rhizobacteria" was coined by Kloepper and Schroth (1978), based on their experiments with radishes, and they defined these bacteria as a community that competitively colonizes plant root and enhances their growth and also reduces plant diseases. Few properties strictly

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associated with PGPR are their properties of aggressive colonization and plant growth stimulation and their biocontrol ability (Weller et al. 2002; Vessey 2003). Rhizobacteria show all positive, negative, and neutral interaction with plants (Whipps 2001). PGPR are further classified as extracellular plant growth rhizobacteria or intracellular plant growth rhizobacteria depending upon their intimacy in interaction with plants (Martinez-Viveros et al. 2010). These are designated as ePGPR and iPGPR. The ePGPR is mainly existing in rhizosphere, rhizoplane, or between cells of root cortex include generally bacteria from genera like *Azotobacter*, *Chromobacterium*, *Agrobacterium*, *Caulobacter*, etc. (Gray and Smith 2005). Specialized nodular structures for root cells are home for iPGPR which includes endophytes (*Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, etc.) and *Frankia* species (Verma et al. 2010; Wang and Martinez-Romero 2000).

Studies have reported that application of PGPR increases nodulation and nitrogen fixation in many plants including soybean (*Glycine max* (L.) Merr.) (Zhang et al. 1996). PGPR have both direct and indirect mechanisms to promote growth and yield of crop plants. Rhizosphere colonization accounts for siderophore (Schippers et al. 1988), antibiotic (Weller 1988), and hydrogen cyanide (Stutz et al. 1986) production.

The objective of this chapter is to understand the mechanisms of plant growth promotion by rhizobacteria and to know about the state of the art of this wide area of study.

1.2 Plant–Microbe Interaction

The interaction of plants with microbes occurs at three different layers, namely, endosphere, phyllosphere, and rhizosphere. The region of contact between root and soil is rhizosphere. This region is a cloud of microbes which literally surrounds plant roots and is vital for the plant's survival and growth. The term "rhizosphere" was coined by Lorenz Hiltner in 1904. Clark proposed the term "rhizoplane" for the external root surface and closely adhering particles of soil and debris. The influence of root exudates on the proliferation of soil microorganisms around and inside roots (Hartmann et al. 2008) and interactions between soil microorganisms, rhizosphere colonists, and plant hosts (Dennis et al. 2010; Friesen et al. 2011; Berendsen et al. 2012) has been widely studied. In rhizosphere, the microbial population differs both quantitatively and qualitatively from that in the soil. As per the hypothesis, most of the plant roots are surrounded by mycorrhizae. Hence, it is appropriate to use the word mycorrhizosphere instead of rhizosphere (Shrivastava et al. 2014). Amino acids and sugars released as plant exudates are rich sources of energy and nutrition. Plant root interaction in the rhizosphere is a combinatorial effect of root–root interaction, root–microbe interaction, and root–insect interaction.

Studies based on molecular techniques have estimated about 4,000 microbial species per gram of soil sample (Montesinos 2003). One of the most important communities in rhizosphere microbiota is filamentous actinomycetes (Benizri et al. 2001). Rhizosphere microbial colonies have dynamic association with biogeochemical cycling of nutrients (C, P, N, and S) and production of phytohormones or antibiotics (Cardoso and Freitas 1992). PGPR are well known to colonize plant roots and stimulate plant growth (Andrews and Harris 2000). *Azospirillum* sp., *Bacillus subtilis* sp., and *Pseudomonas* sp. have been well studied as plant rhizosphere-colonizing microorganisms (Steenhoudt and Vanderleyden 2000; Trivedi et al. 2005). Soil microorganisms (free-living, associative, and symbiotic rhizobacteria) belonging to the genera like *Acinetobacter, Burkholderia, Enterobacter, Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Erwinia, Flavobacterium, Rhizobium, Serratia, Xanthomonas, Proteus,* and *Pseudomonas* are the integral parts of rhizosphere biota (Glick 1995; Kaymak 2011) and exhibit successful rhizosphere colonization. Rhizospheric colonization is a crucial step in the application of microorganisms for beneficial purposes such as biofertilization, phytostimulation, biocontrol, and phytoremediation, although the colonization of rhizosphere by PGPR is not a uniform process.

1.3 PGPR in Agriculture

1.3.1 PGPR as Biofertilizer

Biofertilizers are the substances prepared from living microorganisms which, when applied to the seeds or plant surfaces adjacent to soil, can colonize rhizosphere or the interior parts of the plants and thereby promote root growth. *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium*, and *Sinorhizobium* are reported as the potent PGPR strains for their ability to act as biofertilizers (Vessey 2003). In rhizospheric relationship, the PGPR can colonize the rhizosphere, the surface of the root, or even the superficial intercellular spaces of plant roots (McCully 2001). It is only due to the changes in different physicochemical properties of rhizospheric soil such as soil pH, water potential and partial pressure of O_2, and plant exudation as compared to the bulk soil that in turn can affect the ability of PGPR strains to colonize the rhizosphere (Griffiths et al. 1999). In endophytic relationship, PGPR reside within the apoplastic spaces inside the host plants. There is a direct evidence of existence of endophytes in the apoplastic intercellular spaces of parenchyma tissue (Dong et al. 1997) and xylem vessel (James et al. 2001). The best examples can be cited from legume–rhizobia symbioses in leguminous plants (Vessey 2003).

1.3.2 Plant Growth Regulator by PGPR

PGPR can alter root architecture and promote plant development with the production of different phytohormones like IAA, gibberellic acid, and cytokinins (Kloepper et al. 2007). Several PGPR as well as some pathogenic, symbiotic, and free-living rhizobacterial species are reported to produce IAA and gibberellic acid in the rhizospheric soil and thereby play a significant role in increasing the root surface area and number of root tips in many plants (Han et al. 2005). Recent investigations on auxin synthesizing rhizobacteria (Spaepen et al. 2007) as phytohormone producer demonstrated that the rhizobacteria can synthesize IAA from tryptophan by different pathways, although the general mechanism of auxin synthesis was basically concentrated on the tryptophan-independent pathways.

1.3.3 PGPR as Phosphorous Solubilizers

Phosphorus is one of the most essential nutrient requirements in plants. Ironically, soils may have large reservoir of total phosphorus (P) but the amounts available to plants are usually a tiny proportion of this total. This low availability of phosphorus to plants is because of the vast majority of soil P found in insoluble forms, while the plants can only absorb it in two soluble forms, the monobasic ($H_2PO_4^-$) and the diabasic (HPO_4^{2-}) ions (Glass 1989). Several phosphate-solubilizing microorganisms (PSMs) are now recorded to convert the insoluble form of phosphorus to soluble form through acidification, secretion of organic acids or protons (Richardson et al. 2009), and chelation and exchange reactions (Hameeda et al. 2008). Saprophytic bacteria and fungi are reported for the chelation-mediated mechanisms (Whitelaw 2000) to solubilize phosphate in soil. Release of plant root exudates such as organic ligands can also alter the concentration of P in soil solution (Hinsinger 2001).

1.3.4 PGPR as Producers of Volatile Organic Compounds

The discovery of rhizobacterial-produced volatile organic compounds (VOCs) constitutes an important mechanism for the elicitation of plant growth by rhizobacteria. Ryu et al. (2003) recorded some PGPR strains, namely, *Bacillus subtilis* GB03, *B. amyloliquefaciens* IN937a, and *Enterobacter cloacae* JM22 that released a blend of volatile components, particularly, 2,3-butanediol and acetoin, which promoted growth of *Arabidopsis thaliana*, suggesting that synthesis of bioactive VOCs is a strain-specific phenomenon. Acetoin-forming enzymes have been identified earlier (Forlani et al. 1999) in certain crops like tobacco, carrot, maize, and rice although their possible functions in plants were not properly

established in that period. It has now been established that the VOCs produced by the rhizobacterial strains can act as signaling molecule to mediate plant–microbe interactions as volatiles produced by PGPR colonizing roots are generated at sufficient concentrations to trigger the plant responses (Ryu et al. 2003). Farmer (2001) identified low molecular weight plant volatiles such as terpenes, jasmonates, and green leaf components as potent signal molecules for living organisms in different trophic levels. However, to acquire a clear appreciation on the mechanisms of VOCs in signaling plants to register plant defense, more investigations into the volatile components in plant–rhizobacteria system should follow.

1.3.5 PGPR as Biotic Elicitors

Elicitors are chemicals or biofactors of various sources that can trigger physiological and morphological responses and phytoalexin accumulation in plants. It may be abiotic elicitors such as metal ions or inorganic compounds and biotic elicitors, basically derived from fungi, bacteria, viruses, plant cell wall components, and chemicals that are released due to antagonistic reaction of plants against phytopathogens or herbivore attack. It has now been observed that the treatment of plants with biotic elicitors can cause an array of defense reactions including the accumulation of a range of plant defensive bioactive molecules such as phytoalexins in the intact plants. Thus, elicitation is being used to induce the expression of genes responsible for the synthesis of antimicrobial metabolites. Rhizosphere microbes are best known to act as biotic elicitors, which can induce the synthesis of secondary products in plants (Sekar and Kandavel 2010). Signal perception is the first committed step toward the biotic elicitor signal transduction pathway in plants. Jasmonic acid and its methyl ester are the signal transducers in a wide range of plant cell cultures that could accumulate rapidly when the suspension cultures of *Rauvolfia canescens* L. and *Eschscholzia californica* Cham. are treated with a yeast elicitor (Roberts and Shuler 1997). Ajmalicine, serpentine, picrocrocin, crocetin, hyoscyamine and scopolamine, safranal compounds, and tanshinone are recorded as the important metabolites produced by PGPR species in eliciting the physiological and morphological responses in crop plants.

1.3.6 Induction of Systemic Disease Resistance by PGPR

Application of mixtures of different PGPR strains to the seeds or seedlings of certain plants has resulted in increased efficiency of induced systemic resistance (ISR) against several pathogens (Ramamoorthy et al. 2001). Various nonpathogenic PGPR strains have the ability to induce systemic disease resistance in plants against broad-spectrum phytopathogens (Kloepper et al. 2004; Elbadry et al. 2006). Induction of systemic disease resistance in faba bean (*Vicia faba* L.) against bean yellow

mosaic virus (BYMV) via seed bacterization with *Pseudomonas fluorescens* and *Rhizobium leguminosarum* has been investigated by Elbadry et al. (2006). They isolated PGPR strains from the roots of faba bean and examined singly or in combination for the induction of resistance in faba bean against BYMV. The results established a pronounced and significant reduction in percent disease incidence (PDI) as well as in virus concentration (ELISA) in plants treated with *Pseudomonas fluorescens* and *Rhizobium leguminosarum* as compared to the non-bacterized plants. Similarly, induction of systemic resistance by *Pseudomonas putida* strain 89B-27 and *Serratia marcescens* strain 90–166 against *Fusarium* wilt of cucumber incited by *Fusarium oxysporum* f. sp. *cucumerinum* has been investigated by Liu et al. (1995). Alstroem (1991) observed induced systemic protection of PGPR against the bacterial diseases. He reported that the bean seeds when treated with *Pseudomonas fluorescens* protected the plant against the halo blight disease caused by *Pseudomonas syringae* pv. *phaseolicola*. Kloepper et al. (1993) treated cucumber seeds with rhizobacterial strains like *Pseudomonas putida* 89 B-27 and *Serratia marcescens* 90–166 and recorded a significant decrease in incidence of bacterial wilt. Similar investigations on the treatment of cucumber seeds against angular leaf spot disease caused by *Pseudomonas syringae* pv. *lachrymans*, with a large number of PGPR strains such as *Pseudomonas putida* 89B-27, *Flavimonas oryzihabitans* INR-5, *Serratia marcescens* 90–166, and *Bacillus pumilus* INR-7, have been made by Wei et al. (1996). They observed more systemic protection in the plants (indicated by the reduction of total lesion diameter) whose seeds are inoculated with the strains of PGPR as compared to the uninoculated plants. Pieterse et al. (2001) studied rhizobacterial strain *Pseudomonas fluorescens* to enhance the defensive capacity in plants against broad-spectrum foliar pathogens (Fig. 1.1). Based on their experiments they concluded that *Pseudomonas fluorescens* strain WCS417r could elicit systemic disease resistance in plants through a variety of signal translocation pathways like SA-independent JA-ethylene-dependent signaling, ISR-related gene expression, NPR 1-dependent signaling, etc. Recently, interactions between *Bacillus* spp. and plants with special reference to induced systemic disease resistance have been elicited by Choudhary and Johri (2009). Several strains of *Bacillus* like *B. amyloliquefaciens, B. subtilis, B. pasteurii, B. cereus, B. pumilus, B. mycoides*, and *B. sphaericus* (Ryu et al. 2004) are presently recorded to elicit significant reduction in disease incidence on diversity of hosts. Elicitation of resistance by the strains has been demonstrated both in greenhouse and field trials on tomato, bell pepper, muskmelon, watermelon, sugar beet, tobacco, and cucumber. Through the activation of various defense-related enzymes like chitinases, β-1, 3-glucanase, peroxidise (PO), phenylalanine ammonia-lyase (PAL), and polyphenol oxidase (PPO), PGPR strains can induce this type of systemic resistance in plants (Bharathi 2004).

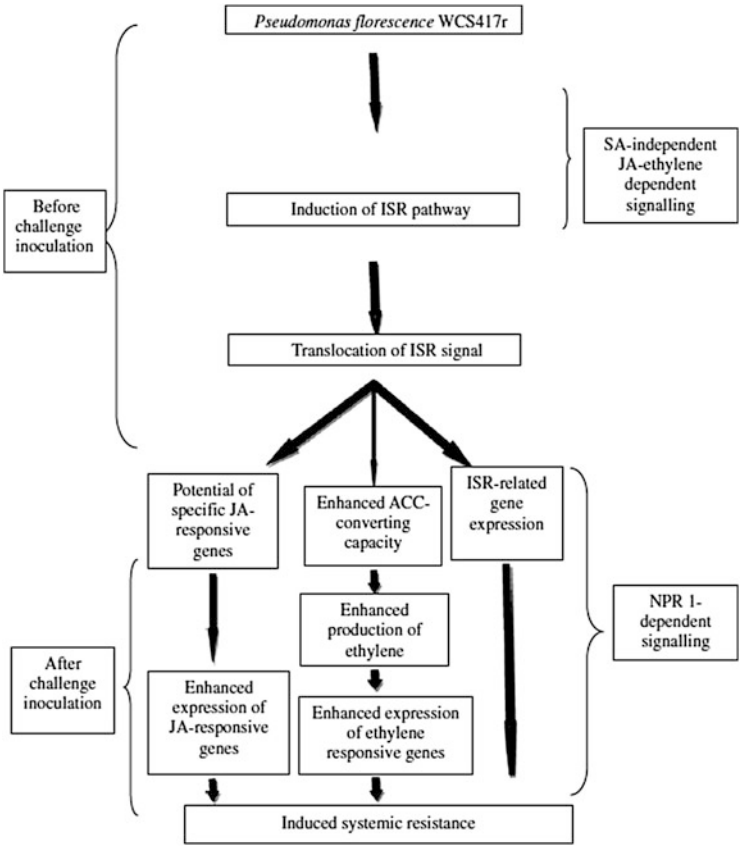

Fig. 1.1 Possible involvement of jasmonic acid and ethylene in *Pseudomonas fluorescens* WCS417r-mediated induced systemic resistance in *Arabidopsis* (Adapted from Pieterse et al. 2001)

1.3.7 Nitrogen Fixation

Nitrogen is a principal plant nutrient. Apart from being the most important, it is also a limiting factor in the agricultural ecosystem due to its loss by rainfall and mineral leaching. PGPR strains such as *Klebsiella pneumoniae, Pantoea agglomerans*, and *Rhizobium* sp. are reported to fix atmospheric N_2 in soil and avail it to plants (Antoun et al. 1998; Riggs et al. 2001). Fluorescent *Pseudomonades* and *Pseudomonas fluorescens* have been reported to promote nodulation in chickpea (Parmar and Dadarwal 1999) and tomatoes (Minorsky 2008). They promote enhanced plant height and increased fruiting and flowering capability. Ability of microorganisms to fix nitrogen symbiotically or nonsymbiotically in soil and enhance crop yield could replace the use of nitrogen fertilizers (Vessey 2003). Symbiotic N_2 fixation to legume crops with the inoculation of effective PGPR is well known (Dobereiner

1997; Barea et al. 2005; Esitken et al. 2006). Symbiotic N_2 fixation is mostly done by *Azotobacter* spp., *Bacillus* spp., *Beijerinckia* spp., etc. and is limited to leguminous plants, trees, and shrubs that form actinorhizal roots with *Frankia*, whereas nonsymbiotic nitrogen fixation is carried out by free-living diazotrophs like *Azospirillum* (Bashan and de-Bashan 2010), *Burkholderia* (Estrada de los Santos et al. 2001), *Azoarcus* (Reinhold-Hurek et al. 1993), *Gluconacetobacter* (Fuentes-Ramirez et al. 2001), and *Pseudomonas* (Mirza et al. 2006). Researchers have also studied the effect of combined inoculation of symbiotic and nonsymbiotic microorganisms on plant growth enhancement. Combined inoculations of *Bradyrhizobium* sp. with *Pseudomonas striata* have established enhanced nodule occupancy in soybean resulting in more biological N_2 fixation (Dubey 1996).

1.3.8 PGPR as Plant Growth Enhancement

Enormous PGPR are known to promote plant growth, crop yield, seed emergence, etc., thus promoting agriculture (Minorsky 2008). Plant properties like leaf area, chlorophyll content, total biomass, etc. are enhanced by inoculation of PGPR (Baset Mia et al. 2010). They also studied the effect of PGPR on external layers of root cortex of maize and wheat seedlings. Increasing demand for food and improving environmental quality have focused on the importance of PGPR in agriculture. Dobbelaere et al. (2001) assessed the inoculation effect of *Azospirillum* sp. on the development of agriculturally important plants and observed a noteworthy boost in the dry weight of both the root system and aerial parts of the PGPR-inoculated plants, resulting in better progress and flowering. Foliar applications of rhizobacterial microbes in mulberry and apricot and their better development in leaf area and chlorophyll production were investigated by Esitken et al. (2003). *Bacillus subtilis, B. licheniformis, Achromobacter xylosoxidans*, *B. pumilus, Brevibacterium halotolerans*, and *Pseudomonas putida* are identified as having critical roles in cell elongation, escalating ACC deaminase activity, and plant growth promotion (Sgroy et al. 2009). The effect of *Pseudomonas fluorescens* on tomato and cucumber roots was studied by Saravanakumar and Samiyappan (2007). Seeds of various crops and ornamental plants bacterized with a mixture of PGPR and rhizobia before planting resulted in enhanced growth and disease resistance (Zehnder et al. 2001). Growth responses in wheat after the inoculation with rhizobacteria basically depends on various factors like plant genotype, nature of PGPR inoculants, as well as environmental conditions as observed by Khalid et al. (2004). The root inoculation of apple tree with *Bacillus* M3 and *Microbacterium* FS01 (Karlidag et al. 2007) and the effect of arbuscular mycorrhizal (AM) fungi and PGPR in soils differing in nitrogen concentration (Ahanthem and Jha 2007) are few other important studies in this field. It was found that enhancing apple tree growth in the study might be due to enhanced production of plant growth regulators and mobilization of available nutrients by PGPR.

Ahanthem and Jha (2008) also studied the interactions between *Acaulospora* and *Azospirillum* and their synergistic effect on rice growth at different sources.

1.3.9 Maintenance of Soil Fertility and Nutrient Uptake by PGPR

Plant physiology and nutritional and physical properties of rhizospheric soil are all altered by PGPR. Rhizobacteria are reported to increase uptake of nutrient elements like Ca, K, Fe, Cu, Mn, and Zn through proton pump ATPase (Mantelin and Touraine 2004). *Bacillus* and *Microbacterium* inoculants improve uptake of mineral elements by crop plants (Karlidag et al. 2007). The importance of rhizobacterial activities on maintaining soil fertility is well studied by many scientists (Phillips 1980; Forde 2000; Glass et al. 2002). Rhizobacteria also help in solubilizing unavailable forms of nutrients and facilitating its transport in plants (Glick 1995).

1.3.10 Enhancement of Resistance to Water Stress

PGPR are beneficial to the wide variety of plants growing in water-stressed conditions (Aroca and Ruiz-Lozano 2009). Drought stress causes limitation to the plant growth and productivity of agricultural crops particularly in arid and semiarid areas. Figueiredo et al. (2008) suggested that inoculation of plants with PGPR can enhance the drought tolerance that might be due to the production of IAA, cytokinins, antioxidants, and ACC deaminase and inoculation of seeds of *Phragmites australis* with *Pseudomonas asplenii* improved germination and protects the plants from growth inhibition (Bashan et al. 2008).

1.4 Commercialization of PGPR

Commercialization of PGPR is important for its beneficial usage and this very aspect requires a proper tuning between scientific organization and industries. Different stages in the process of commercialization include isolation of antagonist strains, screening, pot tests and field efficacy, mass production and formulation development, fermentation methods, formulation viability, toxicology, industrial linkages, and quality control (Nandakumar et al. 2001). Isolation of effective strain is the prime criteria for better agricultural development (Nakkeeran et al. 2005), and then selection of the best antagonistic strain can be carried out by screening for antimicrobial action against soilborne pathogens. The next stage of study is when

the plant, pathogen, and antagonists are tested for their efficacy in field trials along with recommended fungicides (Pengnoo et al. 2000). Mass production is achieved through liquid (Manjula and Podile 2001), semisolid, and solid fermentation requirement for entrepreneurship requires a patent application of the identified strain.

The next crucial step to retain the confidence of farmers on efficacy of antagonistic strain is quality control. The first commercial product of *Bacillus subtilis* was developed in 1985 in the USA. 60–75% of cotton, peanut, soybean, corn, vegetables, and small grain crops raised in the USA are now treated with commercial product of *B. subtilis*, which become effective against soilborne pathogens such as *Fusarium* and *Rhizoctonia* (Nakkeeran et al. 2005). The potential of *Bacillus* spp. has also been widely studied by Backman et al. (1997). Besides *Bacillus* spp., certain other PGPR strains belonging to the genera such as *Agrobacterium, Azospirillum*, *Burkholderia*, *Pseudomonas*, and *Streptomyces* are also used for the production of several commercial products, which are generally being applied against several target pathogens like *Botrytis cinerea, Penicillium* spp., *Pythium* sp., *Geotrichum candidum*, *Mucor piriformis*, *Erwinia amylovora*, russet-inducing bacteria, *Fusarium* sp., *Rhizoctonia* sp., *Fusarium* sp., *Phytophthora* sp., and *P. tolaasii* (Nakkeeran et al. 2005).

Chet and Chernin (2002) studied a wide variety of PGPR and have also been successful in developing formulations for commercialization of products.

1.5 Future Prospects and Challenges

PGPR inoculants can fulfill diverse beneficial interactions in plants. Applications of rhizosphere soil with desirable bacterial populations have established considerable promises in both the laboratory and greenhouse experiments. Combined applications of transgenic plants with PGPR have proved another promising future (Ali and Hj 2010) in advancing rhizoremediation technologies. Rationalizing the understanding of PGPR may promote plant growth, leading to its use as biofertilizer at a wide level. Denton (2007) worked on the use of PGPR to remediate complex contaminated soil which could result in increased crop yield. The rhizobacterial community can be specifically engineered to target various pollutants at co-contaminated sites to provide customized rhizoremediation system (Wu et al. 2006). Production of transgenic plants and then inoculating it with PGPR has also increased efficiency (Zhuang et al. 2007; Farwell et al. 2007). Modern technology based on the transformations of 1-aminocyclopropane-1-carboxylic acid deaminase gene, which directly stimulates plant growth by cleaving the immediate precursor of plant ethylene into *Pseudomonas fluorescens* CHAO, not only increased the plant growth but also accelerated biocontrol properties of PGPR species (Holguin and Glick 2001). Genomic tinkering of naturally occurring PGPR strains with effective genes (Nakkeeran et al. 2005) could lead to accentuated expression of genomic products, thereby alleviating the attack of both pests

and diseases on field crops that would further facilitate for better introduction of a single bacterium with multiple modes of action to benefit the growers.

1.6 Conclusions

PGPR enhance plant growth by direct and indirect means, but the specific mechanisms involved have not all been well characterized. The present review indicates the advances and formulations of PGPR in biological promotion of different characteristics of plant growth. Most PGPR isolates significantly increase plant height, root length, and dry matter production in various agricultural crops like potato, tomato, maize, wheat, etc. One of the promising approaches of replacing the use of chemical fertilizers is developing stable formulation of antagonistic PGPR in sustainable agricultural systems. Another approach is through activation of octadecanoid, shikimate, and terpenoid pathways which in turn assists the plant growth promotion. Plenty of research in this field is going on and various are fruitful too. It can be concluded that vigilantly controlled field trials of crop plants inoculated along with rhizobacteria are necessary for utmost commercial exploitation of PGPR strains.

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Part I
Plant Improvement

Chapter 2
Rhizosphere Microbes Interactions in Medicinal Plants

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2.1 Introduction

The diversity and functions of microbes in the rhizosphere, a narrow region around the root, are related to the root exudates (proteins and sugars), biogeochemical reactions and respiration (Narula et al. 2009). The rhizosphere contains abundant bacteria, fungi, protozoa and nematodes. Some nematodes are feeding on bacteria and fungi. The root exudates in the rhizosphere may control disease suppression and help in nutrient cycling. The different compounds secreted by plant roots into the rhizosphere perform multiple functions. For example, strigolactones stimulate the colonisation of the mycorrhiza fungi and germination of the parasitic plant such as *Striga*. The flavonoids secreted by the roots of leguminous plants increase the growth of symbiotic and non-symbiotic nitrogen-fixing bacteria, root nodules and nitrogen uptake by plants. Allelochemicals can inhibit the growth of other micro-organisms in the rhizosphere, and therefore interactions are complex.

In the mycorrhizosphere around the mycorrhiza-colonised roots, most of the actively absorbing rootlets are extended to the surrounding soil for nutrient uptake (Johansson et al. 2004). Since mycorrhizal fungi stimulated by some root exudates may modify root morphology and metabolic functions, the volume of the mycorrhizosphere soil is larger than the rhizosphere soil (Linderman 1988), and root exudates in the mycorrhizosphere is quantitatively and qualitatively different from that in the rhizosphere (Leyval and Berthelin 1993; Rygiewicz and Andersen 1994) producing the 'mycorrhizosphere effect' (Linderman 1988). In addition, mycorrhizal fungi can produce antibiotics that may reduce bacterial activity in sandy soil (Olsson et al. 1996).

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A wide range of organic compounds secreted by plant roots in the rhizosphere provide a food source for microorganisms increasing microbial density and activity in the rhizosphere than in the bulk soil (the soil away from the rhizosphere is known as bulk soil). Most of the microorganisms in the rhizosphere are related to plant species that can efficiently solubilise poorly soluble inorganic P and mineralise organic P sources (unaccessible to plants) and markedly increase plant growth in soils with low P availability. However, the contribution of the plant-specific microorganisms to plant P uptake in soils with low P availability is poorly understood. The arbuscular mycorrhizal (AM) fungi form symbioses with more than 80 % of all land plant species and can help for plant P acquisition via the fungal hyphae (Jasper et al. 1989; Smith and Read 2008).

Medicinal plants are a rich source of bioactive compounds (Toussaint et al. 2007), and these are thought to be safe to human beings and the environment compared to the synthetic medicines for the treatment of cancer and many other diseases (Nema et al. 2013). The use of medicines of plant origin has a long tradition in Europe and Asia such as traditional Chinese medicine, Indian Ayurvedic medicine and herbal medicine. More than 600 medicinal plants, comprising more than 30 % of known plant species, are recorded in the Chinese *Materia Medica*, citing the first use of medicinal herbals in China as early as 1100 BC (Cragg et al. 1997; Joy et al. 1998). With the increased population pressure, costs and side effects and the development of resistance to allopathic drugs for infectious diseases, the uses of medicines of plant sources for a wide variety of human ailments are increasing. So, large-scale productions of medicinal plants using modern cultivation technologies are being practised across Asian countries, to meet the demand of medicinal plants. The pests and diseases of plant are hampering the growth and quality of medicinal plants. In addition, excessive use of pesticides may degrade the quality of medicinal plant products. Therefore, the development of innovative technologies for cultivation of medicinal plants is required.

Many recent research works have indicated that mycorrhizal colonisation is common in most of the medicinal plants in Fiji Island and Hawaii, America (Taber and Trappe 1982), Pakistan (Waheed 1982; Gorsi 2002; Haq and Hussain 1995; Iqbal and Nasim 1986), China (Wei and Wang 1989), Japan (Udea et al. 1992) and many other areas that play many significant roles in increasing soil structure, nutrient uptake by plants, plant growth, productivity and biodiversity in the diverse agroecosystems (Smith and Read 2008). The AM fungi are the most widely distributed symbioses out of all types of mycorrhizas such as arbuscular mycorrhiza, ectomycorrhiza, ectoendomycorrhiza, ericoid, orchid, arbutoid and monotropoid mycorrhiza (Smith and Read 2008). Many researches have focused on the AM fungal community and diversity in the rhizosphere of medicinal plants (e.g. Kumar et al. 2010; Wubet et al. 2003; Zeng et al. 2013) and improved plant growth (Karthikeyan et al. 2009; Chandra et al. 2010) and medicinal values by AM fungal colonisation (e.g. Copetta et al. 2006, Yuan et al. 2007; Morone-Fortunato and Avato 2008; Toussaint et al. 2008; Sasanelli et al. 2009; Koeberl et al. 2013). However, the microbes in the rhizosphere of medicinal plants are largely unexplored. Therefore, further research is recommended to provide the novel

insights on (1) the microbiome of medicinal plants, (2) plant- and microbe-derived ingredients of medicinal plants and (3) plant growth promotion and plant protection for pests and diseases.

2.2 Microbial Diversity in the Rhizosphere of Medicinal Plants

2.2.1 Bacterial Diversity

The study of rhizosphere bacteria from the important medicinal plants is very crucial, as they are well known to have impact on plant growth and also produce industrially important metabolites and improve quality of medicinal product (Bafana and Lohiya 2013). A significant number of bacteria produce the phytotherapeutic compounds (Koeberl et al. 2013) and increase the growth of the medicinal plants when they are associated with rhizosphere of plants that are listed in Table 2.1. This information will be useful in developing a biofertiliser consortium for commercially grown medicinal plants.

Gram-negative, nonmotile, catalase-positive and oxidase-negative short rods and exopolysaccharide-producing bacterium, designated as strain DRP 35 (T) (Whang et al. 2014) and DR-9(T) (Lee et al. 2013), were isolated from the rhizosphere soil of a medicinal herb, *Angelica sinensis*. The phylogenetic analyses based on 16S rRNA gene sequences indicated that strain DRP 35(T) belongs to the genus *Terriglobus* in the phylum *Acidobacteria* with a similarity to *Terriglobus saanensis* SP1PR4(T) and *Terriglobus roseus* KBS63(T), while strain DR-9(T) formed a lineage within the genus *Mucilaginibacter* and was closely related to *Mucilaginibacter polysaccharеus* DRP28(T), *Mucilaginibacter myungsuensis* HMD1056(T), *Mucilaginibacter ximonensis* XM-003(T) and *Mucilaginibacter boryungensis* BDR-9(T).

The soil microbes in the rhizosphere of three medicinal plants (*Matricaria chamomilla* L., *Calendula officinalis* L. and *Solanum distichum* Schumach. & Thonn.) grown on the desert ecosystem had a high abundance of Gram-positive bacteria of prime importance for pathogen suppression (Koeberl et al. 2013). For all three plants, a plant-specific selection of the microbes as well as highly specific diazotrophic communities was found. The results identified that the plant species were important drivers in structural and functional diversity. Furthermore, the native *Bacillus* strains promoted the plant growth and elevated the plants' flavonoid production. Among 28 endophytic bacterial isolates from different organs of *Plectranthus tenuiflorus* medicinal plant, 8 isolates were *Bacillus* sp., *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus licheniformis*, *Micrococcus luteus*, *Paenibacillus* sp., *Pseudomonas* sp. and *Acinetobacter calcoaceticus* (El-Deeb et al. 2013). Li et al. (2013) found the great differences in the endophytic bacterial diversity in the three medicinal plant species of *Codonopsis pilosula*, *Ephedra*

Table 2.1 Medicinal plants and rhizosphere-associated bacteria

Plant species	Microorganisms	References
Angelica sinensis	*Terriglobus saanensis* *Mucilaginibacter polysacchareus* *Mucilaginibacter myungsuensis*, *Mucilaginibacter ximonensis*	Whang et al. (2014) Lee et al. (2013)
Matricaria chamomilla Calendula officinal, *Solanum distichum*	*Bacillus* sp.	Koeberl et al. (2013)
Rumex patientia	*Proteobacterium* *Bacteroidetes* *Acidobacteria* *Gemmatimonadetes* *Verrucomicrobia* *Planctomycetes* *Actinobacteria* *Firmicutes* *Chloroflexi*	Qi et al. (2013)
Atractylodes lancea	Gram-negative bacteria	Dai et al. (2013)
Plectranthus tenuiflorus	*Bacillus* sp. *Bacillus megaterium* *Bacillus pumilus* *Bacillus licheniformis* *Micrococcus luteus* *Paenibacillus* sp. *Pseudomonas* sp. *Acinetobacter calcoaceticus*	El-Deeb et al. (2013)
Origanum vulgare	*Pseudomonas*, *Stenotrophomonas*	Bafana and Lohiya (2013)
Typhonium giganteum	*Kribbella flavida* *K. karoonensis* *K. alba*	Xu et al. (2012)
Ginseng plants	Actinomycetes	Zhang et al. (2013)
Hypericum silenoides	*Acinetobacter* *Enterobacter* *Pseudomonas* *Sphingobium* *Stenotrophomonas* *Agrobacterium* *Pantoea* *Serratia*	Lopez-Fuentes et al. (2012)
Ajuga bracteosa	*Pseudomonas*	Kumar et al. (2012)
Nerium indicum	*Pontibacter*	Raichand et al. (2011)
Fritillaria thunbergii	*Proteobacteria* *Acidobacteria* *Actinobacteria* *Bacteroidetes*	Shi et al. (2011)

(continued)

Table 2.1 (continued)

Plant species	Microorganisms	References
Astragalus membranaceus	*Geodermatophilus obscurus*	Zhang et al. (2011a)
Phytolacca acinosa	*Aspergillus fumigatus*	Guo et al. (2010)
Agathosma betulina	*Cryptococcus laurentii*	Cloete et al. (2010)
Ocimum sanctum, *Coleus forskohlii*, *Catharanthus roseus*, *Aloe vera*	*Azospirillum* *Azotobacter* *Pseudomonas*	Karthikeyan et al. (2008)
Annona squamosa *Eclipta alba* *Cassia auriculata*	*Bacillus* *Pseudomonas* *Enterobacter*, *Corynebacterium*, *Micrococcus* *Serratia*	Tamilarasi et al. (2008)

sinica and *Lamiophlomis rotata*. Zhao et al. (2013) explored the microbial diversity from the rhizosphere soils of some medicinal plants and found a total of 50 strains identified into 7 genera, *Myxococcus* (18), *Corallococcus* (11), *Cystobacter* (7), *Archangium* (8), *Stigmatella* (1), *Chondromyces* (4) and *Pyxidicoccus* (1) with the dominant genera of *Myxococcus* and *Corallococcus*.

The continuous cropping of *Rehmannia glutinosa*, an important medicinal plant, on the same land decreases its productivity (Qi et al. 2009). An alteration of soil microbial community following *R. glutinosa* cropping might be an important reason for the constraints associated with continuous cropping. There were several characteristic differences in the microbial community composition and activities in the rhizosphere following *Rehmannia glutinosa* monoculture (Qi et al. 2009; Wu et al. 2013). However, the interactions among plant, soil and microflora are crucial for the productivity and quality of *Rehmannia glutinosa* in consecutive monoculture system (Wu et al. 2011). The relative proportion of bacterial communities in rhizosphere soils of the wild medicinal plant *Rumex patientia* was similar to non-rhizosphere soils in five phylogenetic groups (*Acidobacteria, Actinobacteria, Chloroflexi, Planctomycetes* and *Proteobacteria*), but there were differences in five other phylogenetic groups (*Bacteroidetes, Firmicutes, Gemmatimonadetes, Verrucomicrobia* and unclassified bacteria) (Qi et al. 2012). Qi et al. (2013) identified a total of 83 unique phylotypes classified as *Proteobacterium* (43.37 %), *Bacteroidetes* (13.25 %), *Acidobacteria* (10.84 %), unclassified bacteria (9.64 %), *Gemmatimonadetes* (7.23 %), *Verrucomicrobia* (4.82 %), *Planctomycetes* (4.82 %), *Actinobacteria* (3.61 %), *Firmicutes* (1.20 %) and *Chloroflexi* (1.20 %) in the rhizosphere soil of *Rumex patientia*.

The peanut production in continuous monocrop farming system is affected by various environmental factors that deteriorate soil microbial communities, especially decrease in fungal diversity and increase in fungal pathogens. Whereas, the peanut production was increased by the improved soil microcosm environment and

the fungal diversity and decreased fungal pathogens such as *Fusarium* sp. and *Verticillium* sp. when peanut was intercropped with *Atractylodes lancea* and *Euphorbia pekinensis*, traditional Chinese medicinal plants (Dai et al. 2009, 2013). The increase in the Gram-negative bacterial population and the decrease of phenolic allelochemicals resulted in the promotion of peanut growth and increased peanut yield in the intercropping treatments.

The *Origanum vulgare* is a perennial medicinal aromatic plant rich in phenolic antioxidants. Bafana and Lohiya (2013) isolated both root endophytes and rhizospheric soil bacteria with a total of 120 morphologically different isolates grouped into 21 phylotypes. Majority of the isolates belonged to *Firmicutes* and gamma-*Proteobacteria*. *Pseudomonas* and *Stenotrophomonas* were the most dominant species and together constituted 27.5 % of the total isolates. Lopez-Fuentes et al. (2012) isolated and identified the 103 bacterial communities in the rhizosphere and roots of *Hypericum silenoides* Juss, mostly belonging to the genera *Acinetobacter, Agrobacterium, Enterobacter, Pseudomonas, Sphingobium, Stenotrophomonas, Pantoea and Serratia*. In order to determine their plant growth-promoting and biotechnological potential, Kumar et al. (2012) isolated a total of 123 morphologically different bacteria from the rhizospheric soil and roots of the medicinal plant *Ajuga bracteosa* that belonged to alpha- and gamma-*Proteobacteria*, with *Pseudomonas* constituting the most dominant species. The endophytic bacterial community consisted almost exclusively of *Firmicutes*.

Raichand et al. (2011) isolated a Gram-negative, pink pigmented bacterium strain from the rhizosphere of an Indian medicinal plant, *Nerium indicum* (*Chuvanna arali*), that matched with most of the phenotypic and chemotaxonomic properties of the genus *Pontibacter* and represents a novel species. The main bacterial population found in the rhizosphere of medicinal plant *Fritillaria thunbergii* was *Proteobacteria* (55 %), *Acidobacteria* (12 %), *Actinobacteria* (12 %) and *Bacteroidetes* (18 %) (Shi et al. 2011). The bacterial diversity of *Indigofera tinctoria* and *Pueraria mirifica* rhizospheres was significantly different from that of *Derris elliptica* Benth rhizosphere (Nimnoi et al. 2011). The microbial population is more in the rhizosphere soil compared to non-rhizosphere soil of the medicinal plants *Ocimum sanctum* L., *Coleus forskohlii* Briq., *Catharanthus roseus* (L.) G. Don and *Aloe vera*. The diazotrophic bacterial population studied includes *Azospirillum*, *Azotobacter* and *Pseudomonas* (Karthikeyan et al. 2008).

The actinobacterial biocontrol strains in medicinal plants are important as they can be a source of potent antibiotics. Zhao et al. (2012) analysed the actinobacterial diversity in the rhizosphere of seven traditional medicinal plant species and found 18 actinobacterial genera. In particular, Diels hosted a diverse selection of *Actinobacteria*. Xu et al. (2012) isolated an actinomycete, designated XMU 198 (T), from the rhizosphere soil of a pharmaceutical plant, *Typhonium giganteum* Engl., exhibiting highest sequence similarities with *Kribbella flavida, K. karoonensis* and *K. alba*. Zhang et al. (2011a) isolated a novel actinobacterial strain, CPCC 201356(T), from a rhizosphere soil sample of the medicinal plant *Astragalus membranaceus* that belonged to the family *Geodermatophilaceae*.

Table 2.2 Medicinal plants and rhizosphere-associated fungi

Plant species	Microorganisms	References
Atractylodes lancea Dioscorea zingiberensis, *Euphorbia pekinensis Ophiopogon platyphyllum*, *Pinellia ternata*	*Fusarium* sp. *Verticillium* sp.	Dai et al. (2009)
Andrographis paniculata	*Acaulospora scrobiculata*, *Glomus aggregatum*	Radhika and Rodrigues (2010)
Hemidesmus indicus	*Ambispora leptoticha* *G. maculosum* *G. geosporum* *G. multicaule* *G. fasciculatum*	Radhika and Rodrigues (2010)
Aloe vera	*G. maculosum* *G. multicaule* *G. geosporum*	Radhika and Rodrigues (2010)
Azadirachta indica	*A. scrobiculata* *G. fasciculatum* *Gi. albida* *S. calospora*	Radhika and Rodrigues (2010)
Naregamia alata	*A. scrobiculata* *Am. Leptoticha* *A. nicolsonii* *G. rubiforme* *G. maculosum* *G. fasciculatum* *S. verrucosa*	Radhika and Rodrigues (2010)
Physalis minima	*A. rehmi* *G. fasciculatum* *G. multicaule* *G. maculosum* *G. geosporum* *G. rubiforme*	Radhika and Rodrigues (2010)
Centella asiatica	*G. multicaule*, *G. clarum*, *G. fasciculatum*, *A. delicate*, *S. scutata*	Radhika and Rodrigues (2010)
Panax ginseng	*A. cavernata*, *A. spinosa*, *G. fasciculatum*, *G. geosporum*, *G. macrocarpum*, *G. microaggregatum*, *G. mosseae*	Cho et al. (2009)
Panax notoginseng	*G. versiforme*, *G. monosporum*, *G. mosseae*, *G. constrictum*, *G. claroideum*	Zhang et al. (2011b)
Arnica montana	*G. geosporum*, *G. constrictum*, *G. intraradices*, *G. mosseae*, *G. macrocarpum*, *G. fasciculatum*, *G. versiforme*	Jurkiewicz et al. (2010)
Echinacea purpurea	*G. intraradices*	Araim et al. (2009)

(continued)

Table 2.2 (continued)

Plant species	Microorganisms	References
Cercidiphyllum japonicum	*G. aggregatum*, *G. constrictum*, *G. dimorphicum*, *G. fasciculatum*, *G. flavisporum*, *G. intraradices*, *G. mosseae*, *S. aurigloba*, *Archaeospora leptoticha*	Wang et al. (2008)
Hippophae rhamnoides	*G. albidum*, *G. claroideum*, *G. constrictum*, *G. coronatum*, *G. intraradices*	Tang et al. (2004)
Ziziphus jujuba Mill. var. *inermis*	*G. coronatum*, *G. intraradices*, *G. monosporum*, *G. reticulatum*	Tang et al. (2004)
Lycium barbarum	*Gi. margarita*, *G. albidum*	Tang et al. (2004)
Taxus chinensis	*G. aggregatum*, *G. ambisporum*, *G. clarum*, *G. constrictum*, *G. fasciculatum*, *G. geosporum*, *G. magnicaule*, *G. reticulatum*, *G. verruculosum*, *G. viscosum*, *A. denticulate*	Wang et al. (2008)
Euptelea pleiosperma	*G. ambisporum*, *G. constrictum*, *G. fasciculatum*, *G. geosporum*, *G. hyderabadensis*, *G. intraradices*, *S. verrucosa*	Wang et al. (2008)
Cassia alata *C. occidentalis* *C. sophera*	*Glomus* spp.	Chatterjee et al. (2010)
Curcuma mangga	*Alternaria brassicicola*, *Colletotrichum gloeosporioides* *Fusarium oxysporum*, *Penicillium digitatum*, *Sclerotium rolfsii*	Khamna et al. (2009)
Centella asiatica and Ocimum sanctum	AM and endophytic fungi	Sagar and Kumari (2009)
Paeonia suffruticosa	*Glomus* *Acaulospora* *Scutellospora*	Shi et al. (2013)
Artemisia annua	*Glomus mosseae* *G. aggregatum* *G. fasciculatum* *G. intraradices*	Awasthi et al. (2011)
Magnolia cylindrica	*Acaulospora* *Glomus* *Gigaspora* *Scutellospora*	Yang et al. (2011)
Bacopa monnieri	*Glomus mosseae* *Glomus intraradices*	Khaliel et al. (2011)
Leptadenia reticulata *Mitragyna parvifolia* *Withania coagulans*	*G. constrictum* *G. fasciculatum* *G. geosporum* *G. intraradices* *G. mosseae* *G. rubiforme*	Panwar and Tarafdar (2006)

(continued)

Table 2.2 (continued)

Plant species	Microorganisms	References
Sorghum bicolor	*G. mosseae* *G. intraradices*	Sun and Tang (2013)
Curculigo orchioides	*G. geosporum* *G. microcarpum*	Sharma et al. (2008)
Ginseng plants	Soil fungi	Zhang et al. (2013)

Zhang et al. (2010) determined that allelochemicals released by the medicinal plant *Scutellaria baicalensis* Georgi negatively affected *S. baicalensis* directly by inducing autotoxicity and indirectly by increasing pathogen activity in the soil.

2.2.2 Fungal Diversity

AM fungal colonisations in the medicinal plants have been reported widely. However, the diversity of AM fungal species and the extent of colonisation in the rhizosphere of medicinal plants may vary depending on host plant species, growing season, soil properties, local climate and environmental factors. Various informations of medicinal plants and rhizosphere-associated fungi are stated in Table 2.2.

The Egyptian henbane (*Hyoscyamus muticus* L.), a medicinal plant of family Solanaceae native to the desert producing pharmaceutically important compounds (tropane alkaloids) as secondary metabolites, is colonised by a higher number of fungal species and endophytic fungi (El-Zayat et al. 2008). Rhizosphere soil of the medicinal plants (*Centella asiatica* and *Ocimum sanctum*) revealed the presence of 16–17 species of fungi (Sagar and Kumari 2009). The endophytic fungi were also isolated from the roots and leaves of *Centella asiatica* and *Ocimum sanctum*. There was a massive variation in the AM fungi spore population and root colonisation in the rhizosphere of ten medicinal plant species(*Aloe barbadensis*, *Centella asiatica, Emblica officinalis, Euphoria longan, Mimosa pudica, Rauvolfia tetraphylla, Rauwolfia serpentina, Sapindus trifoliatus, Smilax* sp. and *Trachyspermum copticum*) in spite of their growth in similar climatic conditions (Hussain and Srinivas 2013). Chatterjee et al. (2010) surveyed the mycorrhizal status in three different species of *Cassia* plants such as *C. alata, C. occidentalis* and *C. sophera*. *Cassia alata* possesses maximum root colonisation by the AM fungus that belongs mostly to the *Glomus* species followed by *C. occidentalis* and *C. sophera*. It seems that *C. alata* is the most potent species for having significant antimicrobial activity.

Mycorrhizal plants (colonised by *Glomus mosseae* or *Glomus intraradices*) of Sorghum (*Sorghum bicolor*) compared with non-mycorrhizal plants contained more alcohols, alkenes, ethers and acids (Sun and Tang 2013). The AM fungi can alter the profile of volatile organic carbon released by roots as well as the root morphology of sorghum plants to adapt to the soil environments. The rhizosphere of

14 common cultivars of tree peony (*Paeonia suffruticosa*) was colonised by AM fungi (Shi et al. 2013). A total of 31 AM fungi species belonging to 3 genera were identified in the rhizospheric soil. *Glomus* (21) was the dominant genus, followed by *Acaulospora* (7) and *Scutellospora* (3). The Paris-type, 17 species of AM fungi and fungal colonisation structures (hyphae, hyphal coils and vesicles) were present in roots of medicinal plant Huangshan magnolia (*Magnolia cylindrica*) (Yang et al. 2011). The species were from the genera *Acaulospora* (6 species), *Glomus* (8 species), *Gigaspora* (1 species) and *Scutellospora* (2 species).

AM fungi (colonised by *Glomus mosseae* and *Glomus intraradices*) have increased plant growth and salinity tolerance by various mechanisms in *B. monnieri*, an important medicinal plant (Khaliel et al. 2011). Sundar et al. (2011) identified 21 AM fungal species in roots of the medicinal plants such as *Eclipta prostrata*, *Indigofera aspalathoides* and *I. tinctoria*. The mean AM fungi colonisation and diversity pattern was dependant on edaphic factors and type of vegetation. Panwar and Tarafdar (2006) identified 5 genera of AM fungi in the rhizosphere of 3 medicinal plant species (*Leptadenia reticulata*, *Mitragyna parvifolia*, *Withania coagulans*). The association with AM fungi of these plant species native to the extreme environmental conditions of the Indian Thar Desert may play a significant role in the re-establishment and conservation of these medicinal plants.

The *Artemisia annua* L. (Asteraceae) is an important medicinal plant whose secondary metabolite artemisinin is used for the treatment of cerebral malaria. Awasthi et al. (2011) found the compatibility and synergy between AM fungus *Glomus mosseae* and *Bacillus subtilis* bacteria and suggested the use of this microbial consortium in *Artemisia annua* L. (Asteraceae) for enhancing growth and the content and yield of artemisinin. Zubek and Blaszkowski (2009) and Zubek et al. (2011) studied AM fungi and dark septate endophyte (DSE) associations in 36 medicinal plant species from 33 genera and 17 families. AM was found in 34 of 36 plant species, and the abundance of AM fungi hyphae in roots varied with particular species, ranging from 2.5 % (*Helianthus tuberosus*) to 77.9 % (*Convallaria majalis*). The mycelium of DSE was observed in 13 plant species; however, the percentage of root colonisation by these fungi was low.

Khamna et al. (2009) obtained a total of 445 actinomycete isolates from 16 medicinal plant rhizosphere soils. Among them, 23 *Streptomyces* isolates showed activity against phytopathogenic fungi. The consecutive monoculture of *Rehmannia glutinosa* L. could be a causative agent to decrease the diversity of fungal community in the rhizosphere soil (Zhang et al. 2011b). Sharma et al. (2008) suggest the use of mixed consortium of AM fungi (*Glomus geosporum*, *G. microcarpum* and one crude consortium of AM fungal spores) over monospecific cultures for the sustainable cultivation and conservation of endangered medicinal plant such as *Curculigo orchioides*.

The 76 medicinal plants were reported to have AM fungi in Pakistan (Gorsi 2002). Radhika and Rodrigues (2010) found that 30 out of 36 medicinal plant species were mycorrhizal in Goa region, India. The molecular diversity of AM fungi associated with *Prunus africana* revealed that 109 sequences obtained belong

to the members of the *Glomeromycota* (Wubet et al. 2003), and subsequent 5.8S/ITS2 rDNA sequence analysis indicated high AM fungal diversity and dominance of *Glomus* species. Appoloni et al. (2008) analysed AM fungi community in roots of *Dichanthelium lanuginosum* and found that 18S rDNA phylotypes belong to the genera *Acaulospora*, *Archaeospora*, *Glomus*, *Paraglomus* and *Scutellospora*. The most diverse and abundant AM fungi were from the genera *Glomus*, with the most frequent phylotype corresponding to *Glomus intraradices*. The AM fungal community in the rhizosphere of *Phellodendron amurense* showed three general groups of *Glomus*, *Scutellospora* and *Hyponectria*, respectively (Cai et al. 2009).

2.3 Effect of Microbial Inoculation on the Growth of Medicinal Plants

More than 24 genera of nonpathogenic rhizobacteria have been identified till today. Plant growth-promoting rhizobacteria, first defined by Kloepper and Schroth (1978), after being inoculated on seeds, could successfully colonise plant roots and positively enhance plant growth. Besides this, the plant root-secreted growth-promoting compounds (e.g. auxins or cytokinins) and improvement in mineral nutrient uptake (e.g. siderophore) can increase the plant growth. The synthesis of antibiotics or secondary metabolite-mediated induced systemic resistance can control the pathogens (biocontrol) and promote the plant growth (van Loon 2007).

AM could promote nutrient uptake, improve the functional diversity and activity of microbes in the rhizosphere of *Atractylodes lancea* medicinal plant and influence the composition of the organic matter leading to the growth of *A. lancea*, but not to the quality (Guo et al. 2006). The root-nodulating bacterium, *Rhizobium meliloti*, isolated from the medicinal plant, *Mucuna pruriens*, produced siderophores and thus promotes the plant growth (Arora et al. 2001). The medicinal sclerophyll, *Agathosma betulina* (Berg.) Pillans, grown under nutrient-poor conditions was colonised by *Cryptococcus laurentii* soil yeast as a plant nutrient-scavenging microsymbiont (Cloete et al. 2010). Guo et al. (2010) screened and exploited molluscicidal microorganisms against *Oncomelania hupensis* from the rhizosphere of medicinal plant *Phytolacca acinosa* Roxb. that had a higher similarity to *Aspergillus fumigatus*. The symbiotic interaction between the common soil yeast, *Cryptococcus laurentii*, and medicinal plant *Agathosma betulina* (Berg) Pillans helped the plant growth on nutrient-poor soils (Cloete et al. 2009). The addition of *Streptomyces pactum* (Act12) could improve the soil microbial activity which, eventually, enhances the resistance and root activity of ginseng plant and could increase yield and its quality (Zhang et al. 2013). The medicinal plants forming association with various microorganisms can be formulated as biofertiliser and biocontrol tools. Therefore, it is very important to identify, characterise and use rhizospheric microorganisms associated with medicinal plants (Vasudha et al. 2013).

The rhizobacterial strain Jdm2 (*Bacillus subtilis*) isolated from the rhizosphere of the traditional Chinese medicinal herb *Trichosanthes kirilowii* enhances plant growth and inhibits the activity of nematode and has the potential to be a safe and effective microbial pesticide (Wei et al. 2014). The bacterial endophytes isolated from medicinal plant *Annona squamosa* L. showed antimicrobial activity (Baker and Satish 2013), and the bacterium belonged to the genus *Pseudomonas* sp., identified by using 16s rRNA and biochemical tests. Yang et al. (2012) discussed the mechanisms involved in controlling the soilborne disease of medicinal plants by different species of microorganisms as biocontrol agents from the following aspects: improving host plant nutrient uptake, the nutrient and space competition with the pathogenic bacteria, changing anatomical structure and the morphology of roots, balancing the host plants' endogenous hormones, activating the host plants' defence system and restoring the balance of host rhizosphere soil conditions. Plant growth-promoting rhizobacteria (PGPR) isolated from the medicinal weed, *Cassia occidentalis*, are an attractive ecofriendly alternative to chemicals in agriculture and open up possibilities for the utilisation of these in plant growth increase and subsequent boost of yield for agricultural crops (Arun et al. 2012).

The mycorrhizal medicinal plants have higher nutrient uptake capacity and growth than non-mycorrhizal plants (e.g. Karagiannidisa et al. 2011; Nisha and Rajeshkumar 2010). The mycorrhizal inoculation increased the dry matter of five medicinal plants (*Abelmoschus moschatus*, *Clitoria ternatea*, *Plumbago zeylanica*, *Psoralea corylifolia* and *Withania somnifera*) grown in five different types of soil (Chandra et al. 2010). The shoot height and root biomass of *Poncirus trifoliata*, *Piper longum*, *Salvia officinalis* and *Plectranthus amboinicus* medicinal plants were promoted by mycorrhizal colonisation (Wang et al. 2006; Rajeshkumar et al. 2008; Geneva et al. 2010; Gogoi and Singh 2011).

2.4 Effect of Rhizosphere Microbes on P Solubility and Availability to Medicinal Plants

The *Aspergillus niger*, *A. fumigatus* and *Penicillium pinophilum* fungal isolates, identified in the rhizosphere of different plants, can effectively solubilise rock phosphate or tricalcium phosphate (Wahid and Mehana 2000) and increase the uptake of phosphorus (P) by the growth of plants. *Pseudomonas aeruginosa* is a plant growth-promoting rhizobacterium. The application of *P. aeruginosa* with a medicinal plant *Launaea nudicaulis* as soil amendment resulted in maximum reduction in *Macrophomina phaseolina* infection on mung bean roots (Mansoor et al. 2007). The endophytic strain of *Bacillus pumilus* isolated from tissues of the medicinal plant *Ocimum sanctum* can be used as a bioinoculant to enhance plant growth and also as a probiotic (Murugappan et al. 2013). Gupta et al. (2011) evaluated the potential of phosphate-solubilising bacteria, *Burkholderia gladioli*, *Enterobacter aerogenes* and *Serratia marcescens*, for utilising Mussoorie rock

phosphate to enhance the medicinal plant growth as biofertiliser because some medicinal plants are less dependent on chemical fertilisers. The strains differed in the extent of rhizosphere colonisation, carbon source utilisation pattern and whole cell fatty acid methyl esters composition.

Despite the high concentrations of total P in soil, its P concentration in the soil solution and uptake by plants is very low (Marschner et al. 2006) due to the low availability of inorganic and organic P compounds and poorly available inorganic P forms (Ca phosphates, Fe/Al phosphates and P adsorbed onto Fe/Al oxides and organic matter) (Schachtman et al. 1998; Richardson and Hadobas 1997). The microbial biomass is another important P pool in soil ranging from 1 to more than 10 % of total soil P (Richardson 2001), because plants and microorganisms compete for P uptake. The microbial biomass may also represent a slow sustained source of available P through decomposition of dead microbial cells (Oberson et al. 2001). Plant P uptake causes depletion of available P in the rhizosphere due to the low solubility and slow diffusion of P in soils (Jungk and Claassen 1986). The plants with the assistance of rhizosphere microorganisms can develop various strategies to increase P uptake and overcome the low P availability in soils. Bacterial and mycorrhizal fungi symbiosis can increase the plant P uptake and P acquisition efficiency (Smith and Read 2008; Rengel 1999) by increasing root growth, mineralisation of organic P by phosphatase enzymes released by roots and microorganisms (Tarafdar and Jungk 1987) and by excretion of organic acids into the rhizosphere and/or changing the rhizosphere pH (Gerke and Meyer 1995; Imas et al. 1997). The microbe in the rhizosphere has different capacity to solubilise or mineralise poorly available P (Banik and Dey 1983) and therefore could affect P availability to medicinal plants.

2.5 Effect of Rhizosphere Microbes on Nutrient Uptake and Stress Tolerance

The AM fungal inoculation has played a significant positive role on plant growth via improved acquisition of nutrients of low mobility, especially P in low-nutrient and constrained soils. AM fungi increase plant uptake of nutrients such as P, Zn, Cu, Mn and Fe in poor soils (Chen and Zhao 2009; Hosamani et al. 2011) and increase the shoot dry weight of plants (Gupta and Janardhanan 1991; Hosamani et al. 2011). The external hyphae of AM fungi can also increase NH_4^+ and NO_3^- uptake by plants and assimilate these molecules into free amino acids (Johansen et al. 1996). However, the effectiveness of AM fungi differs with the plant species, soil fertility and plant growth environments (Smith and Smith 2011). For example, Zhao and Yan (2006) reported that leaf nitrogen contents were lower in the mycorrhizal *Camptotheca acuminata* than its non-mycorrhizal counterpart.

AM fungi-colonised plants have greater tolerance capacity over non-mycorrhizal plants to several biotic and abiotic stresses, such as toxic metals,

root pathogens, drought, high soil temperature, saline soils (Khaliel et al. 2011), adverse soil pH and transplanting shock (Evelin et al. 2009; Lu et al. 2003; Tang et al. 1999). Inoculation with AM fungi enhanced tolerance of *Rosa multiflora* to HCO_3^- as indicated by greater nutrient uptake and leaf chlorophyll and lower root iron reductase activity and alkaline phosphatase activity (Cartmill 2004). The possible drought-induced genes may enhance the tolerance of AM plants to water deficit (Fan and Liu 2011; Ruiz-Lozano et al. 2008). The AM colonisations may alleviate metal stress of plants showing capability in binding heavy metals (Joner et al. 2000; Salvaraj and Kim 2004; Prasad et al. 2011), even though the mechanisms involved in metal tolerance of AM plants are still poorly understood (Hildebrandt et al. 2007) and need to be explored.

2.6 Effect of Rhizosphere Microbes on Quantity and Quality Medicinal Compounds

Bacteria and AM fungi can improve secondary metabolite contents in medicinal plants via improving plant phosphorus status or an altered hormonal balance of the plants (Koeberl et al. 2013; Toussaint 2007). Root diseases (rot and wilt) caused by a complex involving *Fusarium chlamydosporum* (Frag. & Cif.) and *Ralstonia solanacearum* (Smith) are serious diseases affecting the cultivation of *Coleus forskohlii*, a medicinal plant producing forskolin compound (Singh et al. 2013). Coinoculation of *Pseudomonas monteilii* with *Glomus fasciculatum* significantly improved the AM root colonisation and spore numbers, and *Pseudomonas monteilii* can be a mycorrhiza helper bacterium. The forskolin content of tubers was significantly increased by the inoculation treatments of *G. fasciculatum*, *P. monteilii* and *P. monteilii* + *G. fasciculatum*.

Terpenoids, phenolics and alkaloids are the three major groups of secondary plant metabolites and natural medicinal products used for pharmacological and therapeutical purposes. Essential oils mostly consisting of monoterpenes, sesquiterpenes and phenylpropanoids are often used as flavours and fragrances, antimicrobials and antioxidants and medicines (Deans and Waterman 1993). AM fungi increased the content of essential oil and alterations of its composition, such as in the medicinal plant basil (*O. basilicum*) (Copetta et al. 2006). *Andrographis paniculata* that has been used to treat gastrointestinal tract, upper respiratory infections, fever, herpes, sore throat and other chronic and infectious diseases in Asian countries from ancient time contains the primary medicinal compound of andrographolide, a colourless diterpene lactone with a bitter taste. The AM symbiosis after inoculation with *Gigaspora albida* produced the high concentration of andrographolide in the leaf extracts of *A. paniculata* (Radhika and Rodrigues 2011), mostly at flowering growth stage.

The inoculation of *Glomus intraradices*, either alone or in a mixture with *G. mosseae*, significantly increased total phenolic content in leaves and flower heads of *Cynara cardunculus* (Ceccarelli et al. 2010). The AM fungi colonisation

increased the concentrations of isoflavone in roots of legume plants (Catford et al. 2006); flavonoid in white clover (*Trifolium repens*) (Ponce et al. 2004), *Bupleurum chinense*, *Ginkgo biloba* and *Astragalus membranaceus* (Meng and He 2011); rosmarinic acid, a highly antioxidant phenolic compound, in basil (Toussaint et al. 2008); and total coumarin and imperatorin in *Angelica dahurica* (Zhao and He 2011). The AM fungal colonisation could induce two different signalling pathways in the accumulation of phenylpropanoid metabolism: one is through the induction of phenylalanine ammonia-lyase and chalcone synthase, and the other is through the suppression of isoflavone reductase (Zhao and Yan 2006).

The camptothecin in *Camptotheca acuminata* and vinca alkaloids in vinca (*Catharanthus roseus*) are two important anticancer compounds (Rosa-Mera et al. 2011). The castanospermine is effectively used in the treatments against AIDS and cancers (Spearman et al. 1991). Sweet basil has been traditionally used for the treatment of headaches, coughs and diarrhoea (Jayasinghe et al. 2003). AM fungal inoculation significantly enhanced plant growth and the total content of vinblastine in Vinca leaves (Rosa-Mera et al. 2011), castanospermine content in seeds and leaves of *Castanospermum australe* (Abu-zeyad et al. 1999), rosmarinic acid (antioxidant activity) in sweet basil shoots (Toussaint et al. 2007), camptothecin in *Camptotheca acuminata*, vinca alkaloids in vinca (Rosa-Mera et al. 2011) and total phenols, ortho-dihydroxyphenols, flavonoids, alkaloids and tannins in the root and leaf of *O. basilicum* and *Coleus amboinicus* (Hemalatha 2002).

However, a few other studies reported some controversial results for mycorrhizal effects on phenolic contents in medicinal plants. Zeng et al. (2013) showed neutral effects of AM colonisation on the composition of phenolic ingredients. AM symbiosis did not alter the total concentrations of phenolic and rosmarinic acid in roots of *Salvia officinalis* (Nell et al. 2009) and the polyphenolic profile in leaves and stems of basil (Lee and Scagel 2009) after AM fungal inoculation.

2.7 Conclusions

The quality of medicinal plants (active compound content) is largely influenced by abiotic and biotic factors of the rhizosphere. The rhizosphere microbes play an important role in improving medicinal values of medicinal plants. The role of microbes in plant growth, nutrient availability, disease resistance, yield and quality of medicinal compounds is demonstrated in medicinal plants. There are increasing interests in the research of the interaction between medicinal plant and their rhizosphere microbes for the improvement of medicinal plants. A wide variety of bacteria and fungi diversity including AM fungi is recognised in the rhizosphere of medicinal plants that have high significance in plant nutrient acquisition and secondary metabolite alteration. The inoculation of PGPR and/or AM fungi is a sustainable technology to enhance the quantity and quality of the medicinal plant compounds. However, selecting and inoculating specific and efficient bacteria and/or fungi for a particular plant are essential for the cultivation of medicinal

plants in order to obtain the high-quality secondary plant metabolites. Therefore, further research is recommended to better understand the diversity and function of rhizosphere bacteria and/or fungi and their uses in the increased production of medicinal plants by identifying relationship between genetic and functional diversity of bacteria and/or fungal species.

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Chapter 3
Enhanced Efficiency of Medicinal and Aromatic Plants by PGPRs

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3.1 Introduction

Other than nutritional value for human and livestock, plants have gained significant attention in recent years due to their secondary metabolites, which are widely used in aromatic, therapeutic, or chemical industries. Higher plants use primary metabolites such as carbohydrates, lipids, and amino acids to synthesize various secondary metabolites that serve a variety of functions including plant defense against herbivores and microbes, protection against environmental stresses, and contribution to specific odors, tastes, and colors in plants (Seigler 1998). Plant secondary metabolites are unique sources for food additives, flavors, fragrances, and pharmaceuticals (Bennett and Wallsgrove 1994; Ravishankar and Rao 2000). Plants accumulate secondary metabolites mostly under stress conditions in response to various biotic and abiotic elicitors or signal molecules. Physiological traits and genetic diversity, environmental conditions, geographic variation, and evolution are among the main factors affecting the accumulation and composition of secondary metabolites (Figueiredo et al. 2008). Moreover, infection by microorganisms and abiotic factors such as osmotic stresses can induce particular secondary metabolite pathways in plants (Sanchez et al. 2004).

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Plant growth-promoting rhizobacteria (PGPRs) are a specific group of soil bacteria that aggressively colonize the rhizosphere and rhizoplane, and substantially improve plant growth and productivity. PGPRs function as plant growth promoters and biological control agents via direct or indirect mechanisms. Direct mechanisms by PGPRs include the provision of bioavailable phosphorus and nitrogen for plant uptake, sequestration of iron by siderophores, production of plant hormones like auxins, cytokinins, and gibberellins, and lowering ethylene levels inside plants using ACC deaminase that accumulate in plants subjected to biotic and abiotic stresses (Glick 1995; Glick et al. 1999; Mayak et al. 2004). The indirect mechanisms include the production of antibiotics, reducing iron availability for phytopathogens in the rhizosphere, enzymatic lysis of fungal cell wall and insect-gut membrane secreting chitinase enzyme for the hydrolysis of chitin layer of the eggshell of nematodes, competition with detrimental microorganisms for sites on plant roots, and induction of systemic resistance in plants against various pathogens and pests (Ramamoorthy et al. 2001). Bacterial strains showing PGPR activity have been reported for diverse bacterial taxa including *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcus, Pseudomonas,* and *Serratia* (Gray and Smith 2005).

To date, PGPRs have been shown to promote the growth of cereals, ornamentals, vegetables, and food crops (Vessey 2003; Lugtenberg and Kamilova 2009; Mishra et al. 2010). However, a limited number of studies have been undertaken regarding the interactions between PGPRs and medicinal or aromatic plants. This chapter, therefore, aims to introduce proven or putative mechanisms by which PGPRs promote seed germination, growth, nutrient acquisition, and production of primary and secondary metabolites in aromatic and medicinal plants.

3.2 Seed Germination and Vigor Index in Medicinal Plants Under PGPRs Inoculation

Medicinal plants, native to the arid lands, often readily germinate within their native environment, while low germination rates have been observed under laboratory or field conditions (Gupta 2003). Recent advances in ex situ propagation methods, however, encourage the cultivation of these plants and reduce the pressure on their natural environment. Seeds and clones (produced by micro-propagation) are the most common means of propagation in medicinal plants. For the majority of species, seed is considered as the most effective and convenient propagation method.

Use of PGPRs as stimulants of seed germination in medicinal and aromatic species can provide more uniformity in germination, seedling emergence, and other growth stages in particular flowering, which is a critical time to achieve more bioactive secondary metabolites. PGPRs are able to increase the rate of seed

germination and seedling emergence and improve plant growth (Shaukat et al. 2006). The development stage of the plant organ (leaf, flower, and fruit ontogeny) can be a determinant factor for the composition of volatiles (Figueiredo et al. 1997; Badalamenti 2004). Several studies have reported increases in the yield of the volatiles from the flower bud to the mature flower. Concomitantly, the composition of secondary metabolites can undergo major changes, some components varying from traces to 10 % in the initial stages, and 50–70 % in the full flowering stage (Figueiredo et al. 2008). However, there are also reports indicating that the volatiles are largely accumulated before the organ is fully expanded (Figueiredo et al. 2008). Therefore, uneven or poor germination and subsequently inhomogeneous seedling growth can lead to the production of plants with variable content and composition of secondary metabolites.

Although PGPRs have been broadly used to improve seed germination and overall yield of many crops in different agro-ecosystems, there is a lack of literature on seed germination and vigor index in medicinal and aromatic species. Recently, the role of PGPRs on growth and phytochemical parameters, from seed germination to the mature flower stage, was evaluated in two types of medicinal plants containing different classes of secondary metabolites including alkaloids and essential oils (Ghorbanpour et al. 2013a, b, 2014). Inoculation of *Hyoscyamus niger* seedling radicles with 20 PGPR strains belonging to *Pseudomonas putida* (PP) and *P. fluorescens* (PF) on vigor index [seedling length (root length + shoot length) × germination %] under two conditions, in vitro (with agar media) or sand culture tubes, indicated that PGPRs can have contrasting effects on vigor index. The most efficient strains were shown to be those producing the optimum auxin level (PP-168 and PF-187). The PF-187 strain increased root and shoot elongation by 73 and 51 % compared with uninoculated controls, respectively. Moreover, two PP strains (PP-4 and PP-11) had negative effects on vigor index when compared with the uninoculated controls. Under both assay conditions, PF-187 and PP-168 strains were the most effective strains for early seedling development (Ghorbanpour and Hatami 2013). The fluorescent pseudomonads used had substantial effects on plant growth under various conditions particularly via auxin secretion. However, the production of this phytohormone at the amount beyond that is needed for plant produces additional levels of ACC, the immediate precursor of ethylene production, which significantly inhibits root elongation and decreases vigor index and plant growth (Fig. 3.1) (Glick et al. 1998).

Similar to *H. niger*, treatment of *Salvia officinalis* seeds by PGPRs including *P. fluorescens* (PF-23) and *P. putida* (PP-41, PP-108 and PP-159) differently affected the germination and vigor parameters (Ghorbanpour and Hatami 2014). The maximum (78.5 %) and minimum (16.75 %) germination percentages were recorded for PP-41 and PF-23 treatments, respectively. Also, the highest germination rate, root and shoot length, seedling vigor index, and the lowest mean germination time were recorded in seeds inoculated with PP-41, a strain with the ability to produce moderate auxin, when compared to other treatments (Fig. 3.2 and Table 3.1). According to the studies mentioned above, the net effect of plant–

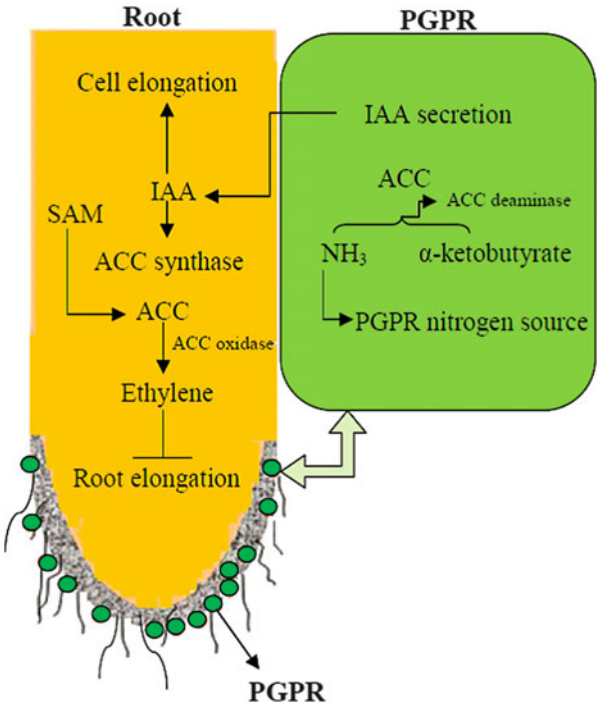

Fig. 3.1 Schematic model of PGPRs' effects on ethylene synthesis and inhibition of root elongation. *IAA* Indole acetic acid, *ACC* 1-aminocyclopropane 1-carboxylic acid, and *SAM* S-adenosylmethionine (Ghorbanpour and Hatami 2014)

PGPRs interactions on seed germination, root elongation, and subsequently vigor index could be positive, neutral, or negative.

Jahanian et al. (2012) studied the effects of seed inoculation of artichoke (*Cynara scolymus*) with different PGPR strains (*Azotobacter, Azospirillum, P. putida* PP-41, and PP-168) on seed germination and plant early growth characteristics. The combination of PP-168, *Azotobacter,* and *Azospirillum* strains was the most effective treatment in increasing the germination percentage, number of normal plants, radicle and shoot weight, shoot length, and vigority and in decreasing the mean time of germination. Moreover, either sole or the integrated application of phosphate-solubilizing bacteria along with nitrogen-fixing ones led to significant increases in radicle and shoot length, shoot weight, coefficient of velocity of germination, seedling vigor index, and significant decrease in mean germination time compared to uninoculated controls.

The PGPR strain P-35 with multiple PGPR activities (like IAA, ammonia, HCN, and catalase) was subjected to seed germination test for *Withania somnifera* plants. The results established a significant enhancement in seed germination rate as well as root and shoot growth of this valuable medicinal plant (Rathaur et al. 2012).

A commercial soil amendment containing a mixture of four PGPR strains (*Azospirillum lipoferum, Azotobacter chroococcum, P. fluorescens, and Bacillus megaterium*) was evaluated for impact on germination and initial growth of *Catharanthus roseus* (Lenin and Jayanthi 2012). The results indicated that

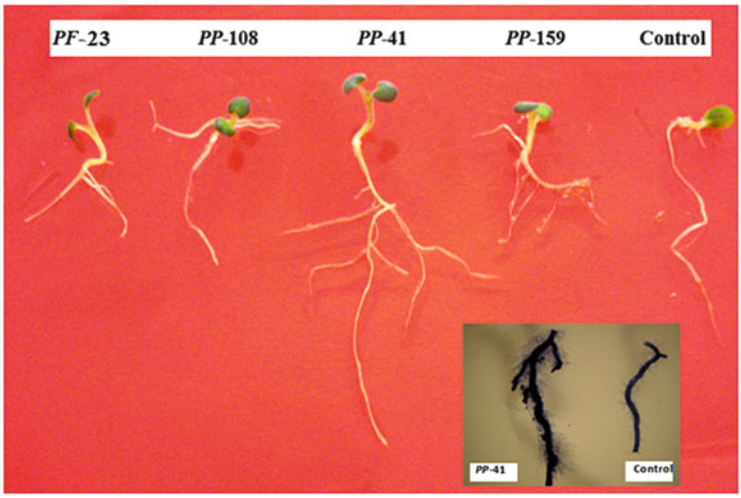

Fig. 3.2 Effects of *Pseudomonas putida* (PP-41, PP-108 and PP-159 strains) and *P. fluorescens* (PF-23 strain) on root morphology, root hair formation, and seedling vigor index in *Salvia officinalis* L. (Ghorbanpour and Hatami 2014)

Table 3.1 Seed germination behaviors and seedling vigor index in *Salvia officinalis* plants inoculated with *Pseudomonas putida* (PP-41 and PP-159) and *P. fluorescens* (PF-23) strains (Ghorbanpour and Hatami 2014)

PGPR strain treatment	Germination characteristics					
	Germination percentage (%)	Mean germination time (day)	Germination rate (seed/day)	Root length (cm)	Shoot length (cm)	Vigor index
Control	41.25^{c}	$7.75^{a, b}$	0.66^{c}	3.92^{c}	2.32^{c}	257.03^{c}
PP-41	78.50^{a}	4.25^{c}	1.05^{a}	8.45^{a}	4.20^{a}	992.13^{a}
PP-159	57.75^{b}	7.5^{b}	0.83^{b}	6.47^{b}	3.37^{b}	570.40^{b}
PP-23	16.75^{d}	9.75^{a}	0.17^{d}	2.25^{d}	1.87^{c}	69.63^{d}

In each column, values followed by different letters differ significantly ($P < 0.01$) according to Duncan's multiple range test

inoculation by PGPR strains significantly increased germination rate and vigor index. Harish Kumar and Maheshwari (2011) studied five bacterial strains (TR1–TR5) isolated from the root nodules of fenugreek (*Trigonella foenum-graecum*) for their plant growth promontory traits. The TR2 isolate was identified as *Rhizobium leguminosarum,* and the other four strains were *Ensifer meliloti*. The maximum increments in vigor index, nodule number, and root and shoot biomass were recorded for seeds inoculated with consortium (TR1 + TR2) followed by single inoculation as compared to uninoculated fenugreek plants. In addition, seed treatment of two *Acacia senegal* genotypes with *B. licheniformis* or *Sinorhizobium saheli,* either individually or in combination, had positive effects on the phenotypic traits of germination (Singh et al. 2011). However, inhibition of seed germination

has also been recorded when *Ambrosia artemisiifolia* seeds were inoculated with *P. fluorescens* (Vrbnicanin et al. 2011). Moreover, *P. fluorescens* has been classified as either deleterious rhizobacteria (DRB) (Zdor et al. 2005) or PGPR (Jaleel et al. 2007), depending on the experimental conditions in which bacterial cultures develop.

Growth promotion and beneficial effects conferred by PGPRs may involve various mechanisms of action. Direct growth promotion by PGPRs is regarded as one of the most important mechanisms of action, which include the production of plant growth regulators such as indole acetic acid (IAA) (Mishra et al. 2010), gibberellic acid (Narula et al. 2006), cytokinins (Castro et al. 2008), and ethylene (Saleem et al. 2007). The improved germination rate in plants inoculated by PGPRs (Nelson 2004) may be due to the increased synthesis of hormones like gibberellins, which would have triggered the activity of specific enzymes such as a-amylase that promote early germination by facilitating starch assimilation (Bharathi 2004). Moreover, significant increases in seedling vigor have been attributed to better synthesis of auxins (Bharathi 2004).

3.3 PGPRs Stimulate Plant Growth and Modify Enzyme Activities in Medicinal and Aromatic Plants Under Normal or Stress Conditions

Plant growth in the field is a reflection of diverse interactions with physiochemical and biological components that exist in the soil and modulated by environmental conditions. Microorganisms are a driving force for fundamental metabolic processes in soil. The genetic and functional diversities of the extensive microbial populations have a critical impact on soil function and plant growth (Nannipieri et al. 2003). Plant production and health are negatively affected by a large number of both biotic and abiotic stresses through the formation of reactive oxygen species (ROS). The induction of ROS-scavenging enzymes including superoxide dismutase (SOD), peroxidase (POX), and catalase (CAT) is the most common mechanism for detoxifying ROS synthesized under stress conditions (Munns and Termaat 1986). To deal with these biotic and abiotic stresses, chemical inputs have been extensively used during the past few decades to achieve high yields, causing harmful effects on the environment. Sustainable agriculture needs to be further promoted as a key strategy to counteract the rapid decline in environmental quality via the gradual reduction in the use of chemical fertilizers and pesticides accompanied by greater use of the biological and genetic potential of plant species and microbial communities in order to gain sustainable high yields. The plant's ability to modify its physiology and metabolism to either avoid or partially overcome the environmental stresses can be improved by the aid of certain microorganisms existing in the rhizosphere (Govindasamy et al. 2008). Here, we introduce several detailed

mechanistic studies exploring the positive effects of PGPRs on medicinal plants under normal or stress conditions.

The effects of inoculation with PGPR strains *P. putida* (PP-168) and *P. fluorescens* (PF-187) on growth parameters, proline and chlorophyll content, leaf relative water content (RWC), as well as antioxidant enzymes activity (SOD, POX, and CAT) were investigated in *Hyoscyamus niger* under three water-deficit stress (WDS) levels of 30 % (W1), 60 % (W2), and 90 % (W3) water depletion of field capacity (Ghorbanpour et al. 2013a). Inoculation with PP and PF strains minimized the deleterious effects of WDS on growth parameters, whereas uninoculated plants had a grave reduction in plant growth. The number of leaves, leaf area, and leaf greenness decreased with the increase in water stress levels, but PP- and PF-inoculated plants had lower reduction percentages compared to uninoculated control plants. The greatest accumulation of proline was found in PF-inoculated plants against severe WDS. In contrast, proline accumulation in PP-inoculated plants and in uninoculated control plants was observed only up to the W2 treatment level, and it later started to decline, particularly in the uninoculated control plants. Furthermore, inoculation with PP and PF strains significantly improved the chlorophyll content of plants. The results also unearthed that the RWC was significantly higher in plants subjected to PP and PF strains under all WDS conditions than their respective controls. This effect may be associated with the hydraulic nature of branch root junctions, which facilitate the radial flow of water (Kothari et al. 1990). The advantageous effects of PGPRs and common adaptation mechanisms of plants exposed to WDS are always mutually related to exceptional changes in root morphological and anatomical traits such as root branching networks and biomass (Fig. 3.3). The PF strain had higher efficiency than the PP strain in plants growing under moderate (W2) and severe (W3) WDS conditions. This outstanding capacity might be linked with the geographical origin of the PF strain as it was isolated from the wheat rhizosphere in rainfed wheat fields (dry land farming), where water is restricted and dry periods often take place. In contrast, the PP strain was isolated from wheat rhizosphere in irrigated wheat fields showing no phosphate-solubilizing activity and inferior performance under limited water supply compared to the PF strain (Ghorbanpour et al. 2013a).

Thus, it can be concluded that selection of PGPR strains should be based on multiple plant growth-promoting characteristics and their ecological adaptation with respect to the potential abiotic stresses of the host plant.

Inoculation with PGPRs can stimulate the activities of antioxidant enzymes and increase proline accumulation under drought stress conditions (Kohler et al. 2008) and induce systemic resistance against fungal plant diseases through the activation of various defense-related enzymes (Bharathi 2004). The activities of SOD and POX in root and leaf tissues of *Hyoscyamus niger* plant increased to a significant extent after inoculation with PP and PF strains (and with WDS treatment as well), while CAT activity decreased with increasing WDS, except in PF-inoculated plants (Ghorbanpour et al. 2013a). This is in keeping with a report by Kohler et al. (2008), suggesting that the overexpression of SOD, if accompanied by enhanced

Fig. 3.3 Effects of seed inoculation with *Pseudomonas putida* (PP-168) and *P. fluorescens* (PF-187) on root and shoot growth in *Hyoscyamus niger* (Ghorbanpour et al. 2013a)

H_2O_2-scavenging mechanisms like CAT and POX activities, is an important antidrought mechanism to cope with oxidative stress during WDS conditions. The effects of water stress and PGPR strains *Pseudomonades* sp., *B. lentus,* and *Azospirillum brasilense* were assessed on proline, soluble carbohydrates, chlorophyll, and mineral content in basil (*Ocimum basilicum* L.) plants (Heidari et al. 2011). The proline and soluble carbohydrate content increased significantly with increasing water stress in plants inoculated with *Pseudomonas* sp. and *B. lentus*, respectively. The chlorophyll content was also increased in all plants inoculated with the PGPR strains.

PGPR-mediated resistance has been documented against certain biotic stresses. Bacterization of seeds of cucumber plants with different PGPR strains resulted in enhanced growth and efficiency, and also induced resistance against bacterial wilt, which is caused by *Erwinia tracheiphila* and transmitted by a beetle vector (Zehnder et al. 2001). In this case, the induced systemic resistance was attributed to a reduction in cucurbitacin (a secondary metabolite stimulating the beetle feeding) and induction of other plant defense mechanisms.

The general mechanisms by which PGPRs enhance plant growth and productivity are given in Fig. 3.4 which are as follows: (1) producing plant growth regulators including IAA (Mishra et al. 2010), gibberellic acid (Narula et al. 2006), cytokinins and analogs (Castro et al. 2008), jasmonate, salicylate, and volatile growth stimulants such as ethylene and 2,3-butanediol (Saleem et al. 2007; Vessey 2003); (2) producing ACC deaminase (1-amino-cyclopropyl-carboxylic acid) to reduce the ethylene levels in the roots of developing plants (Dey et al. 2004); (3) asymbiotic nitrogen fixation (Ardakani et al. 2010); (4) exhibition of antagonistic activity against plant pathogens by producing iron-chelating

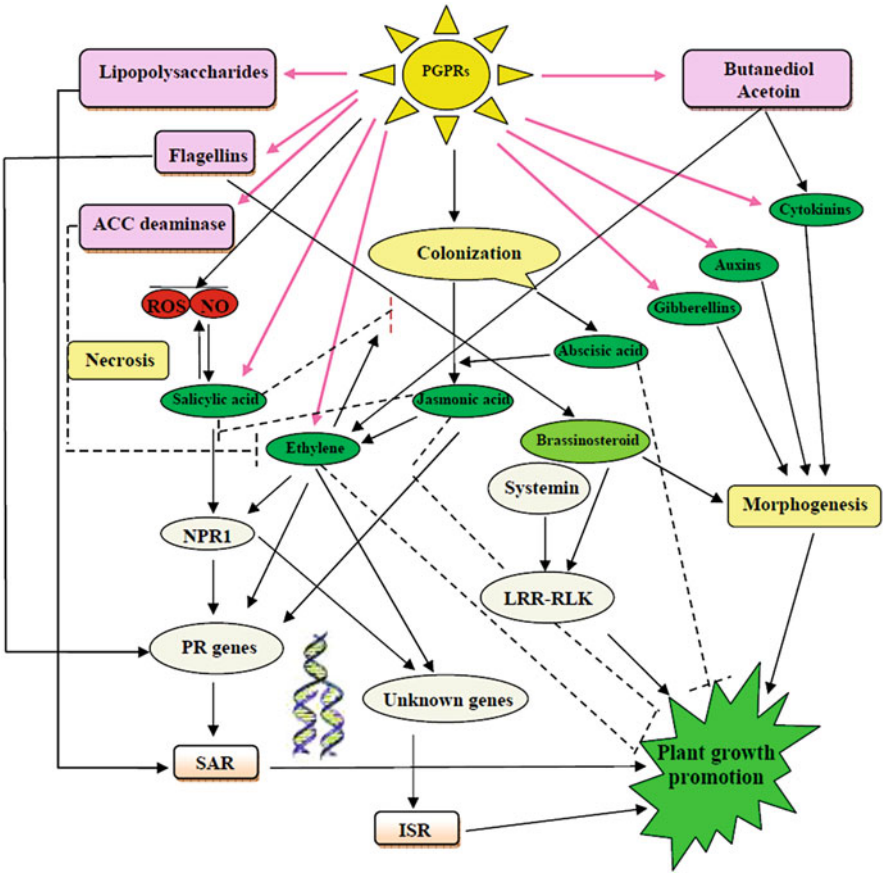

Fig. 3.4 A diagram of signaling cascades involved in plant growth promotion by PGPRs. *Thick pink arrows* indicate secretions or bioactive components from the PGPRs. *Dark green ovals* are phytohormones; *pink boxes* show the exudate elicitors in the signaling cascades; *solid black arrows* represent active signaling pathways; *broken lines* indicate inhibitory effects [modified from Ping and Boland (2004)]. *Abbreviations*: *ISR* induced systemic resistance, *SAR* systemic acquired resistance, *NO* nitric oxide, *NPR1* nonexpressor of PR genes, *PR* pathogenesis-related proteins, *ROS* reactive oxygen species, *LRR-RLK* leucine-rich repeat receptor-like kinases, *ACC* 1-Aminocyclopropane-1-carboxylate deaminase

siderophores, ß-1,3-glucanase and chitinase enzymes, antibiotics, fluorescent pigments, and cyanide (Pathma et al. 2011); and (5) solubilization of phosphate and other nutrients (Hayat et al. 2010). PGPRs may simultaneously apply a combination of these mechanisms to improve the plant's performance (van Loon 2007; Martinez-Viveros et al. 2010). However, regardless of the type of mechanism involved, PGPRs must colonize the rhizosphere or root itself (Glick 1995).

Lenin and Jayanthi (2012) observed that root inoculation of *Catharanthus roseus* with different PGPR strains (*Azospirillum lipoferum, Azotobacter chroococcum, P. fluorescens,* and *B. megaterium*) increased chlorophyll and nutrient (N, P, and K) content. They concluded that PGPRs in combination have a greater potential to increase plant growth, nutrient uptake, and yield. Similarly, vegetative growth and chemical composition in *Catharanthus roseus* were promoted by the combined treatment of *Azotobacter* and phosphate-solubilizing bacteria (Attia and Saad 2001). Together, inoculation with diverse PGPR strains can contribute to maintaining good soil health and fertility in order to achieve sustainable high yields and high-quality products.

Arbuscular mycorrhizal (AM) fungi interact synergistically with other soil microorganisms such as nitrogen-fixing bacteria (Barea and Azcon-Aguilar 1983), phosphate-solubilizing bacteria (Villegas and Fortin 2002), and biocontrol agents (Abdel-Fattah and Mohamedin 2000) to favor plant growth. This interaction could be direct by providing niche and/or habitat or indirect by modifying physiology of the host plant (Bianciotto et al. 2000; Walley and Germida 1997). PGPRs in combination with other beneficial microorganisms such as AM fungi can induce plants to produce certain metabolites making the rhizosphere a more suitable environment for their stay (Dutta and Podile 2010). Certain PGPRs have been reported to enhance the activity of AM fungi and plant growth, consequently (Jayanthi et al. 2003). It appears that PGPRs and AM fungi establish mutually beneficial relationships in rhizosphere to support their co-existence and promote the plant's performance.

Investigations into the interaction between the AM fungus *G. aggregatum* and the PGPR strain *B. coagulans* and *T. harzianum* in soil and their consequent effects on growth, nutrition, and enzyme activity of *Solanum viarum* seedlings demonstrated that plant biomass and nutrient (P, Fe, Zn, Cu, K, and Mn) content were maximum in the plants co-inoculated with all three microorganisms (Hemashenpagam and Selvaraj 2011). The positive effects were probably due to the enhanced mycorrhizal colonization resulting in efficient nutrient uptake. The results also showed that acid phosphatase, alkaline phosphatase, and dehydrogenase activities in the root zone soil of all the inoculated seedlings were significantly higher compared to those in uninoculated control plants. Moreover, root zone soil of plants co-inoculated with all three microbes had higher *B. coagulans* population suggesting the stimulatory effect and synergistic activity between the organisms involved. Here, the mycorrhiza helper bacteria enhanced the activity of *G. aggregatum* presumably by producing organic acids which serve as a carbon source to the fungus or by producing hydrolytic enzymes enabling the AM fungus to penetrate and ramify in the root system of the host plant (Lakshmipathy

et al. 2002; Selvaraj et al. 2008). In another study, inoculation of pot marigold (*Calendula officinalis* L.) seeds with PGPR strains (*Azotobacter, Pseudomonas,* and *Azospirillum*) and the AM inocula substantially increased growth parameters, root and shoot dry weight, photosynthetic pigments (chlorophyll a and b, carotene, and xanthophylls), and the content of N, P, and K in leaves and roots (Hosseinzadah et al. 2011).

Kohler et al. (2008) investigated the effects of inoculation with the PGPR strain *P. mendocina* Palleroni, alone or in combination with an AM fungus (*G. intraradices* or *G. mosseae*), on activities of antioxidant enzymes (SOD, CAT, and POX), phosphatase and nitrate reductase, and solute accumulation in leaves of *Lactuca sativa* L. cv. Tafalla under different levels of water stress. At moderate drought, PGPR and AM inoculation with *G. intraradices,* alone or in combination, significantly stimulated the nitrate reductase activity. At severe drought, inorganic fertilization and *P. mendocina* inoculation, alone or in combination with either of the selected AM fungi, significantly increased phosphatase activity in lettuce roots and proline accumulation in leaves. Inorganic fertilization and combined treatment of PGPR with either AM fungus showed the highest values of leaf POX activity under severe drought conditions. The highest CAT activity was recorded in the fertilized plants inoculated by *P. mendocina* grown under severe stress conditions. These results highlight the potential capacity of PGPRs to alleviate the oxidative damage produced under WDS (Kohler et al. 2008). Similarly, Liddycoat et al. (2009) demonstrated the remarkable effects of PGPRs on plant vigor and productivity under stress conditions. The effects of PGPRs (*Pseudomonas* sp.) treatment on 3-week-old seedlings and seeds of two asparagus (*Asparagus officinalis* L.) cultivars (Guelph Millennium and Jersey Giant) were studied. According to the results, single inoculation of seedlings resulted in positive growth response under optimal and drought stress for both cultivars tested. Seed inoculation led to enhanced growth for Guelph Millennium under optimal conditions, while no positive response was observed for the Jersey Giant cultivar under either normal or stress treatments.

The literature noted above highlights the key role of PGPRs to improve nutrition and productivity in various plants with therapeutic and industrial significance. The potential biofertilizer activities of PGPRs could be divided into five distinct areas including biological N_2 fixation, increasing the nutrient availability in rhizosphere, increasing the root surface area, enhancing beneficial symbioses of the host plant, and finally the combinations of all these mechanisms (Bhattacharyya and Jha 2012). However, the effectiveness of PGPRs–plant interactions depends on soil biological components, the genetic and physiological properties of the organisms involved, and their adaptation to the existing ecosystem-related constraints.

3.4 PGPRs Function as Biotic Elicitors in the Biosynthesis of Plant Secondary Metabolites

Plant secondary metabolites are unique sources for pharmaceuticals, fragrances, flavors, food additives, and other industrially important compounds. The major roles of plant secondary metabolites are to protect plants from attack by insect pests, herbivores, and phytopathogens or to help plants survive other biotic and abiotic stresses. The environmental stresses (microbial, physical, or chemical factors) can function as biotic/abiotic elicitors leading to an increase in the production of secondary metabolites (Radman et al. 2003; Ghorbanpour et al. 2013a, b, 2014). The biotic elicitors have biological origin and are derived from microorganisms such as fungi, bacteria, viruses, or plant cell wall components and chemicals that are released by plants against phytopathogens or herbivore attack. Thus, elicitors could be employed for improving the production of plant valuable secondary metabolites (Namdeo 2007).

Rhizosphere microbes such as PGPRs are best known as biotic elicitors, which have the potential to induce the synthesis of secondary metabolites in plants. Below, we summarize some of the recent studies dealing with the major role of PGPRs to improve the production of secondary metabolites in plants. The effects of PGPR strains *P. putida* (PP-168) and *P. fluorescens* (PF-187) were studied on the root and shoot content and yield of two tropane alkaloids hyoscyamine (HYO) and scopolamine (SCO) in black henbane (*Hyoscyamus niger*) under different WDS levels (30, 60, and 90 % water depletion of field capacity; W1, W2, and W3, respectively) at vegetative, full flowering, and seed-ripening stages (Ghorbanpour et al. 2013a, b). The SCO content of roots in PGPR-inoculated and uninoculated control plants increased significantly with increasing WDS up to W2 treatment, and later it started to decline, except for PF-inoculated plants, which kept an upward trend continuously. The highest root SCO content was observed in the PF-inoculated plants under W3 conditions. Also, the maximum root HYO content was observed in W3 treatment, where both PP and PF strains had identical effects in this regard. In shoots, however, HYO content significantly increased with increasing WDS in both PGPR treatments. The SCO content of shoots in all employed treatments had same changes as root, and was mildly increased with increasing WDS only under PF treatment. Inoculation of *H. niger* plants with the PF strain promoted HYO and SCO accumulation in both root and shoot organs. Almost the same trend was observed for alkaloid yield in both tissues under all employed treatments. Although shoot HYO yield decreased with increasing WDS in both PGPR-inoculated and uninoculated control plants, the reduction percentage in PGPR treatments was lower than uninoculated controls. Shoot SCO yield also decreased with increasing WDS in PP-inoculated and uninoculated control plants, but in plants inoculated with the PF strain showed unchanged. The largest total alkaloids (HYO + SCO) yield belonged to the PP treatment under W1 conditions. Accordingly, it can be concluded that an integrative use of effective PGPRs (biotic elicitor) and WDS (abiotic elicitor) could be an encouraging and eco-friendly strategy for increasing

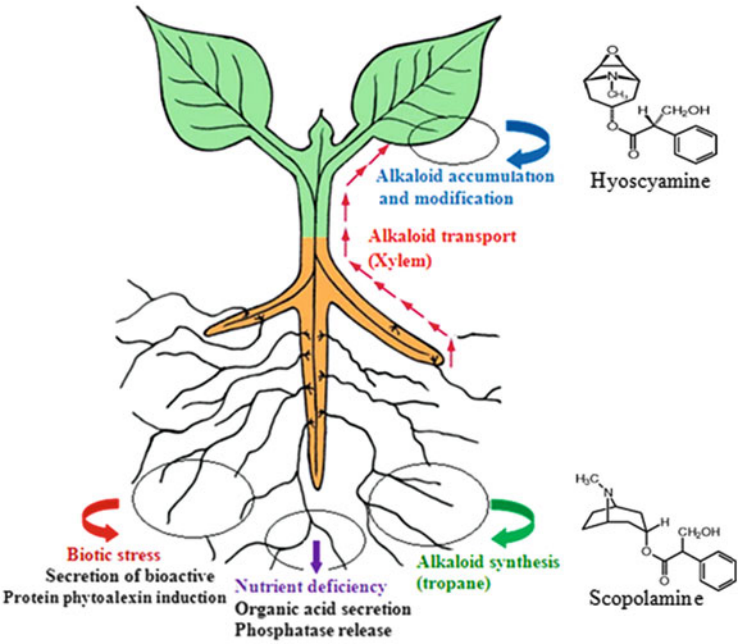

Fig. 3.5 Diagram of a generic tropane alkaloid-producing plant like *Hyoscyamus niger*. The diagram highlights the key role of plant roots in determining the spatial and temporal patterns of bioactive secondary metabolites such as hyoscyamine and scopolamine synthesis in response to biotic and abiotic (nutrient deficiency) stresses

the yield and content of these two alkaloids in *H. niger* organs (Ghorbanpour et al. 2013a). Furthermore, PP-inoculated plants under W1 conditions had higher proportion of fine roots compared to other treatments. Plant fine roots without secondary growth have been found to be the principal site for production of tropane alkaloids and the location for main enzymes involved in their biosynthesis pathway (Suzuki et al. 1999). Although root is known to be the location for the biosynthesis of tropane alkaloids in Solanaceae, these alkaloids may be transported through the xylem to the aboveground parts of the plant (Figs. 3.5 and 3.6).

Higher plants activate various defense mechanisms when attacked by microbial pathogens such as fungi, bacteria, or viruses. These defense responses include suicide of the attacked host cell (hypersensitive response); the production of antimicrobial secondary metabolites (phytoalexins); the production of pathogenesis-related (PR) proteins with potential antimicrobial properties; and the production and oxidative cross-linking of cell wall polymers (Penninckx et al. 1998). The effective defense system is a result of a synchronized expression of a series of these defense responses (Ayers et al. 1976). Ghorbanpour et al. (2013a, b) observed that *Hyoscyamus niger* plants inoculated with PGPRs had higher values of HYO than SCO, which could be due to the high antimicrobial activity of HYO. Abdel-Motal et al. (2009) investigated the antifungal activity of HYO and SCO against

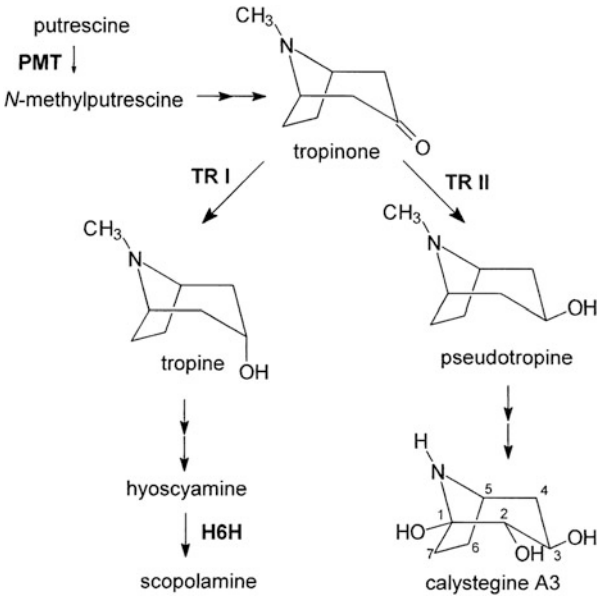

Fig. 3.6 Biosynthetic steps for tropane alkaloids. *PMT* putrescine N-methyltransferase; *TR I* tropine-forming tropinone reductase; *TR II* pseudotropine-forming tropinone reductase; *H6H* hyoscyamine-6-Hydroxylase

40 fungal strains associated with *Hyoscyamus muticus* and found that all fungal strains were tolerant to SCO but sensitive to HYO.

The essential oils (EOs) production can also be positively affected by PGPRs. Treatment of cuttings and foliage of *Salvia officinalis* plants with PGPR strains *P. fluorescens* (PF-23) and *P. putida* (PP-41 and PP-159) significantly affected the EOs content, yield, and composition (Ghorbanpour et al. 2014). The highest (3.95 g/plant) and lowest (1.22 g/plant) EOs yields were observed for PP-159 and uninoculated plants, respectively. Plants inoculated with PP-159 or PP-41 showed significant increases in total EOs yield of 69.1 and 68.5 % compared to uninoculated controls, respectively. The increases in total essential oil yield in response to PGPRs inoculation were due to both increased plant dry weight and the biosynthesis of terpenes. The increased EOs yield was associated with a significantly larger density of trichomes, the main structure for EOs secretion (Fig. 3.7). Totally, 27 different compounds were identified in the EOs of *Saliva officinalis* plants under all employed treatments. Inoculation with PGPRs (PP-159 in particular) stimulated the production of certain monoterpenes such as *cis*-thujone, camphor, and 1,8-cineol.

Essential oils serve important ecological roles. The reported increases in the synthesis of EOs can be considered as a defensive response to colonization by harmful microorganisms, since several EOs exhibit antimicrobial properties (Sangwan et al. 2001). The EOs compounds rich in *cis*-thujone, camphor, and 1,8-cineole (eucalyptol) are well-known chemicals with strong antimicrobial activity against different pathogenic bacteria (Tzakou et al. 2001; Cha et al. 2005). Ghorbanpour et al. (2014) proved that the EOs of *Saliva officinalis* plants under

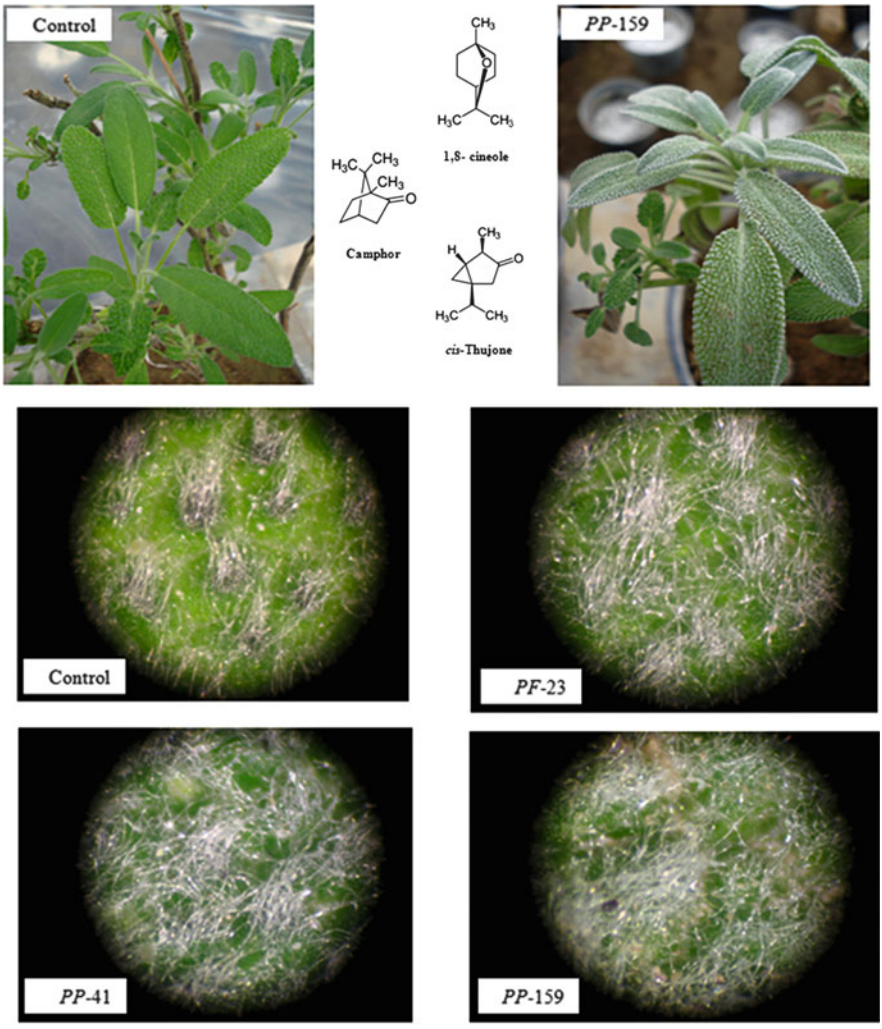

Fig. 3.7 Effect of foliar application of *Pseudomonas putida* (PP-41 and PP-159 strains) and *P. fluorescens* (PF-23 strain) on density of trichomes in *Salvia officinalis* plants grown in pot cultures under greenhouse conditions (Ghorbanpour et al. 2014)

PGPRs treatment (PP-159) have strong antibacterial activity (disc diffusion) against the test pathogenic microorganisms including gram-positive (*Staphylococcus aureus, S. epidermidis, Enterococcus faecalis,* and *Streptococcus agalactiae)* and gram-negative (*Escherichia coli*) bacteria (Tables 3.2 and 3.3). The EOs obtained from PP-inoculated plants showed the maximum antibacterial activity with 23.44-mm inhibition zone against *Staphylococcus aureus*. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values for *Escherichia coli* were 5 and 10 µl for EOs obtained from control plants, while

Table 3.2 Antibacterial activity of *Salvia officinalis* essential oils against test microorganisms in plants inoculated with a PGPR strain (PP-159) and uninoculated control plants (Ghorbanpour et al. 2014)

Pathogenic bacteria	Inhibition zone (mm)		EOs (μL) in 5 ml of pathogen suspension							
			EOs of control plants				EOs of PP-159-inoculated plants			
	EOs of control plants	EOs of PP-159 treated plants	5	10	20	30	5	10	20	30
Staphylococcus aureus	19.54 ± 1.61	23.44 ± 1.74	−	+	+ +	+ + +	+	+ +	++ +	++ ++
Staphylococcus epidermidis	14.32 ± 1.42	18.36 ± 1.14	−	+	+ +	+ + +	−	+	++	++ +
Escherichia coli	8.65 ± 0.76	11.78 ± 0.65	−	−	−	+	−	−	+	++
Enterococcus faecalis	12.62 ± 0.98	13.54 ± 1.12	−	+	+ +	+ + +	−	+	++	++ +
Streptococcus agalactiae	11.23 ± 1.01	10.42 ± 0.93	−	+	+ +	+ + +	−	+	++	++ +

Antimicrobial activities were expressed as inhibition diameter zones in millimeters (mm), − (negative) = 0 mm; + (weak) = 1–4 mm; ++ (moderate) = 5–10 mm; +++ (strong) = 10–15 mm and ++++ (very strong) ≥16 mm

Table 3.3 Antibacterial activity of *Salvia officinalis* essential oils (MIC and MBC, μg/ml) against test microorganisms in plants inoculated with a PGPR strain (PP-159) and uninoculated plants using the disc diffusion method (Ghorbanpour et al. 2014)

Pathogenic bacteria	EOs of control plants		EOs of PP-159-inoculated plants	
	MIC	MBC	MIC	MBC
Staphylococcus aureus	2	4	1	2
Staphylococcus epidermidis	3	5	3	4
Escherichia coli	5	10<	3	6
Enterococcus faecalis	3	4	2	4
Streptococcus agalactiae	3	6	4	6

MIC minimal inhibitory concentration; *MBC* minimal bactericidal concentration

these values were 3 and 6 μl for plants inoculated with PP-159, respectively (Table 3.3).

Banchio et al. (2008) studied the effects of PGPR strains *P. fluorescens, B. subtilis, Sinorhizobium meliloti, and Bradyrhizobium* sp. on qualitative and quantitative composition of EOs in *Origanum majorana*. The results demonstrated that inoculation with PGPRs can increase the production of certain terpenes. Plants

inoculated with *Bradyrhizobium* sp. or *P. fluorescens* showed significant increases in total EOs yield by 10- and 24-fold, respectively. Based on the results, they suggested that increases in total EOs yield in response to inoculation were not merely due to increased biomass, and might have resulted from increased biosynthesis of terpenes. The main compounds affected by inoculation with *P. fluorescens* were terpinene-4-ol, *cis*-sabinene hydrate, *trans*-sabinene hydrate, and a-terpineol as the concentrations of these compounds in inoculated plants were >1,000-fold higher than uninoculated controls. Furthermore, the lack of effect of *B. subtilis* and *S. meliloti* strains tested was attributed to their poor adaptation to root exudates and/or insufficient root colonization.

The synergistic effects of combined inoculation of PGPRs with AM fungi have been reported on the production of EOs in plants. According to Prasad et al. (2012), the chemical composition of geranium oil was significantly affected by inoculation with phosphate-solubilizing bacteria (PSB) and/or AM fungi and phosphate fertilization. The content of linalool, geranial, 10-epi-γ-eudesmol, and citronellol in geranium oil increased and that of *cis*- and *trans*-rose oxide decreased by inoculation with PSB alone or in combination with AM fungi as compared to uninoculated controls. The changes in various constituents in the EOs of all inoculated and fertilized geranium plants could be related to the enhanced uptake of P and divalent metallic cations in plant tissues (Prasad et al. 2012).

In a study by Vafadar et al. (2013), tissue culture-regenerated plantlets of *Stevia rebaudiana* Bertoni were transferred to pots and subsequently inoculated with PGPR strains (*B. polymyxa, P. putida,* and *Azotobacter chroococcum*) and an AM fungus (*G. intraradices*). Although inoculation with a single microorganism significantly increased the stevioside content, the highest stevioside value was obtained in plants dually inoculated with *G. intraradices* + *Azotobacter chroococcum,* followed by *G. intraradices* + *B. polymyxa* and *Azotobacter chroococcum* + *P. putida*. Triple inoculations had less positive effects compared to dual inoculations, probably due to higher competition between microorganisms (Vafadar et al. 2013).

The root system of Italian oregano (Origanum × majoricum) was subjected to inoculation with three PGPR strains (*P. fluorescens, B. subtilis,* and *Azospirillum brasilense*), and the EOs content was measured (Banchio et al. 2010). The total EOs yield for plants inoculated with *P. fluorescens* or *Azospirillum brasilense* was approximately 2.5-fold higher than controls, without change of quantitative oil composition. The major EOs compounds *cis*- and *trans*-sabinene hydrate, γ-terpinene, carvacrol, and thymol showed increased biosynthesis.

Nonpathogenic PGPRs have been shown to stimulate the biosynthesis of secondary metabolites in plants through a mechanism termed ISR (induced systemic resistance) (Van Loon and Glick 2004). It is well established that biological agents can act as effective elicitors of key enzymes involved in biosynthetic pathways of secondary metabolites (Chen et al. 2000), which are clearly related to plants' defense responses against pathogenic agents despite being induced by nonpathogenic bacteria (Kloepper 1993).

Biosynthesis of terpenoids depends on primary metabolism, e.g., photosynthesis, and oxidative pathways for carbon and energy supply (Singh et al. 1990).

Giri et al. (2003) found that net photosynthesis of PGPRs hosting plants increases as a result of improved nutritional status. Factors linked with increased dry matter production may influence the interrelationship between primary and secondary metabolism, leading to increased biosynthesis of secondary metabolites (Shulka et al. 1992). The increased plant biomass appears to be correlated with a greater availability of substrate for monoterpene biosynthesis (Harrewijn et al. 2001). The increased concentration of monoterpenes in PGPR-inoculated plants may be due to growth-promoting substances produced by the microorganisms, which affect metabolic pathways in plants.

The effect of combined inoculation of *Begonia malabarica* Lam. (Begoniaceae) plants by an AM fungus (*G. mosseae*), a PGPR strain (*B. coagulans*), and *T. viride* was studied on the production of secondary metabolites (Selvaraj et al. 2008). Plants inoculated with microbial consortium consisting of *G. mosseae* + *B. coagulans* + *T. viride* showed the highest increase in leaf secondary metabolites (total phenols, ortho dihydroxy phenols, flavonoids, alkaloids, and tannins) followed by the plants dually inoculated with *G. mosseae* + *B. coagulans*.

In a similar study, the effects of the AM fungus *G. aggregatum,* the PGPR strain *B. coagulans,* and *T. harzianum* were evaluated on secondary metabolites content of *Solanum viarum* seedlings (Hemashenpagam and Selvaraj 2011). Triple inoculation of *G. aggregatum* + *B. coagulans* + *T. harzainum* resulted in maximum secondary metabolites including total phenols, orthodihydroxy phenols, flavonoids, alkaloids, saponins, and tannins. Here, the higher secondary metabolites values in inoculated plants were attributed to the enhanced mycorrhizal colonization and improved nutrient status of the host plants.

Cappellari et al. (2013) investigated the effects of single inoculation and co-inoculation with two PGPR strains (*P. fluorescens* and *Azospirillum brasilense*) on EOs composition and phenolic content in Mexican marigold (*Tagetes minuta*) and observed that EOs yield increased by 70 % in *P. fluorescens*-inoculated and co-inoculated plants in comparison with uninoculated controls, without altering the EOs composition. The biosynthesis of major EOs components increased in inoculated plants. The total phenolic content was two-fold higher in singly inoculated or co-inoculated treatments than in uninoculated control plants. Accordingly, they suggested that considering the economic importance of monoterpenes and phenolic compounds for a variety of applications in food and cosmetic industries, *P. fluorescens* and other suitable PGPRs have clear potential for improving EOs yield and productivity of cultivated medicinal plants.

Employing microorganisms as coculture in biotization has been another important area of research in recent decades. Biotization is a metabolic response of in vitro-grown plant materials to microbial inoculants leading to developmental and physiological changes of the derived propagules, which enhances resistance against biotic and abiotic stresses in plants. Here, plantlets are usually cocultured with PGPRs to achieve higher biomass and secondary metabolites. For instance, *Origanum vulgare* L. plantlets cocultured with *Pseudomonas* spp. produced more phenolics and chlorophyll than nonbacterized control plants (Nowak 1998).

The use of biotic elicitors is one of the effective strategies to increase the production of important secondary metabolites in plants. Secretions or bioactive

components from PGPRs (Fig. 3.4), besides being involved in plant growth promotion, are the components that were found to work in an elicitor signal transduction network. On the other hand, indole acetic acids (IAAs), cytokinins (CTKs), gibberellins (GAs), brassinosteroids (BRs), salicylic acid (SA), jasmonic acid (JA or analogs), ethylene, abscisic acid (ABA), nitric oxide (NO), and ROS which increase plant immunity by activating defense pathways, have long been observed to be transducers of elicitor signals in the production of plant secondary metabolites. Multiple signaling pathways and important mechanisms of action of elicitors in the biosynthesis of plant secondary metabolites are shown in Fig. 3.8. Signal perception is regarded as the first committed step of the elicitor signal transduction pathways in plants. Following perception, plant receptors are activated initially, and then in turn they activate their effectors such as ion channels, GTP-binding proteins (G-proteins), and protein kinases. The activated effectors transfer the elicitor signals to secondary messengers, which further amplify the elicitor signal to other downstream reactions (Ebel and Mithoefer 1998; Blume et al. 2000). The sequentially occurring events and reactions in elicitor-induced defense pathways can be organized as follows: perception of elicitor by a receptor, reversible phosphorylation and dephosphorylation of plasma membrane proteins and cytosolic proteins, cytosolic [Ca^{2+}] cyt spiking, plasma membrane depolarization, Cl^- and K^+ efflux/H^+ influx, extracellular alkalinization and cytoplasmic acidification, mitogen-activated protein kinase (MAPK) activation, NADPH oxidase activation and ROS production, early defense gene expression, ethylene and jasmonate production, late defense response gene expression, and accumulation of secondary metabolites (Zhao et al. 2005).

Different molecules produced by PGPRs play a crucial role in pathways linked to the biosynthesis of secondary metabolites. Salicylic acid (SA) is a well-known inducer of plant's systematic acquired resistance (SAR) in plant–microbe interactions through inducing expression of genes related to the biosynthesis of certain classes of secondary metabolites in plants (Taguchi et al. 2001). For example, indole alkaloids can be induced in *C. roseus* cell cultures by acetylsalicylic acid, an analog of SA (Zhao et al. 2000). Nitric oxide (NO), besides its effects on root branching and architecture, serves as a signal molecule for plant growth, development, and defense (Neill et al. 2002). Transcriptional profiling studies showed that NO treatment induces some stress- and disease-related signal transduction component genes along with defense genes, implying that the NO signal pathway(s) could be involved in secondary metabolism (Aziz et al. 2003). In addition, fungal elicitors were shown to stimulate saponin production in ginseng cell cultures, and this is partially mediated by NO, with NO biosynthesis also being induced by the fungal elicitor (Hu et al. 2003).

Exposure of plant cell culture or intact plant to jasmonic acid (JA), methyl jasmonate, as well as their conjugated compounds can stimulate the biosynthesis of secondary metabolites (Tamogami et al. 1997). The JA signaling pathway is generally regarded as an integral signal for the biosynthesis of many plant secondary products including terpenoids, flavonoids, alkaloids, and phenylpropanoids. Many elicitors (like pathogens and PGPRs) stimulate endogenous JA biosynthesis in plants, so the JA signaling pathway functions as a transducer or mediator for

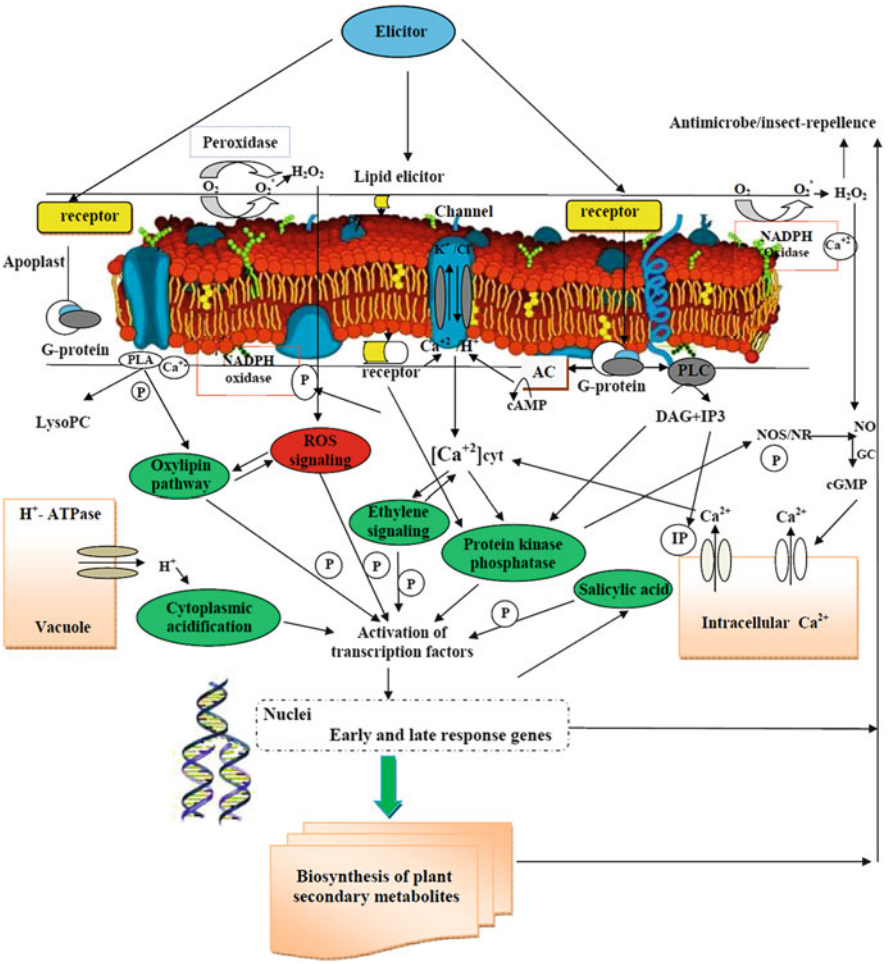

Fig. 3.8 A schematic model of signal transduction events by elicitors, leading to the expression of genes encoding enzymes involved in the biosynthesis of secondary metabolites in plants. Different elicitors are perceived by distinct membrane receptors, though they may activate the same signaling pathways. The activated receptors may then activate ion channels and G-proteins and subsequently activate phospholipases (such as PLA_2), through Ca^{2+} signaling or by G-protein coupling. Phospholipases hydrolyze phospholipids such as PC, into fatty acid and lysoPC; the former can function as a precursor for biosynthesis of JA and related oxylipins via the octadecanoid pathway, or peroxidized by reactive oxygen species (ROS) to produce another class of pentacyclic oxylipin phytoprostanes. Elicitors also activate mitogen-activated PK (MAPK) cascades that phosphorylate transcription factors regulating the expression of early and late response genes. All these pentacyclic oxylipins can activate biosynthesis of secondary metabolites in plants (modified from Zhao et al. 2005)

elicitor signaling pathways, leading to the accumulation of secondary metabolites in plants (Mueller et al. 1993). Application of methyl-jasmonate (0.5 mM) significantly increased the quantity of monoterpenes in basil (*Ocimum basilicum*) via increasing the number of transcripts of the enzymes linked to metabolic pathways of monoterpenes (Kim et al. 2003).

Ethylene is another phytohormone that regulates a wide range of plant processes including growth, development, and defense responses, and its production can be induced by various stresses and microbial infections like PGPRs. However, the concentration of ethylene in the culture is critical for acquiring desirable effects, i.e., low concentrations can promote the elicitor-induced production of secondary metabolites, whereas high concentrations may have inhibitory effects (Zhao et al. 2004).

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Growth regulators and plant hormones stimulate plant growth and terpene biosynthesis in a broad number of aromatic plant species, which result in beneficial changes in terpene quality and quantity (Farooqi and Sharma 1988).

Secretion of volatile organic compounds (VOCs) by PGPRs can be another possible mechanism for enhancing the production of plant secondary metabolites. All organisms produce VOCs, which are characterized by low molecular weight and high vapor pressure, and play important roles in communication within and between organisms. Bacterial VOCs have been reported as a rich source for new natural compounds that may increase crop productivity and EOs yield in medicinal and aromatic plants. Santoro et al. (2011) studied the effects of VOCs released by three PGPR strains (*P. fluorescens*, *B. subtilis,* and *Azospirillum brasilense*) on EOs composition of *Mentha piperita* (peppermint). The results showed that the production of monoterpenes increased two-fold in plants inoculated with *P. fluorescens,* which also increased biosynthesis of the two major EOs, (+) pulegone and (−) menthone. Menthol in *Azospirillum brasilense*-inoculated plants was the only major EOs constituent that showed a significant decrease. It has also been reported that the PGPR strain *B. subtilis* GB03 releases VOCs that elevate EOs accumulation in *Ocimum basilicum* (Banchio et al. 2009). Two major EOs components, R-terpineol and eugenol, increased by two- and ten-fold, respectively. This was seen in plants exposed to GB03 VOCs or with root inoculation, as compared to uninoculated controls. Some of the PGPRs proven to be biotic elicitors for the production of secondary metabolites in medicinal and aromatic plants are presented in Table 3.4.

Table 3.4 Efficient biotic elicitors used for the production of secondary metabolites in different plant species

PGPRs as elicitors	Plant species	Elicitation of secondary metabolites	Reference
Pseudomonas putida and *fluorescens*	*Hyoscyamus niger* L.	Hyoscyamine and scopolamine	Ghorbanpour et al. (2013a, b)
Pseudomonas putida and *fluorescens*	*Salvia officinalis* L.	*Cis*-thujone, camphor, 1,8-cineole	Ghorbanpour et al. (2014)
Glomus aggregatum, Bacillus coagulans, and *Trichoderma harzianum*	*Solanum viarum*	Total phenols, ortho-dihydroxy phenols, tannins, flavonoids, saponins, and alkaloids	Hemashenpagam and Selvaraj (2011)
Pseudomonas fluorescens	*Catharanthus roseus*	Ajmalicine	Jaleel et al. (2007)
Pseudomonas fluorescens	*Catharanthus roseus*	Serpentine	Jaleel et al. (2009)
Pseudomonas fluorescens, Bacillus subtilis, and *Azospirillum brasilense*	*Mentha piperita*	(+) pulegone and (−) menthone	Santoro et al. (2011)
Bacillus cereus	*Salvia miltiorrhiza* Bunge	Tanshinone	Zhao et al. (2010)
Trichoderma viride	*Catharanthus roseus*	Ajmalicine	Namdeo et al. (2002)
Glomus mosseae, Bacillus coagulans, and *Trichoderma viride*	*Begonia malabarica* Lam	Total phenols, ortho-dihydroxy phenols, tannins, flavonoids, and alkaloids	Selvaraj et al. (2008)
Pseudomonas fluorescens and *Bradyrhizobium* sp.	*Origanum majorana* L.	Terpinene- 4-o1, *cis*-sabinene hydrate, *trans*-sabinene hydrate, and α-terpineol	Banchio et al. (2008)
Pseudomonas fluorescens, Bacillus subtilis, and *Azospirillum brasilense*	*Origanum* × *majoricum*	*Cis*- and *trans*-sabinene hydrate, gamma-terpinene, carvacrol, and thymol	Banchio et al. (2010)
Hormonema ssp. homogenates	*Brugmansia candida*	Hyoscyamine and scopolamine	Pitta-Alvarez et al. (2000)
Bacillus polymyxa, Pseudomonas putida, Azotobacter chroococcum, and *Glomus intraradices*	*Stevia rebaudiana*	Stevioside	Vafadar et al. (2013)
Arbuscular mycorrhizal and phosphate-solubilizing bacteria	Rose-scented geranium (*Pelargonium* sp.)	Citronellol, geraniol, geraniol, and 10-epi-γ eudesmol	Prasad et al. (2012)

(continued)

Table 3.4 (continued)

PGPRs as elicitors	Plant species	Elicitation of secondary metabolites	Reference
Pseudomonas aeruginosa and *Pseudomonas fluorescens*	*Pisum sativum*	Phenolic compounds (gallic, cinnamic, and ferulic acid)	Bahadur et al. (2007)
Bacillus subtilis and *Pseudomonas fluorescens*	*Pelargonium graveolens*	Essential oil yield	Mishra et al. (2010)
Bacillus subtilis GB03	*Ocimum basilicum*	α-terpineol and eugenol	Banchio et al. (2009)
Pseudomonas fluorescens and *Azospirillum brasilense*	*Tagetes minuta*	Monoterpenes and phenolic compounds	Cappellari et al. (2013)

3.5 Conclusions

Infection by microorganisms as well as physiological and genetic factors and environmental conditions are the main agents affecting the accumulation and composition of secondary metabolites in plants. Among these, PGPRs seem to be a promising candidate considering their well-established role in plant nutrition, tolerance against biotic and abiotic stresses and enhancing the yield of different classes of secondary metabolites. As an environmentally friendly strategy, PGPRs should be considered to achieve sustainable high yields of industrially important secondary metabolites in plants using minimum chemical inputs.

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Chapter 4
Plant Growth-Promoting Microbes from Herbal Vermicompost

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4.1 Introduction

Overreliance on chemical pesticides and fertilizers has resulted in problems including safety risks, outbreaks of secondary pests normally held in check by natural enemies, insecticide resistance, environmental contamination, and decrease in biodiversity (Lacey and Shapiro-Ilan 2008). The increasing costs and negative effects of pesticides and fertilizers necessitate the idea of biological options of crop protection and production. This includes the use of animal manure, crop residues, microbial inoculum (*Rhizobium*, *Azotobacter*, *Azospirillum,* and blue green algae), and composts. They provide natural nutrition, reduce the use of inorganic fertilizers, develop biodiversity, increase soil biological activity, maintain soil physical properties, and improve environmental health (Hue and Silva 2000; Vessey 2003).

On the other hand, a progressive increase in world's population, intensive industrialization of food and beverage processing, and animal husbandry production leads to the generation of large volumes of organic wastes. As per the estimation of World Bank, municipal solid waste alone from the urban areas of Asia is projected to be 1.8 million tonnes/day in 2025 (Chandrappa and Das 2012). These can be disposed by landfilling, pelletization, incineration, biomethanization, and composting. Organic wastes act as a major source of environmental pollution and create serious disposal problem, release odor and ammonia into air, contaminate groundwater, and thereby pose health risks (Inbar et al. 1993). This problem can be solved by vermicomposting, a process of decomposing organic wastes into a valuable product of organic fertilizer and soil conditioner by the use of earthworms.

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Vermicomposting is an enhanced bio-oxidative and nonthermophilic organic decomposition process by the joint action of earthworms and microorganisms which involves a wide range of organic wastes such as horticultural and agricultural residues, weeds, dry leaves, cow dung, animal droppings, brewery wastes, sericulture wastes, municipal sewage sludge, industrial wastes, paper mills and dairy plants sludge, and domestic and kitchen wastes (Kumar 2005; Chitrapriya et al. 2013). The resultant product of vermicomposting is a stabilized, uniformly sized substance with a characteristic earthy appearance known as "vermicast/vermicompost." Vermicompost exhibits better performance on various plants during field application due to its enrichment with various macro- and microelements, enzymes, hormones, plant growth regulators, and antibiotics (Makulec 2002; Tilak et al. 2010). Detailed methods of vermicomposting have been documented by many authors (Domínguez 2004; Nagavallemma et al. 2004).

Vermicomposting accelerates decomposition rates which further leads to higher nutrient turnover (Mikola and Setälä 1998; Sampedro and Domínguez 2008) than the traditionally prepared compost which involves the action of microorganisms alone. Though microorganisms act as primary partner for the biochemical decomposition of organic matter, the earthworms, as secondary partner, are crucial drivers for the process and they are broadly grouped into three ecological categories: (1) anecics such as *Lumbricus terrestris*, *L. polyphemus,* and *Aporrectodea longa* are geophagous in nature and live in deep soils; (2) endogeics such as *A. caliginosa*, *Octolasion cyaneum*, *Pontoscolex corethrurus,* and *Aminthas* sp. reside just below the soil surface and feed the organic materials in soils, which were further subdivided into polyhumic, mesohumic, and oligohumic endogeic earthworms; and (3) epigeics such as *Eisenia foetida, L. rubellus,* and *Eiseniella tetraedra* live in the upper surface of soils and feed mainly on plant litter and other organic debris available on the soil surface. The details about different earthworm species, their ecological niches, characteristic features, and beneficial actions on decomposition have been reviewed by Pathma and Sakthivel (2012). Since the epigeic earthworms are consumers of a variety of organic matters, they are most suitable for vermicomposting process; however, the use of anecic and endogeic earthworms has also been reported (Lavelle and Martin 1992).

Each earthworm has its own characteristic features on decomposition of organic matter, and they are sensitive to fluctuating climatic and environmental conditions. For instance, *Eudrillus eugeniae* known as the "African night crawler" can decompose large quantities of organic wastes rapidly as it has higher growth and reproduction rates. Hence it is applied widely for vermicomposting and also in combination with other earthworms such as *E. foetida* and *Perionyx excavates* (Pattnaik and Reddy 2010); *P. excavates*, a commercially produced tropical earthworm known as "blues/Indian blues," is useful for vermicomposting in tropical and subtropical regions (Chaudhuri and Bhattacharjee 2002); *L. terrestris*, an introduced species of North America, is a long-living, cold-tolerant species which makes deep burrows beneath the frost line (Joschko et al. 1989). Domínguez (2004) reported different earthworm species, the factors affecting earthworm

survival (moisture content, temperature, pH, aeration, and ammonia), and also the process of vermicomposting.

Earthworms harbor a variety of decomposer microbes in their gut and excrete them along with nutrients in their excreta, and both are found to be mutual partners. Various enzymes and intestinal mucus in the earthworm's intestinal tract play a key role in the breakdown of organic macromolecules, which in turn results in a greater increment of the available surface area for microbial colonization, their biological activity, and higher nutrient retention. So, vermicompost is a hotspot for the isolation of beneficial microorganisms, including saprophytic bacteria and fungi, protozoa, nematodes, and microarthropods. Maintenance of mesophilic conditions throughout the entire process is another contributing factor (Domínguez et al. 2010). These microorganisms directly or indirectly offer many agriculturally favorable traits to the vermicompost, but exploration of those microbes has not been studied in detail, though enough reports are available for the microbial diversity of vermicompost (Huang et al. 2013; Pathma and Sakthivel 2012). An overview on the effect of vermicompost and associated microbes on agriculturally useful traits is depicted in Fig. 4.1.

Microbes with agriculturally favorable traits were categorized as plant growth-promoting (PGP) microbes—a heterogeneous group of beneficial bacteria/fungi/actinomycetes which promotes plant growth either directly (nitrogen fixation, phosphate solubilization, iron chelation, and phytohormone production) or

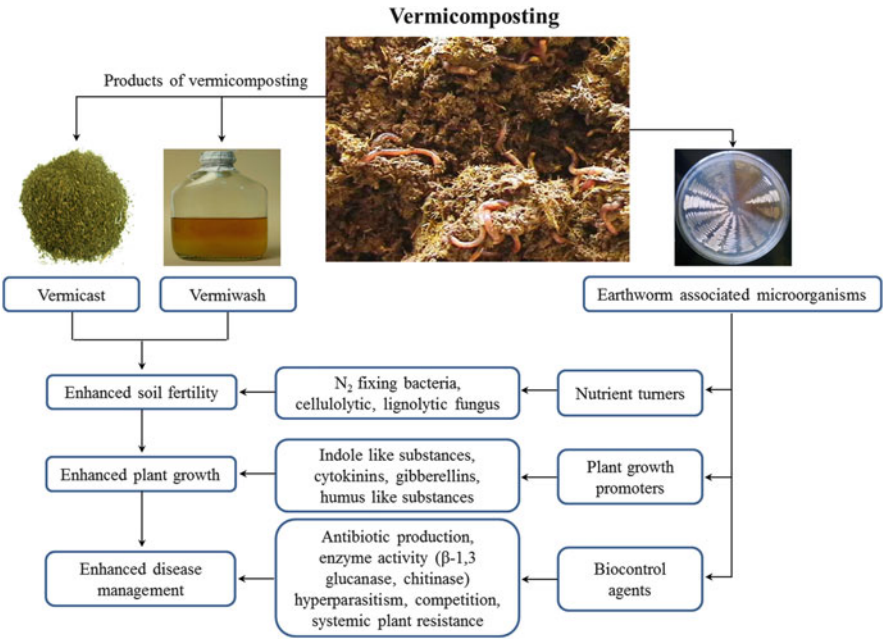

Fig. 4.1 Overview of vermicompost and its associated microbes on plant growth

indirectly (suppression of plant pathogenic organisms, induction of resistance in host plants against plant pathogens, and abiotic stresses).

PGP microbes include *Bacillus, Pseudomonas, Erwinia, Caulobacter, Serratia, Arthrobacter, Micrococcus, Flavobacterium, Chromobacterium, Agrobacterium, Hyphomycrobium,* and free-living nitrogen-fixing bacteria and also the members of the family Rhizobiaceae such as *Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium, Mesorhizobium,* and *Allorhizobium.* The practice of using such PGP microorganisms to agriculturally important crops as inoculants is getting attraction as it has a wide range of applications including the substantial reduction of the use of chemical fertilizers/pesticides, increased soil health, inhibitory activity against phytopathogens/insects, and enhanced crop yield (Bhattacharyya and Jha 2012; Mehboob et al. 2012). Hence, in this chapter, we intend to deliberate the usefulness of vermicompost and the associated microorganisms in enhancing soil health and agricultural productivity.

4.2 Microbial Diversity of Earthworms and Vermicomposts

Microbial communities including bacteria, actinomycetes, filamentous fungi, and yeast have been reported in earthworms such as *L. terrestris, Allolobophora caliginosa,* and *A. terrestris* (Parle 1963a, b), and most of them are mesophilic bacteria, fungi, and actinomycetes (Benitez et al. 1999; Sen and Chandra 2009; Vivas et al. 2009), which have been illustrated in Table 4.1. It is noticed that, earthworm's age hasn't showed any influence on microbial community (Fernández-Gómez et al. 2012), but the microbial counts between the earthworm species may vary due to their different ability to digest and assimilate microbial biomass, their ecological group, food, and environmental conditions in which earthworms live (Brown and Doube 2004). These factors make the vermicompost a hotspot of microbes. Unique indigenous gut-associated microflora has been documented in *E. foetida* (Toyota and Kimura 2000). In contrary, microbes living in traditional compost undergo a selection process during the heating phase, where the organic material is decomposed by specially adapted thermophilic bacteria (Dees and Ghiorse 2001). The microbial community which resides in the finished traditional compost are the facultative thermophiles, which form spores during the hot phase and recolonize during the mesophilic stage.

Microbial count in the ingested material of earthworms can be increased up to 1,000-fold while passing through their gut (Edwards and Fletcher 1988). Devi et al. (2009) have given a distinction on the microbial count of vermicomposts and of normal composts of fruit and vegetable waste, cow dung, and groundnut husk for bacteria, fungi, and actinomycetes. A similar trend of supporting evidence has been given by many research groups (Pedersen and Hendriksen 1993; Devliegher and Verstraete 1995). Microbial biomass and activity were also

Table 4.1 Microbial diversity of earthworms

S. no	Microorganisms	Earthworm	References
1	An oxalate-degrading *Pseudomonas oxalaticus*	*Pheretima*	Khambata and Bhat (1953)
2	Anaerobic N_2-fixing bacteria—*Clostridium butyricum, C. beijerinckii, and C. paraputrificum*	*E. foetida*	Citernesi et al. (1977)
3	*Streptomyces lipmanii* and *Streptomyces* spp.	*E. lucens*	Contreras (1980)
4	Actinobacteria	*L. rubellus*	Krištüfek et al. (1993)
5	Fluorescent pseudomonads	*L. terrestris*	Devliegher and Verstraete (1997)
6	*Aeromonas hydrophila*	*E. foetida*	Toyota and Kimura (2000)
7	Gammaproteobacteria, firmicutes, and actinobacteria	*L. rubellus*	Furlong et al. (2002)
8	*Pseudomonas, Paenibacillus, Azoarcus, Burkholderia, Spiroplasma, Acaligenes,* and *Acidobacterium*	*L. rubellus*	Singleton et al. (2003)
9	Novel nephridial symbiont, *Verminephrobacter eiseniae*	*E. foetida*	Pinel et al. (2008)
10	Gammaproteobacteria	*L. rubellus*	Knapp et al. (2009)
11	Acidobacteria, actinobacteria, bacteroidetes, chloroflexi, cyanobacteria, firmicutes, Gemmatimonadetes, nitrospirae, planctomycetes, proteobacteria, tenericutes, and verrucomicrobia	*L. terrestris*	Wüst et al. (2011)
12	Aeromonadaceae, comamonadaceae, enterobacteriaceae, flavobacteriaceae, moraxellaceae, "paenibacillaceae," pseudomonadaceae, rhodocyclaceae, sphingobacteriaceae, and actinobacteria	*A. caliginosa*	Ihssen et al. (2003), Horn et al. (2003)

significantly increased in vermicasts over composts (Brown and Doube 2004; Aira et al. 2006; Monroy et al. 2009). Earthworms' interaction with physical, chemical, and biological components affects the structural features of the microflora and microfauna in vermicompost (Domínguez et al. 2003; Lores et al. 2006; Monroy et al. 2009).

A recent study by Huang et al. (2013) on the bacterial communities of the earthworm *E. foetida* showed different phyla including Bacteroidetes, Firmicutes, Actinomycetes, Chlorobi, Planctomycetes, and Proteobacteria in vegetable waste compost, in which Bacteroidetes were predominant. Enrichment of Bacteroidetes (anaerobic group of microorganisms) in the vermicompost is probably due to the anaerobic conditions in the earthworm's gut (Karsten and Drake 1995). In contrast, Pathma and Sakthivel (2013) noticed *Bacillus* as the dominating genus followed by *Pseudomonas* and *Microbacterium* in goat manure compost. Bacterial diversity analysis of commercial composts (poultry litter, sewage sludge, and municipal solid waste) and homemade composts (vermicompost from food wastes) has been

registered with the groups such as Firmicutes: *Bacillus benzoevorans, B. cereus, B. licheniformis, B. megaterium, B. pumilus, B. subtilis,* and *B. macroides*; Actinobacteria: *Cellulosimicrobium cellulans, Microbacterium* spp., and *M. oxydans*; Proteobacteria: *Pseudomonas* spp. and *P. libaniensis*; ungrouped genotypes: *Sphingomonas* spp. and *Kocuria palustris;* and yeasts: *Geotrichum* spp. and *Williopsis californica* (Vaz-Moreira et al. 2008). Fischer et al. (1995) observed variations in the bacterial community of vermicasts and guts (including foregut, midgut, and hindgut) of earthworms in which the bacterial count of α, β, and γ subgroups of proteobacteria increased significantly toward the end of the gut and remained high in the cast. Among the subgroups, α-proteobacteria was higher in the hindgut and casts, and β- and γ- proteobacteria were predominant in the fore- and hindgut. Similar studies conducted by Nechitaylo et al. (2010) revealed the presence of Bacteroidetes, Alphaproteobacteria, Betaproteobacteria, and representatives of classes Flavobacteria, Sphingobacteria (Bacteroidetes), *Pseudomonas* spp., and unclassified Sphingomonadaceae (Alphaproteobacteria) and *Alcaligenes* spp. (Betaproteobacteria) in earthworm (*L. terrestris* and *A. caliginosa*), casts, and soil.

In addition to bacteria, several studies have also been reported for fungal diversity in vermicompost and earthworms. The phyla of Saccharomycetes, Lecanoromycetes, and Tremellomycetes dominated in the initial substrate of vermicompost (Bonito et al. 2010). The compost without earthworm was reported to have less fungal diversity, whereas during earthworm treatment, the fungal diversity has increased with Sordariomycetes, followed by Agaricomycetes, Pezizomycetes, Eurotiomycetes, Saccharomycetes, and Orbiliomycetes (Bonito et al. 2010; Huang et al. 2013). Besides this, other beneficial fungi in the vermicompost have also been noticed and some of the identified populations include *Paecilomyces* spp. and *Dactylaria biseptata* (Siddiqui and Mahmood 1996), *Cephaliophora tropica* (Morikawa et al. 1993), and *Trichoderma* spp. (Harman 2006). A study by Anastasi et al. (2005) also revealed the differentiation of fungal diversity in compost and vermicompost. Among the 194 fungal species isolated, 66 were common to both the compost and vermicompost, whereas 118 were obtained from compost and 142 from vermicompost. This concludes that fungal diversity is found more in vermicompost than in compost.

Next to bacteria, actinomycetes are the major gut flora of earthworm and have been reported widely in the literature (Parle 1963a, b; Ravasz et al. 1987; Ravasz and Tóth 1990; Jayasinghe and Parkinson 2009). It is noticed that vermicompost has higher actinomycetes than fungus in the final product, which might be due to the antagonistic activity of the former group against the latter group (Jayasinghe and Parkinson 2009). For instance, Yasir et al. (2009) and Huang et al. (2013) detected *Streptomyces* and *Rhodococcus,* the genera which have the ability to kill plant pathogens from vermicompost and fresh sludge. The actinomycetes present in the form of cell aggregates or individual cells and most of them belong to *Streptomyces* spp., the well-known antibiotic producers (Krištüfek et al. 1993, 1994, 1995). Other actinomycetes such as *Micromonospora* spp. were also recorded (Krištüfek et al. 1990; Polyanskaya et al. 1996).

Earthworms have food preference for substances colonized by certain fungal (Tiwari and Mishra 1993; Moody et al. 1995; Marfenina and Ishchenko 1997) and bacterial species (Wright 1972). Their food preference for actinomycetes has been demonstrated by Polyanskaya et al. (1996) on *E. foetida*, which actively consumed the spores of *S. caeruleus* than other actinomycete spores. Even though a substantial quantity of actinomycetes is digested in the foregut of the earthworms, the undigested remaining actinomycetes are able to develop rapidly in the earthworm's mid- and hindgut. Hence, the chances of survival for actinomycetes were found to be higher in earthworm's hindgut (Krištüfek et al. 1992; Polyanskaya et al. 1996; Zenova et al. 1996). These ingested actinomycetes inhibit the growth of other microorganisms particularly litter-decomposing and pathogenic fungi and Gram-positive bacteria in the earthworm's gut. This leads to the predominance of other actinomycetes and other antibiotic-resistant microorganisms and hence the biocontrol properties against various phytopathogens (Doube et al. 1994a, b; Stephens et al. 1994). Though the microbial community of bacteria/fungi/actinomycetes varies with the earthworm species/vermicompost, it also depends on the initial substrate of vermicompost.

4.3 Nutritional Values of Vermicompost

The nutritional quality of the vermicompost depends on the type of the initial substrate, earthworm species (epigeic, endogeic, and anecic), microbial population (cellulolytic, lignolytic, and N_2-fixers), and environmental conditions like aeration, humidity, pH, and temperature. The nutrient composition of vermicomposts has been documented with organic carbon 9.2–17.9 %, total nitrogen 0.5–1.5 %, available phosphorus 0.1–0.3 %, available potassium 0.1–0.2 %, calcium and magnesium 22–70 mg/100 g, copper 2–9.3 ppm, zinc 5.7–11.5 ppm, and available sulfur 128–548 ppm (Kale 1995). Vermicompost has higher concentrations of exchangeable Ca^{2+}, Mg^{2+}, and K^{+} than the initial substrate, which indicates the conversion of nutrients to plant-available forms during the passage in the earthworm's gut and associated microorganisms. Apart from the nutritional indices, the earthworm's activity also enhances the soil's physical qualities like bulk density, pore size, water infiltration rate, soil water content, and water-holding capacity (Edwards 1998).

A detailed study on the effect of substrate (cow dung, grass, aquatic weeds, and municipal solid waste), liming (enhances earthworm activity and microbial population), and microbial community (*Trichoderma viride, Phenerocrete crysosporium*—lignolytic fungus and *Bacillus polymyxa*—free-living nitrogen-fixing bacteria) on the nutritional status of vermicompost has been reported by Pramanik et al. (2007). They found that the usage of cow dung, *B. polymyxa,* and lime concentration of 5 g/kg was found to be the best combination in increasing NPK values, humic acid content, and enzyme activities like urease and phosphatase; however, *T. viridae* has shown equal nutrient effects irrespective of the lime content. Ghosh et al. (1999) demonstrated the difference in composting of organic

wastes such as cow dung, poultry droppings, kitchen wastes, municipal wastes, and dry leaves with and without *E. foetida* and observed higher availability of macro- and micronutrients in vermicast than compost without earthworms. Similarly, three- to fourfold increased NPK and micronutrient content on cow dung vermicompost than the noncomposted parental material was also noticed. Recent studies also concluded the nutritional enrichment of vermicompost over normal compost (Atiyeh et al. 2000; Hashemimajda et al. 2004; Lazcano et al. 2008). Hence, it can be concluded that the extensive usage of vermicompost can reduce the application of chemical fertilizers without affecting crop yield.

Vermicast has been documented with various enzyme activities including cellulase, amylase, invertase, protease, peroxidase, urease, phosphatase, and dehydrogenase in which the maximum enzyme activity is contributed by gut microbes (Sharpley and Syers 1976; Edwards and Bohlen 1996; Devi et al. 2009). Though vermicomposts have a wide range of enzyme activities, fluctuations are there during the composting period that the maximum enzyme activities were observed during 21–35 days in vermicomposting, whereas in conventional composting it was noticed on 42–49 days (Devi et al. 2009). This might be due to higher microbial count and activity in vermicomposts than the conventional composts. Since earthworms influence soil physical, chemical, and biological properties, they have been considered as soil engineers and as indicators of soil quality (Muys and Granval 1997; Jouquet et al. 2006).

4.4 Plant Growth Promoters of Vermicompost

Vermicompost was found to increase the growth of various vegetable, fruit, flower, and food crops not only by their macro- and microelement composition of the vermicast but also by their plant growth-promoting substances like growth hormones and enzymes. Microbes residing in the earthworm are the major contributors of such known and other unknown growth-promoting elements. *Rhizobium*, one of the PGP bacterium in soil that fixes nitrogen, was reported to disperse in soil by the earthworm *A. trapezoids* (Bernard et al. 1994). The first report on the identification of plant growth-promoting substances in earthworms was done by Nielson (1965). He identified indole-like substances in the tissue extracts of *A. caliginosa, L. rubellus,* and *E. foetida* and observed enhanced growth rate of garden pea. Various researchers reported substantial quantities of plant growth promoters such as auxins, gibberellins, cytokinins of microbial origin (Grappelli et al. 1985, 1987; Krishnamoorthy and Vajranabhaiah 1986; Tomati et al. 1988; Muscolo et al. 1999), and humic acids (Masciandaro et al. 1997; Atiyeh et al. 2002) in vermicomposts.

Vermiwash, the aqueous extracts of vermicompost, is a collection of excretory compounds of earthworms and also the associated microbes. It serves as a fertilizer and also a biocide due to the presence of macro- and micronutrients and antibiosis compounds. Hence, the use of vermiwash also registered increased plant growth on

a par with the use of hormones such as auxins, gibberellins, and cytokinins on plants such as *Petunia*, *Begonia,* and *Coleus* (Grappelli et al. 1987; Tomati et al. 1987, 1988). Nagavallemma et al. (2004) showed a marked difference in the plumule length of maize seedlings dipped in vermiwash than normal water. Comparative studies on the impact of vermiwash and urea solution on seed germination and on root and shoot length in cluster bean, *Cyamopsis tertagonoloba,* demonstrated the enhanced growth in vermiwash solution which might be due to hormone-like substances (Suthar 2010). HPLC and GC–MS analyses of the vermiwash of cattle waste-derived vermicompost showed the presence of significant amounts of indole acetic acid (IAA), gibberellins, and cytokinins (Edwards et al. 2004). Thus, it was demonstrated that both vermicompost and vermiwash are rich source of plant growth-promoting substances.

4.5 Biocontrol Properties of Vermicompost

Microbial population in vermicompost acts as powerful biocontrol agents due to the production of antibiotics and secretion of extracellular enzymes such as chitinase and lipase which cause the lysis of fungal and bacterial phytopathogens. Vermicompost is a valuable source of antagonistic bacteria and/or actinomycetes; several research reports are available to augment the biocontrol properties of vermicompost against phytopathogens such as *Botrytis cineria* (Singh et al. 2008), *Fusarium* spp. (Yeates 1981; Moody et al. 1996), *Gaeumannomyces* spp. (Clapperton et al. 2001), *Rhizoctonia* spp. (Doube et al. 1994a; Hoitink et al. 1997; Stephens et al. 1994; Stephens and Davoren 1997), *Phytophthora* (Ersahin et al. 2009), *Plasmodiophora brassicae* (Nakamura 1996), and *P. infestans* (Kostecka et al. 1996). Control of powdery mildew in barley (Weltzien 1989), balsam, and pea by vermicompost application has been demonstrated under field conditions (Singh et al. 2003). Pathogen control has been demonstrated in other crops like clover, cabbage, cucumber, grapes, tomatoes, radish, and strawberry (Jack 2011). Besides the biocontrol properties of vermicompost, vermiwash was also found to have biocontrol traits against *B. cineria, Sclerotinia sclerotiorum, Corticium rolfsii, R. solani, F. oxysporum* (Nakasone et al. 1999), *Erysiphe cichoracearum,* and *E. pisi* (Singh et al. 2003). Systemic plant resistance, microbial competition, antibiosis, enzyme activity, and hyperparasitism are the suspected reasons for pathogenic control (Hoitink and Grebus 1997). Yasir et al. (2009) documented the presence of chitinolytic bacteria *Nocardioides oleivorans, Streptomyces* spp., and *Staphylococcus epidermidis* from vermicompost with inhibitory activity against phytopathogens such as *R. solani, Colletotrichum coccodes, Pythium ultimum, P. capsici,* and *F. moniliforme*. Similarly, antibiotic heliomycin-producing *S. olivocinereus* has been isolated from *E. foetida's* gut (Polyanskaya et al. 1996). The dispersed actinomycetes from earthworms act as potential biocontrol agents against plant pathogenic fungi (Doube et al. 1994a, b; Stephens et al. 1994) due to their production capacity for a wide range of secondary

metabolites and antibiosis compounds. Besides pathogen control, insects or pests such as jassids, aphids, spider mites, mealy bugs, sucking pests, caterpillars, and beetles have also been controlled by vermicompost application (Edwards et al. 2007; Biradar et al. 1998; Rao et al. 2001; Rao 2002, 2003) under greenhouse and field conditions.

4.6 PGP Research at ICRISAT

ICRISAT has identified over 1,500 microbes including bacteria and actinomycetes, isolated from various composts and rhizospheric soil, in which at least one out of six has documented either single or multiple agriculturally favorable traits. Our research group has a collection of 137 actinomycetes isolated from 25 herbal vermicomposts prepared from *Jatropha curcas*, *Annona squamosa*, *Parthenium hysterophorus*, *Oryza sativa*, *Gliricidia sepium*, *Adhatoda vasica*, *Azadirachta indica*, *Capsicum annuum*, *Calotropis gigantea*, *Calotropis procera*, *Datura metal*, *Allium sativum*, *Zingiber officinale*, *Ipomoea batatas*, *Momordica charantia*, *Moringa oleifera*, *Argyranthemum frutescens*, *Nerium indicum*, *Allium cepa*, *Curcuma aromatica*, *Pongamia pinnata*, *Abacopteris multilineata*, *Nicotiana tabacum*, *Tridax procumbens,* and *Vitex negundo* using the epigeic earthworm *E. foetida* (Gopalakrishnan et al. 2013a) and demonstrated plant growth-promoting and biocontrol properties under laboratory, greenhouse, and field conditions.

Among them, actinomycetes, *Streptomyces* spp., *S. caviscabies, S. globisporus* sub sp. *caucasicus,* and *S. griseorubens* isolated from herbal vermicomposts, have registered in vitro PGP traits such as IAA and siderophore production and also documented their positive effect on the upregulation of PGP genes such as IAA and siderophore-producing genes. They proved these in vitro potentials by enhanced growth performance on rice under field conditions via increased tiller numbers, panicle numbers, filled grain numbers and weight, stover yield, grain yield, total dry matter, root length, root volume (Fig. 4.2), and root dry weight. In addition, they significantly enhanced rhizospheric total nitrogen, available phosphorous, % organic carbon, microbial biomass carbon, microbial biomass nitrogen, and dehydrogenase activity over the uninoculated control. Apart from the PGP traits, they also have the capacity to act as biocontrol agents due to the production of hydrogen cyanide and enzymes such as lipase, chitinase, and β–1,3 glucanase (Gopalakrishnan et al. 2012, 2013b, 2014). PGP actinomycetes such as *Streptomyces* spp., *S. tsusimaensis, S. caviscabies, S. setonii,* and *S. africanus* isolated from herbal vermicomposts have proved this by their inhibitory activity against *Fusarium oxysporum* f. sp. *ciceri* (FOC) (Gopalakrishnan et al. 2011a) and *Macrophomina phaseolina*, a causative agent for the charcoal rot of sorghum (Gopalakrishnan et al. 2011b) under greenhouse conditions. Antagonistic activity of these actinomycetes on Fusarium wilt-sick fields has also been demonstrated.

Besides the biocontrol activity of microbes isolated from herbal vermicomposts, washings of vermicompost, "vermiwash or biowash," were also demonstrated to

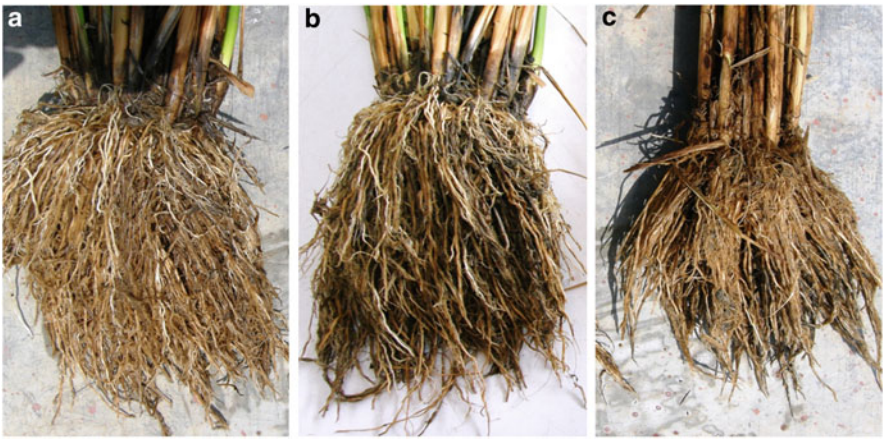

Fig. 4.2 Effect of PGP actinomycetes (**a**) *S. caviscabies* and (**b**) *S. globisporus* sub sp. *caucasicus* on root development of rice over (**c**) uninoculated control (Gopalakrishnan et al. 2014)

have inhibitory activity against phytopathogens. Crude biowash and partially purified extracts of vermicompost prepared from *Jatropha curcas*, *Annona squamosa,* and *Parthenium hysterophorus* marked their fungicidal activity on FOC, *S. rolfsii,* and *M. phaseolina* (Gopalakrishnan et al. 2010). Additionally, insecticidal activity has been registered by biowash and microbes isolated from herbal vermicomposts. Our investigation proved this via the biowash of *Annona, Datura, Jatropha*, Neem, *Parthenium, Pongamia*, and isolated PGP bacteria *B. subtilis*, *B. megaterium, Serratia mercescens,* and *Pseudomonas* spp.; fungus *Metarhizium anisopliae* and actinomycetes *S. cavourensis* sub sp. *cavourensis, S. albolongus, S. hydrogenans, S. antibioticus, S. cyaneofuscatus, S. carpaticus, S. bacillaris,* and *Streptomyces* spp. which were found to have broad-spectrum insecticidal properties against lepidopteran pests such as *Helicoverpa armigera, Spodoptera litura,* and *Chilo partellus* (Gopalakrishnan et al. 2011c; Vijayabharathi et al. 2014).

Besides the contribution of actinomycetes, bacteria have also been registered with PGP activity. Phosphate-solubilizing bacteria and *Azotobacter* have been isolated from the vermicompost of cow dung and saw dust with earthworms *E. eugeniae* and *P. excavatus* (Chitrapriya et al. 2013). Similarly, *Bacillus, Pseudomonas, Rhizobium,* and *Azotobacter* with in vitro PGP traits such as IAA, ammonia, and siderophore production were isolated from the vermicompost of paper mill sludge, leaf litter, and press mud with *E. foetida* (Prakash and Hemalatha 2013). A detailed study by Pathma and Sakthivel (2013), on vermicompost produced from straw and goat manure with *E. foetida*, identified 193 bacteria with antagonistic and/or biofertilizing potential. The dominance of identified bacteria was found to be in the order of *Bacillus* (57 %) > *Pseudomonas* (15 %) > *Microbacterium* (12 %) > *Acinetobacter* (5 %) > *Chryseobacterium* (3 %) with the other members such as *Arthrobacter, Pseudoxanthomonas, Stenotrophomonas, Paenibacillus, Rhodococcus, Enterobacter, Rheinheimera,* and *Cellulomonas*. Functional analyses of

these microbes have registered in vitro PGP traits such as phosphate solubilization, nitrate reduction, assimilation of different carbon sources, and production of IAA, siderophore, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, chitinase, lipase, and HCN. Besides this, they have also been reported with the production of commercially important enzymes protease, cellulase, amylase, xylanase, and Dnase. These studies thus conclude that vermicomposting organisms and biowash have the potency to promote plant growth, control the infectious diseases, and restrict pest attack. Hence, these PGP microorganisms are expected to replace inorganic fertilizer, pesticides, and artificial plant growth regulators which have numerous side effects to sustainable agriculture.

4.7 Conclusions

This chapter was intended to summarize the current knowledge on plant growth-promoting microbes associated with vermicompost. Vermicompost, vermiwash, and earthworm, in specific earthworm gut, nephridia and alimentary canal, have complex group of beneficial microorganisms. These microorganisms directly or indirectly contribute to the beneficial properties of vermicompost and vermiwash in enhancing soil health, plant growth, and hence agricultural productivity. Plenty of literatures are available for the presence/diversity of bacteria, fungi, and actinomycetes in vermicompost and earthworm and also for the enhanced plant growth by vermicompost application. However, studies related to the exploration of such potential microbes with plant growth-promoting properties are scarce. So, investigation on the isolation, identification, and characterization of plant growth-promoting microbes and their active metabolites from vermicompost will be useful for sustainable agriculture.

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Chapter 5
Effect of AM Fungi and Plant Growth-Promoting Rhizobacteria (PGPR) Potential Bioinoculants on Growth and Yield of *Coleus forskohlii*

Uliyan Sakthivel and Balathandayutham Karthikeyan

5.1 Introduction

Coleus forskohlii (family Lamiaceae) grows perennially in the tropical and subtropical regions of India, Pakistan, Sri Lanka, East Africa, and Brazil. Its roots are the source of a labdane diterpene compound called forskolin having a unique property to stimulate adenylate cyclase. Forskolin is also a potent vasodilatory, antihypertensive, and inotropic agent (Seamon 1984). The crop has a great potential due to the expected increase in demand for forskolin, which is widely used for the treatment of glaucoma, cardiac problems, and also certain types of cancers (Shah et al. 1980; Kavitha et al. 2010). Its ethnomedicinal uses for the relief of cough, eczema, skin infections, tumors, and boils have also been recorded (De Souza et al. 1986). Because of the continuous collection of roots from wild sources, this plant has been included in the list of endangered species (Boby and Bagyaraj 2003; Singh et al. 2009a). Recently, its cultivation has picked up as a crop with an annual production of about 100 t from 700 ha in India (Shivkumar et al. 2006).

Arbuscular mycorrhizal (AM) fungi of the phylum Glomeromycota are ubiquitous component of most agroecosystems, where they provide several benefits to their host plant, including better phosphorus nutrition (Toro et al. 1998; Parniske 2008), increased drought tolerance (Ruiz-Lozano and Azcon 1995), increased uptake of water (Graham and Sylvertsen 1984), and increased disease resistance (Pozo et al. 1999; Whipps 2004). Evidences are being accumulated to show that the inoculated AM fungi are an important component of organic farming (Powell and Bagyaraj 1984; Gosling et al. 2006) and can benefit annual crops, temperate fruit trees or shrubs, tropical plantation crops, ornamentals, spices, and medicinal and aromatic plants (Azcon-Aguilar and Barea 1997; Barea et al. 2004; Vestberg

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et al. 2002; Arpana et al. 2008; Singh et al. 2009a, b). Karthikeyan et al. (2007) also reported the occurrence of vesicle AM fungi in certain medicinal plants of the coastal plains of Tamil Nadu.

An intensive practice that warrants high yield and quality requires the extensive use of chemical fertilizers, which are costly and may create environmental problems. Therefore, more recently, there has been a resurgence of interest in environmental friendly, sustainable, and organic agricultural practice (Esitken et al. 2005). In this context, the use of biofertilizers containing plant growth-promoting rhizobacteria (PGPR) strains instead of synthetic chemicals may serve as an effective alternative and environmental friendly practice to improve plant growth through the supply of plant nutrients and soil productivity. Moreover, it has been found that exploiting these PGPR strains for growth promotion could reduce the need for chemical fertilizers as well as the cost of cultivation.

PGPRs have gained considerable interest in recent years. Many rhizosphere-colonizing bacteria especially PGPRs, including *Azotobacter, Azospirillum, Bacillus,* and *Pseudomonas*, typically produce substances that stimulate plant growth or inhibit root pathogens (Kloepper et al. 1992; Glick 1995; Mantelin and Touraine 2004; Compant et al. 2005; Weyens et al. 2009; Karthikeyan et al. 2008, 2009; Sakthivel and Karthikeyan 2012). Although numerous reports suggest the growth-promoting activities of bioinoculants, their use in the fields has not become popular or effective. The main limitation being the effective delivery system of bioinoculants particularly in case of vegetative propagated crops (multiplied by vegetative cuttings) for maintaining sufficient populations in the rhizosphere, though inoculation of seeds has been found effective in case of *Rhizobium* (Ben Rebah et al. 2002). A few reports exist on the symbiotic growth and yield response of patchouli to bioinoculants (Manjunatha et al. 2002; Arpana et al. 2008). Because of current public concerns about the harmful effects of agrochemicals, there is an increasing interest in improving the understanding of cooperative activities among rhizospheric plant-beneficial microbial populations and how these might be applied to agriculture (Kennedy 1998; Bowen and Rovira 1999; Barea et al. 2004; Lucy et al. 2004; Malik et al. 2009).

Among the different groups of plant growth-promoting rhizobacteria, nitrogen-fixing and phosphorus-solubilizing/phosphate-mobilizing organisms may be considered to be important since they improve plant nutrition by increasing N and P uptake by plants, and they play a significant role as PGPR in the biofertilizers of crops (Karthikeyan et al. 2008, 2013).

Certain cooperative microbial activities can be exploited as low-input biotechnology, and form a basis for a strategy to help sustainable, environmentally friendly practices fundamental to the stability and productivity of both agricultural systems and natural ecosystems (Kennedy and Smith 1995). A plant-beneficial symbiosis may be obtained by the preinoculation of plants with the desired bioinoculants. Preinoculation with AM fungi/bioinoculants is an obvious management practice in crops established as transplants and may lead to increased yield (Sorensen et al. 2008), provided sufficient numbers are transferred and get established in the rhizosphere. Nurseries can realize two main benefits from introducing

bioinoculants to their plants: effective and stronger establishment resulting in superior growth of the plants in the nursery and improved performance after their transplanting in field (Gianinazzi et al. 2001).

5.2 Distribution and Population Dynamics of AM Fungi and PGPR in *Coleus forskohlii*

The occurrence of Arbuscular Mycorrhizal fungi (AMF) in the roots of several medicinal plants was noticed by Govinda Rao et al. (1989). The occurrence of spore and colonization in the roots of *Coleus forskohlii* is also reported. The percentage of mycorrhizal root colonization was significantly greater in plants inoculated with AM fungi compared to uninoculated plants. Maximum colonization was observed in plants inoculated with *G. bagyarajii*, which was significantly different from all other treatments, the next best being with *S. calospora*. Spore numbers in the root zone soil followed a similar trend. There was a positive correlation between the intensity of mycorrhizal colonization and growth response (Earanna et al. 2002).

Karthikeyan et al. (2007) also reported that certain species of medicinal plants were screened for their VAM association by collecting rhizosphere soil samples of individual species along with fine root from coastal plains. Fifteen species of medicinal plants screened were found to colonize with VAM fungi. Among 15 species, Thulasi (*Ocimum sanctum*) recorded the maximum (58 %) VAM fungal colonization followed by Nithya Kalyani (*Catharanthus roseus*). All the species of VAM fungi such as *Glomus* sp., *Gigaspora* sp., and *Acaulospora* were found.

5.3 Growth Promotion by AM Fungi and PGPR

The association between mycorrhizae and medicinal herbs could result in better plant growth as well as an increase in phytochemical concentrations. AM fungi are known to play a pivotal role in the nutrition and growth of plants in many production-oriented agricultural systems, but little is known about their potential effect on secondary metabolites in medicinal and aromatic plants (Kapoor et al. 2002a, b, 2004; Copetta et al. 2006; Khaosaad et al. 2006).

Mycorrhizal inoculation had a significant effect on the quality and quantity of essential oils of *Coriandrum sativum* (Kapoor et al. 2002b). Variations in plant growth and active principles in mycorrhizae-inoculated plants have been reported for many other medicinal plants (Sailo and Bagyaraj 2005; Copetta et al. 2006). The inoculation of AM fungi and other beneficial soil microorganisms significantly increased the biomass of different medicinal plants (Sena and Das 1998; Kothari et al. 1999).

The role of mycorrhizae on plant growth has often been related to the increase in the uptake of immobile nutrients, such as phosphorus. Inoculation with AM fungi improved phosphorus uptake in *Coleus aromaticus* (Earanna et al. 2001). *Coleus forskohlii* showed an increase in plant height, number of branches, biomass, P content, and forskolin content when it was inoculated with *Glomus bagyarajii* (Sailo and Bagyaraj 2005).

The mode of interaction between AM fungi and PGPR is a universally recognized interaction, marketing each symbiont as an individual entity capable of inducing growth. PGPRs interact with host plants and indigenous Rhizobia through endosymbiosis and release stimulatory control compounds, while AM fungi interact by forming infection sites (spores) on host plant's roots, increasing the susceptibility for Rhizobia and PGPR induction, all the while increasing the surface area through hyphal extensions (Bianciotto and Bonfante 2002). On coinoculation, AM fungi and PGPR initiate morphological, physiological, and biological changes in the rhizosphere and mycorrhizosphere with aims of attaining prolonged growth and fertility in various types of soil conditions. Such parameters are generated through interactions which promote nutrient acquisition, nitrogen fixation, phosphorus capture, exudates' secretion, and release of antipathogenic compounds (Barea et al. 2005). It was observed that AM fungi, in association with nitrogen-fixing bacteria, *Azospirillum brasilense*, increase plant productivity by stimulating AM fungi root colonization, thereby increasing the number of internal vesicles relaying nutrient capture and flow (Linderman and Paulitz 1990). Furthermore, inoculation of *Rhizobium* sp. with phosphate-solubilizing microorganism (PSM) *Pseudomonas striata* and AM fungi species *Glomus fasciculatum* enhanced plant yield and nutrient and phosphorus uptake for chickpea plants in phosphorus-deficient sandy clay loam soils (Zaidi et al. 2001).

In fact, the postinoculation period between 45 and 90 days was marked by significant levels of growth through collective combinations of PSM on root infection and spore density (Zaidi et al. 2001). This persistent symbiotic behavior between AM fungi, PGPR, and rhizobia suggested that similar results can be obtained in environmentally stressed soils where viable growth is hindered due to source availability. AM fungi species *Glomus fasciculatum* as a coinoculant with *P. fluorescens* exhibited varying deficit intensities. Individually, in water-deprived soil, *P. fluorescens* (Pf) had limited grain and biomass production, while coinoculation with AM fungi increased the assimilation of phosphorus treatment. However, when inoculated in water-deficient soil, dual inoculation with phosphorus fertilizer and AM + *P. fluorescens* inoculation significantly increased grain phosphorus and nitrogen concentrations as compared to uninoculated well-watered treatments (control). Root colonization was significantly higher in applications with dual inoculants, against control (uninoculated) and phosphorus fertilizer treatment in well-watered soils (Ehteshami et al. 2007). Such increased levels of colonization coincide with increased ACC-deaminase and chitinase activity (Shaharoona et al. 2006). Further, Ehteshami et al. (2007) suggest these gains market proliferation through the aid of plant hormones (phytohormones) and release of regulatory metabolites to counteract and maintain vitality during erratic intensities of water

deficit (Ehteshami et al. 2007). Earlier, Subramanian et al. (2006) suggested that the increased adsorptive surface area and densely proliferated root growth in the mycorrhizosphere complement increased root colonization and infection. These characteristics support the use of bioinoculants as potential remediation tools to combat water-deficit stresses. However, water uptake through a plant vascular system can be hindered if severe stresses disrupt root architecture and distribution, thereby affecting the rate of water absorption per unit root (Auge 2001).

Arpana and Bagyaraj (2007) reported the influence of inoculation with the arbuscular mycorrhizal (AM) fungus *Glomus mosseae* and the plant growth-promoting rhizobacteria microorganisms (PGPR) *Trichoderma harzianum* singly and in combination on the growth and yield of Kalmegh (*Andrographis paniculata*) at two levels of P fertilizer application, i.e., at the recommended level and 75 % of the recommended level. The plant height, plant spread, number of branches per plant, number of leaves per plant, leaf area, plant dry matter, plant P content, and andrographolide concentration were significantly higher in plants inoculated with both the organisms, at both the levels of P as compared to uninoculated plants.

The effect of mycorrhization on growth and development has been observed in other plant species, including members of Asteraceae. Mycorrhization of *Annona Squamosa* has been reported to have a marked effect on the height of the plant and fresh and dry weight of the roots and shoots compared to their respective controls (Ojha et al. 2008).

Karthikeyan et al. (2008) evaluated the effectiveness of AMF and phosphorus levels (100, 150, and 200 kg) for increasing biomass yield and ajmalicine content in a medicinal plant (*Catharanthus roseus*). The plants treated 200 kg P_2O_5/ha along with AMF had the maximum plant height, number of leaves, root biomass, phosphorus content, root colonization, spore count, and ajmalicine content on 120 days after planting when compared with the control plants.

5.4 Influence of AM Fungi and PGPR for the Growth and Yield of *Coleus forskohlii*

Sakthivel and Karthikeyan (2012) reported that the plant height of *Coleus forskohlii* significantly increased due to the inoculated *G. fasciculatum* and PGPR strains. The *G. fasciculatum* with PGPR consortium treatment (T_4) at 180 DAP recorded the maximum plant height of 68.5 cm/plant followed by the treatments T_1, T_3, and T_2. The uninoculated control treatment T_5 recorded the minimum plant height for all the sampling periods (Table 5.1 and Fig. 5.1).

The number of tubers per plant of *Coleus forskohlii* significantly increased due to the inoculated *G. fasciculatum* and PGPR strains. *G. fasciculatum* with PGPR consortium treatment (T_4) at 180 DAP recorded the maximum number of tubers per plant of 21.6 per plant followed by the treatments T_1, T_3, and T_2. The uninoculated

Table 5.1 Effect of AM fungi and plant growth-promoting rhizobacteria (PGPR) inoculation on plant height of *Coleus forskohlii*

Treatments	Plant height (cm/plant)			
	45 DAP	90 DAP	135 DAP	180 DAP
T_1—*Glomus fasciculatum*	23.8b	42.5b	55.2b	64.5b
T_2—*Achromobacter xylosoxidans*	21.6c	38.3d	49.6d	58.3d
T_3—*Azospirillum lipoferum*	22.5c	40.2c	52.9c	60.2c
T_4—Consortium (*G. fasciculatum* + *A. xylosoxidans* + *A. lipoferum*)	27.3a	49.7a	59.7a	68.5a
T_5—Control (uninoculated)	15.7d	31.4e	44.5e	51.3e

Means of trials; the mean values in vertical columns followed by the same letter do not differ statistically between themselves at $P \leq 0.05$

Fig. 5.1 Overall view of *Coleus forskohlii* in pot culture experiment

control treatment T_5 recorded the minimum number of tubers for all the sampling periods (Table 5.2).

The number of tuber length per plant of *Coleus forskohlii* significantly increased due to the inoculated *G. fasciculatum* and PGPR strains. The *G. fasciculatum* with PGPR consortium treatment (T_4) at 180 DAP recorded the maximum number of tuber length per plant of 19.5 per plant followed by the treatments T_1, T_3, and T_2. The uninoculated control treatment T_5 recorded the minimum tuber length for all the sampling periods (Table 5.3).

Plants inoculated with *G. fasciculatum* and PGPR strains performed equally well, showing that significant increases in treatment T_4 recorded the tuber wet weight and

Table 5.2 Effect of AM fungi and plant growth-promoting rhizobacteria (PGPR) inoculation on number of tubers per plant of *Coleus forskohlii*

Treatments	Number of tubers/plant			
	45 DAP	90 DAP	135 DAP	180 DAP
T_1—*Glomus fasciculatum*	7.5b	13.5b	16.6b	19.2b
T_2—*Achromobacter xylosoxidans*	6.2c	12.0c	14.0d	16.6d
T_3—*Azospirillum lipoferum*	6.5c	12.3c	15.5c	17.4c
T_4—Consortium (*G. fasciculatum* + *A. xylosoxidans* + *A. lipoferum*)	9.8a	16.4a	18.9a	21.6a
T_5—Control (uninoculated)	4.3d	9.8d	12.5e	14.3e

Means of trials; the mean values in vertical columns followed by the same letter do not differ statistically between themselves at $P \leq 0.05$

Table 5.3 Effect of AM fungi and plant growth-promoting rhizobacteria (PGPR) inoculation on tuber length per plant of *Coleus forskohlii*

Treatments	Tuber length (cm/plant)			
	45 DAP	90 DAP	135 DAP	180 DAP
T_1—*Glomus fasciculatum*	5.4b	9.2b	13.4b	16.9b
T_2—*Achromobacter xylosoxidans*	4.5c	7.4d	10.7d	13.5d
T_3—*Azospirillum lipoferum*	4.8c	8.6c	12.3c	14.8c
T_4—Consortium (*G. fasciculatum* + *A. xylosoxidans* + *A. lipoferum*)	6.5a	11.2a	16.9a	19.5a
T_5—Control (uninoculated)	2.0d	5.5e	9.0e	12.6e

Means of trials; the mean values in vertical columns followed by the same letter do not differ statistically between themselves at $P \leq 0.05$

dry weight of, respectively, 137.42 and 67.60 g/plant at 180 DAP followed by the treatments T_1, T_3, and T_2, compared with controls.

Forskolin concentration was not affected by any of the bioinoculant treatments, but as a result of higher tuber yields, the total forskolin yield (calculated) was significantly higher in plants treated with treatment T_4—*G. fasciculatum* + PGPR strains (97.0 %), T_1—*G. fasciculatum* (88.0 %), T_3—*Azospirillum lipoferum* (82.0 %), and T_2—*Achromobacter xylosoxidans* (78.0 %) than in T_5— controls (Table 5.4 and Figs. 5.2 and 5.3).

The results clearly indicate that efficient bioinoculants (*G. fasciculatum* and *Achromobacter xylosoxidans* + *Azospirillum lipoferum*) significantly improved plant growth parameters of *C. forskohlii*, in pot conditions, a finding also supported by Earanna et al. (2001).

Tuberous roots are the main economic part of *C. forskohlii*. *Glomus fasciculatum*, *Achromobacter xylosoxidans,* and *Azospirillum lipoferum* produced significantly higher dry root yields. Earlier reports indicating the usefulness of bioinoculants in improving growth and yield support these results (Earanna et al. 1999; Singh et al. 2009a). Higher root yields might also be caused by the

Table 5.4 Effect of AM fungi and plant growth-promoting rhizobacteria (PGPR) inoculation on tuber yield and forskolin content of *Coleus forskohlii*

Treatments	Tuber wet weight (g/plant)				Tuber dry weight (g/plant)				Forskolin content (%)
	45 DAP	90 DAP	135 DAP	180 DAP	45 DAP	90 DAP	135 DAP	180 DAP	
T_1—*Glomus fasciculatum*	23.35b	42.45a	69.20b	95.55b	15.30b	23.45a	35.20b	48.38b	88.0b
T_2—*Achromobacter xylosoxidans*	19.88c	36.61b	52.84c	76.10d	11.00c	18.30b	27.44c	38.65c	78.0d
T_3—*Azospirillum lipoferum*	20.56b	39.10b	55.72c	83.65c	13.15c	20.12b	32.38b	42.05b	82.0c
T_4—Consortium (*G. fasciculatum* + *A. xylosoxidans* + *A. lipoferum*)	36.44a	48.33a	86.25a	137.42a	18.85a	27.25a	46.65a	67.60a	97.0a
T_5—Control (uninoculated)	16.80c	28.15c	49.30d	64.88e	8.66d	14.20c	23.22c	29.40d	65.0e

Values in each column followed by different letters are significantly different at $P \leq 0.05$

Fig. 5.2 Effect of AM fungi and plant growth-promoting rhizobacteria (PGPR) inoculation on tuber yield of *Coleus forskohlii* (Pot culture experiment)

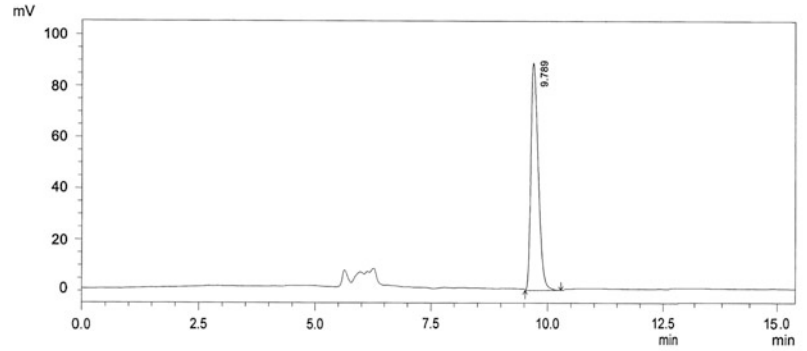
effectiveness of these bioinoculants and neem cake in controlling plant pathogens (Singh et al. 1980, 2011) and in providing nutrition to the plants. Bioinoculants also increased forskolin yield, which is supported by the results of Boby and Bagyaraj (2003). Sakthivel and Karthikeyan (2012) reported that PGPR inoculation of *Coleus forskohlii* significantly increased plant height, number of tubers, tuber length, tuber yield, and forskolin yield on 180th day after planting.

Singh et al. (2013) reported that higher tuber yields in *Coleus forskohlii* plants inoculated with *Glomus fasciculatum* and/or *Pseudomonas monteilii* under field conditions may result from the effectiveness of the bioinoculants, improving the availability of nutrients to the plants.

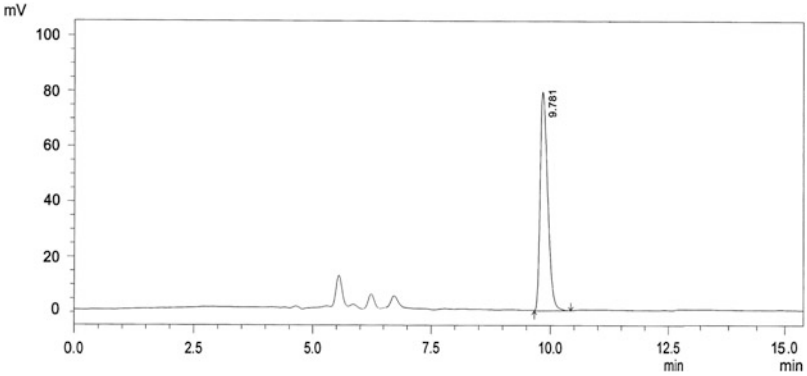
Santosh Dharana et al. (2006) reported the application of bioinoculants (*Glomus intraradices, G. fasciculatum, G. monosporum, G. mosseae, Sclerocystis dussii, Gigaspora margarita,* and a consortium of *A. chroococcum, A. lipoferum, P. striata,* and *trichoderma harzianum*) to significantly increased the plant height, plant spread, tuber yield, and forskolin yield in *Coleus forskohlii*.

Singh et al. (2009a) reported that treatments with AM fungus *Glomus fasciculatum* and *P. fluorescens* were the most effective that reduced the severity of root rot and wilt of *Coleus forskohlii* under lower and higher levels of pathogen *F. chlamydosporum*. *Glomus fasciculatum* increased the dry shoot and root weight, while in plants treated with *P. fluorescens,* an increase of dry shoot and root weight of *Coleus forskohlii*. A positive effect of *Glomus mosseae* and phosphorus levels was observed on growth, biomass yield, and ajmalicine content of *Catharanthus roseus* (Karthikeyan et al. 2008).

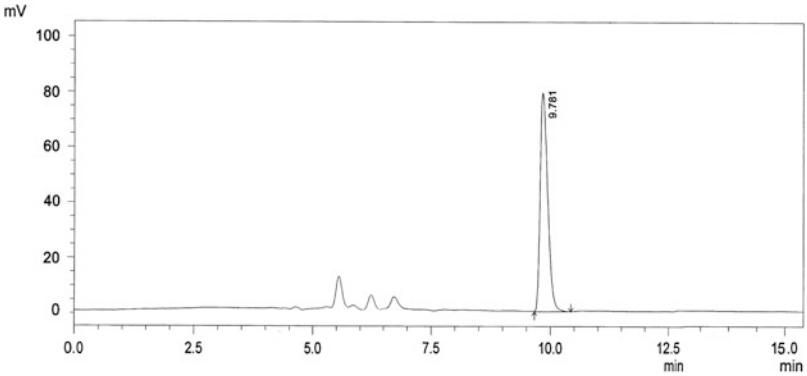
An investigation made by Karthikeyan et al. (2009) about the response of vesicular mycorrhizal fungi of *Glomus fasciculatum* on *Ocimum sanctum,*



T_1- *Glomus fasciculatum*



T_2-*Achromobacter xylosoxidans*



T_3- *Azospirillum lipoferum*

Fig. 3 (continued)

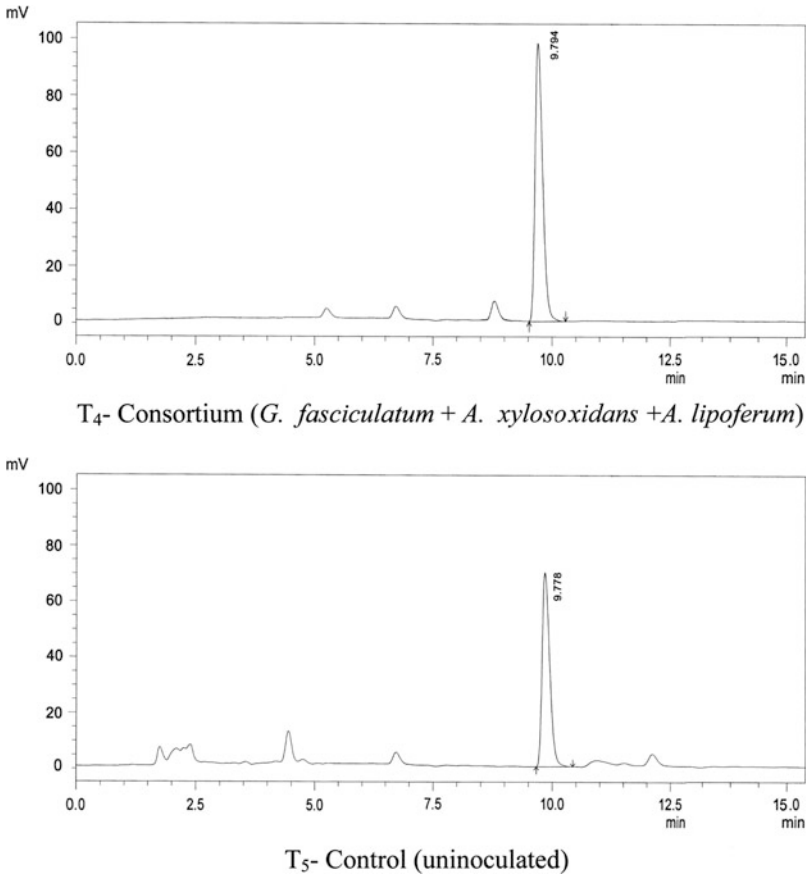


Fig. 5.3 Effect of AM fungi and plant growth-promoting rhizobacteria (PGPR) inoculation on forskolin content of *Coleus forskohlii* tubers by HPLC. T_1—*Glomus fasciculatum,* T_2—*Achromobacter xylosoxidans,* T_3—*Azospirillum lipoferum,* T_4—Consortium (*G. fasciculatum* + *A. xylosoxidans* + *A. lipoferum*), T_5—Control (uninoculated)

Catharanthus roseus, Coleus forskohlii, and *Cymbopogon flexuosus* revealed an increase in total dry matter production (shoot and root dry weight), protein content, and total chlorophyll contents in mycorrhizae-inoculated plants.

Sailo and Bagyaraj (2005) reported the AM fungi (*Glomus bagyarajii*) to significantly increase plant height, number of branches, length of fresh root, tuber dry weight, P uptake, and forskolin content of *Coleus forskohlii*. A study carried out by Senthilkumar et al. (2009) on *Artemisia palleus* has shown that the combined application of nitrogen, phosphorus, and *Azospirillum* resulted in the highest number of laterals per plants, increase in fresh and dry weight, and increase in photosynthetic efficiency of the crop.

5.5 The Role of AM Fungi in Soil and as a Potential Bioinoculant

When considering fungi as a source of soil inoculums, often negative connotations propelled by the intensive degradation by fungal species (e.g., *Fusarium oxysporum*) are contributing factors to agricultural condemnation. However, recent advances toward biotechnology have identified fungal species capable of promoting successive growth and increasing soil fertility (Sharif and Moawad 2006). The major groups of fungi that establish mutualistic symbiosis are categorized for their ability to interact with the roots of various plant species, referred to as mycorrhizal symbiosis (Ahmad et al. 2008a). AMF have been identified as existing entities in most agroecosystems, colonizing the root cortex biotrophically and establishing a mycelium bridge (hyphal network), connecting root to surrounding microhabitats (Egamberdieva et al. 2004). AM fungi are considered as obligate microbial symbionts, dependent on the colonization of host plants to maintain viability in the system. This mutually exclusive relationship benefits the host through correspondence with the mycorrhizal hyphal network, providing a large surface area for the absorption of essential immobile ions such as phosphate, copper, and zinc needed by the plant for sustaining growth (Paraskevopoulou Paroussi et al. 1997; Masoumeh et al. 2009). Mycorrhizal symbiosis also provides the plant with versatility against various biotic and abiotic stresses through the formation of stable soil aggregates, selective proliferation of synergistic microbial colonies, and formation of macropore structures in soil to facilitate aeration and water penetration to deep surface layers (Piotrowski et al. 2004). These compositional structure modifications and branching complexes allow nutrients to be sequestered from various deep soil reserves, mandating a push toward plant fitness and tolerance, increasing the probability of survival when subsurface nutrient concentrations are limited or faced with harsh environmental conditions (Ahmad et al. 2008b).

Macrophomina phaseolina (tassi) is a common root rot fungus, infecting about 500 plant species, one of which being *Cicer arietinum* (Srivastava et al. 2001). *Rhizobia* provide an initial barrier to fungal pathogens; however, through the use of AM fungus species, the potential for remediating pathogenesis while promoting growth is possible (Siddiqui and Akhtar 2009; Ozgonen and Erkilic 2007; Akkopru and Demir 2005). Akhtar and Siddiqui (2010) studied the influence of four AM fungi species, *Glomus intraradices, G. aggregatum, G. claroideum,* and *Glomus* sp., for the biocontrol of *M. phaseolina* on *Cicer arietinum* pod growth, nodulation, chlorophyll, nitrogen, phosphorus, potassium concentrations, and effectiveness of controlling root rot. The experimental design consisted of five randomized blocks, each with different treatments of *G. intraradices*, *G. aggregatum, G. claroideum*, *Glomus* sp., and Control in the presence and absence of *M. phaseolina*. The plants were harvested 90 days after inoculation and grown in sandy loam soil mixed with washed river sand and farm yard manure at the ratio 3:2:1. The inoculation of all four AM fungi species without treatment of *M. phaseolina* exercised all growth parameters as compared to the uninoculated control. Increases in shoot dry weight,

number of pods per plant, the number of nodules per root system, nitrogen, potassium, phosphorus, chlorophyll, and degree of root colonization by AM fungi were all exhibited after the 90-day harvest period, with *G. intraradices* optimizing greatest yields. The influence of *M. phaseolina*, interestingly enough shoot dry weight also increased, recording higher percentages, and then control and nonpathogen treatment. This gain corresponded to the increased shoot dry weight of pathogenic fungus manifested through AM fungi colonization; however, this also resulted in considerable decreases in the number of pods per plant as compared to non-*M. phaseolina* treatment (Akhtar and Siddiqui 2010). The number of nodules per root system stayed relatively the same, while root colonization of AM fungus was found to be considerably lower, suggesting the formulation of spores and/or the activation of plant defense mechanisms, inhibiting growth and colonization (Demir and Akkopru 2005). Through the influence of AM fungi on *M. phaseolina*-treated plants, a reduction in root rot index was seen, suggesting that the uninoculated control (index of 4) was less effective in secreting enzymes and biocontrol compounds necessary to maintain viability after infection (Pozo et al. 1999).

Arpana and Bagyaraj (2007) reported that the highest mycorrhizal root colonization in Kalmegh plant was observed when *G. mosseae* was coinoculated with *T. harzianum* at both levels of P (75 and 100 %). Among the single inoculated treatments, highest mycorrhizal colonization was observed in plants inoculated with the AM fungus *G. mosseae*, thus supporting the well-documented fact that inoculation with effective AM fungi enhances mycorrhizal root colonization (Rajan et al. 2000).

5.6 AM Fungi Interactions with PGPR as a Potential Bioinoculant

Diversity in the rhizosphere and surrounding microhabitats is marked by various interactive microfloras, stimulating mechanisms to promote or suppress microbial activity. AM fungi establish host specificity by infecting the host cortical cells, forming arbuscules along the plant root architecture. In this, the soil-dwelling *Rhizobium* and PGPR bacteria interact through endosymbiosis, forming an AM fungal endophytic bacteria capable of promoting rhizobial interactions with mycorrhizae and plant (Bianciotto and Bonfante 2002). The typical rhizobacteria–AM fungi interaction describes PGPR as the "mycorrhizae-helper microorganism/bacteria," active in stimulating mycelial growth and/or enhancing mycorrhizal formation (Garbaye 1994). PGPR or soil-dwelling Rhizobia interact with the mycorrhizal fungi by adhering to fungal spores and hyphal structures, initiating exposure and spread to other microorganisms capable of symbiosis within the rhizosphere (Bianciotto and Bonfante 2002). As PGPRs or rhizobia interact with the host plant, the rate of exudate expulsion increases. When aided by the presence of AM

fungi, the secretion of root exudates stimulates mycelial growth in the rhizosphere and initiates root penetration by the fungus (Azcon-Aguilar and Barea 1992).

Furthermore, as Azcon-Aguilar and Barea (1992, 1995) observed, the rhizobial interaction influences presymbiotic stages of AM fungal development such as spore germination and mycelia growth when coupled by the release of plant hormones, instigating AM establishment within the rhizosphere and root cortex. Such morphological transformations induce physiological changes within the plant and the surrounding environment to complement the interaction. Symbiosis alters the chemical composition of root exudates through changes in host's physiology, establishing shifts in mineral nutrient deposition of plant tissues, carbon allocation and utilization, and hormonal balances. However, physical development of AM mycelium in the rhizosphere/rhizoplane induces the synthesis and metabolism of essential plant and microbial parameters by acting as an abundant source of carbon (Barea et al. 2005). Secretion, uptake, and availability of root exudates, phytoalexins, and phenolic compounds become more abundant, prompting soil composition to become systemically modified to accommodate elevated interactions (Duponnois et al. 2005), thereby inducing physiological changes in the rhizobial community, marketing both quantitative and qualitative production of viable active symbionts, such as PGPR (Barea et al. 2005). This well-nourished and rich region of interaction and growth of mycorrhizae and mycelia is referred to as the mycorrhizosphere (Linderman 1988; Gryndler 2000). In the mycorrhizosphere, the principle of interaction is oriented toward promoting phosphorus uptake. Through the extensive branching between AM fungal mycelium and host root structures, access to phosphate ions in soil can be elevated, extending beyond the phosphate-depleting zone and into deeper regions in soil (Smith and Read 1997). Besides providing the vessel for transport and available carbon, AM fungi contributed to phosphorus capture by linking the biotic and geochemical portions of the soil ecosystem, thereby affecting both phosphorus cycling rates and patterns (Jeffries and Barea 2001).

Supplementing artificial phosphate feeds with aims of enriching soil content and interactions has shown mediocre gains. It has been suggested that through ecological soil exploration, the naturally occurring uptake of phosphate from bulk soils produces greater levels of activation and response between indigenous microflora and host plant parameters (Gupta et al. 2007). Because the availability of appropriate enzymes and secretion of stimulated growth factors promote rhizobial and soil competency, physiological and adaptive traits catered toward synchronizing symbiosis are induced (Barea et al. 2005). However, large doses of phosphorus fertilizer may potentially inhibit or hinder mycorrhizal growth and efficiency. As the surface area is more prevalent, host and PGPR may absorb more phosphorus at higher rates; however, biological response to meet the surplus may be overwhelmed and may hinder escalation to appropriate metabolite requirements without taxing the plant of other essential compounds (Gupta et al. 2007).

5.7 Conclusions

This study clearly indicated that the growth and yield of *C. forskohlii* could be reduced by soil amendments such as bioinoculants such as *G. fasciculatum, Achromobacter xylosoxidans*, and *Azospirillum lipoferum*. This management approach will be particularly useful under organic farming conditions, especially for medicinal plants, where the use of chemicals is restricted because of health and residue considerations.

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Chapter 6
Plant Growth-Promoting Rhizobacteria (PGPR): Emergence and Future Facets in Medicinal Plants

Shivesh Sharma, Vasudha Singh, Vivek Kumar, Shikha Devi, Keshav Prasad Shukla, Ashish Tiwari, Jyoti Singh, and Sandeep Bisht

6.1 Introduction

Medicinal plants are source of many potent and effective drugs which are used in different countries for their different therapeutic purposes (Mahesh and Satish 2008). Medicinal plants are easily accessible healthcare alternative, and approximately 60–80 % of the world's population still relies on these medicinal plants for the treatment of common illnesses (Menghani et al. 2011). According to the World Health Organization, more than 80 % of the world's population relies on traditional medicine for their primary healthcare needs (Shetty and Singh 1993; Goto et al. 1998). Humans depend on more than 9,000 plant species for food, clothing, shelter, medicines, forages, and industry, and about 1,200 herbal plants are mentioned in ancient Indian texts (Bairoch 2000; Yuan et al. 2010). About 900 species have been domesticated for agriculture, and from these about 168 species are specifically cultivated for food and agriculture (Bansal and Woolverton 2003).

India is a varietal emporium of medicinal plants, and it is one of the richest countries in the world as regards genetic resources of medicinal plants (Alluri et al. 2005). It is rich in its biological resources and has century's old heritage of medicinal plants and herbal medicines for curing human illness and promotion of

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health in tribal and rural areas. Its ethnic people and tribals living in the remote forest areas still depend to a great extent on the indigenous systems of medicine (Dutta and Dutta 2005). A wide range in topography and climate is exhibited in India which results in different types of vegetation and floristic composition. Moreover the agroclimatical conditions are conducive for introducing and domesticating new exotic plant varieties (Alluri et al. 2005). In India, out of 18,864 species of higher plants, 1,100 species are used in different systems (Das et al. 2009). The number of higher plant species (angiosperms and gymnosperms) is estimated between 215,000 and 500,000 species. Of these, only about 6 % have been screened for biological activity, and a reported 15 % have been evaluated phytochemically (Fabricant and Farnsworth 2001; Verpoorte 2000). Natural medicines are in great demand in the developed world for primary health care because of their efficacy, safety, and lesser side effects (News 2010). Medicinal plants represent a rich source of antimicrobial agents (Das et al. 2009).

Many researchers have reported the activity of various medicinal plants from various regions which are now being used as an alternative source for drugs (Rajakumar and Shivanna 2009). In studies carried out by Sharma et al. (2009a, b), it is observed that plants produce a wide variety of secondary metabolites which are used either directly as precursors or as lead compounds in the pharmaceutical industry, and it is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens. These active compounds inhibit the growth of disease causing microbes either singly or in combination (Cowan 1999). Inhibition of the growth of microbes by these active compounds is brought about by lysing the cell wall, breaking the peptide bonds, acting as chelating agents, binding their surface proteins, altering their biochemical systematics, or preventing utilization of available nutrients to the microorganisms (Cowan 1999; Maji et al. 2010; Zafar et al. 1999).

Though the screening of Indian medicinal plants has revealed varying degrees of activity against pathogenic microorganisms, due to lack of experimental scientific studies, confirmation of the antimicrobial properties of a great number of these remedies is not possible (Sharma et al. 2009a, b; Ahmed and Beg 2001). The most important bioactive constituents of plants are alkaloids, tannin, flavonoid, and phenolic compounds (Shihabudeen et al. 2010). Alternative sources for more natural and environmentally friendly antibiotics, antimicrobials, crop protection agents, and antioxidants are being searched by various industries; hence medicinal plants are being investigated thoroughly for their bioactivity for different pharmacological purposes. They are mainly interested in the discovery of active chemical structures from which they can develop and prepare synthetic analogues which are more controllable from the point of reproducibility, patentability, and safety and are more economically viable (Svoboda and Hampson 1999). Some researchers have observed that volatile oils of many plants are known to have antimicrobial activity (Henikoff et al. 1995). Plant essential oils also act as antioxidant which has been researched in detail with the view to investigate their protective role for highly unsaturated lipids in animal tissues (Henikoff et al. 1995; Deans et al. 1993; Ushimaru et al. 2007). The multidrug-resistant strain of many microorganisms

has revealed exploration of alternative antimicrobial agent. As reported by Bhaskarwar et al. (2008), *Jatropha podagrica* of family Euphorbiaceae is known for many biological activities such as antitumor, antimicrobial, molluscicidal, and anti-insect. *Jatropha podagrica* is also used as an antipyretic, diuretic, choleretic, and purgative (Kupchan et al. 1970; Sigrist et al. 2002). Medicinal plants have become the focus of intense study in terms of validation of their traditional uses through the determination of their actual pharmacological effects (Sen et al. 2008; Muniappan and Ignacimuthu 2011; Bhaskarwar et al. 2008; Nair and Chanda 2007; Pullaiah 2002). This can be brought about by using different computational approaches for identifying promising lead candidates for the development and study of the bioactive substances of medicinal plants.

The plant kingdom is a very rich resource for discovering new antimicrobial compounds for human medicine as well as many other applications such as food preservation, disease management in agriculture, veterinary disease control, and the coatings of household products (Fikret et al. 2000; Jagtap et al. 2009). Although molds, actinomycetes, and bacteria are the chief sources of antibiotics, antibacterial agents are also present in higher plants (Nimet 2002). Plants that possess therapeutic properties on the animal or plant body are generally designated as medicinal plants. With the development of microorganisms resistant to chemicals applied indiscriminately to crops, research has been done with the goal to search for alternative and safe forms of agrochemical pest control without causing any damage to environment and to humans, maintaining the crop qualitatively and quantitatively (Babalola 2010). The use of phytochemicals as natural antimicrobial agents commonly called biocides is gaining popularity (Menghani et al. 2011; Smid and Gorris 1999). The undocumented medicinal plants and practices of a specific community are known as ethnobotanical knowledge which is under the threat of habitat destruction and biopiracy. Unsustainable harvesting of these medicinal plants has led to exploitation and decrease of the species. Systematic efforts to exploit the valuable potential are still lacking (Rai 2004). The large-scale deforestation of green forest wealth, a renewable resource, is leading to an accelerated loss of valuable or potentially valuable biodiversity, extinction of species, and genetic erosion. It has been reported by Botanical Survey of India that around 93 % of medicinal plants of India now belong to endangered species. The soil factors also have very important effects on the quality and quantity of genuine regional drug (Ren et al. 2005).

The maintenance of a high diversity of plant species requires a correspondingly high level of diversity in the soil microbial community (Wardle 1992, 2002; Wardle and Nicholson 1996; Lugtenberg and Dekkers 1999). Plant growth-promoting bacteria are associated with many, if not all, plant species and are commonly present in many environments (Schroeder and Schwitzguebel 2004). Despite inhabiting different niches, rhizosphere-associated bacteria share some mechanisms that improve plant growth and/or protect them from soilborne deleterious organisms (Jain and Mudgal 1999).

6.2 Rhizospheric Bacterial Diversity

Medicinal plants harbor a distinctive microbiome due to their unique and structurally divergent bioactive secondary metabolites that are most likely responsible for the high specificity of the associated microorganisms (Shrivastava 2003; FAO Investment Centre Socio-economic and Production Systems Studies). In general, natural products play a highly considerable role in the drug discovery and development process, as about 26 % of the new chemical entities introduced into the market worldwide from 1981 to 2010 were either natural products or those derived directly therefrom, reaching a high of 50 % in 2010 (Newman and Cragg 2012; Koberl et al. 2013). Plant rhizosphere is a versatile and dynamic ecological environment of intense microbe plant interactions for harnessing essential micro- and macronutrients from a limited nutrient pool (Jeffries et al. 2003). Rhizosphere microorganisms thus provide a critical link between plant and soil environments (Kozhevin 1989). The "rhizosphere effect" is defined as the overall positive influence of interactions between plant roots and rhizoflora on the development of the plant (Manoharachary and Mukerji 2006; Kandeler et al. 2002; Micallef et al. 2009; Soderberg et al. 2002). The magnitude of the rhizosphere effect depends mainly on the nature and amount of root exudates which appear to be related to plant age as well as species on one hand and edaphic and climatic factors on the other hand (Pandey and Palni 2007). The original concept includes the soil surrounding a root in which physical, chemical, and biological properties have been changed by root growth and activity (Tizzard et al. 2006). Plants release organic compounds through root exudates and provide a rich environment for microbial activity (Pandey and Palni 1998).

The root exudates of different plants support the development of different bacterial communities. Root exudates provide a lot of nutrients for the soil microbes and energy materials (Tilak et al. 2005). Microorganisms affect the permeability of root cells, metabolism of roots, and absorption and excretion of certain compounds in root exudates. Norman (1961) found that certain polypeptide antibiotics, for example, polymyxin which is formed by *Bacillus polymyxa* from soil, altered cell permeability and increased leakage. There are two main difficulties in interpreting the significance of their results which show that culture filtrates or products increase the leakiness of plant roots. First, the conditions under which the organisms are grown are quite different both physically and nutritionally from those under which a rhizosphere population grows. Second, as it is not possible to calculate the concentration of biologically active substances in the rhizosphere, the concentrations used for "in vitro" experiments must of necessity be selected rather arbitrarily (Shukla et al. 2011). Interactions between plants and soil microbes are highly dynamic in nature and based on coevolutionary pressures (Dobbelaere et al. 2003; Duffy et al. 2004; Klironomos 2002; Morgan et al. 2005; Morrissey et al. 2004; Reinhart and Callaway 2006). Bacteria are the most abundant microorganisms in the rhizosphere, and they influence the plant physiology to a greater extent because of their ability to compete for root colonization (Glick 1995). The negative rhizosphere

effect shows growth inhibitory relationships or the antagonistic behavior of microbial groups growing around roots of some plants that also result in a reduced or smaller microbial population. This includes various categories of antagonism, such as competition, antibiosis, parasitism, and predation (Chaiharn et al. 2008). These antagonistic activities in a suppressive rhizosphere may maintain a low microbial population in the rhizosphere (Pandey and Palni 2007). The influence of individual plants is reflected in the rhizosphere as the R:S (rhizosphere to non-rhizosphere ratio). R:S ratio determines the relative stimulation of the microorganisms in the rhizosphere of different medicinal plant species (Pandey and Palni 1997). For bacteria and fungi, values commonly range from 5 to 20. Actinomycetes, a somewhat less affected group of microorganisms by the rhizosphere, may reveal R:S ratios between 2 and 12 (Pandey and Palni 1997).

6.3 Plant–Microbe Interaction

One of the most important indexes of soil quality is considered to be the diversity of microbial communities present. Alteration in the activity of microbes is proposed to be a sensitive indicator of anthropogenic effects on soil ecology (Shi et al. 2002; Brookes 1995). Plant growth-promoting rhizobacteria (PGPR) are beneficial soil bacteria, which may facilitate plant growth and development both directly and indirectly (Chernin and Chet 2002). The root surface and surrounding rhizosphere are significant carbon sinks (Kandeler et al. 2002). Photosynthate allocation to this zone can be as high as 40 % (Degenhardt et al. 2003). Thus, along root surfaces, there are various suitable nutrient-rich niches attracting a great diversity of microorganisms, including phytopathogens. Root exudates provide a lot of nutrients for the soil microbes and energy materials. Competition for the nutrients and niches is a fundamental mechanism by which PGPR protects plants from phytopathogens (Asghar et al. 2002; Duffy 2001). Rhizodeposition of various exudates provides an important substrate for the soil microbial community, and there is a complex interplay between this community and the quantity and type of compounds released (Kandeler et al. 2002; Marschner and Baumann 2003). Plant species is considered to be one of the most important factors in shaping rhizobacterial communities, but specific plant microbe interactions in the rhizosphere are still required to be studied to fully understand it (Micallef et al. 2009). Based on their effects on the plant, microbes interacting with plants can be classified as pathogenic, saprophytic, and beneficial (Ben et al. 2002). Various species of bacteria like *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus*, and *Serratia* have been reported to enhance the plant growth (Joseph et al. 2007).

Soil plant microbe interaction has got much importance in recent decades. Many types of microorganisms are known to inhabit soil, especially rhizosphere, and play important role in plant growth and development (Safdar et al. 2011). Direct stimulations by microbes on plants include fixed nitrogen, phytohormones, iron

that has been sequestered by bacterial siderophores, and soluble phosphate, whereas indirect stimulation includes preventing phytopathogens (biocontrol) and this interaction promotes plant growth and development (Juanda 2005). PGPR performs some of these functions through specific enzymes, which provoke physiological changes in plants at molecular level. Microbes interacting with plants can be classified as pathogenic, saprophytic, and beneficial, and beneficial microbes are often used as inoculants (Bloemberg and Lugtenberg 2001). According to scientific reports, 86 % of the bacterial isolates from the rhizosphere of various plants produced phytohormones and also different vitamins (Nelson 2004). Rhizosphere bacteria produce growth-promoting substances in culture media, in the rhizosphere, and in the rhizoplane of forage grasses and many economically important cereals like wheat and barley and vegetables, tomato, and bean plants under cultural conditions (Whipps 2001). They can be classified according to the goal of their application: biofertilizers (such as rhizobia, which have been applied commercially for over a century), phytostimulators (such as auxin-producing, root-elongating *Azospirillum*), rhizoremediators (pollutant degraders which use root exudate as their carbon source), and biopesticides (Glick and Bashan 1997). Plant growth by bacterial synthesis of plant hormones including indole-3-acetic acid, cytokinin, and gibberellins as well as by increased mineral and nitrogen availability in the soil is triggered by PGPR colonization (Saharan and Nehra 2011). Some of these plant hormones are also known to protect their host plant from pathogenic microorganisms. Bacteria that can produce indole-3-acetic acid (IAA) and siderophores and solubilize inorganic phosphate and HCN are capable of stimulating plant growth and help plants to acquire sufficient iron, phosphate, and other essential nutrients for optimal growth (Glick 1995, Idris et al. 2007; Chabot et al. 1996; Rajkumar et al. 2006). However, little work has been done on PGPR activities of forest plants (Chanway et al. 1991).

The role played by PGPR in relation to medicinal plants and their effect on the production of botanicals is an area remaining naïve (Sekar and Kandavel 2010). Plant microbial interactions can be classified into three basic groups: (1) negative (pathogenic) interactions; (2) positive interactions, in which either both partners derive benefits from close association (symbiosis), both partners derive benefits from loose association, or only one partner derives benefits without harming the other (associative); and (3) neutral interactions, where none of the partners derive a direct benefit from interaction and in which neither is harmed (Singh et al. 2004). The indigenous phosphate-solubilizing microorganisms of the selected medicinal plants and their inoculation in the plant rhizosphere can be used practically in increasing the growth of plants (Safdar et al. 2011). Alternate ways of plant growth by PGPR have also been observed like by associative N_2 fixation, solubilizing nutrients, promoting mycorrhizal function, regulating ethylene production in roots, releasing phytohormones, and decreasing heavy metal toxicity (Saharan and Nehra 2011). Scientific studies of PGP activities and biocontrol in medicinal plants are limited. There are two possibilities to influence the antagonistic/plant growth-promoting potential: (1) by managing the indigenous microbial potential, e.g., by the introduction of organic or inorganic amendments (Wardle 2002; Emmert and

Handelsman 1999; Conn and Lazarovits 2000), and (2) by applying autochthonous microorganisms as biocontrol or plant growth-promoting agents (Compant et al. 2005; Weller 2007; Weller et al. 2002; Whipps 2001). Direct plant growth promotion by microbes is based on improved nutrient acquisition and hormonal stimulation. Diverse mechanisms are involved in the suppression of plant pathogens, which is often indirectly connected with plant growth (Barazani and Friedman 1999; Khalid et al. 2004; Ashrafuzzaman et al. 2009; Bertland et al. 2001; De Freitas and Germida 1990; Husen 2003). Beneficial plant microbe interaction leads to the development of microbial inoculants for use in agricultural biotechnology (Berg 2009). Traditional knowledge is one of the most important sources for sustainable development of developing countries in various fields like agriculture, food, and medicine where biological resources are the main components. A wide array of natural products from botanicals are traditionally in use over several years (Janovska et al. 2003). An enhanced production is necessary for the increasing human population as well as basic compounds in industrial processes like in pharmaceutical industry (Berg 2009). It has been observed that physical and chemical properties of soil selected from different medicinal plant varied to some extent from soil to soil (Safdar et al. 2011). The potential PGPR strains have been recognized that can be used to inoculate tree roots in forests that require immediate attention. As suggested by Tizzard et al. (2006), studies are required on investigating the application of PGPR and fungi for commercial forestry operation, especially in the areas of enhancing tree growth and survival of tree seedlings through microbially mediated phytohormone production.

6.4 Plant Growth-Promoting Attributes

PGPRs are usually in contact with the root surface and improve growth of plants by several mechanisms, e.g., enhanced mineral nutrition, phytohormone production, and disease suppression (Kremer et al. 2004; Mauch et al. 1988; Shakilabanu et al. 2012; Schrey and Tarkka 2008; Tarkka et al. 2008; Hrynkiewicz and Baum 2011). Indian researchers have studied the diversity of rhizobacteria in a variety of plants, cereals, legumes, and others along with the assessment of their functionality based on the release of enzymes (soil dehydrogenase, phosphatase, nitrogenase, etc.), metabolites (siderophores, antifungals, HCN, etc.), and growth promoters (IAA, ethylene) and as inducers of systemic disease resistance (ISR) (Teixeira et al. 2007; Singh et al. 2013). Two groups of PGPR were described: one group is involved in the nutrient cycling and plant growth stimulation (biofertilizers) (Vessey 2003), and the second group is involved in the biological control of plant pathogens (biopesticides) (Whipps 2001). Medicinal plant constitutes a segment of the flora which provides raw materials for the use of industries producing pharmaceuticals, cosmetics, fragrance, and biochemicals (Karthikeyan et al. 2008). Medicinal plants like any other plants take nutrients from the soil during growth, and among macroelements, nitrogen results in the largest growth and yields response in

medicinal plants (Ordookhani et al. 2013; Ayub et al. 2011; Cox 1992). The contents of secondary metabolites are mostly increased through the positive effects on the metabolic pathways of active compound synthesis in medicinal plants (Papavizas and Ayers 1974; Ghorbanpour and Hatami 2013).

Biofertilizers are based on living microorganisms which colonize the rhizosphere or the interior of the plant (when applied to seed, plant surface, or soil) and promote growth by increasing the availability of primary nutrients to the host plant (Vessey 2003). There are a number of PGPR inoculants currently commercialized which promote plant growth with at least one mechanism, i.e., suppression of plant disease (bioprotectants), improved nutrient acquisition (biofertilizers), or phytohormones (biostimulants) (Kidoglu et al. 2007).

In summary, bacteria may support the plant growth by several mechanisms, e.g., increasing the ability of nutrients in the rhizosphere (1), inducing root growth and thereby increasing the root surface area (2), and enhancing other beneficial symbioses of the host (3), and by combination of modes of action (Vessey 2003). The occurrence of PGPR (*Azotobacter, Azospirillum, Bacillus*, and *Pseudomonas*) in the rhizosphere of medicinal plants *Catharanthus roseus, Coleus forskohlii, Aloe vera*, and *Ocimum sanctum* has been documented. Tamilarasi et al. (2008) isolated various bacteria from rhizosphere of 50 medicinal plants, which among the isolated bacteria, the dominant species was *Bacillus* followed by *Pseudomonas, Enterobacter, Corynebacterium, Micrococcus*, and *Serratia*. The main reason of microbial specificity toward the various medicinal plants could be due to the exchange of plant metabolites (Garagulia et al. 1974; Ramesh et al. 2012; Ghodsalavi et al. 2013). Although in the recent years several researches were conducted to study the effect of the PGPR on many plants, there is a lack of available reports on medicinal plants.

6.4.1 Phosphate Solubilization

Plant root-associated phosphate-solubilizing bacteria (PSB) have been considered as one of the possible alternatives for inorganic fertilizers for promoting plant growth and yield (Islama et al. 2007). The ability to solubilize various insoluble phosphates is always desirable to be a competent PGPR. Phosphorus is an essential nutrient for plant growth and is one of the most important elements after nitrogen (Nautiyal and Mehta 2001). It exists in organic and inorganic forms in soil and is commonly deficient in most natural soils, especially acidic soils with low pH. It is mostly fixed as insoluble iron and aluminum phosphates in acidic soil and fixed as calcium phosphates in alkaline soils. It is required for several key plant structure compounds like root development, stalk and stem strength, flower and seed formation, and crop maturity and production (Ordookhani et al. 2006). Phosphorus nutrition is important for crop quality and resistance to plant diseases. Different soils have varying phosphorus contents ranging between 0.02 and 0.5 %. Inadequate supply of phosphorus in soil can lead to diminished plant growth and plant

yield (Halford 1997). The quality of crops, vegetables, and fruits can be enhanced by providing sufficient phosphorus that will not only increase its yield but also improve its resistance to diseases. Plant can acquire phosphorus from soil which is available in the form of apatite (rock phosphate). Around 50–70 % phosphorus found in soil is in inorganic form and its uptake by plant is low (Altomare et al. 1999). The establishment and performance of phosphate-solubilizing microorganism is however affected severely under stressed conditions such as high salt, pH, and temperature prevalent in degraded ecosystems represented by alkaline soils with a tendency to fix phosphorus (Moran et al. 2001). The most efficient phosphate-solubilizing microorganism (PSM) belongs to the genera *Bacillus, Rhizobium*, and *Pseudomonas* among bacteria and *Aspergillus* and *Penicillium* among fungi. Among the whole microbial population in soil, phosphate-solubilizing bacteria (PSB) constitute 1–50 %, while phosphate-solubilizing fungi (PSF) are only 0.1–0.5 % (Chen et al. 2006). Bacterial isolates *Pseudomonas* sp. and *Azospirillum* sp. from the rhizosphere soil and root cuttings of *Piper nigrum* L. exhibit high phosphate-solubilizing ability in vitro (Ramachandran et al. 2007). The phosphate-solubilizing microorganisms found in the rhizosphere of the selected medicinal plant can be inoculated in the plant rhizosphere which can be used for increasing the growth of plants (Kidoglu et al. 2007). Bacteria were found to be more active than fungi in conversion of insoluble phosphorus to soluble phosphorus (Alam et al. 2002; Safdar et al. 2011). Several publications have demonstrated that phosphate-solubilizing strains of *Bacillus* sp. and *Pseudomonas* sp. increase growth and phosphorus content of non-leguminous as well as leguminous plants (Antoun et al. 2004; Chabot et al. 1998; Halder et al. 1990). In the study of Malviya and Singh (2012), phosphate-solubilizing bacteria were isolated from soil, and their effect on germination of G*lycine max* seeds as well as seedling growth was studied with an objective to develop a biofertilizer. Safdar et al. (2011) conducted an experiment to characterize the phosphate-solubilizing microorganisms (PSM) isolated from the rhizosphere of selected medicinal plants and their inoculation effect on plant growth and found that phosphate-solubilizing bacteria and fungi constituted 4.14 and 38.2 % of total microbial population, respectively.

6.4.2 Production of Indole-3-Acetic Acid (IAA)

IAA is phytohormone which is known to be involved in root initiation, cell division, and cell enlargement (Salisbury 1994). The physiologically most active auxin in plants is indole-3-acetic acid (IAA), which is known to stimulate both rapid (e.g., increases in cell elongation) and long-term (e.g., cell division and differentiation) responses in plants (Gray and Smith 2005). IAA is the most common and best characterized phytohormone. It has been estimated that 80 % of bacteria isolated from the rhizosphere can produce plant growth regulator IAA (Patten and Glick 1996; Patten and Glick 2002). In addition to IAA, bacteria such as *Paenibacillus polymyxa* and *Azospirillum* also release other compounds in the rhizosphere, like

indole-3-butyric acid (IBA), Trp and tryptophol, or indole-3-ethanol (TOL) that can indirectly contribute to plant growth promotion (Hayat et al. 2010; Lebuhn et al. 1997; El Khawas and Adachi 1999). Patten and Glick (2002) demonstrated that bacterial IAA from *P. putida* played a major role in the development of host plant root system. Similarly, IAA production in *P. fluorescens* HP 72 correlated with suppressing of creeping bent grass brown patch. Patten and Glick (2002) also showed that bacterial IAA stimulates the development of the host plant root system. The advantage for root-associated bacteria is a rich supply of nutrients, as much of the metabolic products of the carbon fixed by plants are lost from roots and move into the rhizosphere as exudates, lysates, and mucilage (Hayat et al. 2010). Independent of the origin (rhizosphere vs. phyllosphere), bacterial strains produced IAA, which accounts for the overall synergistic effect on growth of peas and wheat (Saharan and Nehra 2011). The highest concentration of IAA is produced by bacterial strain *P. fluorescens* and *Kocuria varians* (Egamberdieva 2008). Joseph et al. (2007) found while working on chickpea that all the isolates of *Bacillus, Pseudomonas*, and *Azotobacter* produced IAA, whereas only 85.7 % of *Rhizobium* was able to produce IAA (Joseph et al. 2007). Chaiharn and Lumyong (2011) successfully screened rhizobacteria for in vitro solubilization of inorganic phosphate, IAA production, and their effects on root elongation of bean and maize seedlings and found that *Klebsiella* isolated from rhizosphere was the best IAA producer and produced the highest amount of IAA (291.97 $\pm$0.19 ppm) in culture media supplemented with L-tryptophan. Khamna et al. (2010) isolated *Streptomyces* sp. from the rhizosphere soils of 14 Thai medicinal plants, which were found to produce the plant growth hormone indole-3-acetic acid (IAA) in a yeast malt extract medium supplemented with 2 mg/ml L-tryptophan. However, the effect of IAA on plants depends on the plant sensitivity to IAA and the amount of IAA produced from plant-associated bacteria and induction of other phytohormones (Peck and Kende 1995).

6.4.3 HCN Production

Rhizobacteria can inhibit phytopathogens by the production of hydrogen cyanide (HCN) and/or fungal cell wall-degrading enzymes, e.g., chitinase and β-1, 3-glucanase (Bloemberg and Lugtenberg 2001; Persello Cartieaux et al. 2003; Friedlander et al. 1993). HCN is produced by many rhizobacteria and is postulated to play a role in biological control of pathogens (Defago and Haas 1990). The cyanide ion is exhaled as HCN and metabolized to a lesser degree into other compounds (Alizadeh et al. 2013). HCN first inhibits the electron transport, and the energy supply to the cell is disrupted leading to the death of the organisms. It inhibits proper functioning of enzymes and natural receptors' reversible mechanism of inhibition (Corbett 1974), and it is also known to inhibit the action of cytochrome oxidase (Gehring et al. 1993). Fluorescent *Pseudomonas* strain RRS1 isolated from Rajnigandha (tuberose) produced HCN, and the strain improved seed germination

and root length (Saxena et al. 1996). HCN from *P. fluorescens* strain played a significant role in disease suppression of *F. oxysporum f.* sp. *radicis-lycopersici* in tomato (Duffy et al. 2003). Ramatte et al. (2003) reported that hydrogen cyanide is a broad-spectrum antimicrobial compound involved in biological control of root disease by many plant-associated fluorescent pseudomonads. The production of HCN by certain strains of fluorescent pseudomonads has been involved in the suppression of soilborne pathogens (Voisard et al. 1989). Suppression of black root rots of tobacco (Stutz et al. 1986) and consumption of wheat by *P. fluorescens* strain CHAO were attributed to the production of HCN (Defago and Haas 1990). *Pseudomonas fluorescens* HCN inhibited the mycelial growth of *Pythium* in vitro (Weststeijn 1990). Ahmad et al. (2008) explored for efficient PGPR strains with multiple activities; a total of 72 bacterial isolates belonging to *Azotobacter*, fluorescent *Pseudomonas*, *Mesorhizobium*, and *Bacillus* were isolated from different rhizospheric soils where it was found that HCN production was a more common trait of *Pseudomonas* (88.89 %) and *Bacillus* (50 %). However, the role of cyanide production is contradictory as it may be associated with deleterious as well as beneficial rhizobacteria (Bakker and Schippers 1987; Alstrom and Burns 1989; Ahmad et al. 2008).

6.4.4 Siderophore Production

Indirect plant growth promotion includes the prevention of deleterious effects of phytopathogenic organisms (Schippers et al. 1987; Glick and Pasternak 2003; Dobbelaere et al. 2003). This can be achieved by the production of siderophores, i.e., small iron-binding molecules. In soils, iron is found predominately as ferric ions, a form that cannot be directly assimilated by microorganisms. Siderophore production enables bacteria to compete with pathogens by removing iron from the environment (O'Sullivan and O'Gara 1992; Persello Cartieaux et al. 2003). Siderophore production is very common among *Pseudomonas* (Kozhevin 1989; O'Sullivan and O'Gara 1992; Boyer et al. 1999), and *Streptomyces* sp. has also been shown to produce iron-chelating compounds (Loper and Buyer 1991). Fluorescent *Pseudomonas* are characterized by the production of yellow-green pigments, termed pyoverdines which fluoresce under UV light and function as siderophores (Demange et al. 1987; Kloepper et al. 2004). The role of siderophores produced by fluorescent pseudomonads in plant growth promotion was first reported by Kloepper et al. (1981). *Pseudomonas* culture and purified siderophores showed good antifungal activity against the plant deleterious fungi, viz., *Aspergillus niger, A. flavus*, *A. oryzae, F. oxysporum*, and *Sclerotium rolfsii* (Manwar et al. 2004). Though siderophores are part of primary metabolism (iron is an essential element), on occasions they also behave as antibiotics which are commonly considered to be secondary metabolites (Haas and Defago 2005). Suryakala et al. (2004) have reported that siderophores exerted higher impact on *Fusarium oxysporum* than on *Alternaria* sp. and *Colletotrichum capsici*. Arora et al. (2001) isolated *Rhizobium*

meliloti from medicinal plant, *Mucuna pruriens*, which were able to produce siderophores that not only act as biocontrol agents against *M. phaseolina* but also proved to be plant growth promoter in nature as evidenced in the increase of seedling biomass and fresh nodule weight over uninoculated controls.

6.4.5 Biocontrol Activity

Most sustainable and environmentally acceptable control may be achieved using biocontrol agents due to the effort to reduce the use of agrochemicals and their residues in the environment and in food (Haggag and Abdel-latif 2007). Identifying, understanding, and utilizing microorganisms or microbial products to control plant diseases and to enhance crop production are integral parts of sustainable agriculture. Biological control is a potent means of reducing the damage caused by plant pathogens (Haggag 2002; Jeyarajan and Nakkeeran 2000). Biological control of plant disease can occur through different mechanisms, which are generally classified as antibiosis, competition, suppression, direct parasitism, induced resistance, hypovirulence, and predation (Johnson and Curl 1972; Chaurasia et al. 2005). The antagonistic activity has often been associated with the production of secondary metabolites (Haggag and Abdel-latif 2007; Silva et al. 2001). Plant-associated microorganisms fulfill important functions for plant growth and health. These rhizospheric microorganisms could be exploited for its innumerable properties and active metabolites (Tamilarasi et al. 2008). Biological control of plant disease is defined as "The involvement of the use of beneficial microorganisms, such as specialized fungi or yeast or bacteria, to attack and control the plant pathogens (i.e., fungi, bacteria, nematodes, or weeds) and the diseases they are causing" (Fravel 2005). Biocontrol is a potent means of reducing the damage caused by plant pathogens (Jeyarajan and Nakkeeran 2000). The relationship of PGPR and biocontrol is not only important but also worthwhile. A biocontrol strain should be able to protect the host plant from pathogens and fulfill the requirement for strong colonization. Numerous compounds that are toxic to pathogens, such as HCN, phenazines, pyrrolnitrin, and pyoluteorin, as well as other enzymes, antibiotics, metabolites, and phytohormones are the means by which PGPR acts, just as quorum sensing and chemotaxis which are vital for rhizosphere competence and colonization (Babalola 2010). Mostly *Pseudomonas* sp. and *Bacillus* sp. are known for their antifungal properties; hence, they have great importance in the biological control of a number of plant diseases (Safdar et al. 2011; Milner et al. 1996; Ryder et al. 1999). Anith et al. 2004 reported that when PGPR (*Pseudomonas putida*, *Bacillus pumilus*) and Actigard (acibenzolar-S-methyl) applications were combined, the bacterial wilt incidence caused by *R. solanacearum* was reduced when compared to the untreated control. Ahmadzadeh et al. (2004) reported that antagonistic rhizobacteria more specifically fluorescent pseudomonads and certain *Bacillus* species possessed the ability to inhibit fungal and bacterial root diseases of agricultural crops. In vitro evaluation of the *P. fluorescens* isolates also confirmed their

antagonistic ability against both *Pyricularia grisea* and *Rhizoctonia solani* in dual culture tests. Numerous rhizosphere organisms are capable of producing compounds that are toxic to pathogens (plant diseases) (Ahmadzadeh et al. 2004). *Bacillus subtilis* is one such commercialized PGPR organism, and it acts against a wide variety of pathogenic fungi. Boby and Bagyaraj (2003) carried out a field study to investigate the possibility of controlling the root rot or wilt of medicinal plant, *Coleus forskohlii* using three biocontrol agents, viz., *Glomus mosseae, Pseudomonas fluorescens,* and *Trichoderma viride*, singly and in combination, and observed that *Glomus mosseae* and *Trichoderma viride* in combination not only controlled the disease but also increased the tuber yield and the forskolin content. Some of the ubiquitous microorganisms can be a significant component of management practices to achieve sustainable yields. Literature pertaining to the plant growth promotion, biocontrol activity, and mechanisms of actions of PGPR of medicinal plants is limited.

The selection and use of PGPR should be done taking into account the adaptation of the inoculant to a particular plant and soil in the rhizosphere ecosystem, though the development of effective microbial inoculants remains a major scientific challenge (Richardson 2001). Many researchers suggest that microbial inoculants can be used as an economic input to increase crop productivity and maintain the sustainability of soil (Solanki et al. 2011). Though PGPR has a very good potential in the management of pests and diseases, it cannot be used as cell suspension under field conditions, and so it should be immobilized in certain carriers and should be prepared as formulations for easy application, storage, commercialization, and field use (Nakkeeran et al. 2005). A multipurpose formulation of the screened isolates is prepared with suitable and available agricultural and industrial waste carriers that support the survival of bacteria for a considerable length of time. The carrier must display two fundamental properties; it must support the growth of the target organism and maintain desired population of inoculant strains over the acceptable time period. Carriers may be either organic or nonorganic. It should be economical and easily available and have long shelf life. The carrier should be nearly sterile and chemically and physically uniform, display high water holding capacity and high water retention, be suitable for as many bacterial species and strains as possible, and should support growth and survival. It should be easily manufactured, amendable to nutrient supplement, nearly neutral pH or easily adjustable, and manageable in the mixing, curing, and packaging operations. It should be nontoxic, biodegradable, and nonpolluting and minimize environmental risks such as the dispersal of cells to the atmosphere or to the groundwater.

Different types of carriers used by various researchers are peat and peat plus additives, coal and coal with additives (Crawford and Berryhill 1983), clays and inorganic soil (Kotb and Angle 1986; Chao and Alexander 1984; Smith 1995), compost made from bagasse (Phipotts 1976), soybean meal (Iswaran et al. 1972), wheat bran (Jackson et al. 1991), agricultural waste material, plant debris, vermiculite ground rock phosphate, calcium sulfate, polyacrylamide gels, alginate beads, and synthetic carrier which have been formed by plain lyophilized microbial culture and oil-dried bacteria (Johnston 1962).

The rhizobacteria that is isolated from various agroecological zones of the country based on their bioactivity reflected as control of root and soilborne diseases, improved soil health, and increased crop yields. Effective rhizobacteria have been further field tested with success which was chosen based on primary screening protocols. These effective rhizobacteria are used for making several commercial formulations, mostly based on dry powder (charcoal, lignite, farmyard manure, etc.) which are field tested; however, problems of appropriate shelf life and cell viability are still to be solved (Johri et al. 2003). *Bacillus*-based products are mostly used commercially among several PGPR strains. It is mostly used based on bioformulation with plant growth-promoting activity because they produce endospores which are tolerant to extremes of abiotic environments such as temperature, pH, pesticides, and fertilizers. Several microbial inoculants have already been successfully commercialized (Sharma et al. 2009a, b; Abbasi et al. 2010), but a specific biological control strategy for medicinal plants, which are increasingly affected by different soilborne phytopathogens, has not been available until now (Shrivastava 2003).

The soil factors also have very important effects on the quality and quantity of genuine regional drug (Ren et al. 2005). Only a small subset of potential microbial strains could be definitively attributed to phytotherapeutic properties (Janovska et al. 2003; Pestana-Calsa et al. 2010; Hegde 2007), and their relative contribution to the recognized valuable bioactivity of medicinal plants is not clear as of yet (Shrivastava 2003).

6.5 Conclusions

The indigenous plant growth-promoting microorganisms of the medicinal plants and their inoculation in the plant rhizosphere are very useful in increasing the growth of plants. The large-scale deforestation of green forest wealth, a renewable resource, is leading to an accelerated loss of valuable or potentially valuable biodiversity, extinction of species, and genetic erosion. Hence, alternative and safe forms of preservation and cultivation of naturally occurring medicinal and aromatic plants are required which can be carried out by utilizing the indigenous rhizosphere bacteria of medicinal plants. Indigenous microorganisms of these medicinal plants also influence the quality and quantity of bioactive constituents and its potential in agriculture, pharmaceutical, and medicine. It can also influence the metabolic activity and bioactivity of these medicinal plants. Hence studies are required for the evaluation of the differences of rhizobacterial diversity and the bioactive component of medicinal plants among different habitats which will lead to undermine the relationship between microorganism diversity and the quality of genuine authentic crude drug.

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Part II
Alleviation Plant Stress

Chapter 7
Alleviation of Abiotic Stress in Medicinal Plants by PGPR

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7.1 Introduction

Plants have been a fundamental component of human lives in terms of food, fibre and health since the beginning of human civilisation. The use of medicinal plants and their derived compounds/metabolites to cure various health ailments has been in practice across cultures for thousands of years (Crispin and Wurtele 2013). According to WHO, >80 % of the world's population in developing countries is primarily dependent on medicinal plant-derived herbal medicines for basic healthcare needs (Kamboj 2000). Medicinal plants are known to be rich in secondary metabolites and are potentially useful to produce natural drugs (Briskin 2000; Goldman 2001). The use of herbal medicines in developed countries has also gained popularity in last few years (Sahoo et al. 2010). Identifiable characteristic attributes involving antimicrobial (Taye et al. 2011), antioxidant (Thambiraj et al. 2012) and nutraceutical (Royer et al. 2013) properties of these plants make them a suitable alternative medicine. These plant bioagents control or prevent a

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number of diseases in both human being and livestock (Table 7.1). The growth and productivity of medicinal plants are adversely affected by several biotic and abiotic constraints. These plants are frequently exposed to various stress factors such as salt, drought, low temperature, flooding, heat, oxidative and heavy metal stress (Kirakosyan et al. 2003; Ben Taarit et al. 2012; Ahmad et al. 2013; Flora et al. 2013). Plants subjected to various abiotic stress conditions undergo different physiological and biochemical changes leading to numerous modifications in the structure and functions of cell membranes (Ben Taarit et al. 2010). Prevailing stress factor is capable to induce changes in plant metabolism by affecting plant growth, metabolite synthesis and their qualitative and quantitative composition to a great extent (Ksouri et al. 2007). Several studies have confirmed the negative/positive effect on medicinal plants exposed to various abiotic stress factors (Table 7.2).

In the present chapter, we aim to give an overview about the role of PGPR in a biotic stress alleviation of medicinal plants against different types of stress factors.

7.2 Plant Growth-Promoting Rhizobacteria

The plant rhizosphere is a zone of intense microbial activity and ecological significance where numerous microorganisms colonise in, on and around the roots of growing plants. The diverse groups of bacteria are associated with the root systems of all higher plants (Khalid et al. 2006). These bacteria are considered as efficient microbial competitors in the root zone, and the net effect of plant–microbe associations on plant growth could be positive, neutral or negative (Kennedy 2005; Nadeem et al. 2006; Patel et al. 2008; Khalid et al. 2009; Shahzad et al. 2014). Bacteria having close proximity with plant roots through aggressive colonisation and capable of stimulating plant growth by any mechanism(s) of action are referred to as plant growth-promoting rhizobacteria (PGPR) (Kloepper et al. 1986; Arshad and Frankenberger 1998; Kremer 2006; Böhm et al. 2007; Shahzad et al. 2013).

The plant growth-promoting rhizobacteria are characterised by the following inherent distinctivenesses: (a) they must be proficient to colonise the root surface; (b) they must survive, multiply and compete with other microorganisms, at least for the time needed to express their protection activities; and (c) they must promote plant growth (Kloepper 1994; Ahemada and Kibret 2014). Nearly about 2–5 % of rhizobacteria, when reintroduced by plant inoculation in a soil containing competitive microorganisms, exert a beneficial effect on plant growth and are known as plant growth-promoting rhizobacteria (Kloepper and Schroth 1978; Antoun and Kloepper 2001). As shown by Vessey (2003), soil bacterial species burgeoning in plant rhizosphere which grow in, on or around plant tissues stimulate plant growth by a plethora of mechanisms collectively known as PGPR. Alternatively, Somers et al. (2004) classified PGPR based on their functional activities as (1) biofertilisers (increasing the availability of nutrients to plant), (2) phytostimulators (plant growth promotion, generally through phytohormones), (3) rhizoremediators (degrading organic pollutants) and (4) biopesticides (controlling diseases, mainly by the

Table 7.1 Medicinal plant and their uses against different diseases of human being and animals

Common name	Botanical name	Family	Part used	Medicinal use	References
Amla (T)	*Emblica officinalis*	Euphorbiaceae	Fruit	Vitamin C, cough, diabetes, cold, hyper-acidity, laxative, prevention of cancer	Jacob et al. (1988), Saeed and Tariq (2007), Yokozawa et al. (2007), Baliga and Dsouza (2011)
Anantamool (S)/Indian Sarap sarilla	*Hemidesmus indicus*	Asclepiadaceae	Root/leaf	Appetiser, carminative, aphrodisiac, astringent, wound healing	Deeb et al. (2010), Ganesan et al. (2012)
Ashok (T)	*Saraca asoca*	Caesalpiniaceae	Bark, flower	Diabetes disorder, menstrual pain, uterine problems	Cowen (1984), Varghese et al. (1992), Pradhan et al. (2009)
Ashwagandha (H)	*Withania somnifera*	Solanaceae	Roots, leaves	Restorative tonic, stress, nerves disorder, aphrodisiac, regulation of reproductive hormone	Bucci (2000), Scartezzini and Speroni (2000), Ahmad et al. (2010), Pandit et al. (2013)
Bach (H) Sweet flag	*Acorus calamus*	Araceae	Rhizome	Antifungal, sedative, analgesic, epilepsy, hypertensive, antimutagenic	Ghosh (2006), Jabbar and Hassan (2010)
Bael/bilva (T)	*Aegle marmelos*	Rutaceae	Fruit, bark	Diarrhoea, dysentery, constipation	Sharma et al. (1981), Pattnaik et al. (1996), Brijesh et al. (2009), Pallaty et al. (2011)
Bahada (T)	*Terminalia bellirica*	Combretaceae	Seed, bark	Cough, insomnia, dropsy, vomiting, ulcer, triphala	Sabu and Ramadasan (2002), Kumar et al. (2010)
Benachar (S) Khus/khus	*Vetiveria zizanioides*	Poaceae/ Gramineae	Root	Hyperdipsia, burning, ulcer, skin, vomiting	Singh et al. (1978), Thakur et al. (1989)
Bhumi amla (H)	*Phyllanthus amarus*	Euphorbiaceae	Whole plant	Anaemic, jaundice, dropsy	Calixto et al. (1984), Nishiura et al. (2004), Patel et al. (2011)
Blue snakeweed (P)	*Stachytarpheta cayennensis*	Verbenaceae	Whole plant	Remedy for dysentery, syphilis, gonorrhoea and catarrhal conditions	Gills (1992)
Brahmi (H)	*Bacopa monnieri*	Scrophulariaceae	Whole plant	Nervous disorder, memory enhancer, mental disorder	Singh and Dhawan (1997), Khare (2003), Allan et al. (2007), Rastogi et al. (2012), Aguiar and Borowski (2013)

(continued)

Table 7.1 (continued)

Common name	Botanical name	Family	Part used	Medicinal use	References
Kalahari/glory lily (H)	*Gloriosa superba*	Liliaceae	Seed, tuber	Skin diseases, labour pain, abortion, general debility	Duke (1985), Pawar and Nabar (2010)
Chirata (high altitude) (H)	*Swertia chirata*	Gentianaceae	Whole plant	Skin disease, burning sensation, antimalarial, antiamoebic, fever	Natarajan et al. (1974), Ray et al. (1996), Saha et al. (2005), Tabassum et al. (2012)
Coat buttons/ tridax daisy (P)	*Tridax procumbens*	Asteraceae	Whole plant	Treatment of diarrhoea; stops bleeding, malaria and stomachache; antidiabetic; antiarthritic	Holm et al. (1997), Bhagwat et al. (2008), Petchi et al. (2013)
Dalchini (S)	*Cinnamomum zeylanicum*	Lauraceae	Bark, oil	Bronchitis, asthma, cardiac disorder, fever	Baratta et al. (1998), Gruenwald et al. (2010)
Fireplant (Mexico) (T)	*Euphorbia heterophylla*	Euphorbiaceae	Root, leaves	For erysipelas, cough, bronchial, paroxysmal asthma, hay fever, catarrh	Edeoga and Gomina (2001), Holm et al. (1997), Gills (1992)
Fringed spider flower (H)	*Cleome rutidosperma*	Capparaceae	Leaves	Ear cure for inflammation, anthelmintic and carminative	Burkill (1984), Gills (1992)
Goatweed (T)	*S. dulcis*	Scrophulariaceae	Whole plant	Antiviral, inhibitory and antitumour activity; cough, chest pains and sore throat; gonorrhoea	Hayashi et al. (1993), Gills (1992)
Gokhur (H) crawling puncture vine	*Tribulus terrestris*	Zygophyllaceae	Whole plant	Sweet, cooling, aphrodisiac, appetiser, digestive, urinary	Tomova et al. (1979), Brown et al. (2000), Gauthaman and Ganesan (2008)
Ghritkumari (H)	*Aloe vera*	Liliaceae	Leaves	Laxative, wound healing, skin burns, ulcer	Maenthaisong et al. (2007), Eshun and He (2004)
Gudmar/ madhunasini (C)	*Gymnema sylvestre*	Asclepiadaceae	Leaves	Diabetes, hydrocele, asthma	Sinsheimer et al. (1970), Baskaran et al. (1990), Persaud et al. (1999), Luo et al. (2001), Ramachandran et al. (2003)
Guggul (T)	*Commiphora wightii*	Burseraceae	Gum resin	Rheumatism, arthritis, paralysis, laxative, hyperlipidaemia	Szapary et al. (2003), Sahni et al. (2005), Siddiqui and Mazumder (2012)

Guluchi/giloe (C)	*Tinospora cordifolia*	Menispermaceae	Stem	Gout, pile, general debility, fever, jaundice, anti-tuberculosis	Singh et al. (2003), Badar et al. (2005)
Hangertjie (S)	*Erica coccinea*	Asteraceae	Whole plant	Treatment of fever and convulsions in children, ulcer, craw-craw, ringworm	Burkill (1984)
Harida (T)	*Terminalia chebula*	Combretaceae	Seed	Anti-mutagenic, triphala, wound, ulcer, leprosy, inflammation, cough	Grover and Bala (1992), Carounanidi et al. (2007)
Henna/mehandi (S)	*Lawsonia inermis*	Lythraceae	Leaf, flower, seed	Burning, steam, anti-inflammatory, reduce the secretion of sweat	Stulberg et al. (2002), Stante et al. (2006)
Indian pink/ water weed (H)	*S. anthelmia*	Loganiaceae	Root, bark, leaves	Worm expeller	Gills (1992)
Kaincha/creeper baidanka	*Mucuna pruriens*	Fabaceae	Root, hair, seed, leaf	Nervous disorder, constipation, nephropathy, strangury and dropsy	Iauk et al. (1993), Amin et al. (1996), Salau and Odeleye (2007)
Kalmegh/Bhui neem (H)	*Andrographis paniculata*	Acanthaceae	Whole plant	Fever, weakness, release of gas, anti-inflammatory	Coon and Ernst (2004), Sheeja et al. (2006), Mishra et al. (2007)
Kantakari/ akranti perennial (H)	*Solanum xanthocarpum*	Solanaceae	Whole plant, fruit, seed	Diuretic, anti-inflammatory, appetiser, stomachic	Patel et al. (2012)
Kochila (T)	*Strychnos nux-vomica*	Loganiaceae	Seed	Nervous disorder, paralysis, healing wound	McIntosh (1940), Neetu et al. (2013)
Kurai (S)	*Holarrhena antidysenterica*	Apocynaceae	Bark, seed	Scabies, antipyretic, amoebic dysentery	Gilani et al. (2010), Mehmood et al. (2011)
Long pepper/ pippali (C)	*Piper longum*	Piperaceae	Fruit, root	Appetiser, enlarged spleen, bronchitis, cold, antidote	Pathak et al. (2010), Kumar et al. (2011)
Makoi (H) Kakamachi	*Solanum nigrum*	Solanaceae	Fruit/whole plant	Dropsy, general debility, diuretic, antidysenteric, antitumour	Aslanov (1971), Jian et al. (2008)
Mandukaparni (H) Indian pennywort	*Centella asiatica*	Umbelliferae	Whole plant	Anti-inflammatory, jaundice, diuretic, diarrhoea, anticancer, antiulcer	Nanasombat and Teckchuen (2009), Abdulla et al. (2010)

(continued)

Table 7.1 (continued)

Common name	Botanical name	Family	Part used	Medicinal use	References
Nageswar (T) Nag Champa	*Mesua ferrea*	Guttiferae	Bark, leaf, flower	Asthma, antimicrobial, skin, burning, vomiting, dysentery, piles	Banerjee et al. (1993), Hemalatha et al. (2013)
Neem (T)	1x(111) *Azadirachta indica*	Meliaceae	Rhizome	Sedative, analgesic, epilepsy, hypertensive	Rahim et al. (2010), Kumar and Navaratnam (2013)
Pashan bheda	*Coleus barbatus*	Lamiaceae	Root	Kidney stone, diabetes	Day (1998), Bnouham et al. (2002), Porfírio et al. (2010)
Pashan bheda/ Patharchur (H)	*Coleus barbatus*	Lamiaceae	Root	Kidney stone, calculus	Valdes et al. (1987), Porfírio et al. (2010)
Peppermint (H) perennial	*Mentha piperita*	Lamiaceae	Leaves, flower, oil	Digestive, painkiller	McKay and Blumberg (2006), Cappello et al. (2007)
Rakta chitrak (H)	*Plumbago indica*	Plumbaginaceae	Rootbar	Dyspepsia, colic, inflammation, cough	Figueriedo et al. (2003), Vijayakumar et al. (2006), Eldhose et al. (2013)
Sada Bahar (H) periwinkle/ nayantara	*Vinca rosea/ Catharanthus roseus*	Apocynaceae	Whole plant	Leukaemia, hypotensive, antispasmodic, antidote	Singh et al. (2001), Prajakta and Ghosh (2010)
Sandalwood (T)	*Santalum album*	Santalaceae	Heartwood, oil	Skin disorder, burning sensation, jaundice, cough	Edeoga et al. (2005), Misra and Dey (2012)
Sarpagandha (H)	*Rauvolfia serpentina*	Apocynaceae	Root	Hypertension, insomnia	Obdoni and Ochuko (2001), Edeoga et al. (2005)
Shatavari (C)	*Asparagus racemosus*	Liliaceae	Tuber, root	Enhance lactation, general weakness, fatigue, cough, insulin enhancer	Mathews et al. (2006), Hannan et al. (2007)
Senna (S)	*Cassia angustifolia*	Liliaceae	Dry tubers	Rheumatism, general debility tonic, aphrodisiac	Duncan (1957), Spiller et al. (2003)
Sweet chitrak Perennial (H)	*Plumbago zeylanica*	Plumbaginaceae	Root, rootbar	Appetiser, antibacterial, anticancer, antifertility	Vijayakumar et al. (2006), Annan et al. (2009), Vishnukanta and Rana (2010)

Tulsi (perennial)	*Ocimum sanctum*	Lamiaceae	Leaves/seed	Cough, cold, bronchitis, expectorant, anticancer	Gupta et al. (2003), Baliga et al. (2013)
Vai Vidanka (C)	*Embelia ribes*	Myrsinaceae	Root, fruit, leaves	Skin disease, helminthiasis, cardioprotective, snake bite	Warrier et al. (1995), Suanarunsawat et al. (2010)
Vasa (S)	*Adhatoda vasica*	Acanthaceae	Whole plant	Antispasmodic, antiulcer respiratory, stimulant	Vinothapooshan and Sundar (2011), Sheeba and Mohan (2012), Kumar et al. (2013)
Vringraj (H)	*Eclipta alba*	Compositae	Seed/whole	Anti-inflammatory, digestive, hair tonic	Franca et al. (1995), Roy et al. (2008)
White eye/Brazil pusley (P)	*Richardia brasiliensis*	Rubiaceae	Whole plant	Cure for eczema, treatment of boils, active cure against avian malaria	Burkill (1994), Edeoga et al. (2005)
Wireweed (S)	*S. acuta*	Malvaceae	Whole plant	Stops bleeding, sores and wounds, antipyretic	Egunjiobi (1969)

T Tree, *H* Herb, *C* Climber, *S* Shrub, *P* Flowering plant

Table 7.2 Effect of different abiotic stress factors on therapeutic properties of medicinal plants

Medicinal plant	Plant part	Medicinal use	Stress factor	Impact on plant	Reference
Bunium persicumi	Fruit, leaves, bark and roots	Gastroesophageal reflux disease and heartburn, promote to weight loss, kill cancer cell and treat bronchitis, antibacterial and antifungal activity	Drought stress	Due to water stress, essential oil yield (terpinen-4-ol, β-sesquiphellandrene, bornyl acetate) was significantly decreased, while contents of limonene and proline increased	Saeidnejad et al. (2013)
Datura stramonium	Leaves, powder of seeds	Effective for asthma, analgesic during surgery, stimulation of the urinary tract, respiratory tract	Drought stress	Scopolamine N-oxide 17–20, hydroxy hyoscyamine was significantly reduced, and activation of phenylalanine ammonia-lyase in plants is increased by abiotic stresses	Das et al. (2012), Gaire and Subedi (2013), Da Silva et al. (2012)
Emblica officinalis	Fruit, leaves, roots	Vitamin C, cough, diabetes, cold, hyperacidity, antibacterial, prevention of cancer, norovirus infection, laxative	Salt stress	Salt stress increased the phenolics and ascorbic acid while decreased the 1-diphenyl-2-picryl hydrazyl. Similarly some amino acids decreased and increased as a result of salt stress to plant	
Aloe vera (L.) Burm.f	Leaf parts	Analgesic, wound healing, immune modulating, antitumour activities as well as antibacterial, antifungal and antiviral properties. Its juice lowers cholesterol and diabetic effects	Heavy frost or snow, salt stress	Amino acids, anthraquinones, enzymes, minerals, vitamins, lignins, monosaccharide, polysaccharides, salicylic acid, saponins and sterols	Sheteawi et al. (2001), Nema et al. (2013)
Azadirachta indica	Leaves, fruits, bark, roots	Diabetes mellitus, antihyperglycaemic action, antiviral, antibacterial, antifungal, anti-inflammatory, antipyretic, antiseptic and antiparalitic uses	Salt stress, heavy metal stress	Nimbanene, 6-desacetylnimbinene, nimbandiol, ascorbic acid, n-hexacosanol, amino acid, 7-desacetyl-7-benzoylazadiradione, 7-desacetyl-7-benzoyl-gedunin, 17-hydroxy-azadiradione and nimbiol	Biswas et al. (2002), Ghosh et al. (2009), Lucantoni et al. (2010)

Withania somnifera	Roots, leaves	Restorative tonic, nerves disorder, aphrodisiac, regulation of reproductive hormone	Salt stress (NaCl and $CaCl_2$)	The NaCl with $CaCl_2$-treated plants increased total chlorophyll content and proline oxidase activity and decreased the γ-glutamyl kinase activity in all plant parts compared to NaCl-treated plant	Jaleel and Azooz (2009)
Catharanthus roseus	Shoot, leaves, flowers	Diabetes, malaria, leukaemia and Hodgkin's lymphoma	Water-deficit environments with or without CaCl2	Drought with $CaCl_2$-treated plants showed an increase in total indole alkaloid content in shoots and roots than drought-stressed and well-watered plants	Jaleel et al. (2007)
Arachis hypogaea	Fruit, oil, peanut powder	Reducing the risk of heart disease, without adding to body weight. Regular peanut use increases serum Mg concentrations	Salt stress, water stress	Results showed that shoot dry matter, relative water content, chlorophyll and K^+ decreased significantly with salinity, while Na^+, H_2O_2 and proline increased with salinity level	Hossain et al. (2011)
Moringa oleifera Lam	Leaves, stem bark, roots	Fevers, bronchitis, eye and ear infections, headaches, gastric ulcers, diarrhoea, cardiac and circulatory stimulants, antitumour, antipyretic, cholesterol lowering, antidiabetic, antibacterial and antifungal activities	Salt and drought stress	Due to salinity and drought, stress severely limits plant growth, yield and oil content mainly in semiarid regions around the world	Silveira et al. (2003), Hussein and Abou-Baker (2014)
Santalum album	Leaves, bark, oil	Bronchitis, antiulcer, skin disorders, fever, infection of the urinary tract, mouth, pharynx, liver and gallbladder complaints	Salt stress	Significant decrease in growth attributes was observed while interestingly increase in some amino acids and proline content under stress conditions	Sindhu et al. (2010), Swamy and Prasad (2012)

production of antibiotics and antifungal metabolites) (Antoun and Prévost 2005). Furthermore, in most studied cases, a single PGPR will often reveal multiple modes of action including biological control (Kloepper 2003; Vessey 2003; Nadeem et al. 2013). Furthermore, Gray and Smith (2005) have recently shown that the PGPR associations range in the degree of bacterial proximity to the root and intimacy of association. In general, these can be separated into extracellular plant growth-promoting rhizobacteria (ePGPR), existing in the rhizosphere, on the rhizoplane or in the spaces between cells of the root cortex, and intracellular plant growth-promoting rhizobacteria (iPGPR), which exist inside root cells, generally in specialised nodular structures (Figueiredo et al. 2011; Sundaramoorthy and Balabaskar 2012). Some examples of ePGPR are like *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Pseudomonas*, *Serratia*, etc. Similarly, some examples of the iPGPR are *Azorhizobium, Bradyrhizobium, Mesorhizobium* and *Rhizobium* of the family *Rhizobiaceae* (Bhattacharyya and Jha 2012).

7.2.1 Growth-Promoting Mechanisms of PGPR

Investigations into the nature and types of association exhibited by different plant beneficial microorganisms have so far indicated that these interactions may be beneficial, harmful or neutral for the host plant. Bacteria that facilitate plant growth may do so either by binding to exterior plant surface such as roots (rhizosphere) or leaves (phyllosphere), or they may inhabit the interior surfaces of the plant forming endophytic relationship (Dey et al. 2004; Yadav et al. 2005; Duan et al. 2013). In general, the mechanisms involved in plant growth promotion by PGPR include associative nitrogen fixation, lowering of ethylene levels, production of siderophores and phytohormones, induction of pathogen resistance, solubilisation of nutrients, promotion of mycorrhizal functioning and decreasing pollutant toxicity (Fig. 7.1) (Glick et al. 1999). Moreover, interaction of specific bacterium to facilitate plant growth might be either direct or indirect depending upon growth-promoting traits exhibited by the bacterium (Castro et al. 2009).

Direct stimulation includes biological nitrogen fixation, producing phytohormones like auxins, cytokinins and gibberellins, solubilising minerals like phosphorus and iron, production of siderophores and enzymes and induction of systemic resistance, while indirect stimulation is basically related to biocontrol (Table 7.3), including antibiotic production, chelation of available Fe in the rhizosphere, synthesis of extracellular enzymes to hydrolyze the fungal cell wall and competition for niches within the rhizosphere (Zahir et al. 2004).

PGPR strains, especially, *Pseudomonas fluorescens* and *Bacillus subtilis* are the best noted for PGPR-mediated indirect plant growth stimulations (Damayanti et al. 2007). Besides nitrogen transformation, increasing bioavailability of phosphate, iron acquisition, exhibition of specific enzymatic activity and plant protection from harmful pathogens with the production of antibiotics can also

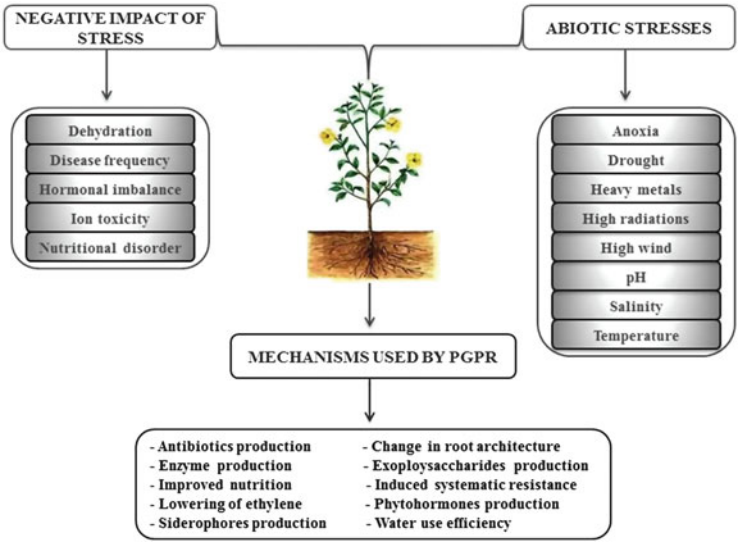

Fig. 7.1 Mechanisms used by PGPR to promote plant growth under abiotic stresses

successfully improve the quality of crops in agriculture (Spaepen et al. 2007). Thus, based on their mechanism of action, PGPR can be categorised into three general forms such as biofertiliser, phytostimulator and biopesticide (Fig. 7.2). The phenomenon of quorum regulation can affect the expression of each of these traits as PGPR are reported for their regular interactions with the resident microbial community in rhizosphere (Lugtenberg and Kamilova 2009). Recent investigations on PGPR revealed that it can promote plant growth mainly by following means: (1) producing 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 (Mishra et al. 2010), gibberellic acid (Narula et al. 2006), cytokinins (Castro et al. 2008) and ethylene (Saleem et al. 2007); (3) a symbiotic nitrogen fixation (Ardakani et al. 2010); (4) exhibition of antagonistic activity against phytopathogenic microorganisms by producing siderophores, b-1,3-glucanase, chitinases, antibiotics, fluorescent pigment and cyanide (Pathma et al. 2011); and (5) solubilisation of mineral phosphates and other nutrients (Hayat et al. 2010). PGPR may use more than one of these mechanisms to enhance plant growth as experimental evidence suggests that the plant growth stimulation is the net result of multiple mechanisms that may be activated simultaneously (Martínez-Viveros et al. 2010). Recently, biochemical and molecular approaches are providing new insight into the genetic basis of these biosynthetic pathways, their regulation and significance as biological tool (Joshi and Bhatt 2011). However, to be more effective in the rhizosphere, PGPR must maintain a critical population density for a longer period, although inoculation of plants with PGPR can temporarily enhance the population size. Although researchers have reported both direct and indirect ways of growth promotion by PGPR, there is no clear separation between these two

Table 7.3 Biocontrol of fungal plant pathogen on medicinal plants through application of PGPR

Medicinal plant	Plant pathogen	Biocontrol agent	Mode of action	Reference
Launaea nudicaulis (Bold-leaf Launaea)	*Macrophomina phaseolina, Fusarium solani and Fusarium oxysporum*	*Pseudomonas aeruginosa*	Siderophores, HCN, diacetyl phloroglucinol, chitinase activity, lytic enzyme production	Mansoor et al. (2007)
Beta vulgaris (sugar beet)	*Pythium ultimum Thielaviopsis basicola*	*Pseudomonas* sp. *Pseudomonas fluorescens* (CHAO)	Diacetyl phloroglucinol, pyrrolnitrin production	Shanahan et al. (1992)
Beta vulgaris (sugar beet)	*Rhizoctonia solani, Pythium ultimum*	*Pseudomonas fluorescens*	Viscosinamide, pyoluteorin, HCN	Nielsen et al. (1998)
Capsicum annuum (chilli)	*Pythium debaryanum*	*Pseudomonas fluorescens*	Production of siderophore, IAA, HCN, phosphate solubilisation, NH_3 and catalase	Ramyasmruthi et al. (2012)
Phyllanthus amarus (Bahupatra, Sanskrit)	*Corynespora cassiicola* (Berk and Curt) Wei	*Bacillus subtilis* (BSCBE4), *Pseudomonas chlororaphis* (PA23), *P. fluorescens* (ENPF1)	Produced both hydroxamate and carboxylate types of siderophores	Mathiyazhagan et al. (2004)
Cynara cardunculus L. (cardoon)	*Alternaria tenuissima*	*Pseudomonas fluorescens* isolates (Q16, B25 and PS2)	Producing phenazines is the secondary metabolites, siderophore, IAA, HCN	Jošić et al. (2012)
Persea americana Mill (avocado)	*Rosellinia necatrix*	*Pseudomonas chlororaphis* PCL1606 and *Bacillus subtilis* CB115	Diacetyl phloroglucinol, pyrrolnitrin, siderophores, kanosamine	González-Sáncheza et al. (2013)
Piper nigrum (black pepper)	*Phytophthora capsici* and *Colletotrichum acutatum*	*P. otitidis* (YJR27), *P. putida* (YJR92),*Tsukamurella tyrosinosolvens* (YJR102) and *Novosphingobium capsulatum* (YJR107)	ACC-deaminase, pyrrolnitrin, siderophores, HCN, ACC-deaminase activity	Sang et al. (2013)
Jerusalem artichoke (sunchoke)	*Aspergillus tamari* (M10), *Fusarium solani* (M9) and *Aspergillus fumigatus* (M2)	*Pseudomonas* spp. strain JK2	Pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol and HCN	Jina et al. (2013)

Cupressus sempervirens (Italian cypress)	*Seiridium cardinale*	*Pseudomonas chlororaphis* subsp. *aureofaciens* strain M71	Production of phenazine-1-carboxylic acid, HCN, chitinase activity	Raioa et al. (2011)
Jatropha curcas L. (Barbados nut, black vomit nut)	*Fusarium oxysporum, Macrophomina phaseolina, Aspergillus versicolor and Aspergillus nidulans*	*Pseudomonas putida* MSC1 and *Pseudomonas pseudoalcaligenes* MSC4	Antibiotic production—pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol and HCN, siderophores	Saraf et al. (2013)
Coleus forskohlii (Makandi, Sanskrit)	*Fusarium chlamydosporum* (Frag. and Cif.) and *Ralstonia solanacearum* (Smith)	*Pseudomonas monteilii*	HCN, siderophores, 2-hydroxyphenazine, protease activity, chitinase activity, L-phenylalanine arylamidase, L-lysine arylamidase	Singh et al. (2012)
Solanum melongena (brinjal)	*Ralstonia solanacearum*	*Pseudomonas fluorescens*	2,4-Diacetylphloroglucinol, HCN, siderophores, antifungal activity	Chakravarty and Kalita (2012)
Panax quinquefolius (panax ginseng)	*Fusarium* cf. *incarnatum*	*Bacillus* species	Pyrrolnitrin, HCN, siderophores, chitinase activity, exopolysaccharides	Song et al. (2014)
Coffea arabica L. and *Coffea robusta* L. (coffee)	*Hemileia vastatrix* Berk	*Bacillus lentimorbus* Dutky and *Bacillus cereus* Frank	Pyrrolnitrin, HCN, bacillomycin, siderophores, antifungal activity	Shiomi et al. (2006)

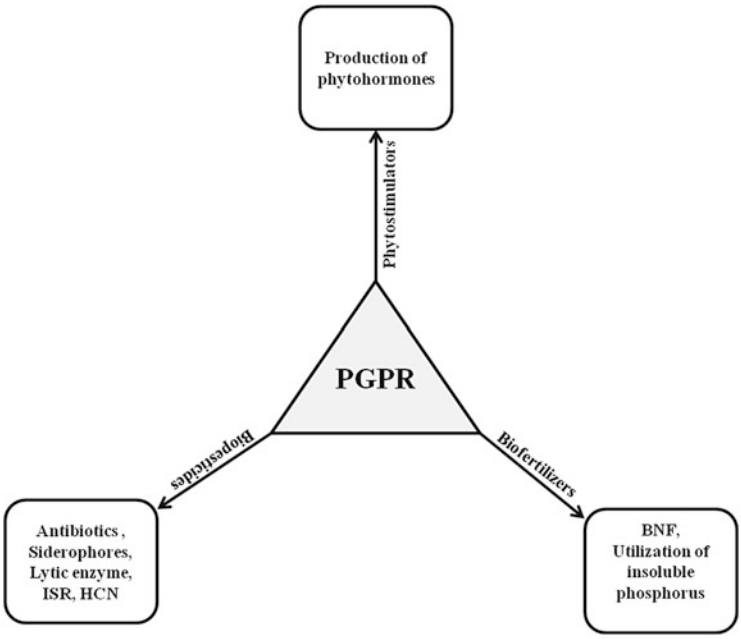

Fig. 7.2 PGPR are categorised into three groups on the basis of their mechanism of action

mechanisms. Some bacteria possess multiple traits to promote plant growth where one trait may dominate the other one (Hafeez et al. 2004; Shaharoona et al. 2008). A bacterium influencing the plant growth by regulating synthesis of plant hormones can also play a role in controlling plant pathogens and diseases (Fig. 7.3) and, vice versa, barriers to the introduction of crop plants into areas that are not suitable for crop cultivation. Drought, salinity, flooding, low temperature, air pollution and heavy metals are key sources of abiotic stress. Depending upon the crop plant exposed to an array of abiotic stress factors, losses in yield and its associated attributes can range from 50 to 82 % (Kang et al. 2014). In semiarid and arid regions of the world, crop yield is limited by increase salinisation of irrigation water as well as soil. Under high salinity, plants exhibit a reduced leaf growth rate due to decreased water uptake, which restricts photosynthetic capacity. Plant undergoes a number of metabolic and physiological changes in response to salt stress and water deficiency (drought) (Han and Lee 2005; Krasensky and Jonak 2012).

Numerous soil beneficial bacteria exhibited strong growth adaptation potential under stressful condition. The long-term goal of improving plant–microbe interactions for salinity-affected fields and crop productivity can be met with an understanding of the mechanism of osmoadaptation in *Azospirillum* sp. The synthesis and activity of nitrogenases in *A. brasilense* is inhibited by salinity stress (Tripathi et al. 2002; Boojar 2009). Tripathi et al. (2002) documented that in *Azospirillum* sp. there is an accumulation of compatible solutes such as glutamate, proline, glycine betaine and trehalose in response to salinity/osmolarity; proline plays a

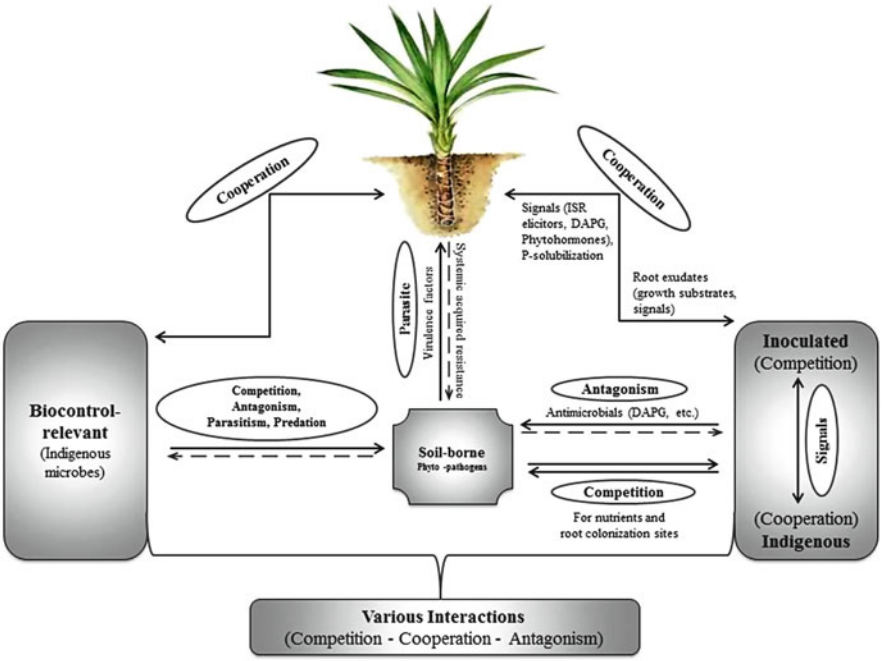

Fig. 7.3 An overview of plant-protection mechanisms in biocontrol agents against soil-borne phytopathogens

major role in osmoadaptation through increase in osmotic stress that shifts the dominant osmolyte from glutamate to proline in *A. brasilense*. *Azospirillum*-inoculated sorghum plants had more water content, higher water potential and lower canopy temperature in their foliage. Hence, they were less drought-stressed over uninoculated plants (Table 7.4).

The PGPR containing ACC deaminase can lower the impact of various environmental stresses such as flooding, heavy metals, soil-borne phytopathogens (Fig. 7.3), drought and high salt on host plant.

The phytohormone ethylene, which is found in all higher plants, is an important regulator of plant growth and development both under normal and stress conditions. However, overproduction of ethylene under stressful conditions can result in the inhibition of plant growth or death, especially in young plant seedlings. PGPR that express ACC deaminase can hydrolyze ACC, the immediate precursor of ethylene, to ά-ketobutyrate and ammonia and in this way promote plant growth by regulating ethylene production in plant. Inoculation of ACC deaminase-containing PGPR in association with plants subjected to a wide range of abiotic stresses results in enhanced plant tolerance against exposed stressors (Stepien and Klobus 2005; Greenberg et al. 2006; Khalid et al. 2006; Shahzad et al. 2014).

PGPR can exert positive effects on seedling vigour and plant productivity under stress conditions. Seed inoculations with PGPR in asparagus (*Asparagus officinalis* L.)

Table 7.4 Effectiveness of PGPR for growth promotion of medicinal plants under stress conditions

Medicinal plant	Type of stress	Bacterial inoculate	PGPR attributes	Response	Reference
Silybum marianum (milk thistle)	Salt	*Pseudomonas extremorientalis* TSAU20	Auxin production, exopolysaccharide, biofilm formation	Significantly increased the root length, shoot length and total biomass of plant	Egamberdieva et al. (2013)
Ociumum basilicm L. (basil)	Drought	*Azospirillum brasilense*	Siderophores of production, P-solubilisation and other nutrients, regulation of ethylene biosynthesis	Significant increase in dry biomass and chlorophyll content was observed over control	Heidari et al. (2011)
Ociumum basilicm L. (basil)	Water stress	*Pseudomonades* sp., *Bacillus lentus, Azospirillum brasilense,* a combination of three bacterial species	ACC-deaminase activity, production of HCN, siderophore, chitinase, IAA production and P-solubilisation	Inoculation of rhizobacteria showed significant increase in antioxidant and photosynthetic pigments, catalase activity in basil plants under water stress	Heidaria and Golpayegani (2012)
Trigonella foenum-graecum L. (fenugreek)	Drought	*Azotobacter chroococcum*	Phosphate solubilisation, exopolysaccharide production, indole-acetic acid	Improving significantly root and shoot biomass and uptake of nutrients	Tank and Saraf (2003)
Arachis hypogaea (groundnut)	Salt	*Pseudomonas fluorescens* TDK1, *Pseudomonas fluorescens* PF2 and *Pseudomonas fluorescens* RMD1	By lowering ethylene production, auxin production, exopolysaccharide	Increasing salt tolerance of *Arachis hypogaea*. The impact of strains was variable and *P. fluorescens* TDK1 proved to be most effective than other ones	Saravanakumar and Samiyappan (2007)
Arachis hypogaea (groundnut)	Salt	*Bacillus licheniformis* A2	Production of NH_3, siderophore, chitinase and HCN and assessment of their antifungal activity, IAA production and P-solubilisation	Showed increase in fresh biomass, total length and root length over respective control	Nautiyal et al. (2013)

Lactuca sativa L. (lettuce)	Drying soil	*Bacillus* spp.	Indole-acetic acid, phosphate solubilisation, lowering ethylene biosynthesis	Significantly increased the root and leaves biomass over untreated control	Arkhipova et al. (2007)
Lactuca sativa L. (lettuce)	Salts	*Azospirillum*	HCN, siderophore, indole-acetic acid, P-solubilisation	Increased the fresh biomass of the plants and enhanced N and P uptake in plant tissues	Barassi et al. (2006)
Brassica napus (rapsi)	Heavy metals	*Pseudomonas tolaasii* ACC23, *P. fluorescens* ACC9, *Alcaligenes* sp. ZN4, *Mycobacterium* sp. ACC14	Siderophores, indole-3-acetic acid, exopolysaccharides	PGPR strains protect canola plant against the inhibitory effects of cadmium	Dell'Amico et al. (2008)
Brassica oxyrrhina (smooth-stem turnip)	Heavy metals	*Bacillus cereus* SRA10	Exopolysaccharides, siderophores, heavy metal mobilisation	Enhanced the metal accumulation in plant tissues by facilitating the release of Ni from soil	Ma et al. (2009)
Ocimum basilicum (sweet basil)	Salt	*Pseudomonas* ssp. and *Bacillus lentus*	Indole-acetic acid, P-solubilisation, ACC-deaminase activity, siderophores production, chitinase activity	Bacteria inoculation alleviated the salinity effects on the antioxidant enzymes ascorbate peroxidase and glutathione reductase, along with mineral content and growth	Golpayegani and Tilebeni (2011)
Capsicum annuum (pepper)	Osmotic stress (45 % PEG)	*Arthrobacter* sp., *Bacillus* sp.	ACC-deaminase activity, IAA, P-solubilisation, siderophores	Inoculation resulted in a significantly reduced upregulation or even downregulation of the stress-inducible genes over control plants	Sziderics et al. (2007)
Vitis vinifera (grapevine)	Temperature	*Burkholderia phytofirmans*	ACC-deaminase activity, phenylalanine ammonia, siderophores, P-solubilisation	Bacterial inoculation showed effective increase in antioxidant and colour pigments, catalase activity in plants under heat stress	Barka et al. (2006)

result in a positive response and enhance plant growth under drought (Liddycoat et al. 2009). On the basis of mutational studies of *Azospirillum*, Kadouri et al. (2003) proved the role of PHB synthesis and accumulation in enduring various stresses, viz. UV irradiation, heat, osmotic pressure, osmotic shock and desiccation. A multi-process phytoremediation system (MPPS) utilises plant/PGPR interactions to mitigate stress ethylene effects, thereby greatly increasing plant biomass, particularly in the rhizosphere, and it also causes the decontamination of persistent petroleum and organic contaminants in soil (Glick and Stearns 2011; Gamalero and Glick 2012a, b).

Drought affects the plant–water relation at cellular and whole plant level causing specific and unspecific reactions and damages. PGPR adapted to endemic sites of low rainfall area or limited water supply are more likely to protect plant from drought stress than similar bacteria from sites where water is more abundant (Mayak et al. 2004). Exopolysaccharides secreted by PGPR formed an organo-mineral sheath around microbial cell, enabling specific bacterium to survive under prevailing stress such as drought and improve drought tolerance in plant through osmotic and intracellular adjustment (Sandhya et al. 2009). Inoculation with exopolysaccharide-producing PGPR revealed drought-exposed barley plant tolerance extended for 2 weeks than uninoculated control plants (Timmusk 2003). It is now widely recognised that most bacteria in natural environments persist as 'biofilm' communities where cells are encased in an extracellular polymeric matrix. The development of biofilm communities is a vital approach employed by bacteria for survival under stress conditions (Fujishige et al. 2006).

Phosphorus is essential for all living cells and organisms. Low soil P availability has profound impact on global agriculture and food production (Song et al. 2014). Low solubility and precipitation of added P source is the major issue of semiarid and arid regions of the world. Some PGPR are characterised for the production of microbial metabolites which results in a decrease in soil pH, which probably plays an important role in the solubilisation of P (Abd-Alla 1994; Rajkumar and Freitas 2008). The phosphate-solubilising microorganisms can interact positively in promoting plant growth as well as P uptake of maize plants, leading to plant tolerance improving under water-deficit stress conditions (Ehteshami et al. 2007). The inoculation of some microorganisms that solubilise the insoluble phosphates into a microcosm containing soil from a barren lakeside area enhances the plant growth significantly and signified the potential capability of these bacteria to be used for the rapid revegetation of barren or disturbed land (Jeon et al. 2003; Paul and Sarma 2006).

The metal-resistant plant growth-promoting bacteria (PGPB) can serve as an effective metal sequestering and growth-promoting bio-inoculant for plants in metal stressed soil (Rajkumar and Freitas 2008). The deleterious effects of heavy metals taken up from the environment on plants can be lessened with the use of PGP bacteria (Belimov et al. 2005; Glick 2010; Ahemada and Kibret 2014). Soil microbes, plant growth-promoting rhizobacteria (PGPR), P-solubilising bacteria, mycorrhizal-helping bacteria (MHB) and arbuscular mycorrhizal fungi (AMF) in the rhizosphere of plants growing on trace metal-contaminated soils play an

important role in phytoremediation (Khan 2005; Gerhardt et al. 2009). Phytoremediation provides a cheap, energy-efficient detoxification method that manipulates intrinsic plant characteristics to concentrate the metal contamination in shoot biomass and reduce the bioavailability of the heavy metals. Soil microbes mitigate toxic effects of heavy metals on the plants through secretion of acids, proteins, phytoantibiotics and other chemicals (Denton 2007). Jing et al. (2007) reviewed recent advances in effect and significance of rhizobacteria in phytoremediation of heavy metal-contaminated soils. Cd in soil induces plant-stress ethylene biosynthesis (Pennasio and Roggero 1992; Gamalero and Glick 2011) and probably contributes to the accumulation of ACC in roots; PGPR protect the plants against the inhibitory effects of cadmium (Amico et al. 2008). ACC deaminase lowers ethylene production under cadmium stress condition when measured in vitro ethylene evolution by wheat seedlings treated with ACC deaminase positive isolates (Govindasamy et al. 2009). Wu et al. (2006) carried out a greenhouse study with *Brassica juncea* to critically evaluate effects of bacterial inoculation on the uptake of heavy metals from Pb–Zn mine tailings by plants. The presence of these beneficial bacteria stimulated plant growth and protected the plant from metal toxicity; it had little influence on the metal concentrations in plant tissues, but produced much larger aboveground biomass and altered metal bioavailability in the soil. As a consequence, higher efficiency of phytoextraction was obtained compared with control treatments. The organism *Pseudomonas putida* is also tolerant to a number of heavy metals at higher levels. These characteristics make *P. putida* an excellent candidate for field application in contaminated soil (Chacko et al. 2009). *Pseudomonas fluorescens* can survive under dry conditions and hyper osmolarity (Schnider-Keel et al. 2001). The hydroxamate siderophores contained in culture filtrates of *S. acidiscabies* E13 promote cowpea growth under nickel contamination by binding iron and nickel, thus playing a dual role of sourcing iron for plant use and protecting against nickel toxicity (Dimkpa et al. 2008; Badri et al. 2009).

The application of microbial biocontrol agents has been shown to be eco-friendly and effective approach against many plant pathogens responsible for various diseases (Gray and Smith 2005). PGPR mediate biological control indirectly by the production of antimicrobial molecules (Ongena et al. 2007; Nithya and Halami 2012), siderophores, and eliciting induced systemic resistance against a number of plant diseases. Plant exposed to various abiotic stress factors are more susceptible to pathogenic infestation due to weaker host defence mechanism as a result of exposed stressor. PGPR mediated biocontrol potential against various pathogenic agents (Beneduzi et al. 2012).

7.3 Recent Advances and Future Prospects of PGPR in the Field of Medicinal Plants

The explicit conclusion from the above discussion is that stressful environments can cause a negative impact on plant growth and development by causing nutritional and hormonal imbalances. However, the stress-induced negative impact on plant growth can be alleviated and/or minimised by naturally occurring microorganisms such as PGPR.

Recently, proteomic-based techniques have provided a powerful tool to reveal the molecular mechanisms of several abiotic stress responses. Several stress-responsive proteins have been proposed for plant using these techniques, and using these proteins and their corresponding genes, it will be possible to change stress-sensitive to stress-tolerant medicinal plants in the near future.

Identification of genes controlling stress tolerance traits of PGPR would enhance our knowledge about the molecular basis of the stress tolerance mechanisms. Most of the in vitro studies lack biochemical and physiological mechanisms involved in stress tolerance. Thus, the work on this aspect will significantly improve the understating of the mechanism.

Another important aspect is to generate transgenic medicinal plants encoding the genes of particular traits of PGPR. The literature shows that these transgenic plants have the ability to withstand stress environment. However, such studies were conducted in controlled conditions. Most of these studies are preliminary investigations which require further verification by performing extensive experimentation. Moreover, information about the molecular mechanisms governing the process of stress tolerance is limited.

Overall, future research should be focused: (1) to mediate PGPR-based metabolite engineering under stressful environments, (2) to explore what strains of PGPR are beneficial for promoting plant growth, (3) to identify target genes for promoting growth under stress and (4) to transfer target genes into plants through biotechnology.

7.4 Conclusions

Numerous agro-biotechnological approaches have been employed to tackle the decline in plant growth and health exposed to various abiotic stresses. One potential way to reduce their drastic effect on plant is the utilisation of microbial bioresource. Plant beneficial microbes (including plant growth-promoting rhizobacteria, i.e. PGPR) and their associative interaction with host plant are termed as plant growth and development stimulus (Shahzad et al. 2013) and have probably been shaped by co-evolutionary mechanisms. In this way, microbial partners could have significant effects on the physiology of the host plant. In recent times, PGPR-mediated stress amelioration has evolved as a vital cog of a biotic stress

management in plant and their potential contribution towards improving growth and productivity.

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Chapter 8
Plant Growth-Promoting Rhizobacteria for Alleviating Abiotic Stresses in Medicinal Plants

Swarnalee Dutta and S.M. Paul Khurana

8.1 Introduction

Plants are exposed to various biotic and abiotic stresses leading to hazardous effect on growth and reduction in yield. Such consequences are serious as plants are the source of food, fodder, feed, fibre and medicines. The ever-increasing population of the world has put tremendous pressure on the agriculture to ensure sufficient and quality food. The accommodation of such explosion in human race has reduced the total arable land leading to apprehensions about availability of adequate food. To top it are the woes of changing climatic and environmental conditions with time. Climatic changes influence the biotic and abiotic factors which are crucial for proper plant growth and potential yields. Environmental changes, with special reference to abiotic stress, can alter the development and productivity of plants and even threaten their survival. Severe changes in the growth, physiology and metabolism of plants caused by abiotic stresses lead to increased accumulation of secondary metabolites. These changes pose challenge or threaten all economically important crops. Harsh climatic conditions such as drought, salinity, extreme temperatures (high and low) and heavy metal contamination significantly affect qualitative and quantitative crop production (Edmeades 2009; Zhu 2002; Lee et al. 2001). Moreover, injudicious use of agrochemicals, pesticides and fertilizers has rendered severe environmental threats including loss of soil fertility. Urbanization and development of industries have led to deforestation and release of toxic

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wastes into the environment posing harmful consequences. Such adversities leading to poor growth of plants are a major concern for agriculturists. Natural adversities, excessive exploitation by human and lack of environment-friendly lifestyles have led to extinction of many plant species.

Of all the economically important plants affected by biotic and abiotic stresses, the medicinal plants are of utmost importance because of the dependency of human population on them for pharmaceutically important metabolites. Chemical synthesis of all the compounds required for various drugs is not possible and also naturally not economical. The production of stable pharmaceutically important compounds is a major challenge for the chemists. The increasing emphasis on herbal treatment of diseases has led to increasing reliance on natural sources rather than chemical compounds. Awareness that the medicinally important plants are more effective and stable when processed minimally has turned the attention of people to include them in their diet. The processing leads to contamination by metals from vessels used and other stabilizing agents used for the medicine. All these result into negative side effects of the processed medicines.

Another problem related to the present-day fast and urbanized lifestyle is the injudicious use of synthetic drugs and antibiotics. Self-medication due to lack of time for consultation and over-the-counter availability of drugs have led to serious complications and development of drug-resistant pathogens. With the increasing awareness about hazards and toxic effects of synthetic drugs, exploitation of medicinal plants for health consideration has become extremely popular.

Plants have evolved to survive the adverse effects of different stresses by initiating a number of molecular, cellular and physiological changes which address the ensuing stress environment. But such alterations may affect the production due to channelizing of the metabolic activities towards acclimatization and adaptation instead of normal growth and yield (Krasensky and Jonak 2012). This could lead to drastic loss especially in case of medicinally important plants where primary and secondary metabolites during normal growth and development are the important sources of drugs. Any alteration in the physiology, biochemistry, genomic, proteomic and metabolic levels caused by abiotic stresses may lead to loss or reduction of the pharmaceutically important chemical production in the plant. Therefore, the changing environmental and climatic conditions along with the increase in global demand for life security emphasize the need for stress-tolerant crop varieties (Newton et al. 2011; Takeda and Matsuoka 2008).

Conventional methods of crop breeding to improve the growth and yield of plants under different environmental threats like biotic and abiotic stresses are time-consuming and not successful in many cases. Use of expensive harmful agrochemicals and pesticides causes severe threat to environment and renders development of resistant pathogens. Nowadays attention has been turned to cost-effective, viable and environment-friendly alternatives such as biological means to improve and facilitate plant growth. Beneficial bacteria, especially in the rhizosphere of plants, have been studied and confirmed to have growth-promoting activities. The beneficial rhizobacteria include the symbiotic *Rhizobium* species, certain actinomycetes and mycorrhizal fungi and free-living bacteria. Plant growth promoting

rhizobacteria (PGPR) are a group of beneficial bacteria which have the potential of improving plant growth and yield besides controlling diseases and rendering tolerance against various abiotic stresses.

Research on the effect of PGPR on medicinal plants is available (Lenin and Jayanthi 2012), but the mechanisms involved have not been completely elucidated as of now. The beneficial effects of PGPR on plants under abiotic stress have also been a topic of research in recent times. A few works have been reported specifically for interaction of PGPR with medicinal plants for alleviation of abiotic stress. The mechanisms reported for interaction with plants in general may also apply for medicinal plants. This chapter is based on reports of plant–PGPR interaction under abiotic stress with special emphasis on plants which are also reported to have medicinal properties. Exclusive reports on PGPR and medicinal plants interaction under abiotic stress are limited.

8.2 Types of Abiotic Stresses

Environmental conditions, like bright light, extreme temperatures, drought, flood, salinity, heavy metals and hypoxia, seriously affect the agricultural production. The changing climatic conditions, whether natural or man-made, are likely to increase the impact of the alterations on crop growth and yield.

8.2.1 Water Stress

Of the various abiotic stresses leading to evolution in plants, availability of water is the most important (Kijne 2006; Zhu 2002). Water stress includes drought, flood as well as salt stress. The impact of drought is a major problem all over the world causing huge loss to farmers and their inputs towards successful cropping. The year 2012 was recorded as the worst drought of the century. While droughts in Europe and the United States had huge impact on commodity markets, shortage of food was the consequence of droughts in Asia. In developing countries like India with its diverse geographical and climatic conditions, farmers are continuously under the threat of abiotic stresses which is a major decisive factor of successful crop yield.

8.2.2 Salinity

Soil salinity is a threat in both developed and developing countries severely affecting agricultural productivity (Jaleel et al. 2007). The agricultural intensification and unfavourable natural conditions have led to increase in soil salinity in the world. The term salt affected refers to soil that are saline (accumulation of salts) or

sodic (too much sodium associated with the negatively charged clay particles) (Rengasamy 2006). It is estimated that more than 800 million hectares of land is affected by salinity throughout the world (FAO 2008). Salinity is of two types—primary and secondary. Naturally occurring salinity in soil and water is known as primary salinity, and those resulting from human activities, such as land development and agriculture, are called secondary salinity.

8.2.3 Extreme Temperatures

Changing temperatures are also a cause of worry for agriculturists all over the world. National Aeronautics and Space Administration (NASA) and National Oceanic and Atmospheric Administration (NOAA) ranked 2012 among the 10 warmest years on record globally. While the truth or myth of global warming is still a topic of debate for scientists, environmentalists, socialists, politicians and economists, there is no doubt about the changing scenario in world temperature affecting the seasonal variations with respective impact on the crops grown. NASA's Goddard Institute for Space Studies (GISS) in New York stated that this decade is warmer than the last decade which was in turn warmer than the previous one. Warmer winters are also a common phenomenon which has serious implications. This alarming issue categorizes temperature as one of the most threatening abiotic stress posing drastic consequences in the forthcoming times.

8.2.4 Heavy Metals

Global industrialization, especially in the field of mining, smelting, manufacturing, fuel production, sewage, municipal wastes and application of fertilizers and pesticides, has significantly contributed to the increase in heavy metal contamination leading to environmental pollution. In contrast to organic pollutants, metals cannot be degraded to harmless products, and they continue to remain in the environment entering water beds and agricultural lands. Common heavy metal contaminants include cadmium, nickel, zinc, chromium, mercury, silver, lead, cobalt and copper. Plants uptake the metals as these are common ingredients of macro- and micro-elements, and this is the basis of phytoremediation of heavy metals. However, assimilation of heavy metals into plants leads to invasion of these harmful elements into the food cycle leading to health threats of human and animals.

8.3 Plant Response to Abiotic Stress

Plants have inherent ability to adjust with seasonal variations, but when subjected to stress like harsh and rapid environmental or climatic conditions, a series of morphological, physiological, biochemical and molecular changes occur leading to alteration in development and yield (Wang et al. 2001). Abiotic stress leads to dehydration and osmotic imbalance of the cells. Almost all types of abiotic stress lead to similar alterations in the plant's biochemical and physiological status.

Some of the medicinal plants, their economically important secondary metabolites and the threatening abiotic stresses have been listed in Table 8.1.

The primary effect of abiotic stress in plants is imbalance of ions and hyperosmotic stress which enhances the accumulation of reactive oxygen species (ROS). Impairment between production of ROS and antioxidant defence leads to disruption of cellular structures and drastic physiological changes like denaturation of proteins, lipids, carbohydrates and DNA (Debnath et al. 2011a). These changes subsequently cause inhibition of photosynthesis and metabolic dysfunction leading to reduced growth and fertility, premature senescence and low yield.

Salt tolerance is a common phenomenon in plants. Salinity induces a number of processes in plants to alleviate osmotic and ionic imbalance. Excessive exposure to salt for a longer period leads to inhibitory effects on growth and yield (Manaa et al. 2011). In vitro studies showed reduced growth under high salinity for medicinal plants *Chlorophytum borivilianum* (Debnath et al. 2011b), *Bacopa monnieri*, *Catharanthus roseus* (Wang et al. 2008) and *Jatropha curcas* (De Oliveira Campos et al. 2012; Gao et al. 2008). Growth and herb yield are comparatively higher in plants under primary salinity than in secondary salinity and plants tend to adapt to gradual increase in salinity after the harm of initial exposure. Salt stress can lead to stomatal closure reducing CO_2 availability in the leaves and inhibits carbon fixation which leads to excessive excitation energy exposure of chloroplasts, thereby generating ROS and oxidative stress (Parvaiz and Satyawati 2008). Excessive generation of ROS induces toxicity causing damage to protein structures, inhibition of many important enzymes of metabolic pathways and oxidation of macromolecules like lipids and DNA which may eventually lead to cell death (Kar 2011; Gill and Tuteja 2010). ROS-initiated formation of oxylipins represents endogenous signals of abiotic stress (Mithofer et al. 2004). Growth inhibition, stimulation of secondary metabolism and lignification leading to cell death occur as a consequence of disturbed redox state of the cell (Schutzendubel and Polle 2002). As a defence response to ROS, plants under salt stress show reduced photosynthetic activity and transpiration rate (Koca et al. 2007). The antioxidant system of cell is composed of radical scavenging metabolites like glutathione and ascorbate along with the protective enzymes. Glutathione donates an electron to unstable molecules of ROS to make them less reactive. It acts as a redox buffer for ascorbic acid to recycle from its oxidized state to the reduced form by the enzyme dehydroascorbate reductase (Jozefczak et al. 2012). The protective antioxidant enzymes include superoxide dismutase (SOD), peroxidase (POD),

Table 8.1 Medicinal plants under abiotic stress (Debnath et al. 2011a)

Medicinal plant	Uses	Secondary metabolites	Abiotic stress	References
Pluchea lanceolata	Bronchitis, dyspepsia and rheumatoid arthritis	Quercetin	Heavy metals	Kumar et al. (2004)
Dioscorea bulbifera	Antispasmodic, analgesic, aphrodisiac and diuretic	Diosgenin	Metal	Narula et al. (2005)
Catharanthus roseus	Cancer and diabetes mellitus	Vinblastine	Salinity	Jaleel (2009)
Ocimum sp.	Cancers, antifertility and adaptogenic	Eugenol	Drought	Khalid (2006)
Jatropha curcas	Skin diseases and rheumatism, piles	Curcin	Salinity	Gao et al. (2008)
Orthosiphon stamineus	Antiallergenic, antihypertensive Anti-inflammatory and nephritis	Polyphenols	Salinity	Ting et al. (2009)
Melissa officinalis L.	Insomnia and anxiety	Quercetin	Salinity and water	Ozturk et al. (2004)
Thymus maroccanus Ball.	Antitussive, antiseptic, antispasmodic and antihelminthic	Thymol	Salinity	Belaqziz et al. (2009)
Matricaria chamomilla	Mucositis and irritable bowel syndrome	Umbelliferone	$CuCl_2$	Eliasova et al. (2004)
Bacopa monnieri	Antioxidant, epilepsy, loss of memory and asthma	Bacoside	Salinity and drought	Debnath (2008)
Olea europaea L.	Antipruritic, antiseptic, astringent and cholagogue	Oleosides	Salinity	Rejskova et al. (2007)
Populus euphratica	Anodyne, anti-inflammatory, febrifuge and vermifuge	Gallic acid	Salinity	Zhang et al. (2004)
Matricaria chamomilla	Sore stomach, irritable bowel syndrome and oral mucositis	Apigenin	Salinity	Razmjoo et al. (2008)
Ziziphora clinopodioides	Antibacterial, sedative, stomachache, carminative, antiseptic and wound healing	Leucoanthocyanins	Salinity and defoliation	Koocheki et al. (2008)
Dioscorea dregeana	Sedative and anti-inflammation	Paclobutrazol	Smoke, temperature	Kulkarni et al. (2007)
Datura innoxia Mill	Anodyne, antispasmodic, hallucinogenic, hypnotic and narcotic	Scopolamine	Light, dark and HCHO deprivation	Laszlo et al. (1998)

catalase (CAT), tryptophan decarboxylase (TDC), reductase, redoxin and phenylalanine ammonia-lyase (PAL). An increase in activity of SOD, POD, CAT, TDC and PAL has been reported in plants under salt stress (Gao et al. 2008).

Salt stress also affects proteins related to cell wall biogenesis, nitrogen, carbohydrate and lipid metabolism (Veeranagamallaiah et al. 2008). However, metabolic adjustments in plants depend on the severity of the stress and on the cultivar.

Different genotypes and plants at different stages of growth also vary in their response to salinity. Salt stress stimulates different response from different layers of cells, and different genes were expressed throughout the duration of stress (Hines 2008). Cellular dehydration occurs in plants under salt stress leading to osmotic stress and removal of water from the cytoplasm which results in reduction of the cytosolic and vacuolar volumes. Ionic and osmotic imbalances due to intercellular accumulation of Na^+ disturb the K^+ nutrition leading to cell toxicity and inhibition of many crucial enzymes (Jaleel et al. 2008).

Similar to salinity, drought or water stress can induce various morphological, biochemical and physiological changes in plants. Some of the alterations caused by drought which inhibit growth include structural changes of stomata, reduced transpiration and photosynthesis, decreased water potential in tissues and membrane disruption. Different stress-responsive genes are activated in plants under water stress. Deficit of water or salinity activates the defence mechanisms through chemical signals. Accumulation of abscisic acid (ABA) is a major signal for drought and salinity in plants and reduces transpiration through stomatal closure thereby diminishing the negative effect of water loss. ABA also induces decrease in photosynthesis and photo-inhibition. ABA regulates expression of stress-responsive genes like late embryogenesis abundant proteins which helps in inducing drought tolerance in plants (Aroca et al. 2008). Deficiency of water also induces accumulation of shikimic acid and levels of amino acids such as proline, tryptophan, leucine, isoleucine and valine (Warren et al. 2012; Bowne et al. 2012). The ROS metabolism of plants is also affected by drought as in *Catharanthus roseus* (Jaleel et al. 2008). Since salinity and water stress are mutually inclusive events, there is overlapping response of plants exposed to these stresses.

Very high temperature also leads to drought conditions thereby causing stress in plants. The physiological alterations in plants due to heat are similar to that under water stress. Low temperatures alter the metabolite profile of plants including that of sugars, phenolics and nitrogenous compounds (Janska et al. 2010; Zhang et al. 1997). Low temperatures also affect the secondary metabolite production.

The hazardous effect of heavy metals on human and animal health is already established. Heavy metal contamination in soil and water bodies leads to exposure of plants to metal pollution. Although plants require trace amounts of heavy metals as micronutrients, exposure to high concentration renders physiological stress (Hall 2002). Heavy metal contamination also inhibits the germination and growth of plants due to production of ROS, disturbing the function and composition of biomolecules (Peng et al. 2010). Plants combat the heavy metal contamination by production of phytochelatins (Hall 2002).

Most of the changes in plants on exposure to any kind of abiotic stress are similar. The ionic imbalance due to salinity-, flood- or heat-induced drought is a common phenomenon. All the abiotic stresses including extreme temperatures and heavy metals activate the defence response of plants leading to excessive generation of ROS and ROS-mediated harmful effects on the physiology of plants.

8.4 Plant Growth-Promoting Rhizobacteria

The zone surrounding the roots of plants in which complex relations exist among the plant, the soil microorganisms and the soil is known as the "rhizosphere". The number, diversity and activity of microorganisms in the rhizosphere microenvironment are more than other parts of soil because of the different physical, chemical and biological properties of the root-associated soil (Kennedy 1998). Rhizosphere microflora includes both deleterious and beneficial elements that have the potential to influence plant growth and crop yield significantly (Compant et al. 2005). PGPR are defined as root-colonizing bacteria that exert beneficial effects on plant growth and development (Cakmakci et al. 2006; Persello-Cartieaux et al. 2003). Bacteria of diverse genera were identified as PGPR of which *Bacillus* and *Pseudomonas* spp. are predominant (Podile and Kishore 2006). The root-colonizing bacteria are the most sought-after group for their multifaceted qualities which include plant growth promotion, disease control and bioremediation.

The effect of PGPR and the mechanisms of interaction have been critically studied through years of vigorous research. PGPR can influence the growth of plants either directly or indirectly. Plant hormone production, enhanced iron availability, phosphorus solubilization and nutrient mobilization are some of the direct methods of growth improvement by PGPR. Indirect growth promotion occurs when PGPR promote plant growth by improving growth-restricting conditions. Production of antagonistic substances to eliminate specific harmful microbes from the vicinity of roots and induction of systemic resistance (ISR) provides protection against pathogens thereby enhancing growth-promoting conditions (Weller et al. 2002; Pierson and Thomashow 1992).

8.4.1 Mechanisms for Growth Promotion

PGPR can affect the plants by the production of diverse metabolites including siderophore and hydrocyanic acid (HCN) (Bhatia et al. 2005), plant hormones such as indole acetic acid (IAA) and some other auxins, gibberellins, cytokinins (Persello-Cartieaux et al. 2001, 2003; Patten and Glick 2002; De Salamone et al. 2001) and ethylene (Glick et al. 1995). There are evidences that the yield-increasing bacteria and other *Bacillus* strains produce plant growth regulators in laboratory culture (Chen et al. 1996). PGPR-produced metabolites like gibberellic

acid, IAA and cytokinin like substances are reported to enhance seed germination and radicle length (Persello-Cartieaux et al. 2003). The production of biologically active metabolites, particularly the plant growth regulators by rhizosphere microbiota, affects plant growth directly after being taken up by the plant or indirectly by modifying the rhizosphere environment (Penrose and Glick 2001). Glick et al. (1998) proposed a model in which PGPR bind to the surface of seeds and in response to tryptophan and/or other amino acids exuded from the germinating seeds, synthesize and secrete IAA. This IAA may be taken up by the seeds and, together with the endogenous IAA, stimulate the cell proliferation and cell elongation or induce the synthesis of 1-aminocyclopropane-1-carboxylate (ACC) synthase. De Leij et al. (2002) suggested that 2,4-diacetylphloroglucinol (DAPG) produced by a *P. fluorescens* strain can act as a plant hormone-like substance, inducing physiological and morphological changes in the plant that can lead to enhanced infection and nodulation by *Rhizobium*. Grimes and Mount (1984) observed an increase in nodulation of plants co-inoculated with *P. putida* and *R. phaseoli*. They proposed that the possible mechanisms for this enhancement include either phosphate supply to the bean plant or a plant growth regulator effect of *P. putida*.

Another mechanism by which certain rhizobacteria improve plant growth is by the breakdown of ethylene which is inhibitory to root growth (Glick et al. 1998). Large number of PGPR strains produces the enzyme ACC deaminase, which hydrolyses ACC, the immediate precursor of the plant hormone ethylene (Belimov et al. 2001; Glick et al. 1995). They stimulate root growth of various crop plants (Belimov et al. 2001; Burd et al. 2000; Glick et al. 1997). This mechanism is most effective on plants that are more susceptible to the effects of ethylene especially under such stress conditions as flooding (Grichko and Glick 2001), drought (Lucy et al. 2004) and phytopathogens (Wang et al. 2000). It has been shown that the bacterial ACC deaminase is not induced in cells grown in nutrient medium abundantly supplied with ammonia (Belimov et al. 2001) which suggests that if sufficient nitrogen is provided to the bacteria, production of ACC deaminase is inhibited.

PGPR can promote plant growth indirectly by affecting symbiotic N_2 fixation, nodulation, or nodule occupancy (Okon et al. 1998) by rhizobia. Co-inoculation of some PGPR strains increased the nodulation of legumes by nitrogen-fixing rhizobia (Kloepper et al. 1991), which were designated as nodulation-promoting rhizobacteria (NPR). Tilak et al. (2006) reported that dual inoculation of *P. putida, P. fluorescens* or *B. cereus* with *Rhizobium* increased plant growth, nodulation and enzyme activity over *Rhizobium*-inoculated and *Rhizobium*-uninoculated control plants. In a study where soybean was co-inoculated with *Bradyrhizobium japonicum* and different isolates of rhizobacteria, the increase in the number and weight of nodules formed by *B. japonicum* was observed (Polonenko et al. 1987). Similarly, Fuhrmann and Wollum (1989) reported fluorescent pseudomonads that consistently increased nodule occupancy of *B. japonicum* in soybean grown in a potting medium with low availability of Fe. Inoculation of legumes with root-colonizing bacteria and *Rhizobium* has been demonstrated to

affect symbiotic nitrogen fixation by enhancing root nodule number or mass (Saxena and Tilak 1994). Lucas-Garcia et al. (2004) had proposed the possibility that metabolites other than phytohormones, such as siderophores, phytoalexins and flavonoids from PGPR, might have a role in enhanced nodule formation. Parmar and Dadarwal (1999) reported that PGPR treatment or application of ethyl acetate extract of the culture supernatant increased concentration of flavonoid-like compounds in roots which are known as chemoattractant for rhizobia. They also reported that rhizobacteria themselves are capable of producing fluorescent flavonoids similar to those produced by the host plant. In addition, PGPR are also known for asymbiotic nitrogen fixation (Figueiredo et al. 2008).

Solubilization of mineral phosphates and mobilization of other essential nutrients by PGPR also help in growth improvement of plants (Bertrand et al. 2001; De Freitas et al. 1997). Yield increase of groundnut by *Pseudomonas* isolates from rhizosphere positively correlated with the ability of these strains to increase available soil phosphorus (Dey et al. 2004). The number of nodules in treated plants was also found to be higher than untreated control, and therefore, it was hypothesized that the energy needed for this symbiotic process is facilitated due to availability of high soil phosphorus content.

8.4.2 Mechanisms for Disease Control

In addition to direct mechanisms for growth promotion, enhanced plant growth is also attributed to the suppression of deleterious microflora by the introduced bacteria (Lugtenberg et al. 2001; Kloepper et al. 1991). Antagonism against plant pathogens are due to production of siderophores (Burd et al. 2000), β-1,3-glucanase (Fridlender et al. 1993), antibiotics (Shanahan et al. 1992), chitinase (Renwick et al. 1991) and hydrogen cyanide (Bhatia et al. 2005). Bacterial antagonists have been widely exploited towards the management of plant diseases (Haas and Defago 2005; Commare et al. 2002). These microorganisms can also function as competitors of pathogens for colonization sites and nutrients.

Triggering the defence mechanism in plants may be one of the important factors of ISR in PGPR-treated plants grown in pathogen-infested soil. Increase in l-phenylalanine ammonia-lyase (PAL), peroxidise (POX) and polyphenol oxidase (PPO) activity has been observed in plants treated with PGPR (Dutta et al. 2008; Ongena et al. 2000). The relation between PAL activity and resistance, in different seedling parts, has also been reported (Saikia et al. 2006). According to some reports, the rapid induction of PAL genes in resistant host and its pathogen might be due to the involvement of a signal transduction mechanism, triggered specifically as a result of interaction between elicitor and receptor molecules, thereby showing differential transcriptional rates of PAL in compatible and incompatible interactions (Kale and Choudhary 2001). Biochemical analysis of rice plants raised from seeds treated with *P. fluorescens* showed an early induction of POX, PAL and chitinase (Nandakumar et al. 2001). Sivakumar and Sharma (2003) reported that

PAL, POX and PPO activities are higher in plants raised from *P. fluorescens*-treated seeds than the increase in pathogen inoculated ones. *Bacillus* enhanced the levels of total phenols, PAL, POX and lipoxygenase in the bacterized seedlings, indicating the involvement of ISR in PGPR-mediated disease control (Sailaja et al. 1997).

Siderophore production by the PGPR may also contribute to the disease suppression in bacterized plants (Yeole and Dube 2000). Siderophores produced by *Pseudomonas* exert killing effect on the plant deleterious fungi *F. oxysporum* and *A. flavus* infecting wheat (Manwar et al. 2000). Fluorescent pseudomonads produce pseudobactin (PSB)-type siderophores which are not readily available as an iron source to other rhizosphere bacteria, but they gain an ecological advantage as they are able to utilize a wide variety of other siderophores (Jurkevitch et al. 1993). Mercado-Banco et al. (2001) reported the production of the siderophore pseudomonine in addition to the fluorescent pseudobactin type in biocontrol strain *P. fluorescens* WCS374.

Production of antibiotics has been reported as another important factor for suppression of diseases by PGPR strains. Bakker et al. (2003) reported that antibiotics do have direct effects on plants and, therefore, might induce systemic resistance. A variety of antibiotics have been identified to be produced by pseudomonads (De Souza et al. 2003; Nielson and Sorensen 2003).

A blend of airborne chemicals released from specific strains of PGPR also promotes growth in plants. It has been reported that volatile organic compounds (VOCs) may play a key role in ISR (Ping and Bolland 2004; Ryu et al. 2004). VOCs secreted by *B. subtilis* and *B. amyloliquefaciens* were able to induce ISR in *Arabidopsis* against *Erwinia carotovora* (Ryan et al. 2001). The discovery that VOCs of bacteria trigger enhancement in plant growth constitutes an unreported mechanism for the elicitation of plant growth by rhizobacteria. It is possible that volatiles produced by PGPR colonizing roots are generated at sufficient concentrations to trigger plant responses (Ryu et al. 2003).

8.5 PGPR a Boon Alleviating Abiotic Stress

PGPR from medicinal plants like *Withania somnifera*, *Catharanthus roseus*, *Coleus forskohlii*, *Ocimum sanctum* and *Aloe vera* have been reported to improve growth and yield (Karthikeyan et al. 2008; Attia and Saad 2001). Various PGPR strains belonging to the genera *Azospirillum, Azotobacter, Pseudomonas* and *Bacillus* have been isolated and applied for growth improvement. A formulation of PGPR including *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Pseudomonas fluorescens and Bacillus megaterium* significantly enhanced germination rate, vigour index and chlorophyll content of *Catharanthus roseus* (Lenin and Jayanthi 2012).

Microbes from extreme environments are known to be adaptive to the surrounding environmental conditions. Bacteria from saline conditions can tolerate high salt concentrations, from water-deficit areas can survive in high temperature and low moisture content. Bacteria undergo various morphological, biochemical and

physiological adaptations to survive in the changing environmental conditions. These bacteria can act as a potential source of PGPR as they can survive and establish in the roots of plants growing in harsh environment thereby exerting the beneficial effect on plant growth and disease control under abiotic stress. The influence of PGPR in alleviation of harmful effects caused by abiotic stresses has been reported (Christian et al. 2009) such as drought (Alvarez et al. 1996), waterlogging (Saleem et al. 2007), oxidative stress (Stajner et al. 1995, 1997) and salinity (Weyens et al. 2009; Yang et al. 2009; Venkateswarlu et al. 2008). Bharti et al. (2013) indicated the role of a halotolerant PGPR strain *Exiguobacterium oxidotolerans* against salinity, improving the growth and yield of *Bacopa monnieri*. PGPR improved tomato and pepper growth under water stress (Aroca and Ruiz-Lozano 2009). Tolerance of plants against abiotic stress due to physical and chemical changes induced by PGPR is termed as "induced systemic tolerance" (IST) (Sandhya et al. 2010). Native rhizobacteria from plants under drought conditions like in arid regions are more competent in enhancing tolerance against water stress (Ilyas and Bano 2010; Marulanda et al. 2008).

Application of PGPR has been effective in reducing the harmful effects of drought (Sarig et al. 1992). Crops treated with PGPR such as *Azospirillum, Klebsiella* and *Paenibacillus* under varied agro-climatic conditions have shown improved growth and yield with extensive root growth facilitating better uptake of water and minerals (Dobbelaere et al. 2001; Timmusk and Wagner 1999). PGPR-treated seedlings when exposed to water stress showed better water status (Casanovas et al. 2002; Creus et al. 1998) thereby indicating the efficacy of PGPR in water-deficient soils (Okon 1985). Similar effect of *A. brasilense* was recorded against salinity in wheat seedlings with relatively higher water content (Creus et al. 1997) which could be due to various physiological changes induced by the colonizing bacteria. Creus et al. (2004) showed a higher water status and elastic adjustment in *Azospirillum*-inoculated wheat leading to higher grain yield with better mineral quality. Various mechanisms involved in mitigation of abiotic stress include increase in proline levels (Dimkpa et al. 2009), decrease in excessive ethylene through ACC deaminase (Barnawal et al. 2012; Arshad et al. 2008), reduction in uptake of Na ions through exopolysaccharide (EPS) production (Kohler et al. 2010). The activity of ACC deaminase and production of EPS were strategies of *Bacillus* and *Exiguobacterium* for abiotic stress elimination and growth improvement (Sgroy et al. 2009; Yumoto et al. 2004; Dastager et al. 2010; Selvakumar et al. 2010). EPS production could restrict Na^+ influx into the roots, and accumulation of proline and glutamate could act as osmoprotectant to reduce the negative effects of salinity and water stress in *Azospirillum*-treated plants (Ashraf et al. 2004; Casanovas et al. 2003, 2002). More than one mechanism seems to be involved in mitigating abiotic stress by PGPR.

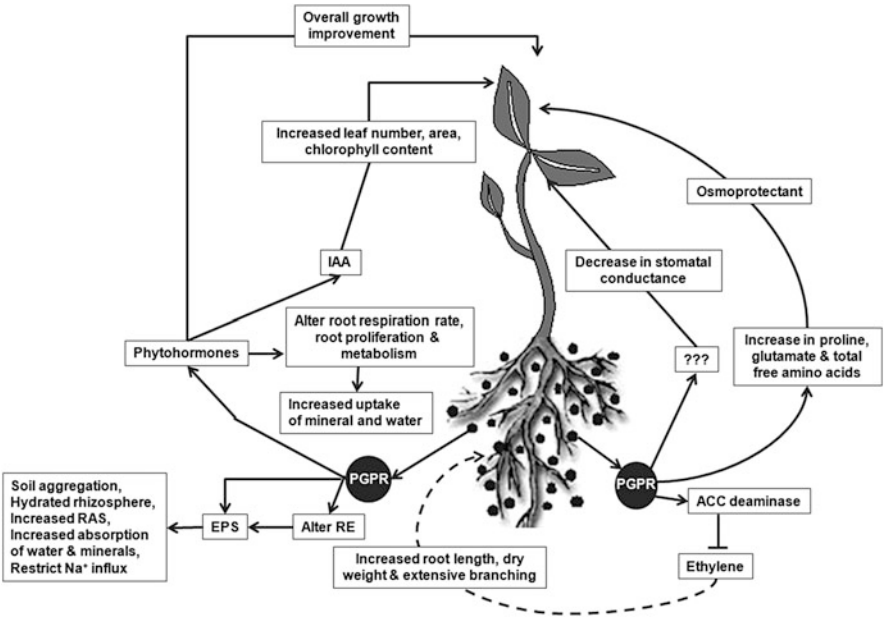

Fig. 8.1 Morphological and physiological changes in plants by application of PGPR leading to abiotic stress tolerance. *IAA* indole acetic acid, *EPS* exopolysaccharide, *RE* root exudate, *ACC* 1-aminocyclopropane-1-carboxylate, *PGPR* plant growth-promoting rhizobacteria, *RAS* root-adhering-soil, ???- unknown mechanism. Production of phytohormones increases the overall growth and also alters root characteristics to facilitate uptake of water and minerals. IAA increases the size of aerial parts of the plants. ACC deaminase reduces the ethylene level to eliminate the negative effect on roots. Production of osmoprotectants by PGPR also contributes towards abiotic stress tolerance. Soil aggregation due to production of EPS or alteration of RE hydrates the rhizosphere and helps in increased uptake of water and minerals

8.5.1 Morphological and Physiological Changes

Fluorescent pseudomonads increased leaf number, leaf area and greenness of black henbane under water-deficit stress conditions which was attributed to release of IAA by the PGPR strains (Ghorbanpour et al. 2013). Exhaustive root system of PGPR-treated plants could help the plants in better assimilation of nutrients and water. Changes in root growth such as increase in root length, dry weight and excessive root branching in PGPR-treated plants (Creus et al. 2005; Marcelo et al. 2000; Okon and Vanderleyden 1997) help the plant to withstand water stress (Fig. 8.1). Thin and branched root system is more efficient in water and mineral uptake. Enhanced root growth due to production of ACC deaminase by PGPR may also render benefit to the plants under stress conditions (Glick et al. 2007; Patten and Glick 2002). Inoculation of plants with ACC deaminase-producing PGPR partially reduced the negative effect of drought on growth, yield and ripening (Arshad et al. 2008).

Changes in stomatal conductance, leaf transpiration and root hydraulic properties are some of the physiological mechanisms of plant to cope with drought conditions to increase the water-use efficiency (Tambussi et al. 2007). This is important for the plant to survive under limited water conditions. Decrease in stomatal conductance is reported by application of PGPR thereby increasing water-use efficiency (Yasmin et al. 2013; Benabdellah et al. 2011) (Fig. 8.1). Highest leaf relative water content and lowest membrane leakage have been recorded in wheat and barley treated with PGPR strains of *Bacillus* and *Azospirillum* (Turan et al. 2012). Several studies report an increased positive effect on plants under drought conditions upon treatment with arbuscular mycorrhizal fungi along with rhizobia or PGPR (Fig. 8.1) like *Enterobacter* and *Bacillus* (Tarafdar and Rao 2007; Valdenegro et al. 2001).

Higher cell wall elasticity and the ability to modify plant hormones are some of the mechanisms induced by *Azospirillum* to combat with salinity and osmotic stress (Creus et al. 1998; Bashan et al. 2004). Similar results of growth improvement due to secretion of phytohormones by PGPR have been reported for *Catharanthus roseus* and *Phaseolus vulgaris* (Jaleel and Panneerselvam 2007; Marcelo et al. 2000). Production of secondary bioactive metabolites by bacteria stimulates plant growth under saline conditions (Dilfuza 2012). Rice plants treated with IAA-producing osmotolerant rhizobacteria showed enhanced root growth and water uptake in drought condition (Yuwono et al. 2005). PGPR-inoculated plants showed altered root respiration rate, root proliferation and metabolism through the production of phytohormones thereby improving mineral and water uptake (Okon and Itzigsohn 1995) (Fig. 8.1). Upregulation of proline biosynthesis pathway and accumulation of total free amino acids in PGPR-inoculated plants maintain the water level in cell thus helping in bearing the salinity and osmotic stress (Yoshiba et al. 1997).

Although the uptake of sodium by plants is not altered by rhizobacteria (Mayak et al. 2004), tomato plants bacterized with *Achromobacter* and exposed to salt stress showed an enhancement in photosynthesis than untreated plants. The exact mechanism is yet to be understood, but it was suggested that increase in phosphorous and potassium uptake might be responsible for this positive effect (Mayak et al. 2004). Increase in chlorophyll and other pigment content of PGPR-treated plants could be a biological strategy to reduce the drought-induced deleterious effect (Ghorbanpour et al. 2013). Salt-stressed maize when inoculated with ACC deaminase containing *Pseudomonas syringae, Enterobacter aerogenes* and *P. fluorescens* resulted in higher K^+/Na^+ ratios, high relative water, chlorophyll and low proline contents (Nadeem et al. 2007) (Fig. 8.1). High K^+/Na^+ ratios were also found in salt-stressed maize in which selectivity for Na^+, K^+ and Ca^{2+} was altered upon inoculation with *Azospirillum* (Hamdia et al. 2004). Moreover, exopolysaccharide-producing bacteria when applied to wheat showed a decrease of Na^+ uptake (Ashraf et al. 2004). This possibly may be due to reduced apoplastic flow of sodium ions into the stele due to formation of soil layer surrounding the root.

8.5.2 Soil Structure Alteration

Soil aggregation is a major factor contributing to retention and movement of water, aeration and temperature which, in turn, affect germination and root growth (Dickson et al. 1990) as unstable aggregates have lower organic matter (Haynes and Swift 1990). EPS production by microbes increase soil aggregation (Lynch and Bragg 1985) and help in maintaining a hydrated microenvironment around the microorganism (Chenu and Roberson 1996). PGPR are also reported to increase root-adhering soil (RAS) due to EPS (Bezzate et al. 2000; Gouzou et al. 1993). Changing the structure and aggregation of RAS by the production of EPS by PGPR can help plants grow and survive under water-limiting conditions (Fig. 8.1). Increase in soil around roots under stress condition to form a mucilaginous layer around cells affects the absorption of water and mineral uptake (Bezzate et al. 2000; Amellal et al. 1998; Gouzou et al. 1993). PGPR may also stimulate exudation of more polysaccharides from root caps as an indirect method of improving soil adhesion and aggregation in the roots. Alteration of plant root exudates by colonizing PGPR strains has been reported earlier (Dardanelli et al. 2009; Kamilova et al. 2006). An increased RAS to root tissue ratio was observed in wheat treated with *Bacillus polymyxa* or *Pantoea agglomerans* irrespective of the water conditions (Amellal et al. 1998; Gouzou et al. 1993). Thus, an increase in RAS and soil aggregation due to EPS helps the rhizosphere to remain hydrated and protect plants against abiotic stresses (Fig. 8.1).

8.5.3 PGPR Against Oxidative Stress

PGPR has been reported to increase drought tolerance in plants by production of IAA, cytokinins, antioxidants and ACC deaminase. Proline along with other non-enzymatic antioxidants induces resistance against salinity by protecting macromolecules from effects of oxidative stress (Galli et al. 1996) (Fig. 8.2).

ROS-mediated activation of lipid peroxidation destabilizes membrane. PGPR is also reported to protect the plants under saline condition (Fig. 8.2) by reducing membrane destabilizing activity in the cell (Khan and Panda 2008). PGPR enhances ROS-scavenging enzymes such as catalase and ascorbate peroxidase (Gururani et al. 2012; Kohler et al. 2010) which may help the plants under salinity and drought stress to balance the harmful effects of ROS (Fig. 8.2). Treatment of pathogen-challenged tomato, hot pepper and pigeon pea with fluorescent pseudomonads increased activities of POX and PPO (Ramamoorthy et al. 2002; Dutta et al. 2008). Thus, increase in ROS-scavenging enzymes as a means of ISR can also help the plants tolerate abiotic stresses (Fig. 8.2).

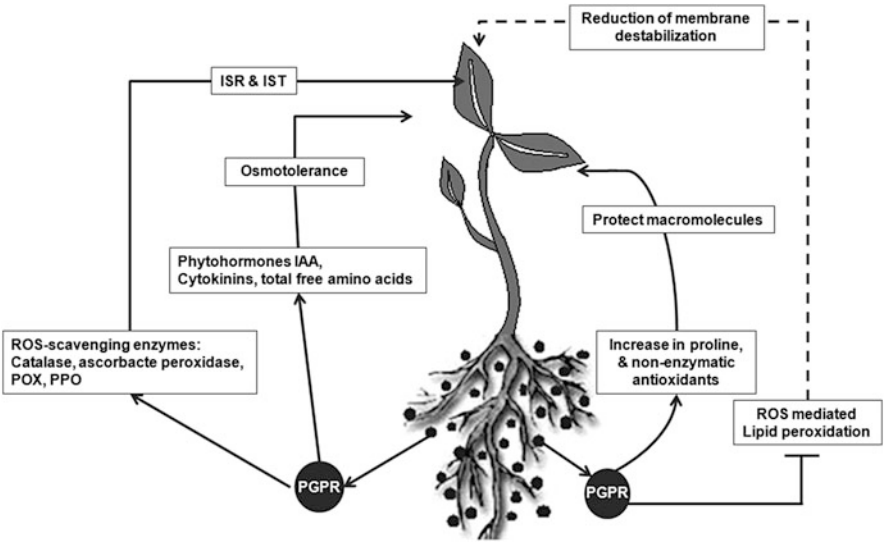

Fig. 8.2 PGPR in mitigating ROS-mediated harmful effects. *ISR* induced systemic resistance, *IST* induced systemic tolerance, *IAA* indole acetic acid, *POX* peroxidase, *PPO* polyphenol oxidase, *PGPR* plant growth-promoting rhizobacteria. Production of phytohormones by PGPR induces osmotolerance. ROS-scavenging enzyme mediated alteration of redox state helps plant tolerate abiotic stress. Increase in proline and non-enzymatic antioxidants protect macromolecules improving plant growth under oxidative stress. PGPR resists ROS-mediated lipid peroxidation reducing membrane destabilization

8.5.4 PGPR for Bioremediation of Soil

PGPR can alleviate soil contamination (Zhuang et al. 2007; Huang et al. 2004, 2005) through mineralization of organic compounds in association with plants (Saleh et al. 2004). PGPR like *Bacillus*, *Pseudomonas* and *Methanobacteria* are used for bioremediation of soil because of their high tolerance to heavy metals (Milton 2007). Bacteria have been reported to remove carbon, nitrogen, phosphorus and toxic metals aromatic compounds, herbicides and pesticides through both aerobic and anaerobic metabolisms (Milton 2007; Zhang et al. 2003). Interaction with other beneficial microbes like mycorrhizal fungi may contribute towards resisting abiotic stress effects on plants (Gamalero et al. 2009; Marulanda et al. 2009) (Fig. 8.3). Natural bioremediation of soil-polluting organic compounds through application of bacterial and fungal isolates has been reported (Juwarkar et al. 2010). Endophytic bacteria, such as *Achromobacter xylosoxidans*, *Bacillus pumilus* and *Corynebacterium flavescens*, prevent plants from the harmful effects of heavy metals and xenobiotics (Glick 2010). Production of pollutant-degrading enzymes, like POX and phosphatase, contributes towards transformation of pollutants (Dowling and Doty 2009; Gerhardt et al. 2009) (Fig. 8.3). PGPR enhances the

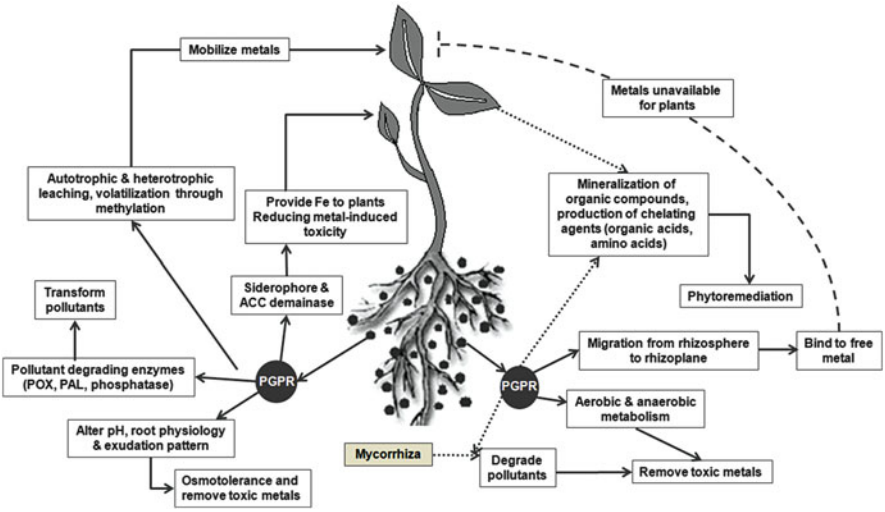

Fig. 8.3 Effect of PGPR in bioremediation. *PAL* phenylalanine ammonia-lyase, *POX* peroxidase, *ACC* 1 aminocyclopropane-1-carboxylate, *PGPR* plant growth-promoting rhizobacteria. Production of pollutant-degrading enzymes like PAL and POX transform pollutants in soil making them unavailable for plants. Alteration of pH in the rhizosphere and exudation pattern increases osmotolerance and removes toxic metals through acidification of soil. Chelating agents siderophores provide sufficient iron (Fe) to the plants reducing metal-induced toxicity. Through aerobic and anaerobic metabolism, PGPR removes toxic metals from rhizosphere. PGPR in combination with mycorrhiza helps in degradation of heavy metal pollutants. Plant and PGPR work towards phytoremediation. Physical movement of PGPR from rhizoplane to rhizosphere facilitates their binding to free metals such as Ni thereby making them unavailable for plants. Metals are mobilized in plants through autotrophic and heterotrophic leaching and volatilization by methylation

PAL activity in plants. This may help the plants thrive under heavy metal contamination such as nickel.

Microorganisms can affect the availability of metals in the rhizosphere by altering the pH and by altering the redox potential (Smith and Read 1997). *Azospirillum brasilense* can alter pH of the rhizosphere (Carrillo et al. 2002), and inoculation with *Azospirillum* may change root physiology and patterns of root exudation (Heulin et al. 1987) (Fig. 8.3). Chelating agents such as organic acids and amino acids by PGPR and associated plants also help in remediation of heavy metal soil contamination (Marchner et al. 1996). Since PGPR can change the root exudates profile such as that of organic acids, the acidification of rhizosphere might affect the metal availability. Tomato treated with biocontrol strain *Pseudomonas fluorescens* showed alteration in the composition of organic acids and sugars in root exudates (Kamilova et al. 2006). Siderophore and ACC deaminase-producing bacterium *Kluyvera ascorbata* protected canola plants from growth inhibitory effects of high concentration of nickel by providing sufficient iron to the plants so as to reduce the toxic effects of nickel and by reducing the nickel-

induced stress ethylene formation (Burd et al. 1998). Also, autotrophic and heterotrophic leaching, volatilization through methylation and release of chelators can mobilize metals (Fig. 8.3). Heavy metal availability can also be reduced due to sorption to cell components followed by intracellular sequestration or precipitation as insoluble organic or inorganic compounds (White and Gadd 1996). *Klebsiella mobilis*-treated barley plants showed increase in grain yield and decrease of Cd contents in grains when grown in Cd-rich soil and simulation of these effects with a mathematical model revealed that the underlying mechanisms might be the migration of bacteria from rhizoplane to rhizosphere (Fig. 8.3) where bacteria bind to free Cd ions forming a complex that cannot be taken up by the plant (Pishchik et al. 2002). This indicates that the PGPR may themselves bind to heavy metals and make them unavailable for plants, produce organic acids, chelators, etc., for removal of heavy metals from the soil and/or may induce the plants to exude these substances in the rhizosphere thereby altering the microenvironment to facilitate degradation of metal pollutants (Fig. 8.3).

8.5.5 *Factors Affecting Against Both Abiotic and Biotic Stresses*

PGPR exerting beneficial effects under abiotic stresses also result in ISR in plants (Barriuso et al. 2008). The priming action during ISR by PGPR could help plants tolerate abiotic stresses. The phenomenon of priming has not been completely elucidated at molecular level but is associated with signaling proteins which remain inactive under normal conditions and start accumulating and transduced in activated form when plants are exposed to stresses (Conrath et al. 2006). Studies on gene expression in *Arabidopsis thaliana* treated with *Paenibacillus polymyxa* when exposed to either biotic or abiotic stress showed co-regulation of genes (Timmusk and Wagner 1999). Similar studies in rice showed the role of cold acclimation transcription factor *Osmyb4*, in enhancing tolerance against both biotic and abiotic stresses in transgenic *A. thaliana* (Vannini et al. 2006). Promoters of osmotins that accumulate under abiotic stress such as salt also activate during pathogen attack (Liu et al. 1995).

However, the regulatory mechanisms involved are very complex and can occur in translational and post-translational levels (La Rosa et al. 1992). Xiong and Yang (2003) confirmed the induction of an ABA-inducible mitogen-activated protein kinase (MAPK) by both abiotic and biotic stresses and, further, tolerance against drought, salinity and cold stress due to over expression of this gene. However, suppression of MAPK enhances against pathogens whereas tolerance to abiotic stresses is drastically reduced.

8.6 Effect of Abiotic Stress on Secondary Metabolites of Plants

Secondary metabolites of medicinal plants are of importance because most of the pharmaceutically important chemicals are produced in the form of secondary metabolites in plants. Secondary metabolites are crucial as they are important for plant growth and development and are needed in plant defence against herbivores and pathogens and confer protection against environmental stresses (Seigler 1998). Secondary plant metabolites such as calcium, abscisic acid (ABA), salicylic acid (SA), polyamines and jasmonates (JA) and nitric oxide accumulate in plants exposed to stress, thus altering the physiology (Tuteja and Sopory 2008; Seigler 1998). Most of these chemicals have medicinal, nutritional and cosmetic value. Drugs like morphine, codeine, cocaine and quinine; alkaloids from *Catharanthus* and belladonna; colchicines, phytostigminine, pilocarpine and reserpine; and steroids like diosgenin, digoxin and digitoxin, flavonoids, phenolics, etc., are some of the economically and industrially important secondary metabolites of plants (Ramakrishna and Ravishankar 2011).

Abiotic stresses increase or decrease the production of secondary metabolites in plants. Elicitation has been used to increase secondary metabolite production in plant cell cultures under in vitro conditions (Dicosmo and Misawa 1985). However, increase or decrease in response to abiotic stress depends on the genotype. Anthocyanins and JA are reported to increase in response to salt stress, but salt-sensitive plants showed a decrease in anthocyanin (Daneshmand et al. 2010; Pedranzani et al. 2003). Anthocyanins are reported to accumulate under drought and cold temperature stress. Similarly, salt-tolerant alfalfa showed an increase in the proline content under salt stress, but the increase was slow in salt-sensitive varieties (Petrusa and Winicov 1997). Alteration in polyphenols and phenolic content in response to abiotic stress has also been reported (Navarro et al. 2006; Dixon and Paiva 1995). Similar alterations in phenolics, carotenoids and flavonoids have also been reported in response to drought (Anjum et al. 2003). Drought induced decrease in chlorophyll content in *Catharanthus roseus* and saponins in *Chenopodium quinoa* (Soliz-Guerrero et al. 2002).

Influence of metal ions on secondary metabolites has been reported. Although trace amounts of nickel (Ni) is required for plant growth, excess of Ni leads to a decrease or inhibition in anthocyanin content (Hawrylak et al. 2007; Krupa et al. 1996). This alteration is caused by the inhibition of PAL activity (Krupa et al. 1996). Similarly, metal ions like Cu^{2+}, Co^{2+}, Zn^{2+} and Cd^{2+} stimulate the production of betalains in *Beta vulgaris* (Trejo-Tapia et al. 2001), betacyanins in callus cultures of *Amaranthus caudatus*, lepidine in cultures of *Lepidium sativum* (Obrenovic 1990) and putrescine in oat and bean plants. However, Cd^{2+} and Cu^{2+} treatment has also been reported to reduce putrescine and spermidine content in sunflower leaf discs (Groppa et al. 2001). Scopolamine and hyoscyamine were elicited by silver or cadmium in cultures of *Brugmansia candida* (Angelova et al. 2006).

Variations in temperature affect the metabolite profile of plants. Low temperatures increase the synthesis of cryoprotectants, soluble sugars, phenolics, anthocyanins and nitrogenous compounds (Janska et al. 2010; Chan et al. 2010; Zhang et al. 1997). Increase in endogenous jasmonates in *Pinus pinaster* and polyamines like putrescine and spermine in carrots and alfalfa in response to low temperatures is also reported (Pedranzani et al. 2003; Lei et al. 2004). In contrast, high temperature reduces the production of anthocyanin in *Perilla frutescens* suspension cultures (Zhong and Yoshida 1993). High temperature enhanced the production of secondary metabolites and storage ginsenoside in root of ginseng *Panax quinquefolius* (Jochum et al. 2007).

8.7 Preference of PGPR over Abiotic Stress as Inducer of Secondary Metabolites

Effect of PGPR on the secondary metabolites of plants has been documented. In medicinal plants *Artemisia annua* and *Centella asiatica*, rhizosphere bacteria enhanced triterpenoids (Satheesan et al. 2012; Awasthi et al. 2011). The increase in secondary metabolite production was suggested because of increase in leaf to stem ratio in PGPR-treated *Bacopa* (Bharti et al. 2013) (Fig. 8.4). Strain-specific effect of PGPR on secondary metabolites of plants has been suggested by Walker et al. (2011). *E. oxidotolerans* was also reported to increase herb yields and content of bacoside A in plants.

Methyl jasmonates (MeJ) and JA are signaling molecules of biotic and abiotic stresses (van der Fits and Memelink 2000). JA and MeJ elicit production of secondary metabolites like alkaloids, terpenoid, phytoalexins, coumarins and taxanes in plants some of which act as defence response. MeJ and growth regulators like 2, 4-D, IAA and NAA supported growth and increased anthocyanin in treated plants (Zhang et al. 2002; Fang et al. 1999). Anthocyanin production was also enhanced through the manipulation of phytohormones in cell suspensions of *Ipomoea batatas* (Nozue et al. 1995) and *Oxalis reclinata* (Makunga et al. 1997). Alteration of phytohormones by PGPR has been reported. PGPR is reported for ISR in plants through JA and ethylene pathway (Spoel and Dong 2012; van Wees et al. 1999). Pieterse et al. (1998) reported that systemic resistance induced by *P. fluorescens* requires responsiveness to ethylene and JA.

As precursors for JA and MeJ, the lipoxygenase (LOX) products also could be involved in ISR (Xu et al. 1994) and secondary metabolite production. The products of LOX pathway contribute to defence reactions by inhibition of pathogen growth and development (Namai et al. 1990), induction of phytoalexin accumulation and/or in signal transduction (Choi and Bostock 1994) (Fig. 8.4). The peroxidation of polyunsaturated fatty acids (PUFAs) by LOXs could be a major source of peroxidases in stressed plant tissue. Peroxidized fatty acids are highly reactive and could be further metabolized to signal molecules such as jasmonates, traumatin

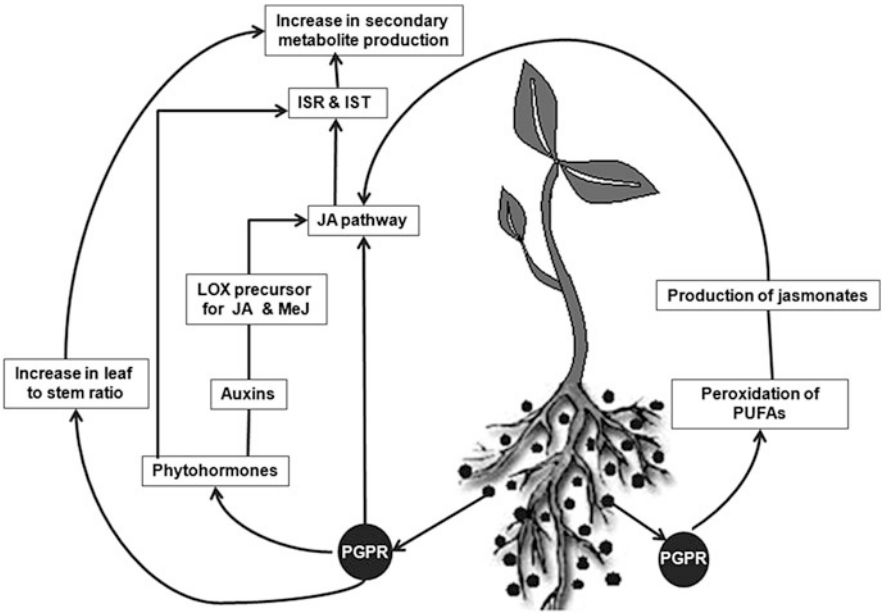

Fig. 8.4 Effect of PGPR on enhancing secondary metabolites in plants. *LOX* lipoxygenase, *ISR* induced systemic resistance, *IST* induced systemic tolerance, *JA* jasmonic acid, *MeJ* methyl jasmonates, *PUFA* polyunsaturated fatty acids, *PGPR* plant growth-promoting rhizobacteria. PGPR is known to induce systemic resistance in plants through the JA pathway which are signals abiotic stress. ISR indirectly prepares the plants to tolerate abiotic stress. Phytohormone such as auxins increase JA and MeJ through the precursor LOX. Peroxidation of PUFAs also produces jasmonates affecting ISR and IST

and hexenals (Croft et al. 1993) (Fig. 8.4). Liu et al. (1991) suggested that the auxins affect the LOX activity. PGPR are known to increase auxin in host plants (Patten and Glick 2002), and *B. subtilis* has been reported to increase the hormone levels, which in turn induce LOX (Sailaja et al. 1997). Thus, auxins produced by PGPR could influence the LOX pathway enhancing JA and MeJ which, in turn, could act as elicitors for increase in secondary metabolite production by plants (Fig. 8.4). Thus, activation of JA by PGPR and production of phytohormones could indirectly result in the production of secondary metabolites in plants besides triggering the defence response in plants and growth promotion.

Abiotic stress has also been applied as elicitor for increasing secondary metabolites like alkaloids in medicinal plants. But plants under abiotic stress such as salinity and water stress have reduced biomass which reduces the overall alkaloid production. Thus, PGPR-induced increase in plant biomass with extensive root branching and increased leaf size and area would lead to increased production of secondary metabolites. Although abiotic stress increases some secondary metabolites, the hazardous effects on plants such as reduced chlorophyll content lead to growth-limiting conditions. PGPR has been reported to increase chlorophyll content in plants. Dutta et al. (2005) reported that a *P. fluorescens* strain increased

germination percentage, dry weight, leaf area and chlorophyll content over the control in mung bean.

Moreover, increase in secondary metabolites production due to abiotic stress is prevalent only at the initial stage of exposure, but PGPR induces long-term tolerance (Egamberdiyeva and Hoflich 2004). Furthermore, most of the studies indicating increase in secondary metabolites due to abiotic stress as elicitors are done under in vitro conditions in cell cultures. The scenario may not resemble exactly in vivo or under field conditions.

8.8 Conclusions

The positive influence of PGPR on growth and yield of plants under biotic and abiotic stresses is well established. Although the mechanisms of action of PGPR for plant growth improvement and disease control has been a vigorous topic of research, complete elucidation of the interaction is yet to be accomplished. Beneficial effect of PGPR on various medicinal plants' growth has been reported. Studies on medicinal plants and PGPR are limited and more so under abiotic stress conditions. The positive effect on secondary metabolite production of medicinal plants by PGPR is also known. The mechanisms such as alteration of phytohormone production, root morphology, ROS-scavenging enzymes and soil aggregation which have been studied in PGPR-treated plants under abiotic stresses like drought, salinity and soils contaminated with heavy metal indicate that the same mechanisms may also be applicable to medicinal plants. However, detailed experimental studies have to be done to establish the exact mechanisms involved. The application of PGPR for improvement of growth and disease control under abiotic stress holds promise. It needs to be exploited for sustainable improvement in growth and production of economically important medicinal plants.

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Chapter 9
Efficiency of Phytohormone-Producing *Pseudomonas* to Improve Salt Stress Tolerance in Jew's Mallow (*Corchorus olitorius* L.)

Dilfuza Egamberdieva and Dilfuza Jabborova

9.1 Introduction

Natural salinity is the result of a long-term natural accumulation of salts in the soil or in surface water, and it is estimated that 33 % of the potentially arable land area of the world is affected by salinity (Ondrasek et al. 2009). Climate change will even increase soil salinity further, since it is accompanied by less rainfall and higher temperatures (Othman et al. 2006). Many studies have demonstrated that salinity inhibits seed germination and growth of various agriculturally important crops, vegetables, and also medicinally important plants (Teixeira da Silva and Egamberdieva 2013; Egamberdieva et al. 2011, 2013a; Jamil et al. 2006; Xu et al. 2011; Khodarahmpour et al. 2012). In aromatic and medicinal plants, growth and synthesis of biological active compounds are influenced by various environmental factors such as salinity, drought, and water stresses (Hasegawa et al. 2000; Parida and Das 2005). Soil salinity inhibits plant growth and the development of *Satureja hortensis* and *Eragrostis curvula* (Colom and Vazzana 2002; Baher et al. 2002), *Citronella* (Kumar and Gill 1995), *Ammolei majus,* and *Hyoscyamus niger* (Ashraf 2004). Several explanations for these effects have been proposed, such as inhibition of the activity of enzymes involved in nucleic acid metabolism (Arbona et al. 2005) and inhibition of biosynthesis of plant hormones within plant tissues (Prakash and Parthapasenan 1990). Debez et al. (2001) observed that salt stress caused by NaCl inhibited the endogenous levels of phytohormones such as gibberellins, abscisic acid, jasmonic acid, and salicylic acid in plants, which correlated with a reduction of root growth in salt bush (*Atriplex halimus* L.). In other study, Figueiredo et al. (2008) reported decreased levels of auxins and gibberellins in the roots of common beans.

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Jew's mallow is, in the tropics and sub-tropics, among the most common plants that thrive nearly anywhere including Middle East, Asia and Africa. The plant is used as food ingredient, herb, and vegetable, and contains acidic polysaccharides, proteins, calcium, thiamin, riboflavin, and dietary fibers (Leung et al. 1968; Tsukui et al. 2004). *C. olitorius* is mostly distributed in arid and stress environment (Fawusi et al. 1984; Chaudhuri and Choudhuri 1997). However its production is reduced by high salinity and poor soil conditions (Velempini et al. 2003). It has been reported that the plant growth and yield of jew's mallow could be improved by Arbuscular mycorrhizal (AM) fungi (Nwangburuka et al. 2012). It has been proposed that the external supply of plant growth regulators produced by root-associated microorganisms under stressed conditions may help plants to cope with abiotic stress (Li et al. 2005).

Most of the root-associated bacteria produce phytohormones such as IAA, GA, abscisic acids, and cytokinins (Egamberdieva et al. 2001, 2004; Egamberdieva and Hoflich 2002; Lyan et al. 2013; Jabborova et al. 2013a; Matiru and Dakora 2004; Hayat et al. 2008). The abilities of PGPR strains to produce plant growth regulators could balance the decrease in the phytohormone levels of the plant roots and alleviate salt stress in plants (Egamberdieva 2009, 2013). The ameliorative effects of PGPR on plant growth under saline conditions have been shown on various plant species, including medicinally important plants (Yildirim and Taylor 2005; Egamberdieva and Lugtenberg 2014). For example, *Pseudomonas* strains alleviated the salinity effects on the growth of basil (*Ocimum basilicum*) (Golpayegani and Tilebeni 2011), goats rue (*Galega officinalis* L.), and milk thistle (*Silybum marianum*) (Egamberdieva et al. 2013a).

This study was conducted to evaluate the effectiveness of phytohormone-producing *Pseudomonas* strain and plant growth regulators such as auxins and gibberellins in improving growth and salt tolerance of jew's mallow (*Corchorus olitorius* L.) under saline conditions.

9.2 Materials and Methods

9.2.1 Plant and Bacteria

The seeds of jew's mallow (*Corchorus olitorius* L.) were obtained from the Department of Botany, Faculty of Biology and Soil Sciences of Uzbekistan. Seeds were sorted to eliminate broken, small, and infected seeds. Seeds were surface-sterilized by immersing them for 1 min in concentrated 10 % v/v NaOCl, followed by 3 min in 70 % ethanol, and rinsed five times with sterile, distilled water. The sterility of seeds was tested on Nutrient agar by incubating plates for 3 days at 28 °C.

The salt-tolerant bacterial strain *Pseudomonas extremorientalis TSAU6*, which produces IAA under saline conditions (7.4 μg/ml) and GA (0.4 μg/ml) was obtained from the culture collection of National University of Uzbekistan. The strain

Pseudomonas fluorescens WCS365, which doesn't produce IAA, was obtained from the culture collection of Leiden University of the Netherlands.

9.2.2 Germination and Seedling Growth

Seed germination was carried out in 85 mm × 15 mm tight fitting plastic petri dishes with 5 ml solution consisting of 0 and 100 mM NaCl. Ten healthy and uniform seeds were sown in each petri plate with three replicates. A filter paper (Whatman No. 2) was soaked in a solution of the respective salt concentrations. To determine the effects of plant growth regulators on seed germination and seedling growth, auxins (IAA) and gibberellic acid (GA) were used at 1 and 0.1 and 0.01 and 0.001 μM concentrations under nonsaline and saline (100 mM NaCl) conditions.

Bacterial strains *Pseudomonas fluorescens* WCS365 and *Pseudomonas extremorientalis* TSAU6 were grown overnight in KB broth. 1 ml of each culture was pelleted by centrifugation, and the supernatant was discarded. Cell pellets were washed with 1 ml phosphate buffered saline (PBS, 20 mM sodium phosphate, 150 mM NaCl, pH 7.4) and suspended in PBS. Cell suspensions were diluted to an optical density of 0.1 at 620 nm, corresponding to a cell density of 10^8 cells/ml. Seeds were placed in the bacterial suspension using sterile forceps and shaken gently for a few seconds. After 10 min, the inoculated seeds were then aseptically placed into *petri dishes* moistened with water, with 100 mM NaCl solution. All germinations were carried out in a plant growth chamber at 28 °C. The lengths of roots and shoots of the germinated seeds which were more than 0.2 mm in length were measured and recorded after 5 days.

9.2.3 Plant Growth in Gnotobiotic Sand Tubes

The effect of seed inoculation with IAA- and GA-producing *Pseudomonas extremorientalis* TSAU6 and *Pseudomonas fluorescens* WCS365 on the growth of jew's mallow seedlings exposed to salt stress (100 mM NaCl) was studied under gnotobiotic conditions. Experiments were carried out in test tubes (25 mm in diameter, 200 mm in length) as described by Simons et al. (1996). The tubes contained 60 g of sterilized high-quality sand (quartz sand 0.1–0.3 mm), which was treated with 10 % Plant Nutrient Solution (PNS) (Kuiper et al. 2001). Salinity conditions were established by adding 100 mM NaCl into the nutrient solution. Bacterial inoculants were grown and prepared, and the sterilized seeds were inoculated as described above. Inoculated seeds were planted into sterile glass tubes, one seed per tube with three replicates. The seedlings were grown in a growth cabinet with a 16-h light period at 22 °C and an 8-h dark period at 16 °C. At harvest after 18 days, the length of the shoots and roots and the fresh weight of whole plants were measured.

9.2.4 Plant Growth in Saline Soils

The effect of *Pseudomonas* strain on plant growth of jew's mallow under saline soil conditions was conducted in plastic pots (12-cm diameter, 15-cm deep). The soil has an EC value of 685 mS m^{-1} and contains 43 ± 9 g sand/kg, 708 ± 12 g silt/kg, and 249 ± 13 g clay/kg. The organic matter content of the soil is 0.694 %; total N, 0.091 %; Ca, 63.5 g/kg; Mg, 20.7 g/kg; K, 6.2 g/kg; P, 1.2 g/kg; Cl, 0.1 g/kg; and Na, 0.7 g/kg, and the pH is 8.0.

The plant seeds were sterilized, allowed to germinate, and coated with bacteria as described above, and the inoculated seedlings were planted in the plastic pots. The inoculation treatments were set up in a randomized design with ten replications. The pot experiment had two treatments: seeds noninoculated with bacteria, and the seeds inoculated with bacteria. Plants were grown at 19–22 °C during the day and 10–11 °C at night, and after 8 weeks the shoot and root length and dry matter of jew's mallow were measured.

9.2.5 Statistical Analysis

Data were tested for statistical significance using the analysis of variance package included in Microsoft Excel 2007. Mean comparisons were conducted using a least significant difference (LSD) test ($P < 0.05$). Standard error and an LSD result were recorded.

9.3 Results and Discussions

9.3.1 Microbial Plant Growth Stimulation

Seed germination is usually the most critical stage in seedling establishment (Almansouri et al. 2001). In this study, salinity (100 mM NaCl) inhibited the germination of jew's mallow seeds by 30 %. Salt-exposed plants exhibited a reduction in shoot and root growth and biomass compared to control plants. NaCl reduced root length by 25 %, shoot length by 20 %, and plant's fresh weight by 25 %. The present result agrees with the work of Gandour (2002) and Vadez et al. (2007) where they observed decreases in percentage germination and seedling emergence of chickpea with increases in salinity. Atak et al. (2006) and Neamatollahi et al. (2009) pointed out that higher saline condition may reduce germination percentage due to higher osmotic pressures. Ashraf (2004) found that increasing salt concentrations caused a significant reduction in the shoot and root growth as well as seed yield of *Ammolei majus* and *Hyoscyamus niger*. Similar results were observed by Razmjoo et al. (2008), where increased salinity and

drought stress caused reduction in the fresh and dry flower weight and essential oil content of *Matricaria chamomila*.

It has been reported that salinity reduces the recovery of diffusible auxins from maize coleoptile tips (Itai et al. 1968). It has been suggested that plants might benefit from external supply of plant growth regulators under stressed conditions (Li et al. 2005).

The root-associated bacteria which produce various phytohormones such as auxins, gibberellins, and cytokinins may help plants to cope with salt stress (Egamberdieva and Tulyasheva 2007; Yue et al. 2007; Egamberdieva et al. 2012). In this study, bacterial strains which produce IAA and GA were also able to alleviate salt stress in plants and improve seed germination of jew's mallow (up to 90 %). They also did reverse the growth-inhibiting effect of salt stress to a certain extent in both shoot and root. The IAA-producing strain *P. extremorientalis* TSAU6 significantly improved root length (66 %), shoot length (43 %), and fresh weight of plants (11 %) under nonsaline conditions, whereas strain *P. fluorescens* WCS365, which doesn't produce IAA, stimulated root and shoot length and fresh weight by 8, 25, and 6 %, respectively (Fig. 9.1a, c).

The inoculated jew's mallow seeds with *P. extremorientalis* TSAU6 significantly increased jew's mallow seedling root growth up to 45 % and shoot growth up to 84 % at 100 mM NaCl compared to control plants (Fig. 9.1b, c). The strain *P. fluorescens* WCS365 was not able to stimulate plant growth under salt stress conditions. There are many reports on the role of phytohormones in changes of root morphology exposed to drought, salinity, temperature, and heavy metal toxicity (Spaepen et al. 2008; Spaepen and Vanderleyden 2010).

In our previous works, we have observed that IAA-producing root-associated bacteria increase root growth, development, and yield of various agricultural crops such as soybean, cotton, wheat, maize, cucumber, and pea (Egamberdieva and Hoflich 2003; Jabborova et al. 2013b; Berg et al. 2010; Egamberdieva and Jabborova 2012, 2013a, b). These results agree with Heidari et al. (2011) who reported that the plant growth and auxin and protein contents of *Ocimum basilicum* inoculated by *Pseudomonas* sp. under drought stress conditions were increased compared to the control. Those reports demonstrated that phytohormones play a major role in improving plant growth and development under saline conditions.

9.3.2 The Effect of Phytohormones on Plant Growth

We also determined the effect of individual phytohormones such as auxins and gibberellins on the plant growth of jew's mallow and development under saline conditions. We observed that seed dormancy enforced by salinity was substantially alleviated and germination was promoted by gibberellins and auxins from 80 to 95 % (data not shown). This finding agrees with other studies in which GA and IAA improved the emergence of rice (Wahyuni et al. 2003), wheat seedlings (Egamberdieva 2009), radishes (Egamberdieva 2008), brinjal (*Solanum melongena* L.) (Gupta 1971),

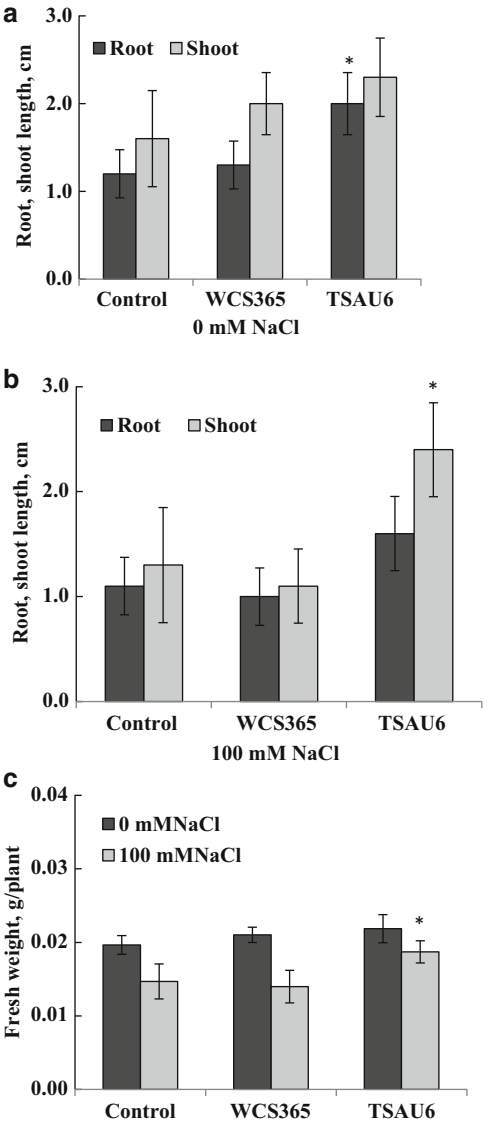

Fig. 9.1 Effect of inoculation of jew's mallow (*Corchorus olitorius* L.) seedlings with *Pseudomonas extremorientalis* TSAU6 (produce IAA and GA) and *Pseudomonas fluorescens* WCS365 (doesn't produce IAA and GA) on (**a**) length of roots, (**b**) length of shoots, and (**c**) fresh weight of whole plants. The seedlings were grown in petri plates with 0 mM and 100 mM NaCl solution. Columns represent means for five seedlings ($N = 5$) with *error bars* showing standard error. Columns with different letters indicate significant differences between treatments at $P < 0.05$ (Tukey's *t*-test)

chayote (***Sechium edule***) (Gregorio et al. 1995), and red sanders (*Pterocarpus santalinus Linn*. F) (Naidu 2001). In previous works, several plant growth regulators such as gibberellins (Afzal et al. 2005), auxins (Khan et al. 2004), and cytokinins (Gul et al. 2000) have been shown to alleviate salinity stress in plants. All concentrations of IAA and GA showed stimulatory effect on the root and shoot growth of jew's mallow seedling under nonsaline and salt stress conditions (Fig. 9.2a–c).

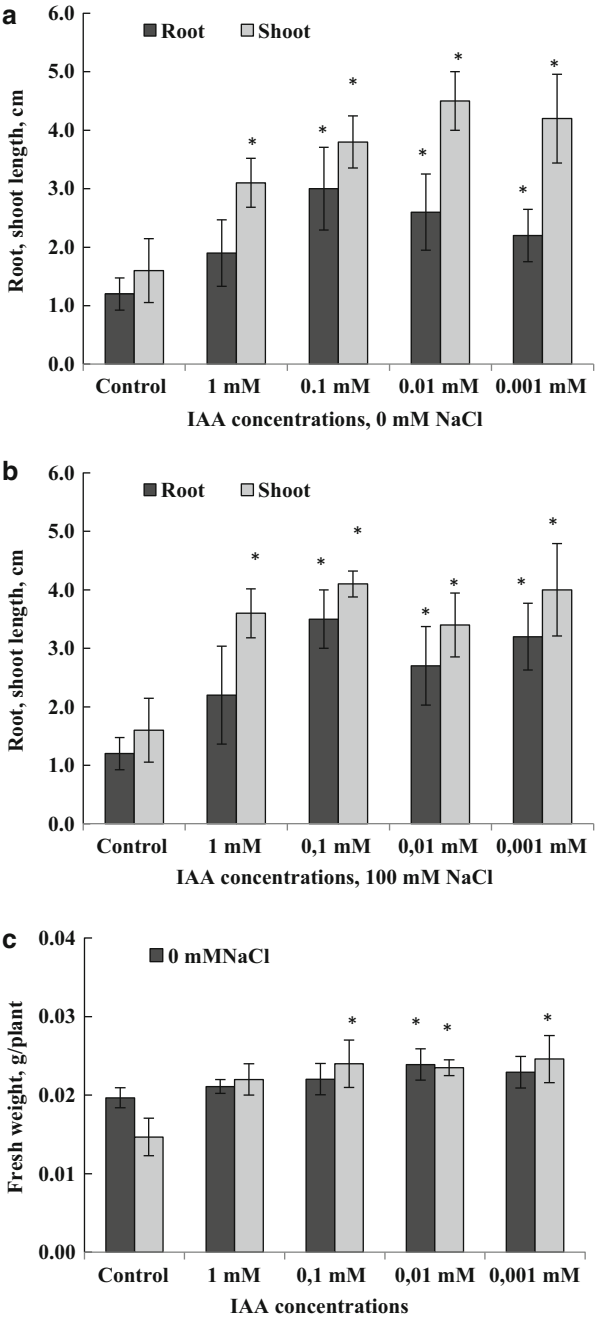

Fig. 9.2 The effect of various concentrations of IAA on the seedling growth of jew's mallow (*Corchorus olitorius* L.): (**a**) length of roots, (**b**) length of shoots, and (**c**) fresh weight of whole plants. The seedlings were grown in petri plates with 0 mM and 100 mM NaCl solution. Columns represent means for five seedlings ($N = 5$) with *error bars* showing standard error. Columns with different letters indicate significant differences between treatments at $P < 0.05$ (Tukey's *t*-test)

We have also observed that GA stimulated the root and/or shoot growth of jew's mallow seedling at concentrations 0.1, 0.01, and 0,001 mM under nonsaline and saline conditions (Fig. 9.3a–c). Lin and Kao (1995) reported that the application of growth regulators such as GA3 and cytokinin on rice seedlings improved seedling growth. Similar results were observed by Gul et al. (2000), where gibberellic acid and zeatin alleviate the effect of salinity on germination and growth of *Ceratoides lanata*, *Salicornia pacifica*, and *Allenrolfea occidentalis* (Khan et al. 2004). Under both nonsaline and saline conditions, lower concentrations of GA (0.1, 0.01, and 0.001 mM) showed higher stimulatory effect compared to control plants. Similar findings were reported by Remans et al. (2007), where low concentration of pure IAA or low titer of IAA-producing bacteria enhanced root growth.

Javid et al. (2011) reviewed the importance of IAA, cytokinins, and gibberellic acid in ameliorating salt stress in various plants. It is also suggested that IAA enhanced different cellular defense systems for protecting plants from external abiotic stresses (Bianco and Defez 2010).

9.3.3 Plant Growth in Gnotobiotic Sand System and Saline Soil

The growth-promoting effect of IAA- and GA-producing *P. extremorientalis* TSAU6 strain was also studied by growing inoculated salt-stressed jew's mallow seedlings for 18 days in a gnotobiotic sand system and 8 weeks in pots with saline soil. The presence of NaCl clearly impaired the plant growth of jew's mallow seedlings. At 100 mM, the length of root, length of shoot, and fresh weight of whole plants were inhibited by 54, 59, and 45 % than those of nonstressed seedlings. The coinoculation of salt-stressed jew's mallow exposed to 100 mM NaCl with *P. extremorientalis* TSAU6 significantly improved fresh weight of plants (on average by 35 %), length of shoots (by 42 %), and length of roots (by 50 %). Also under nonstressed conditions, the addition of *Pseudomonas* strain significantly enhanced root and shoot growth compared to uninoculated control plants.

Plant growth-promoting properties of the strain in pot experiments with saline soil showed that *P. extremorientalis* TSAU6 significantly increased shoot length by 21 % and dry matter by 18 % (data not shown). These results were somewhat similar to those obtained by Golpayegani and Tilebeni (2011) in which salinity decreased plant growth, photosynthesis, and chlorophyll content of basil, whereas *Pseudomonas* sp. alleviated the effects of salinity on plant growth. In our previous work, we have also observed that IAA-producing *Pseudomonas* strains promoted the enlargement of root system, enhancing nutrient uptake, and growth of goat's rue (*Galega officinalis*) (Egamberdieva et al. 2013a) and milk thistle (*Silybum marianum*) (Egamberdieva et al. 2013b) grown in a salt-affected soil. Similar observations were reported by other authors in which *Pseudomonas fluorescens* stimulated the growth and yield of *Catharanthus roseus* under drought stress (Attia

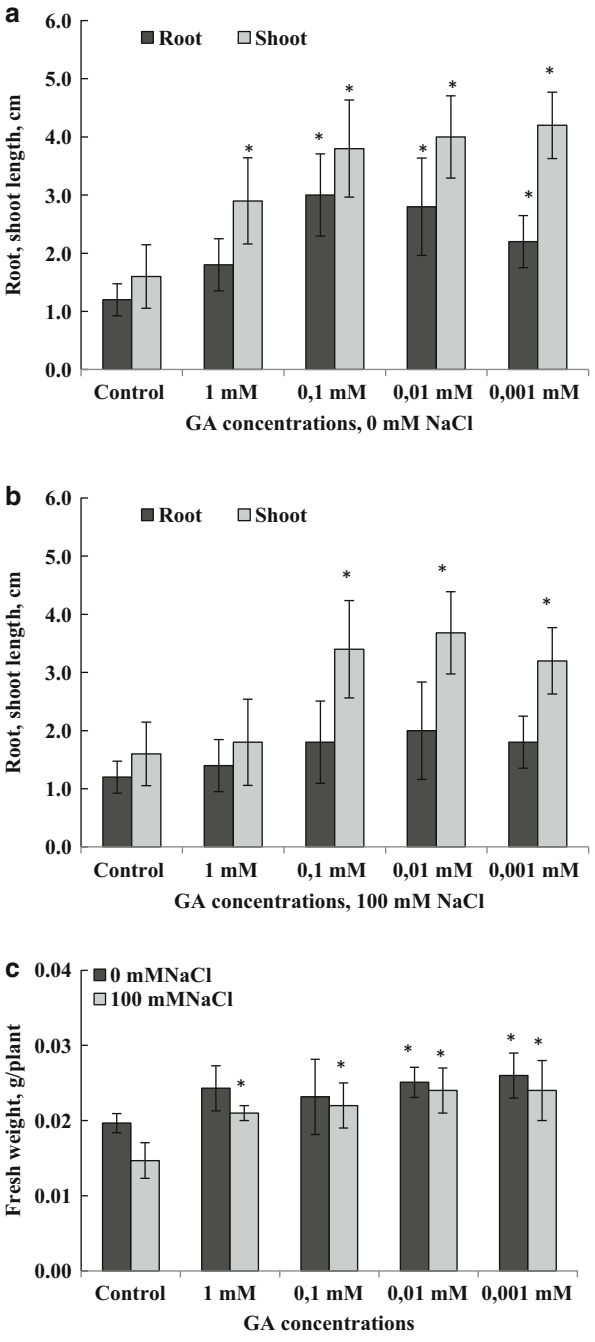

Fig. 9.3 The effect of various concentrations of GA on the seedling growth of jew's mallow (*Corchorus olitorius* L.): (**a**) length of roots, (**b**) length of shoots, and (**c**) fresh weight of whole plants. The seedlings were grown in petri plates with 0 mM and 100 mM NaCl solution. Columns represent means for five seedlings ($N = 5$) with *error bars* showing standard error. Columns with different letters indicate significant differences between treatments at $P < 0.05$ (Tukey's *t*-test)

and Saad 2001; Jaleel et al. 2007). Karthikeyan et al. (2010) reported that PGPR strains *Pseudomonas* significantly increased plant height, root length, root girth, and alkaloid content in Madagascar periwinkle (*Catharanthus roseus*) relative to the control.

9.4 Conclusions

The results presented here make it possible to recommend root-colonizing, phytohormone-producing *P. extremorientalis* TSAU to improve the growth of jew's mallow under saline soil conditions. It is also indicated that plant growth regulators, such as auxins and gibberellins, considerably alleviated salinity stress in plants and stimulated their growth and development.

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Part III
Biological Control

Chapter 10
Ecological Manipulations of *Rhizobacteria* for Curbing Medicinal Plant Diseases

S.K. Singh and Rakesh Pathak

10.1 Introduction

Plants that possess therapeutic properties on the animals or plant body are generally designated as medicinal plants. It is estimated that about 80 % population of the developing countries relies on traditional medicines derived from plants or plant extracts (Farnsworth 1994; Jamil et al. 2007). Naturally occurring soil bacteria that colonize plant roots and have beneficial effects on plant growth are known as plant growth-promoting rhizobacteria (PGPRs) (Vessey 2003). Some of these PGPRs can also enter the root interior and establish as endophytes (Gray and Smith 2005). Many of them are able to enter through the endodermis and establish in the stem, leaf, and other plant parts (Hallmann et al. 1997; Compant et al. 2005). Ultimately an intimate relationship between bacteria and host plant is formed without harming the plant, and they may originate from other sources like phyllosphere, ethnosphere, or spermosphere (Hallmann et al. 1997). The mechanism by which PGPR interact with plants includes production of siderophores, phytochrome-induced resistance, associative nitrogen fixation, solubilization of nutrients, depleting heavy metals, and removal of pollutants (Glick et al. 1999).

10.2 Rhizobacteria Curbing Medicinal Plant Diseases

Atropa belladonna commonly known as Belladonna belongs to the family Solanaceae. Belladonna is a perennial herbaceous plant. The leaf and root are used to make medicine. The active constituents in *Atropa belladonna* are atropine, hyoscyamine, and scopolamine (Hartmann et al. 1986; Rita and Animesh 2011). It is used as a sedative and for treatment of whooping cough, bronchial asthma, hay fever, and Parkinson's disease and as a painkiller (Adler 2008; Moulton and Fryer 2011). Major biotic constraints are *Phytophthora* rot (Middleton 1943) and leafy

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gall formation by the bacterium *Rhodococcus fascians* (Goethals et al. 2001; Nouar et al. 2003). *Cinchona* spp. (*C. pubescens*, *C. officinalis*, *C. rubra*) better known as Cinchona belongs to the family Rubiaceae. Significant phytochemicals are alkaloids, cinchonain, cinchonidine, cinchonine, quinicine, quinine, and quinidine (Staba and Chung 1981; Pachón et al. 2009; Buchberger et al. 2010). It is an important ingredient in medicines to treat malaria (Willcox 2011). Cinchona bark stimulates saliva and gastric juice secretion and possesses health properties like antiarrhythmic, antimalarial, antiparasitic, antiprotozoal, antispasmodic, and cardiotonic (Bareness et al. 2006; Rojas et al. 2006). *Phytophthora cinnamomi* and *Phytophthora quininea* cause stem canker, root rot, and dieback in cinchona (Crandall 1950; Hee et al. 2013).

Plectranthus barbatus commonly known as *Coleus forskohlii*, forskolin, Indian coleus, and false boldo belongs to the family Lamiaceae or Labiatae mint family. The roots contain forskolin which is the active component that activates cellular enzymes and used as a cardiotonic, digestive, and stimulant. It is found in many herbal diet pills and traditionally used to treat high blood pressure, lose weight, lower cholesterol, and improve the immune system. Some of the biotic production constraints of *Coleus forskohlii* are *Fusarium* wilt caused by *Fusarium oxysporum* (Zheng et al. 2012) and root rot/wilt (a complex problem involving *Fusarium chlamydosporum* and *Ralstonia solanacearum*) (Singh et al. 2012). Plants treated with arbuscular mycorrhizal fungus (*Glomus fasciculatum*), neem cake, or PGPR *Pseudomonas fluorescens* showed significantly reduction in the disease incidence and increased forskolin yield (Das et al. 2012).

Withania somnifera, a useful herb of the family Solanaceae, is commonly known as ashwagandha or Indian ginseng. The name ashwagandha is from the Sanskrit language and is a combination of the word ashva, meaning horse, and gandha, meaning smell. The root has a strong aroma that is described as "horselike." The root and berry are used to make medicine. Withanolides from the roots are the main chemical constituent of medicinal significance (Manwar et al. 2012). It is used for the treatment of ulcers, arthritis, anxiety, insomnia, tumors, tuberculosis, asthma, and leukoderma and as a sex stimulant and important component of DPT vaccine (Gautam et al. 2004; Bhatnagar et al. 2005; Rasool and Varalakshmi 2006; Singh et al. 2013). Leaf blight disease caused by the fungus *Alternaria dianthicola* (Militi et al. 2012); root knot/wilt complex caused by soilborne pathogens *Fusarium chlamydosporum*, *Ralstonia solanacearum*, and *Meloidogyne incognita* (Mallesh et al. 2009); and root rot caused by *Fusarium solani* (Bharti et al. 2013) are the major yield constraints of *W. somnifera*.

Foliar application of a talc-based formulation of PGPR *Pseudomonas aeruginosa* strain WS-1 to field-grown *W. somnifera* reduced disease severity by 80 % compared to non-treated control (Militi et al. 2012). PGPR-treated plants provided extended protection against soilborne pathogens *Fusarium chlamydosporum*, *Ralstonia solanacearum*, and *Meloidogyne incognita*, causing root knot/wilt complex, in *Coleus forskohlii* and *W. somnifera* (Mallesh et al. 2009). Soil application of a commercial formulation of PGPR *Pseudomonas fluorescens* resulted in the lowest root-knot nematode *M. incognita* population accompanied

with highest economic yield in *W. somnifera* and *Cassia angustifolia* (Ramakrishnan and Senthilkumar 2009). Combined applications of PGPR *P. fluorescens* and chemical resistance inducers reduced root rot severity by 85 and 88 % and enhanced root yields by 358 and 419 %, respectively, against *Fusarium solani*-induced root rot disease in *W. somnifera*. Reduction in disease severity was correlated with defense-related enzymes peroxidase, polyphenol oxidase, and phenyl ammonium lyase (Bharti et al. 2013).

Panax ginseng is also known by its common names: American ginseng, Asiatic ginseng, Chinese ginseng, five fingers, Japanese ginseng, Korean ginseng, ninjin, oriental ginseng, schinsent, seng and sang, tartar root, and Western ginseng. The roots contain triterpenoid saponins referred to collectively as ginsenosides or panaxosides. It is used by the patients suffering from anemia, diabetes, gastritis, neurasthenia, erectile dysfunction, and asthma.

Dioscorea spp. (*D. villosa*, *D. opposita*, *D. hypoglauca*, *D. macrostachya*, *D. barbasco*) belonging to the family Dioscoreaceae are commonly known as wild yam, colic root, devil's bones, China root, yam, yuma, shan yao, etc. Wild yam is a long perennial vine. The primary active chemical agent in wild yam is the steroidal saponin diosgenin which is a primary source for the important female sex hormone progesterone. Diosgenin is present in the rhizomes and roots of the wild yam as dioscin, which is a steroidal saponin whose aglycone is diosgenin. In addition to their benefits as a healthy vegetable, some species of wild yam are also cultivated for their medicinal and healing values for diseases such as rheumatic arthritis, biliary colic, irritable bowel syndrome, menopausal symptoms, whooping cough, spasms, urinary tract disorders, hypocholesterolemia, menstrual cramps, and pregnancy-related nausea among many others (Hou et al. 2001; Son et al. 2007). *Helminthosporium* or *Cercospora* leaf spots were reported to infect *Dioscorea* sp. (Chandel 2012).

Glycyrrhiza glabra also known as mulethi, jeshthamadh, licorice, liquorice, and sweet licorice belongs to the family Fabaceae. Rhizome contains saponin glycyrrhizin used in treating cervical cancer, kidney and bladder disorders, HIV, hepatitis B, asthma, ulcers, and arthritis (Roshan et al. 2012). Leaf spot diseases caused by *Nigrospora*, *Cylindrosporium*, and *Phyllosticta* are important diseases (Paul and Bhardwaj 1992; Bharat et al. 2002; Verma and Gupta 2008).

Hyoscyamus niger known as black henbane that belongs to the family Solanaceae is a medicinal herb. The leaves and seeds are the parts medicinally used. Chemically henbane is hyosciamia. It is considered better than opium, as it does not produce constipation. Combined with other preparations, it is used for gout, rheumatism, asthma, chronic cough, and neuralgia and has strong analgesic effects (Ghosian et al. 2012). The plant contains the anticholinergic tropane alkaloids (atropine, scopolamine, and hyoscine) (Hashimoto et al. 1991; Eeva et al. 1998). Root infections by *Rhizoctonia solani* alone and in combination with *Meloidogyne incognita* are major threats to *Hyoscyamus niger* cultivation (Kumar et al. 2004).

Papaver somniferum a member of the Papaver family is the species of plant from which opium and poppy seeds are derived. Opium is the source of narcotics,

namely, morphine, thebaine, codeine, papaverine, and noscapine. Morphine is prescribed for relief of severe pain (Singh et al. 2000; Hindson et al. 2007; Baros et al. 2012). Opium is also used for pain relief and sedation and as an antioxidant (Gülcin et al. 2004; Gotti 2011; Njoku et al. 2011).

The crop is attacked by various fungal, bacterial, and viral pathogens. Among fungal diseases downy mildew, damping-off, collar rot (*Rhizoctonia solani*), wilt (*Fusarium solani*), and soft rot (*Pectobacterium carotovorum*) are the most important (Kishore et al. 1985; Sattar et al. 1995, 1999; Aranda and Montes-Borrego 2008).

Catharanthus roseus commonly known as periwinkle is an herb that belongs to the family Dogbane. The aboveground parts are used to make medicine. Active constituents include alkaloid, carbohydrate, flavonoid, tannin, and steroid (Edwin et al. 2008; Siddiqui et al. 2010). The ajmalicine content and biomass in *C. roseus* increased due to *P. fluorescens* treatment under water deficit stress (Jaleel et al. 2007). It is used for improving brain health, tonsillitis, sore throat, and for blood purification (Islam et al. 2009; Siddiqui et al. 2010). Within periwinkle plants, phytoplasmas induce symptoms such as leaf yellowing, growth aberrations (proliferations, internode shortening, stunting), flower malformations, and/or decline (Perica et al. 2007; Chaturvedi et al. 2009).

Plantago major also known as greater plantain, common plantain, rattail plantain, and way-bread is a member of the Plantaginaceae family. It is an herbaceous common garden perennial weed. It contains salicylic, citric, and caffeic acid, mucilage, tannins, proteins, flavonoids, vitamin C, dietary fiber, and potassium (Samuelsen 2000). The plant is diuretic and is used to treat gastroenteritis, asthma, cancer, bladder dysfunctions, etc. (Gomez-Flores et al. 2000). Aqueous extracts of *P. major* inhibited *Mycobacterium tuberculosis* (Gautam et al. 2007). Fungal diseases caused by *Cercospora plantaginis*, *Septoria plantaginis*, and *Phyllosticta plantaginis* and little leaf disease (Witches broom) caused by phytoplasma are major biotic constraints (Farr et al. 1995; Samad et al. 2002; Josic et al. 2012).

Podophyllum peltatum commonly called Indian apple, mayflower, umbrella plant, or mayapple is an herbaceous perennial plant in the family Berberidaceae. The root and rhizome are used for medicinal purpose. It is used for the removal of warts and oral hairy leukoplakia and in the treatment of gynecologic infections and is an anticancer agent (Beutner and Vonkrough 1990; Dwivedi and Dwivedi 2008).

Rauwolfia serpentina also known as sarpagandha belongs to the family Apocynaceae and is a climbing evergreen shrub. Roots possess active alkaloid reserpine, rescinnam, ajmalicine, rescinnamine, yohimbine, and serpentine (Chopra et al. 1980). It is used as a sedative and in the treatment of hypertension, insomnia. Among major diseases are leaf spot caused by *Cercospora rauwolfia*, *Alternaria tenuis*, and *Rhizoctonia solani* and mosaic and root knot; pyralid caterpillar and cockchafer grubs are important insect pests (Mohanthy and Addy 1957; Ganguly and Pandotra 1962; Parashurama and Shivanna 2013).

Cassia angustifolia known by its common names Indian senna, Tinnevelly senna, Cassia senna, and Alexandrian senna is an herb of the family Fabaceae. Leaves and fruits are of medicinal importance. It contains mannitol, sodium,

potassium, tartrate, salicylic acid, volatile oils, resins and calcium oxalate, and chrysophanic acid. Senna is considered to be a laxative and used for the treatment of typhoid, cholera, jaundice, gout, rheumatism, tumors, and bronchitis (Sastry et al. 2000; Pandikumar et al. 2011; Mehrafarin et al. 2012). The seeds are used as an anthelmintic and digestive and to treat piles, skin diseases, and abdominal troubles (Srivastava et al. 2010). Major diseases of Senna include *Alternaria* blight (caused by *Alternaria alternata*) (Tetarwal et al. 2008; Rai and Tetarwal 2010).

Like other cultivated plants, these medicinal plants are also attacked by a few or more fungal, bacterial, viral, and/or nematode diseases and insect pests. Biological control of the insect pests and diseases such as root rots caused by the species of *Phytophthora* and *Rhizoctonia*; wilts by *Fusarium*; leaf blights and spots by *Ralstonia*, *Alternaria, Helminthosporium*, *Cercospora*, *Septoria*, *Nigrospora*, and *Phyllosticta*; leafy gall by the bacterium *Rhodococcus fascians*; soft rot by *Pectobacterium carotovorum*; and nematode root knot by the species *of Meloidogyne* reported by the application of PGPRs in other cultivated plants can also be allied to manage sustainable cultivation of medicinal plants.

10.3 Investigating Plant-Microbe Interaction

The alternative approaches like investigating plant-microbe interactions with medicinal plants and to produce enhanced levels of phytochemicals have recently been reviewed (Sekar and Kandavel 2010; Singh et al. 2013). A thin layer of soil surrounding plant roots and active area of root activity and metabolism is known as the rhizosphere. Plant-microbe interactions can be negative, positive, or neutral. PGPR have positive interactions and affect plant growth directly or indirectly. In indirect mode, it prevents the harmful effects of phytopathogenic microorganisms, whereas direct effects are by facilitating the uptake of nutrients.

Certain species belonging to PGPR genera *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Rhizobium*, *Erwinia*, *Mycobacterium*, *Mesorhizobium*, *Flavobacterium*, *Klebsiella*, *Alcaligenes*, *Arthrobacter*, and *Serratia* have been reported to exhibit plant growth-promoting activities by a variety of mechanisms (Saharan and Nehra 2011; Singh 2013).

PGPR enhance plant growth by suppression of phytopathogens by producing siderophores that chelate iron that makes it unavailable to pathogens. Under scarcity of bioavailable iron, PGPR produce low molecular weight compounds called siderophores. They are small, high-affinity iron-chelating compounds secreted by microorganisms (Neilands 1995). PGPR convert iron from mineral phase by converting them to soluble ferric complexes that are absorbed by the plants. The bacteria belonging to *Pseudomonas*, *Enterobacter*, *Bacillus*, and *Rhodococcus* produce siderophores and suppress phytopathogens (Tian et al. 2009). Some PGPR draw iron from heterologous siderophores produced by other microorganisms (Loper and Henkels 1999; Whipps 2001). Siderophore-mediated antagonism against

species of *Aspergillus*, *Colletotrichum*, and *Fusarium* by *Acinetobacter calcoaceticus* has been observed (Prashant et al. 2009).

PGPRs have the capacity to synthesize antifungal metabolites such as antibiotics, fungal cell wall-degrading enzymes, hydrogen cyanide, etc. which suppress the growth of fungal pathogens. Nowak-Thompson et al. (1994) reported that *P. fluorescens* suppress the growth of phytopathogenic fungi by production of 2,4-diacetylphloroglucinol. Certain PGPRs have specific mechanism to suppress or even prevent phytopathogens, e.g., by degrading fusaric acid produced by *Fusarium* spp. (Toyoda and Utsumi 1991), by lysis of fungal mycelium by *P. stutzeri* (Mauch et al. 1988), by rapid colonization of rhizosphere and available nutrition otherwise to be utilized by plant pathogens (Dowling and O'Gara 1994), and by production of volatile compounds (Hassanein et al. 2009). They exhibit antifungal activities against *Rhizoctonia solani*, *R. bataticola*, *F. oxysporum*, and *Macrophomina phaseolina* and root-knot nematodes (Pal et al. 2000; Siddiqui et al. 2005).

PGPR also increases plant growth by changing the structure and composition of microbial community in rhizosphere (Piromyou et al. 2011). Detoxification and degradation of virulence factor of the pathogens by PGPR is another mechanism of biological control of phytopathogens (Zhang and Birch 1996). Of late it has been discovered that certain PGPR quench pathogen quorum-sensing capacity by degrading autoinducer signals, thereby blocking expression of virulence genes (Molina et al. 2003; Dong et al. 2004).

Endophytic bacteria colonize the internal tissue of the plant showing no external sign of infection or deleterious effect on host (Schulz and Boyle 2006). The bacterial endophytes are potential PGPRs and can form symbiotic, mutualistic, commensalistic, and/or trophobiotic relationships with their host plants. Most of the endophytes colonize in rhizosphere or phyllosphere, or some may be transmitted through the seeds. Their ability to control or suppress plant pathogens, insects, and nematodes has been demonstrated (Krishnamurthy and Gnanamanickam 1997; Hallmann et al. 1998; Azevedo et al. 2000; Ryan et al. 2008). It has been shown that prior inoculation with endophytes can reduce diseases caused by fungi, bacteria, and virus (Sturz et al. 2000; Berg and Hallmann 2006).

10.4 Induced and Acquired Systemic Resistance

Induced systemic resistance (ISR) or systemic acquired resistance (SAR) is defined as the activation of chemical or physical defense mechanism of the host plant by an inducer leading to the control of several pathogens (Kloepper et al. 1992). Application of mixture of different PGPRs to the seeds or seedlings of certain plants has regulated in increased efficiency of ISR against several pathogens (Ramamurthy et al. 2001). ISR against yellow mosaic *Potyvirus* upon seed bacterization with *P. fluorescens* and *Rhizobium leguminosarum* has been achieved with significant reduction in percent disease incidence in faba beans (Elbadry et al. 2006). Several

PGPR traits and metabolites have been shown to trigger ISR such as volatile secreted by *B. subtilis* (Ryan et al. 2001), salicylic acid (SA) independent pathways involving jasmonate and ethylene signals (Pieterse et al. 1998; Pettersson and Baath 2004), thickening of cortical cell wall (Duijff et al. 1997), accumulation of phenolic compounds at the site of pathogen attack (M'Piga et al. 1997), and induced accumulation of pathogenesis-related proteins (PR-proteins) (Park and Kloepper 2000).

Nevertheless, there are several constraints in using PGPRs indiscriminately: (1) The interaction between associative PGPR and plant can be unstable. The good result obtained in vitro can always not be dependably reproduced under field conditions (Chanway and Holl 1993). (2) Some failures derived from the use of bio-fertilizers containing PGPRs may be due to interspecific genetic interactions by the rhizobacteria and the host plant, i.e., different cultures and plant species may produce different types of root exudates which may or may not support PGPRs to produce biologically active substances required to promote plant growth or suppress phytopathogens. (3) Major constraints of massive commercial use of PGPRs are regarding registration and marketing of products of PGPRs (Mathre et al. 1999). (4) Currently, bio-fertilizers with PGPR are still not a reality of extensive commercialization due to lack of consistent response in different host cultivars (Remans et al. 2008). (5) Dry powder-based commercial formulations often lack appropriate shelf life and cell viability (Johri et al. 2003).

Having learnt from the constraints and with the advent and excess to modern biotechnological tools and techniques, there are opportunities to develop, explore, and/or exploit: (1) Stable formulations of antagonistic PGPRs in sustainable agricultural system to replace the use of chemical fertilizers. (2) Eco-friendly biopesticides derived from microorganisms. (3) Soil microbial diversity for PGPRs having combination of plant growth-promoting activities and well adapted to particular soil environment. (4) Multi-strain inocula of PGPR with multiple modes of action, multiple pathogens, and temporal and spatial variability to increase crop production and health (Jetiyanon and Kloepper 2002; Siddiqui and Shaukat 2002; Adesemoye et al. 2008). (5) The application of molecular tools is enhancing our ability to understand and manage the rhizosphere and will lead to new products and improved effectiveness of PGPRs (Nelson 2004). (6) Improvement of efficient PGPRs strains by creating transgenic that combine multiple mechanisms of action (Chin-A-Woeng et al. 2001). (7) Combination of PGPR strains, bacteria with bacteria, or bacteria with fungi to suppress phytopathogens with broader spectrum of microbial weapons (Duffy et al. 1996; Kilic-Ekici and Yuen 2004; Lutz et al. 2004; Olivain et al. 2004). (8) Future studies on endophytes and rhizobacteria to promote the sustainable production of biomass and bioenergy crops in conjunction with phytoremediation of soil and contamination (Ryan et al. 2008). (9) Endophyte-plant interaction by identifying gene governing colonization and establishment of endophyte bacteria in plants can promote sustainable production of biomass by suppressing phytopathogens and by phytoremediation of soil contamination. (10) Interdisciplinary studies on rhizosphere biology, microbiology, and ecology of medicinal plants should be strengthened to enhance biomass

production enriched with phytochemicals making use of PGPRs under organic conditions.

Besides the phytochemical study to determine active principles and biochemicals present in medicinal plants, attempts ought to be made to protect and multiply endangered species of medicinal plants that are about to be extinct.

10.5 Conclusions

The demand for medicinal plants is ever increasing due to growing population and health awareness as plant products are nontoxic and have no side effects. Deforestation has caused irreparable loss to valuable biodiversity resulting in inclusion of many medicinal plants in the list of endangered species of which some are at the verge of extinction. The global plant-based drugs are projected between US$30 and 60 billion with 7 % annual growth rate (Prabhuji et al. 2009). *Atropa belladonna*, *Cinchona* spp., *Plectranthus barbatus*, *Withania somnifera*, *Panax ginseng*, *Dioscorea* spp., *Glycyrrhiza glabra*, *Hyoscyamus niger*, *Papaver somniferum*, *Catharanthus roseus*, *Cassia angustifolia*, *Podophyllum peltatum*, *Rauwolfia serpentina*, and *Plantago major* are major medicinal plants. Several constraints in using PGPRs indiscriminately and opportunities to develop, explore, and/or exploit multi-strain stable formulations of antagonistic PGPRs in sustainable mode to replace the use of chemical fertilizers have been advocated.

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Chapter 11
Mechanism of Prevention and Control of Medicinal Plant-Associated Diseases

Ram Kumar Pundir and Pranay Jain

11.1 Introduction

Worldwide, over three quarters of the world population relies mainly on plants and plant extracts for health care. It is estimated that world market for plant-derived drugs may account for about Rs. 2,00,000 crores. Presently, Indian contribution is less than Rs. 2,000 crores. Indian export of raw drugs has steadily grown at 26 % to Rs. 165 crores in 1994–1995 from Rs. 130 crores in 1991–1992. The annual production of medicinal and aromatic plant's raw material is worth about Rs. 200 crores. This is likely to touch US$5 trillion by 2050. Of the 2,50,000 higher plant species on earth, more than 80,000 are medicinal. India's diversity is unmatched due to the presence of 16 different agro-climatic zones, 10 vegetation zones, 25 biotic provinces, and 426 biomes (habitats of specific species) (Joy et al. 1998).

Of these, about 15,000–20,000 plants have good medicinal potentials. However, only 7,000–7,500 species are used for their medicinal potentials by traditional communities. In India, drugs of herbal origin have been used in traditional systems of medicines such as *Unani* and *Ayurveda* since ancient times. The *Ayurveda* system of medicine uses about 700 species, *Unani* 700, *Siddha* 600, Amchi 600, and modern medicine around 30 species. The drugs are derived either from the whole plant or from different organs, like leaves, stem, bark, root, flower, seed, etc. Some drugs are prepared from excretory plant product such as gum, resins, and latex. Some important chemical intermediates needed for manufacturing the

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Table 11.1 List of most priced medicinal plants of some states in India

State	Species
Gujarat	*Rauwolfia serpentina*, karaya gum
Maharashtra	*Rauwolfia serpentina*
Karnataka	Sandalwood oil, *Phyllanthus emblica*
Tamil Nadu	*Terminalia chebula*, *Terminalia belerica*
Kerala	Gum, fibers, roots of rosewood
Orissa	Sandalwood, rosewood
Uttar Pradesh	Gum, chiraunji

modern drugs are also obtained from plants (e.g., diosgenin, solasodine, b-ionone). Plant-derived drug offers a stable market worldwide, but also plants continue to be an important source for new drugs (Joy et al. 1998).

The term "medicinal plants" include various types of plants used in herbalism, and some of these plants have medicinal activities. These medicinal plants are considered rich resources of ingredients which can be used in drug development and synthesis. Worldwide, these plants play an important role in the development of human cultures. Medicinal plants have a promising future because there are about half a million plants around the world, and most of their medical activities have not been investigated yet and could be decisive in the treatment of present or future studies. Table 11.1 shows a list of most priced medicinal plants of some states in India.

11.2 Alternative Medicine

Nowadays, the term "alternative medicine" became very common in western culture; it focuses on the idea of using the plants for medicinal purposes. Currently, medicines which come in capsules or pills are the only medicines that we can trust and use. Even so most of these pills and capsules we take and use during our daily life came from plants. Medicinal plants are frequently used as raw materials for the extraction of active ingredients which are used in the synthesis of different plant-based drugs such as laxatives, blood thinners, antibiotics, and antimalaria medications. Moreover, the active ingredients of Taxol, vincristine, and morphine were isolated from foxglove, periwinkle, yew, and opium poppy, respectively (Hassan 2012).

11.3 Characteristics of Medicinal Plants

Medicinal plants have many characteristics when used as a treatment, as follows:

1. *Synergic medicine*—the ingredients of plants all interact simultaneously, so their uses can complement or damage others or neutralize their possible negative effects.

2. *Support of official medicine*—these are used to treat the complex cases like cancer diseases.
3. *Preventive medicine*—it has been proven that the component of the plants is also characterized by their ability to prevent the appearance of some diseases. This will help to reduce the use of the chemical remedies which will be used when the disease is already present, i.e., reduce the side effect of synthetic treatment (webpage).

According to Kumar et al. (1997), the major medicinal plants such as *Acorus calamus*, *Aconitum* sp., *Adhatoda vasica*, *Aloe vera*, *Ammi majus*, *Atropa acuminata*, *Berberis aristata*, *Carica papaya*, *Catharanthus roseus*, *Cassia senna*, *Cephaelis ipecacuanha*, *Cinchona* spp., *Dioscorea* spp., *Glycyrrhiza glabra Hedychium spicatum*, *Heracleum candicans*, *Hyoscyamus* sp. *muticus*, *Inula racemosa*, *Juglans regia*, *Juniperus* spp., *Papaver somniferum*, *Plantago ovata*, *Podophyllum emodi*, *Rauvolfia serpentina*, *Rheum emodi*, *Saussurea lappa*, *Swertia chirata*, *Urginea indica*, *Valeriana wallichii*, *Zingiber officinale*, *Bacopa monnieri*, *Boerhaavia diffusa*, *Duboisia myoporoides*, *Eclipta alba*, *Gymnema sylvestre*, *Phyllanthus amarus*, *Piper retrofractum*, *Panax quinquefolius*, *Silybum marianum*, and *Matricaria chamomilla* can be cultivated in India and have established demand for their raw materials. Kumar et al. (1997) also stated that medicinal plants in which significant research leads have been obtained with respect to their pharmaceutical potential for which processing and agrotechnology need to be established, include such as *Andrographis paniculata*, *Artemisia annum*, *Boswellia serrata*, *Centella asiatica*, *Coleus forskohlii*, *Commiphora wightii*, *Curcuma longa*, *Phyllanthus amarus*, *Picrorhiza kurroa*, *Sida rhombifolia*, *Taxus baccata*, and *Withania somnifera*. Plants which delay aging process and form healthy food ingredients in several Ayurvedic formulations belong to *Allium sativum*, *Aloe barbadensis*, *Asparagus racemosus*, *Cassia senna*, *Curculigo orchioides*, *Commiphora wightii*, *Centella asiatica*, *Capsicum annuum*, *Chlorophytum arundinaceum*, *Eclipta alba*, *Fagopyrum esculentum*, *Glycyrrhiza glabra*, *Oenothera biennis*, *Panax pseudoginseng*, *Plantago ovata*, and *Withania somnifera*.

There are many diseases that occur in plants caused by living organisms. Plant diseases need to be controlled to maintain the quality and abundance of food, feed, and fiber produced by growers around the world. Different approaches may be used to prevent, mitigate, or control plant diseases. The use of pesticides has contributed significantly to the spectacular improvements in crop productivity and quality over the past 100 years. However, the environmental pollution caused by excessive use and misuse of agrochemicals has led to considerable changes in people's attitudes towards the use of pesticides in agriculture.

Plant diseases are the result of interactions among the components of disease triangle, i.e., host, pathogen, and environment (Fig. 11.1a). It also includes the time (Fig. 11.1b).

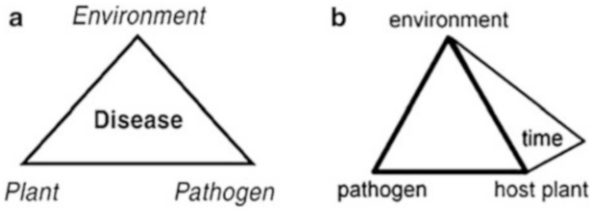

Fig. 11.1 Interactions among the components of plant diseases triangle, (**a**) Host plant, pathogen and environment, (**b**) Host plant, pathogen, environment and time

India is one of the leading countries in Asia in terms of the wealth of traditional knowledge systems related to the use of plant species. India is also known to harbor a rich diversity of higher plant species (about 17,000 species) of which 7,500 are known as medicinal plants (Kala 2005; Shiva 1996).

Medicinal plants are attacked regularly by insects, mites, nematodes, bacteria, fungi, and viruses. A plant disease caused by a pathogen particularly by fungal pathogen is often recognizable from the particular plant organ infected and the type of symptom produced. On this basis, the following general types of fungal diseases can be distinguished.

- Damping-off diseases
- Root and foot rots
- Vascular wilts
- Downy mildews
- Powdery mildews
- Leaf spots and blights
- Rusts
- Smuts
- Anthracnoses
- Galls
- Dieback
- Postharvest diseases

11.4 Bacterial Plant Diseases and Their Control

Most plant pathogenic bacteria belong to the following genera: *Erwinia*, *Pectobacterium*, *Pantoea*, *Agrobacterium*, *Pseudomonas*, *Ralstonia*, *Burkholderia*, *Acidovorax*, *Xanthomonas*, *Clavibacter*, *Streptomyces*, *Xylella*, *Spiroplasma*, and *Phytoplasma*. Plant pathogenic bacteria cause many different kinds of symptoms that include galls and overgrowths, wilts, leaf spots, specks and blights, soft rots, scabs, and cankers. In contrast to viruses, which are inside the host cells, walled bacteria grow in the spaces between cells and do not invade them. The means by which plant pathogenic bacteria cause disease is as varied as the types of symptoms they cause. Some plant pathogenic bacteria produce toxins or inject special proteins

that lead to host cell death, or they produce enzymes that break down key structural components of plant cells and their walls.

11.5 Control

To control bacterial diseases in plants is very difficult. The emphasis is on preventing the spread of the bacteria rather than on curing the plant. There are various integrated management measures for bacterial plant pathogens, which include the following.

11.5.1 Genetic Host Resistance

It includes resistant varieties, cultivars, or hybrids as the most important control procedure.

11.5.2 Cultural Practices

It includes the bacteria-free seed or propagation materials; sanitation, particularly disinfestation of pruning tools; and either eliminating or reducing sources of bacterial contamination, such as crop rotation to reduce over-wintering, preventing surface wounds that permit the entrance of bacteria into the inner tissues, propagating only bacteria-free nursery stock, and prolonged exposure to dry air, heat, and sunlight, which will sometimes kill bacteria in plant material.

11.5.3 Chemical Applications

There are many chemicals used to control bacterial diseases which include applications of copper-containing compounds or Bordeaux mixture (copper sulfate and lime). Antibiotics, streptomycin and/or oxytetracycline, may also help kill or suppress plant pathogenic bacteria prior to infection and reduce the spread of the disease, but they will not cure plants that are already diseased, and antibiotics are also used to treat diseases caused by fastidious vascular bacteria. *Phytoplasma* and *Spiroplasma* are susceptible to certain antibiotics, particularly tetracycline, which has been used to treat pear trees with the pear decline disease. Tetracycline must be injected into mature trees on a routine or therapeutic schedule to be effective and even then only appears to suppress the development of symptoms rather than curing the infected plant. Applications made during the early stages of infection tend to be

more effective than in the later stages of disease development, and insect control will help to eliminate vectors or reduce feeding wounds that can provide points of entry.

11.5.4 Biological Control

The use of antagonistic or biological control products such as BlightBan and Agrosin K84 may also be effective for managing bacterial diseases of plants.

11.5.5 Government Regulatory Measures

It includes the implementation of strict quarantines that exclude or restrict the introduction or movement of fungal and FLO pathogens or infected plant material.

11.6 Nutrients as Plant Disease-Controlling Agents

Nutrients play an important role on growth and development of plants and also microorganisms, and they are important factors in disease control (Agrios 2005). All the essential nutrients can affect disease severity (Huber and Graham 1999). However, there is no general rule, as a particular nutrient can not only decrease the severity of a disease but can also increase the severity and the disease incidence of other diseases or have a completely opposite effect in a different environment (Graham and Webb 1991; Huber 1980). Despite the fact that the importance of nutrients in disease control has been recognized for some of the most severe diseases, the correct management of nutrients in order to control disease in sustainable agriculture has received little attention (Huber and Graham 1999).

11.7 Fungal Plant Diseases and Their Control

There are many fungal species such as *Aecidium withaniae*, *Mucor mucedo*, *Fusarium solani*, *Alternaria alternata*, *Aspergillus niger*, *Rhizopus solani*, *Alternaria alternata*, *A. tenuissima*, *Fusarium* spp., *Aspergillus verocosa*, *Fusarium oxysporum*, *Curvularia cragrotidis*, *Aspergillus flavus*, *Penicillium citrinum*, *F. culmorum*, *Verticillium dahliae*, *V. albo-atrum*, *Rhizoctonia solani*, *Erysiphe cichoracearum*, *Sphaerotheca fuliginea*, *Leveillula guttiferatum*, *E. hypersici*, *E. artemisiae*, *E. beceleate*, *E. communis*, and *L. malvacearum* that cause diseases in medicinal plants, namely, *Withania somnifera*, *Aloe vera*, *Datura metel*,

Lavandula angustifolia, *Rosmarinus officinalis*, *Borago officinalis*, *Salvia officinalis*, *Arctium lappa*, *Melissa officinalis* L., *Cucurbita pepo* var. *sterica*, *Hypericum perforatum*, *Artemisia dracunculus*, *Solanum dulcamara*, *Descurainia sophia*, *Althaea officinalis*, *Malva sylvestris*, *Glycyrrhiza glabra*, *Anethum graveolens*, *Coriandrum sativum*, *Spinacia oleracea*, *Satureja hortensis*, *Thymus serpyllum*, *Mentha pulegium*, and *Mentha piperita* (Chavan and Korekar 2011).

In a study, the medicinal plant *Withania somnifera* Dunal is widely used in Ayurvedic medicine, the traditional medical system of India. It is an ingredient in many formulations prescribed for a variety of musculoskeletal conditions (e.g., arthritis, rheumatism), and as a general tonic to increase energy, it improves overall health and longevity and prevents disease in athletes, the elderly, and during pregnancy. Many herbal drugs and drinks have been formulated from *A. vera* plants for the maintenance of good health (Davis and Moro 1989). *A. vera* gel has been reported to be very effective for the treatment of sore and wounds, skin cancer, skin disease, cold and cough, constipation, pile, fungal infection, etc. (Gill 1992; Kafaru 1994; Daodu 2000; Djeraba and Quere 2000; Olusegun 2000). The use of *Aloe* plants in the treatment of other diseases such as asthma, ulcer, and diabetes has also been reported (Davis and Moro 1989). In cosmetic industries, *Aloe* is used in the production of soap for bathing, shampoo, hair wash, tooth paste, and body creams (Daodu 2000). *Datura metel* L. is another important and widely available medicinal plant of this region. It has a parasympatholytic with anticholinergic property, it reduces secretion, and it is also an antidote in opium and chloral hydrate overdose (Jarald 2006). These medicinally important plants are facing serious problems of the fungal attack. Here are leaf, root, and seed diseases, namely, leaf spot, lead rust, root spot, and seed spot caused by fungal pathogens that adversely affect the medicinal plant parts and decrease the medicinal value of the part.

Using these infected parts as a medicine may be harmful to the human body. Among all the plant pathogens, the fungal group is the major one. The above three important medicinal plants, diseases, and their causal fungal agents are listed as follows:

1. *Withania somnifera* (ashwagandha/Indian ginseng/poison gooseberry/or winter cherry)

   Leaf rust: *Aecidium withaniae* and *Mucor mucedo*
   Leaf spot: *Fusarium solani*, *Alternaria alternata*, and *Aspergillus niger*
   Root: *Rhizopus solani*

2. *Aloe vera* (ghee kawar)

   Leaf spot: *Alternaria alternata*, *A. tenuissima*, and *Fusarium* spp.
   Root: *Aspergillus verocosa* and *Fusarium oxysporum*

3. *Datura metel* (dhatura)

   Leaf spot: *Alternaria alternata* and *Curvularia cragrotidis*
   Seed spot: *Aspergillus flavus* and *Penicillium citrinum*

11.8 Management of Plant-Associated Diseases

11.8.1 Recent Advances in Management of Fungal Pathogens

There are various ways to manage the fungal pathogens as follows:

Cultural practices

1. Heat treatment
2. Fumigation
3. Ionization radiation and UV illumination
4. Chemically impregnated wrapper
5. Antagonism
6. Biocontrol: integrated approaches
7. Induced resistant
8. Host defense through gene silencing
9. Plantibodies
10. Induced resistance
11. Disease-resistant transgenic plant

11.8.2 Management Practices

11.8.2.1 Cultural Management

1. Sanitation—clean environment; remove or reduce sources of inoculum (weed and alternative hosts, insect vectors, debris)
2. Pruning—remove infected tissue, promote more vigorous growth, and increase air circulation
3. Watering—avoid overwatering or underwatering and flooding soils
4. Planting date—unfavorable conditions for pathogen and favorable for host
5. Fertility—avoid overfertilization or underfertilization
6. Rotation—nonhost plants and resistant varieties; reduce soilborne pathogen populations
7. Trap plants and antagonistic plants—e.g., marigolds
8. Quarantines, restrictions on moving plant materials across county, state, or national borders

11.8.2.2 Chemical Management

There is a chemical barrier to protect the host plant and/or eradicate an existing infection. Pesticides typically cannot "cure" heavily diseased plants. The types of pesticides are fungicides, bactericides, nematicides, insecticides, and biocides.

Contact fungicide: It is effective only at the site of application (protectant) and must be applied before pathogen infects the plant; new growth emerging after application is not protected, for example, mancozeb, coppers, chlorothalonil, and captan.

Systemic fungicide: It is absorbed and translocated (moved from application site) by the plant locally and systemically by moving short distances (towards the leaf margin) within the plant from the site of application (e.g., benomyl, triforine).

Systemic: It moves further within the plant from the site of application (e.g., metalaxyl moves from roots up to shoots and foliage).

11.8.2.3 Genetic Resistance

Most plants resist infection by the majority of microorganisms. The degree of resistance/susceptibility varies among plant species and varieties. Resistance is dynamic (changes)—races or strains of a pathogen vary in pathogenicity (how severe a pathogen), and the environment affects host resistance.

11.8.2.4 Physical Management

There are three important physical factors which are responsible for many of the plant diseases:

1. Heat treatment: It is due to steam sterilization of soil/materials, soil solarization, and heat treatments
2. Cold treatment: It is possible due to refrigeration (postharvest).
3. Moisture management: To reduce humidity, dry out bulbs, tubers, etc., for winter storage.

11.8.2.5 Biological Management

Biocontrol of plant diseases involves the use of an organism or organisms to inhibit the pathogen and reduce disease (Cook and Baker 1983). There are many definitions for biological control; however, the basic idea involves a strategy for reducing disease incidence or severity by direct or indirect manipulation of microorganisms (Baker and Cook 1974; Maloy 1993). Consequently, understanding the mechanisms of biological control of plant diseases through the interactions between biocontrol agent and pathogen may allow us to manipulate the soil environment to create conditions conducive for successful biocontrol or to improve biocontrol strategies (Handelsman and Parke 1989).

Recently several methodologies for genetic analysis, such as the approach of mutant analysis, have provided promise for the study of mechanisms of biocontrol agents and their targets. Handelsman and Parke (1989) have suggested the application of Koch's postulates to demonstrate a cause-effect relationship in the

involvement of a particular mechanism in biocontrol because it may not be adequate to demonstrate that a mechanism exists in vitro (Wilhite et al. 1994). The following steps suggested by Handelsman and Parke should be demonstrated in either biocontrol agents or their targets to ascertain the role of a particular mechanism. These steps are as follows:

1. The activity must be associated with a strain that is effective as a bioprotectant, or a metabolite must be identified in situ, such as in the disease situation.
2. The gene(s) coding for the particular product or process must be cloned.
3. The activity of the mutant should be less effective than the wild-type parent if the particular gene(s) is deleted.
4. Replacing the gene(s) encoding for the activity should restore the biocontrol activity.
5. Mutants of the pathogen that are not affected by the activity of the metabolite or process should be able to incite disease in the presence of the biocontrol agent.
6. Restoring sensitivity of the pathogen to the activity should reduce its ability to cause disease. In addition, other steps such as transformation of the gene and expression in heterologous organisms or induced overexpression in the same bioprotectant also may be adequate to demonstrate the particular mechanism (Handelsman and Parke 1989).

Various mechanisms employed by the biocontrol agents in controlling the plant diseases are broadly classified into direct and indirect antagonism. Direct antagonism results from the physical contact and/or high degree of selectivity for the pathogens by biocontrol agent. In such a scheme, hyperparasitism by obligate parasites of a plant pathogen would be considered the most direct type of antagonism because the activities of no other organism would be required to exert a suppressive effect.

Indirect antagonisms result from activities that do not involve sensing or targeting a pathogen by the BCA(s). The stimulation of plant host defense pathways by nonpathogenic BCAs is the most indirect form of antagonism. However, in the natural environment, most described mechanisms of pathogen suppression will be modulated by the relative occurrence of other organisms in addition to the pathogen. While many investigations have attempted to establish the importance of specific mechanisms of biocontrol to particular pathosystems, all of the mechanisms described below are likely to be operating to some extent in all natural and managed ecosystems. And, the most effective BCAs studied to date appear to antagonize pathogens using multiple mechanisms. For instance, pseudomonads known to produce the antibiotic 2,4-diacetylphloroglucinol (DAPG) may also induce host defenses (Iavicoli et al. 2003). Additionally, DAPG producers can aggressively colonize roots, a trait that might further contribute to their ability to suppress pathogen activity in the rhizosphere of wheat through competition for organic nutrients Pal and Gardener (2006).

Hyperparasitism

Hyperparasitism is the most considered and the most direct form of antagonism Pal and Gardener (2006). Hyperparasitism involves tropic growth of biocontrol agent towards the target organism, coiling, final attack, and dissolution of target pathogen's cell wall or membrane by the activity of enzymes. It is one of the main mechanisms involved in *Trichoderma* (Sharma 1996). *Trichoderma harzianum* exhibits excellent mycoparasitic activity against *Rhizoctonia solani* hyphae (Altomare et al. 1999).

Mycoparasitism is under the control of enzymes. Harman (2000) reported the involvement of chitinase and β-1,3-glucanase in the *Trichoderma*-mediated biological control. Since enzymes are the products of genes, slight change in the structure of gene can lead to the production of different enzymes. Gupta et al. (1995) reported that a strain of *Trichoderma* deficient in the ability to produce endochitinase had reduced the ability to control *Botrytis cinerea* but shows increased ability to control *Rhizoctonia solani*.

A single fungal pathogen can be attacked by multiple hyperparasites, e.g., *Acremonium alternatum*, *Acrodontium crateriforme*, *Ampelomyces quisqualis*, and *Gliocladium virens* are few of the fungi that have the capacity to parasitize powdery mildew pathogens (Kiss 2003).

Competition

From the microbial perspective, soils and living plant surfaces are frequently nutrient-limited environment. So to colonize the phytosphere, a microbe must effectively compete for the available nutrients Pal and Gardener (2006). Both the biocontrol agents and the pathogens compete with one another for the nutrients and space to get established in the environment. This process of competition is considered to be an indirect interaction between the pathogen and the biocontrol agent, whereby the pathogens are excluded by the depletion of food base and by physical occupation of site (Lorito et al. 1994).

So far as the competition for nutrients is concerned, biocontrol agents compete for the rare but essential micronutrients, such as iron and manganese, especially in highly oxidized and aerated soils. In these soils iron is present in ferric form, which is insoluble in water and where the concentration may be as low as 10–8 M, too low to sport the microbial growth. Competition for micronutrients exists because biocontrol agents have more efficient utilizing uptake system for the substances than the pathogens (Nelson 1990). This property can be attributed to the production of iron binding ligands called siderophores as in *Erwinia carotovora* (Kloepper et al. 1980). Siderophores chelate the Fe (II) ions and the membrane bind protein receptors specifically recognize and take up the siderophore-Fe complex (Mukhopadhyay and Mukherjee 1998). This results in making iron unavailable to the pathogen, which produces less siderophores with lower binding power. The result is less pathogen infection and biological control.

Biocontrol agents also compete with the pathogen for physical occupation of site and thereby reduce or delay the root colonization by the pathogen. For example, spray the pine sumps with the spore suspension of infection by *Heterobasidion annosum*. Because the pathogen cannot gain a foothold for establishment on host, biocontrol can thus reduce the severity of root rot of pine (Maloy 1993).

Some plant pathogens depend on growth substances or stimulants to overcome their dormancy before they can cause infection, and biocontrol agents are known to exert competition for these stimulants, thereby reducing their disease-causing ability. These substances include fatty acids or peroxidation products of fatty acids (Harman and Nelson 1994) and volatile compounds such as ethanol and acetaldehyde (Paulitz 1991).

11.9 Antibiosis

Antibiosis is the antagonism resulting from the production by one microorganism of a secondary metabolite toxic to another microorganism. It is a very common phenomenon responsible for the activities of many biological control agents such as *Pseudomonas*, *Bacillus*, *Streptomyces*, and *Trichoderma* spp.

It also refers to the production of low-molecular-weight compounds or an antibiotic by microorganisms that have a direct effect on the growth of plant pathogen (Weller 1988). In situ production of antibiotics by several different biocontrol agents has been measured (Thomashow et al. 2002). However, the effective quantities are difficult to estimate because of the small quantities produced relative to the other less toxic, organic compounds present in the phytosphere. An efficient biocontrol agent is one that produces sufficient quantities of antibiotics in the vicinity of the plant pathogen (Chaube et al. 2003).

Most of the bioagents perform well in the laboratory conditions but fail to perform to their fullest once applied to the soil. This is probably attributed to the physiological and ecological constraints that limit the efficacy of bioagents. To overcome this problem, genetic engineering and other molecular tools offer a new possibility for improving the selection and characterization of biocontrol agents. Various methods that can contribute to increase the efficacy of bioagent include mutation or protoplasm fusion utilizing polyethylene glycol. There is also an urgent need to mass produce the bioagents, understand their mechanism of action, and evaluate the environmental factors that favor the rapid growth of biocontrol agents.

11.10 Medicinal Plant Extracts Used to Control Plant Diseases

The increasing incidence of pesticide resistance is further fueling the need for new generation of pesticides which are eco-friendly. A green plant represents a reservoir of effective novel chemotherapeutants with different modes of action and can provide valuable sources of natural pesticides against resistance pathogens (Newman et al. 2003). The popularity of botanical pesticides is once again increasing, and some plant products are being used globally as green pesticides. The body of scientific literature documenting the bioactivity of plant derivatives to different pests continues to expand, yet only a handful of botanicals are currently used in agriculture (Dubey et al. 2008). There are a lot of reports on the use of several plant by-products on several human pathogenic bacteria and fungi, but reports on the management of phytopathogenic bacteria are less. Plant-based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials. Considering the rich diversity of plants, it is expected that screening and scientific evaluation of plant extracts for their antimicrobial activity may provide new antimicrobial substances. In search of better alternatives, natural products are considered to be environmentally safe for the management of plant diseases, and hence the present study was carried out.

Plant extracts used to control the phytopathogens have been obtained mainly from tree species such as eucalyptus and neem (24 % of the studies with extracts) and herbaceous species like garlic, citronella, mint, rue, yarrow, ginger, basil (*Ocimum*), camphor, and turmeric (54 %). Besides these, there are other 237 plants from the Brazilian flora whose antimicrobial potential was tested by Brazilian researchers. With respect to groups of pathogens, the majority of the work is with those that cause disease in the plant canopy (30 % of the works with extracts), like the genera *Alternaria*, *Bipolaris*, *Crinipellis*, *Corynespora*, and *Colletotrichum*, which respond alone for 15 % of the works. The soilborne pathogens represent 20 % of the researches, especially *Rhizoctonia*, *Sclerotium*, *Sclerotinia*, *Fusarium*, and *Phytophthora*. Postharvest pathogens like *Penicillium*, *Aspergillus*, and *Rhizopus* are in 9 % of the works and *Meloidogyne* nematode in 9.5 %. For the host plants, 30 % of the works are with crops like beans, soybeans, coffee, wheat, cotton, and cassava; 20 % with vegetables like cucumber and tomato, which later represent alone 15 % of all the researches with extracts; and 10 % with the fruits like papaya, strawberry, and cocoa.

According to Gahukar (2012), leaf and seed extracts in water (5–10 %), seed cakes (250 kg ha^{-1}), crude oils (0.5–3 %), or essential oils (3,000 ppm) have been effectively used to control inter alia the sap-sucking pests, foliar diseases, and root-knot nematodes. Traditional and commercial products, especially those derived from neem (***Azadirachta indica*** A. Juss.) leaf or kernel, are commonly produced from medicinal crops since the use of plant products including allelochemicals results in reasonably effective, eco-friendly, and cheaper pest and disease management and crude extracts are easy to prepare.

11.11 Conclusions

More research is needed in order to find the nutrients or nutrient combinations which can help to reduce disease severity. It is also necessary to find the best integrated pest management approaches with disease-resistant varieties which can be combined with specific cultural management techniques and can efficiently control plant disease. In addition, more research is required to find how the nutrients increase or decrease disease tolerance or resistance, what the changes are in plant metabolism, and how this can be used to control plant disease. Medicinal plants have a promising future because there are about half a million plants around the world, and most of their medical activities have not been investigated yet and could be decisive in the treatment of present or future studies.

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Chapter 12
Role of PGPR in Soil Fertility and Plant Health

Ram Prasad, Manoj Kumar, and Ajit Varma

12.1 Introduction

Plant growth-promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants health. Their use in crop production can reduce the agro-chemical use and support eco-friendly sustainable food production. Plant growth promotion by PGPR is due to root hair proliferation, root hair deformation and branching, increases in seedling emergence, early nodulation and nodule functioning, enhanced leaf surface area, vigor, biomass, phytohormone, nutrient, water and air uptake, promoted accumulation of carbohydrates, and yield in various plant species (Podile and Kishore 2006). PGPR bring nutrient elements into the ecosystem from atmospheric or mineral reserves in soluble form; the roots take up the nutrients, break down the detritus, and also protect the roots from pathogens. Microorganisms are great potential goldmine for the biotechnology industry because it offers countless new genes and biochemical pathways to probe for enzymes, antibiotics, and other useful molecules.

Soil is the natural habitat for microorganisms beneficial as well as harmful to plant community. They play an important role in soil processes that determine plant productivity. For successful functioning of introduced microbial bioinoculants and their influence on soil health, exhaustive efforts have been made to explore soil microbial diversity of indigenous community, their distribution and behavior in soil habitats. PGPR involved in various beneficial activities within the soil like decomposition of crop residues, mineralization of soil organic matter, immobilization of mineral nutrients, phosphate solubilizers, synthesis of soil organic matter, nitrification, nitrogen fixation, and plant growth promoters including nutrient acquisition (biofertilizers), phytohormone production (biostimulants), and suppression of plant

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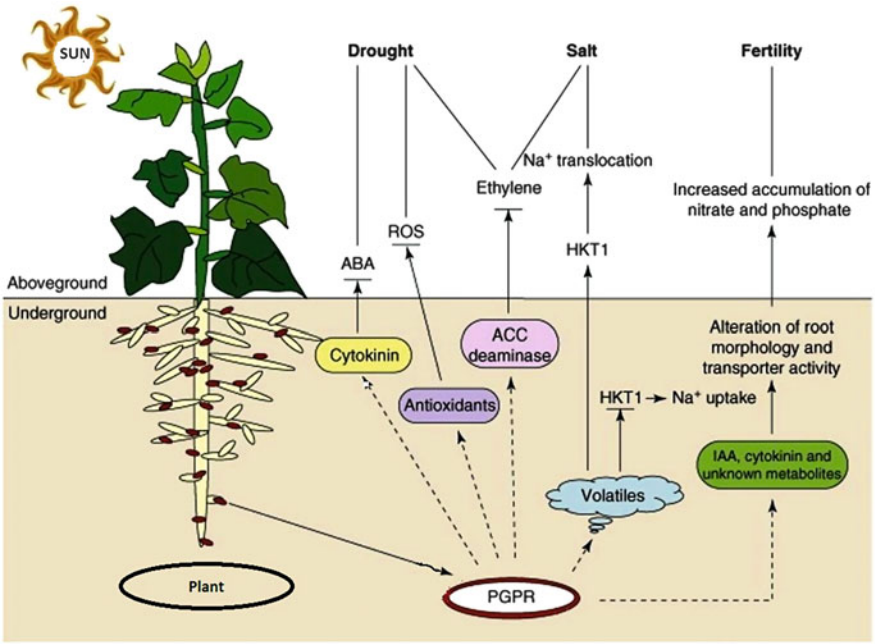

Fig. 12.1 Plant growth-promoting rhizobacteria has potential role in developing sustainable systems in crop production (Courtesy by: PakAgri farming)

disease (termed bioprotectants), which help in crop production and protection. Soil moisture content affects the colonization of the plant rhizosphere by the PGPR after inoculation (Shrivastava et al. 2014). In the recent era of sustainable crop production, the plant–microbe interactions in the rhizosphere play a pivotal role in transformation, mobilization, solubilization, etc. of nutrients from a limited nutrient pool and subsequently uptake of essential nutrients by plants to realize their full genetic potential (Fig. 12.1).

At present, the use of biological approaches is becoming more popular as an additive to chemical fertilizers for improving crop yield in an integrated plant nutrient management system. In this regard, the use of PGPR has found a potential role in developing sustainable systems in crop production. A variety of symbiotic (*Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, *Sinorhizobium*, *Mesorhizobium*) and free-living nitrogen-fixing bacteria or associative nitrogen fixers, viz. *Azospirillum*, *Azotobacter*, *Enterobacter*, *Klebsiella*, and *Pseudomonas*, are now being used in enhancing plant productivity (Cocking 2003). In the rhizosphere, rhizobacteria not only benefit from the nutrients secreted by the plant root but also beneficially influence the plant in a direct or indirect way, resulting in a stimulation of its growth. These PGPR can be classified according to their beneficial effects. For instance, biofertilizers can fix nitrogen, which can subsequently be used by the plant, thereby improving plant growth when the amount of nitrogen in the soil is limiting. Phytostimulators can directly promote the growth of plants,

usually by the production of hormones. Biocontrol agents are able to protect plants from infection by phyto-pathogenic organisms. However, this may be a function of the type of bacterium utilized since high moisture content may decrease the oxygen content of the soil.

12.2 The Rhizosphere

Hiltner (1904) discovered that the rhizosphere, i.e., the layer of soil influenced by the root, is much richer in bacteria than the surrounding bulk soil. These rhizosphere microbes benefit because plant roots secrete metabolites that can be utilized as nutrients. This rhizosphere effect is caused by the fact that a substantial amount of the carbon fixed by the plant, 5–21 %, is secreted mainly as root exudates (Marschner 1995). The rhizosphere is the zone of soil surrounding a plant root where the biology and chemistry of the soil are influenced by the root. As roots grow through soil, they release water-soluble compounds such as amino acids, sugars, and organic acids that supply food for the microorganisms. The food supply means microbiological activity in the rhizosphere is much greater than in soil away from plant roots. In return, the microorganisms provide nutrients for the plants. Some microorganisms, including bacteria and mycorrhizal fungi, form associations with roots that are mutually beneficial to both the plant and the microorganism. The rhizosphere is a center of intense biological activity due to the food supply provided by the root exudates. Most soil microorganisms do not interact with plant roots, possibly due to the constant and diverse secretion of antimicrobial root exudates. However, there are some microorganisms that do interact with specific plants. These interactions can be pathogenic (invade and kill roots and plants), symbiotic (benefit plant growth), harmful (reduce plant growth), saprophytic (live on plant debris), or neutral (no effect on plants). Interactions that are beneficial to agriculture include mycorrhizae, legume nodulation, and production of antimicrobial compounds that inhibit the growth of pathogens (Fig. 12.2). Rhizosphere microorganisms produce vitamins, antibiotics, plant hormones, and communication molecules that all encourage plant growth (Shrivastava et al. 2014).

12.3 Plant Growth-Promoting Rhizobacteria

Rhizosphere represents a nutrient-rich habitat for microorganisms; on the other hand, the microbial colonization of the rhizosphere also affects the whole plant (Hartmann et al. 2008). Kloepper and Schroth (1978) suggest the term “PGPR” for an important group of rhizosphere bacteria that have beneficial effects on plant growth when colonizing roots. Such effects are earlier seedling emergence, and increased vigor, biomass, yield, as well as proliferation of the root system in various plants (Kloepper 1993). PGPR as biological control agents and the ineffectiveness

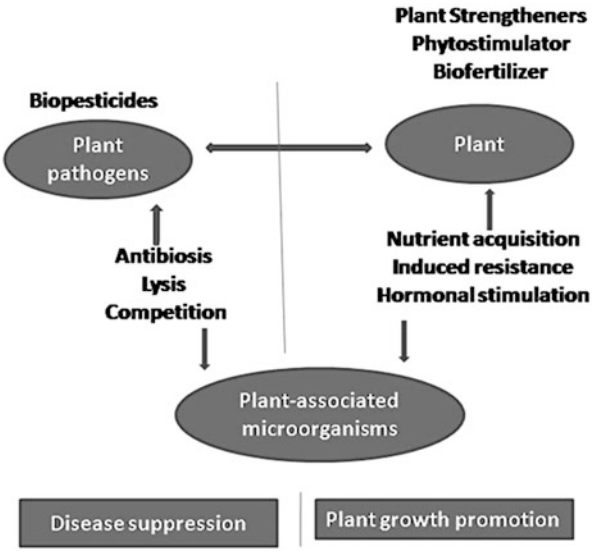

Fig. 12.2 PGPR promoting plant growth and health: mode of action and potential use in biotechnological applications

of PGPR in the field have often been attributed to their inability to colonize plant roots (Bloemberg and Lugtenberg 2001). A variety of bacterial traits and specific genes contribute to this process, but only a few have been identified (Benizri 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 (Lugtenberg et al. 2001). Several rhizospheric bacteria are plant growth promoters stimulating seedling growth and development; while mycorrhizal fungi provide vegetation with increased efficiency of nutrient uptake, increased productivity and abiotic stress may contribute to plant diversity. These facts, among others, are leading to a possible paradigm shift to a more microbial dominated or at least highly reciprocal view of the relationship between plant and associated microbiota. PGPR enhance plant growth either by producing plant hormones or by enhancing nutrient uptake or absence of pathogens (Van Loon 2007).

12.4 Applications of PGPR

PGPR enhance plant growth due to various factors, among which the release of phytohormones, nitrogen fixation, and regulation of ethylene production in roots, solubilizing nutrients such as phosphate, siderophore production, promoting mycorrhizal function, and decreasing heavy metal toxicity are the most important (Whipps 2001). The plant properties that are improved by PGPR during phytoremediation include biomass, contaminant uptake, and plant nutrition and health. Grain yields also an indication of plant health and growth. Plant growth

benefits due to the addition of PGPR include increases in germination rates, root growth, yield including grain, leaf area, chlorophyll content, magnesium content, nitrogen content, protein content, hydraulic activity, tolerance to drought, shoot and root weights, and delayed leaf senescence. Another major benefit of PGPR use is disease resistance conferred to the plant, sometimes known as "biocontrol" (Lucy et al. 2004).

The following genera of endophytes isolated from agricultural crops harbor PGPR-active strains: *Pseudomonas*, *Bacillus*, *Enterobacter*, and *Agrobacterium*. *Pseudomonas* spp. are typical PGPR and their reaction with arbuscular mycorrhizal fungi has been studied by Barea et al. (1998). *Pseudomonas* spp. had a positive effect on the spore germination and mycelial development of AMF in the soil as well as in root colonization. These bacteria (*Pseudomonas* spp.) have been called mycorrhization helper bacteria (Garbaye 1994). PGPR have stimulatory effect on the arbuscular mycorrhizae formation and plant nutrition (Barea et al. 2004). The ability to enter the root interior might help these microorganisms to evade the highly competitive rhizosphere habitat (Whipps 2001).

Siderophores, including salicylic acid, pyochelin, and pyoverdin, which chelate iron and other metals, also contribute to disease suppression by conferring a competitive advantage to biocontrol agents for the limited supply of essential trace minerals in natural habitat (Loper and Hankels 1997). Siderophores produced by PGPR inhibit the root pathogens by creating iron-limiting conditions in the rhizosphere and reduce probability of plant disease (Podile and Kishore 2006). Some siderophores such as pseudobacin and pyoverdin (yellow green fluorescent pigment of *Pseudomonas* bacteria) present high antimicrobial activity and affinity to ions of trivalent iron (Das et al. 2007; Maksimov et al. 2011). Pseudobacin is involved in induced systemic resistance, induction of H_2O_2 local storage, phenol compounds, and strengthening cell wall of rice plants in infection zone. Siderophores may indirectly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to the bacteria. Antibiotics and siderophores may additionally function as stress factors or signal inducing local and systemic host resistance. Biosynthesis of antibiotics and other antifungal compounds is regulated by a cascade of endogenous signals.

12.5 Possible Mechanism of Interaction or Physiology of Interaction

Plant growth promotion can be achieved by the direct interaction between beneficial microbes and their host plant and also indirectly due to their antagonistic activity against plant pathogens. The current status of research, commercial development, and application of PGPR inoculants is to promote plant health and environmental sustainability. In comparison with chemically synthesized pesticides and fertilizers, microbial inoculants have several advantages: they are more safe, show

reduced environmental damage and potentially smaller risk to human health, show much more targeted activity, are effective in small quantities, multiply themselves but are controlled by the plant as well as by the indigenous microbial populations, decompose more quickly than conventional chemical pesticides, resistance development is reduced due to several mechanisms, and can be also used in integrated pest management systems (Gabriele 2009; Chadha et al. 2014; Prasad et al. 2014).

The possible mechanisms by which PGPR aid plant growth include suppression of root pathogens through production of siderophores (compounds secreted by microorganisms that bind iron, making it less available to pathogens) or production of antibiotics (Kloepper et al. 1991), fixation of nitrogen (Chanway and Holl 1991), and production of plant hormones (Holl et al. 1988). PGPR are synergistic with mycorrhizae in stimulating plant growth and root colonization. There has been some success with PGPR in agriculture and commercial preparations are likely to become available (Linderman and Paulitz 1990). Major among them are *Rhizobium* symbiosis with legumes and free-living associative rhizosphere soil bacteria—*Azotobacter* and *Azospirillum*. The other group of beneficial microorganisms includes rhizobacteria, mainly *Pseudomonas*, *Erwinia*, *Flavobacterium*, and *Bacilli*, which improve health and productivity of crop plants through a variety of secondary metabolites and involved in promotion of root growth. Members of the bacterial genera *Azospirillum* and *Rhizobium* are well-studied examples for plant growth promotion; *Bacillus*, *Pseudomonas*, *Serratia*, *Stenotrophomonas*, and *Streptomyces* and the fungal genera *Ampelomyces*, *Coniothyrium*, *Piriformospora indica*, and *Trichoderma* are model organisms to demonstrate influence on plant health (Chadha et al. 2014). Another challenge is that plant-associated bacteria especially those from the rhizosphere play an emerging role as opportunistic human pathogens (Berg et al. 2005). Examples are antagonistic species of the genera *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Ochrobactrum*, *Pseudomonas*, *Serratia*, *Staphylococcus*, and *Stenotrophomonas* that are root-associated bacteria that can enter interactions with plant and human hosts (Ribbeck-Busch et al. 2005; Egamberdieva et al. 2008). Mechanisms involved in the interaction between antagonistic plant-associated bacteria and their host plants are similar to those responsible for the pathogenicity of bacteria to humans (Berg et al. 2005).

For all successful plant–microbe interactions, the competence to root colonize plant habitats is important for beneficial effects on plant growth (Kamilova et al. 2005). Steps of colonization include recognition, adherence, invasion (only endophytes and pathogens), colonization and growth, and several strategies to establish interactions. Plant roots initiate crosstalk with soil microbes by producing signals that are recognized by the microbes, which in turn produce signals that initiate colonization (Bais et al. 2006). To participate and react in this crosstalk, motile organisms are preferred (Lugtenberg et al. 2002). Moreover, there is growing appreciation that the intensity, duration, and outcome of plant–microbe interactions are significantly influenced by the conformation of adherent microbial populations (Danhorn and Fuqua 2004). Bacterial traits, such as pili, outer membrane proteins, and flagella, are involved in the PGPR adherence to plant root surfaces. Not only is the surface of roots colonized but also inner tissues of the

Table 12.1 Production of plant growth regulators (PGRs) by PGPR

PGPR	PGRs	Plant	References
Rhizobium leguminosarum	Indole-3-acetic acid	Rice	Biswas et al. (2000)
Azotobacter sp.	Indole-3-acetic acid	Maize	Zahir et al. (2000)
Pseudomonas fluorescens	Siderophores, indole-3-acetic acid	Groundnut	Dey et al. (2004)
Azospirillum brasilense A3, A4, A7, A10, CDJA	Indole-3-acetic acid	Rice	Thakuria et al. (2004)
Azospirillum lipoferum strains 15	Indole-3-acetic acid	Wheat	Muratova et al. (2005)
Pseudomonas denitrificans	Auxin	Wheat, maize	Egamberdieva (2005)
Azotobacter sp.	Indole-3-acetic acid	Sesbania	Ahmad et al. (2005)
Pseudomonas sp.	Indole-3-acetic acid	Wheat	Roesti et al. (2006)
Bacillus cereus RC 18	Indole-3-acetic acid	Wheat, spinach	Çakmakçi et al. (2007)
Mesorhizobium loti MP6	Chrom-azurol, siderophore, hydrocyanic acid, indole-3-acetic acid	*Brassica*	Chandra et al. (2007)
Pseudomonas tolaasii ACC23	Siderophores, indole-3-acetic acid	*Brassica*	Dell'Amico et al. (2008)
Bacillus sp.	Indole-3-acetic acid	Rice	Beneduzi et al. (2008)
Paenibacillus sp.			

plant. Colonization of the rhizosphere by some nonpathogenic microorganisms can protect the plant from a variety of bacterial, fungal, and viral diseases. This is known as induced systemic resistance. Interaction between the plant and root-colonizing microorganisms triggers signaling pathways and the production of specific gene products that enhance the ability of the plant to resist pathogens. Secondary metabolites involved in these pathways include phenolics, flavonoids, alkaloids, and terpenoids (Table 12.1).

In the processes of plant growth, phytohormones, e.g., production of auxin (IAA), cytokinins, and gibberellins, PGPR can increase root surface and length and promote in this way plant development (Kloepper et al. 2007). Several PGPR as well as symbiotic and free-living rhizobacterial species are reported to produce IAA and gibberellins in the rhizospheric soil and thereby play a significant role in increasing the root surface area and number of root tips in many plants (Bhattacharyya and Jha 2012). A greater root surface area enables the plant to access more nutrients from soil and thus contribute to plant growth promotion (Vessey 2003). These hormones can be synthesized by the plant themselves and also by their associated microorganisms. Furthermore, plant-associated bacteria can influence the hormonal balance of the plant. Ethylene is an important example to

show that the balance is most important for the effect of hormones: at low levels, it can promote plant growth in several plant species including *Arabidopsis thaliana*, while it is normally considered as an inhibitor of plant growth and known as a senescence hormone (Pierik et al. 2006). Interestingly, bacteria are able to reduce the ethylene level by the following way. The compound 1-aminocyclopropane-1-carboyclic acid (ACC) is a precursor of ethylene in plants. As ACC deaminase-producing bacteria are able to degrade this substance, the uptake by and the level in the root is reduced. Thus, these bacteria can increase root growth by lowering the endogenous ACC levels (Glick 2005). Due to the fact that ethylene has also established as a stress hormone, ACC deaminase-producing bacteria have an additional potential to protect plants against biotic and abiotic stress (Saleem et al. 2007). Another example to explain the intimate plant–microbe interaction regarding phytohormones is the root-associated bacterium *Serratia plymuthica* HRO-C48 in which IAA production is surprisingly negatively regulated by quorum sensing (QS) (Müller et al. 2009). Also, low amounts of IAA induced resistance in the plant while IAA is involved in many bacteria-plant signaling, an important role of auxin signaling for plant growth promotion was also shown for *Trichoderma* spp. (Hartmann et al. 2004; Contreras-Cornejo et al. 2009). Besides these mechanisms, improved nutrient acquisition is involved in direct growth promotion. The most well-known example is bacterial nitrogen fixation. The symbiosis between rhizobia and its legume plants is an important example for PGPR. Bacteria of this group metabolize root exudates (carbohydrates) and in turn provide nitrogen to the plant for amino acid synthesis. The ability to fix nitrogen also occurs in free-living bacteria like *Azospirillum*, *Burkholderia*, and *Stenotrophomonas* (Dobbelaere et al. 2003). Another nutrient is sulfate, which can be provided to the plant via oxidation by bacteria (Banerjee and Yesmin 2002). Bacteria may contribute to plant nutrition by liberating phosphorous from organic compounds such as phytates and thus indirectly promote plant growth (Unno et al. 2005). *Azospirillum* treatment resulted in enhancement of root growth and activities (e.g., acidification of the root surroundings) that increases phosphorous and other macroelements and microelements uptake (Dobbelaere and Okon 2007). Mineral supply is also involved in plant growth promotion and includes synthesis of siderophores and siderophore uptake systems (Katiyar and Goel 2004). Poorly soluble inorganic nutrients can be made available through the solubilization of bacterial siderophores and the secretion of organic acids. Recently, de Werra et al. (2009) showed that the ability of *Pseudomonas fluorescens* CHA0 to acidify its environment and to solubilize mineral phosphate is strongly dependent on its ability to produce gluconic acid. Furthermore, the study provides new evidence for a close association of gluconic acid metabolism with antagonistic activity against plant pathogens. Some bacteria, especially *Bacillus* and *Pseudomonas* sp., depress growth and development of filamentous fungi both in vitro and in vivo by secreting lytic enzymes such as chitinases and glucanase. It has been assumed that applying bacteria producing chitinases to biological protection of crops from pathogens, especially those that contain chitin and glucans within their cell wall structure (Maksimov et al. 2011).

Rhizosphere microorganisms, which are able to eliminate or reduce other pathogenic microorganisms, have been defined as biocontrol agents. Important mechanisms of microbial antagonism to plant pathogens are antibiosis, parasitism, and competition for nutrients and/or induced host defense responses (Podile and Kishore 2006). Microbial antagonism include (1) the inhibition of microbial growth by diffusible antibiotics and volatile organic compounds (VOCs), toxins, and biosurfactants (antibiosis); (2) competition for colonization sites and nutrients; (3) competition for minerals, e.g., for iron through production of siderophores or efficient siderophore uptake systems; (4) degradation of pathogenicity factors of the pathogen such as toxins; and (5) parasitism that may involve production of extracellular cell wall-degrading enzymes such as chitinases and β-1,3-glucanase (Whipps 2001; Wheatley 2002; Compant et al. 2005; Haas and Défago 2005; Raaijmakers et al. 2006; Kamal et al. 2008). Plant-associated bacteria can reduce the activity of pathogenic microorganisms not only through microbial antagonisms but also by activating the plant to better defend itself, a phenomenon termed "induced systemic resistance" (Conrath et al. 2002; Van Loon 2007). However, sometimes, the mechanism of ISR elicited by PGPR overlaps partly with that of pathogen-induced systemic acquired resistance (SAR). Both ISR and SAR represent a state of enhanced basal persistence of the plant that depends on the signaling compounds jasmonic acid and salicylic acid (Van Loon 2007). Pathogens are differently sensitive to the resistance activated by these signaling pathways. These interactions are highly specific on each component: the host plant, the pathogen, as well as the PGPR strain. They recognize each other by chemical signaling: root exudates as well as microbial metabolites. The mechanisms of ISR include (1) developmental escape: linked to growth promotion, (2) physiological-tolerance: reduced symptom expression, (3) environmental: associated with microbial antagonisms in the rhizosphere, and (4) biochemical-resistance: induction of cell wall reinforcement, induction of phytoalexins, induction of pathogenesis-related proteins, and "priming" of defense responses (resistance). Substances involved in ISR are partly the same with those involved in microbial antagonisms: siderophores, antibiotics, N-acyl-homoserine lactones, VOCs (e.g., 3-hydroxy-2-butanone (acetoin), and 2, 3-butandiol). Whereas some PGPR activate defense-related gene expression, other examples appear to act solely through priming of effective resistance mechanisms, as reflected by earlier and stronger defense reaction once infection occurs.

PGPR can be used to enhance the growth of plants with natural health products. Pre-inoculation of hosts with PGPR can induce/enhance specific human health promoting compounds in plants; enhance root health; Increase resistance to environmental stress; and increase yield and quality of active ingredient products. Although PGPR have not been used specifically to increase the production of medicinal compounds in plants before, their ability to enhance plant growth and root health has been demonstrated with many crop species (Glick 1995; Van Loon et al. 1998). The use of microbial associations for medicinal plants provides a sustainable approach to improving crop quality and yield and is suitable for use in organic agriculture (Prasad et al. 2008; 2013). It provides the potential to increase

production, value, and export of human health-enhancing crops and products. This will open new avenues products and markets for inoculant manufacturers.

12.6 Ecological Significance of Microbial Interactions

Microorganisms may contribute to the biocontrol of pathogens and improved supply of nutrients, thus maintaining plant health and production. Therefore, understanding of these interactions and the mechanisms could have implications for the progress of sustainable agriculture. Phosphate solubilizing bacteria are widespread in soils and secretion conversion of insoluble forms of phosphorus to plant-available forms (Vessey 2003). The biofertilizer properties of PGPR are frequently attributed to their ability to increase the bioavailability of inorganic and organic phosphorus, and some bacteria have documented synergistically effects on nitrogen fixation and formation of mycorrhizal associations.

PGPR present an alternative to the use of chemicals for plant growth enhancement in many different applications. Extensive research has demonstrated that PGPR could have an important role in agriculture and horticulture in improving crop productivity. In addition, these organisms are also useful in forestry and environmental restoration purposes. Because PGPR, which can fulfill diverse functions in plants, lead to promising solutions for a sustainable, environmentally friendly alternative to chemical fertilizers and pesticides, the use of which is regulated and sometimes forbidden; the market for bioinoculants is still expanding. While inoculants for plant growth promotion and biocontrol already exist, in the future, stress-protecting agents (stress conditions like those generated by salinity, drought, water logging, heavy metals, and pathogenicity) will be of emerging importance not only due to climate change. Furthermore, to improve food quality by PGPR is an important task.

12.7 Conclusions

PGPR are the potential tools for environmentally sustainable approach to increase soil fertility and plant health. PGPR benefit the growth and development of plants directly and indirectly through several mechanisms. The production of secondary metabolites, i.e., plant growth substances, changes root morphology resulting in greater root surface area for the uptake of nutrients, siderophores production, antagonism to soil-borne root pathogens, phosphate solubilization, and di-nitrogen fixation. The root surface area for uptake of nutrients and production of PGPR may help to optimize nutrient cycling in the event of stresses due to unsuitable weather or soil conditions. The beneficial effects of PGPR on plant growth are from changing the root architecture and enhancing nutrient uptake to biocontrol. The application of molecular tools is enhancing our ability to

understand and manage the rhizosphere and will lead to new products with improved effectiveness. The discovery of many traits and genes that are involved in the beneficial effects of PGPR has resulted in a better understanding of the performance of bioinoculants in the field and provides the opportunity to enhance the beneficial effects of PGPR strains by genetic modification.

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Part IV
Mechanism of Action

Chapter 13
Systemic Induction of Secondary Metabolite Biosynthesis in Medicinal Aromatic Plants Mediated by Rhizobacteria

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Abbreviation

EOs Essential oils
PGPR Plant growth-promoting rhizobacteria
VOCs Volatile organic compounds

13.1 Introduction

Bacteria are by far the most abundant organisms in soil, where they play essential roles in nutrient cycling and soil fertility. Root-colonizing bacteria are commonly referred to as "rhizobacteria." Many rhizobacterial strains, collectively termed "plant growth-promoting rhizobacteria" (PGPR), enhance plant growth when inoculated on seeds. PGPR species and strains in the genera *Acetobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Derxia*, *Enterobacter*, *Gluconacetobacter*, *Herbaspirillum*, *Klebsiella*, *Ochrobactrum*, *Pantoea*, *Pseudomonas*, *Rhodococcus*, *Serratia*, *Stenotrophomonas*, and *Zoogloea* have been the subjects of extensive research for many decades (Babalola 2010). PGPR promote plant growth by both direct and indirect mechanisms (Kloepper 1993; Niranjan et al. 2006; Van Loon 2007). Direct mechanisms include production of stimulatory bacterial volatile organic compounds (VOCs) and phytohormones, reduction of ethylene level in plants, improvement of plant nutrient status (release of phosphates and micronutrients from insoluble sources; nonsymbiotic nitrogen fixation), and

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enhancement of disease-resistance mechanisms (induced systemic resistance). Indirect effects of PGPR include functioning as biocontrol agents to reduce diseases, promotion of other beneficial symbioses, and protection of plants by degrading xenobiotics in contaminated soils (Figueiredo et al. 2010). Studies during the past 5 years have shown that some PGPR are capable of releasing functional VOCs that trigger growth promotion and induced resistance (Ryu et al. 2004). Depending on the PGPR species, two or more of the above growth-promoting mechanisms may be present (Vessey 2003).

During the past three decades, medicinal and aromatic plants have undergone a transition from unknown or minor agricultural plantings to major crops that farmers may consider as alternatives to traditional food or feed crops. The steadily increasing agricultural role is driven by consumer interest in these plants for culinary, medicinal, and other anthropogenic applications.

Aromatic plant species are a highly diverse group whose common characteristic is the production of essential oils (EOs) (Guenther 1948). EOs are active compounds that can modify behavioral or physiological responses in other organisms (Langenheim 1994). The major EOs in Lamiaceae, a large plant family that includes many aromatic and medicinal species, are terpenes, particularly monoterpenes (C10 members of the terpenoid class).

Terpenes are responsible for the characteristic fragrances of aromatic plants (Chen et al. 2011) and are typically emitted when plant structures are damaged (Wittstock and Gershenzon 2002). Lamiaceae accumulate EOs in specific structures, glandular trichomes (also termed secretory or peltate trichomes), which are lipophilic glands consisting of secretory cells and a cuticle-enclosed cavity that becomes filled with the secreted compound (Werker 2000). The plastids in glandular trichomes have less-defined membrane structures in comparison with chloroplasts and may be associated with synthesis and/or secretion of secondary metabolites such as terpenoids (Werker 2000).

Monoterpenes are among the best studied plant secondary metabolites with defensive functions. These colorless, lipophilic, volatile substances are the major constituents of plant EOs and display defensive effects (toxic, repellent, antifeeding, anti-ovipositing) against a variety of harmful insects and pathogens (Harrewijn et al. 2001; Chen et al. 2011). Some monoterpenes are involved in plant intraspecific communication (Wittstock and Gershenzon 2002).

Inducible chemical changes are of particular interest in medicinal and aromatic plants, not only in relation to defensive mechanisms as above but also because the altered compounds may have aromatic or therapeutic properties that enhance the economic value of the plant (Banchio et al. 2005). Increased knowledge of factors that affect EO quantity and quality in aromatic plants will be useful for improving production of these natural products and in pest management strategies (Kogan and Fischer 1991).

Chemical fertilizers and pesticides have been used increasingly in recent decades to maximize agricultural production. However, they are responsible for a variety of ecologically and agriculturally deleterious effects, e.g., depletion of nonrenewable energy resources, pollution of watersheds, elimination of beneficial

microorganisms and insects, increasing the susceptibility of the crop to disease, and reducing soil fertility (Babalola 2010).

Interest in environmentally safe, sustainable, and organic agricultural practices that reduce negative environmental effects associated with food and feed production is steadily increasing (Lind et al. 2004). "Organic agriculture" is a production system that avoids or minimizes the use of synthetic fertilizers, pesticides, and growth regulators, relying instead on biofertilization, crop rotation, crop residues, mechanical cultivation, and biological pest control to maintain soil productivity. Reduced yield is a major problem and concern in organic production systems. For many medicinal and aromatic plants that are consumed without further processing following harvest, it is important that synthetic compounds not be present.

Unconventional techniques such as inoculation with PGPR must be considered and investigated in the search for new strategies of plant production with high yield but without undesirable compounds or effects. The effects of PGPR inoculation in medicinal and aromatic plants have received very little research attention to date. New, less aggressive biotechnological methods involving the application of beneficial microorganisms as biofertilizers are a viable alternative to the use of chemical fertilizers. There are economic, environmental, and health-related justifications for research on PGPR strains as inoculants for cultivation of medicinal and aromatic plants. Application of these techniques may contribute to environmental conservation, increased crop productivity, and sustainable agricultural practices.

We present here an integrated summary of our experimental findings on induced responses to PGPR in various aromatic plant species of the families Lamiaceae and Asteraceae. Our focus is on the changes in plant EO/VOC composition (particularly of monoterpenes, the major EOs) induced by inoculation with various PGPR species.

13.2 Materials and Methods

13.2.1 Bacterial Strains, Culture Conditions, Media, and Treatments

Three bacterial strains well known as PGPR were used. *Pseudomonas fluorescens* WCS417r and *Azospirillum brasilense* Sp7 (Van Loon 2007) were grown on LB medium. *Bacillus subtilis* was grown on TSA for routine use and maintained in nutrient broth with 15 % glycerol at −80 °C for long-term storage.

Bacterial cultures were grown overnight at 30 °C with rotation (120 rpm) until reaching exponential phase. Each culture was then washed twice in 0.9 % NaCl by centrifugation (4,300×*g*, 10 min, 4 °C) in an Eppendorf centrifuge, resuspended in sterile water, and adjusted to a final concentration of ~10^9 CFU/ml for use as inoculum. Plants were grown in plastic pots (diameter 12 cm, depth 22 cm) containing 250 g sterilized vermiculite. Seeds were surface sterilized in 70 %

ethanol for 5 min, rinsed 5× with sterile water, dipped in 1 % NaCl for 1 min, rinsed 5× with sterile water, planted in vermiculite (one seed per pot), and inoculated with 1 ml bacterial suspension.

13.2.2 Greenhouse Experiments

Plants were grown in a growth chamber under controlled conditions of light (16/8 h light/dark cycle), temperature (22 ± 2 °C), and relative humidity (~70 %). Bacterial suspensions as described above were applied to experimental seedlings, and sterile water was applied to control seedlings. All plants were watered with Hoagland's nutrient medium (20 ml/pot) once per week (Banchio et al. 2008). All experiments were performed under non-sterile conditions.

Each experiment was replicated (ten pots per treatment; one plant per pot). Pots were arranged randomly in the growth chamber. Ninety days after inoculation, plants were removed from pots, roots were washed to remove vermiculite, and standard growth parameters (leaf number, shoot fresh weight, root dry weight) were measured.

13.2.3 Micropropagation of Plants

Young shoots from *Mentha x piperita* plants grown in Traslasierra Valley (Córdoba province, Argentina) were surface disinfected by soaking for 1 min in 17 % sodium hypochlorite solution and rinsed 3× in sterile distilled water. Disinfected shoots were cultured in 100 ml MS culture medium containing 0.7 % (w/v) agar and 1.5 % (w/v) sucrose (Murashige and Skoog 1962). All culture media contained 30 g/L sucrose and 7.5 g/L agar.

Stage I Initial shoot-tip culture: After 30 days, apical meristems with foliar primordia and no sign of contamination were removed aseptically from terminal buds of shoots obtained as above. Explants were cultured in test tubes containing 40 ml MS medium with 0.66 mg/L indolebutyric acid.

Stage II Growth and in vitro multiplication: Plantlets obtained from shoot tips as above were multiplied by single-node culture, and MS medium was adjusted to pH 5.6–5.8 prior to autoclaving (20 min, 121 °C). Explants were placed in a growth chamber under controlled conditions as in Sect. 13.2.2.

13.2.4 Exposure to VOCs

One node from an aseptically cultured plantlet or one sterilized *O. basilicum* seed was placed on one side of a specialized plastic Petri dish (90 × 15 mm) containing a

center partition (I-plate; Fisher Scientific). Both sides of the dish contained 50 % strength MS solid medium. 20 μL suspension cultures of various PGPR strains in sterile distilled water were applied one drop at a time to the side of the dish opposite the plant node. By this method, plants were exposed to bacterial VOCs without physical contact. Dishes were sealed with Parafilm, arranged in a completely randomized design, and placed in a growth chamber under controlled conditions as in Sect. 13.2.2. Plants were harvested after 30 days. Ten plants were used for each treatment, and experiments were replicated 4× (Santoro et al. 2011).

13.2.5 Extraction of EOs

The shoot samples were individually weighed and subjected to hydrodistillation in a Clevenger-like apparatus for 40 min, and the volatile fraction was collected in dichloromethane. Delta-dodecalactone (0.1 μL in 50 μL ethanol) was added as an internal standard.

The major EOs (accounting for ~75 % of the total EO volume) were identified and quantified relative to the delta-dodecalactone standard. Flame ionization detector (FID) response factors for each compound generated equivalent areas with negligible differences (<5 %).

Chemical analyses were performed using a PerkinElmer Clarus 600 gas chromatograph (GC) equipped with a CBP-1 capillary column (30 m × 0.25 mm, film thickness 0.25 μm) and mass-selective detector. Analytical conditions: injector/detector temperatures 250/270 °C; oven temperature programmed from 60 °C (3 min) to 240 °C at 4 °C/min; carrier gas = helium at constant 0.9 ml/min flow; source 70 eV. EO components were identified based on mass spectra and retention times, in comparison with standards (Banchio et al. 2005). GC analysis was performed using a PerkinElmer Clarus 500 GC fitted with a 30 m × 0.25 mm fused silica capillary column coated with Supelcowax 10 (film thickness 0.25 μm). GC operating conditions: oven temperature programmed from 60 °C (3 min) to 240 °C at 4 °C/min; injector/detector temperature 250 °C; detector FID; carrier gas = nitrogen at 0.9 ml/min constant flow.

13.2.6 Determination of Total Phenols

Total phenols were determined as described by Singleton and Rossi (1965). Plant extracts (each 0.5 ml) or gallic acid (standard phenolic reference compound) were mixed with Folin-Ciocalteu reagent (0.5 ml, diluted with 8 ml distilled water) and aqueous Na_2CO_3 (1 ml, 1 M). After 1 h, the level of total phenols was determined by colorimetry at wavelength 760 nm and expressed in terms of mg gallic acid equivalent per g plant dry weight (Lan et al. 2007).

13.2.7 Statistical Analyses

Data were pooled and subjected to analysis of variance (ANOVA) followed by comparison of multiple treatment levels with controls using Fisher's post hoc LSD (least significant difference) test. Differences between means were considered to be significant for $p < 0.05$. The Infostat software program, version 2008 (Group Infostat, Universidad Nacional de Córdoba, Argentina), was used for all statistical analyses.

13.3 Results

13.3.1 *Sweet Marjoram* (Origanum majorana)

Sweet marjoram is an herb native to Asia Minor (Turkey) and now abundant throughout the Mediterranean region and southern Europe. It is a small woody-stemmed shrub that grows best in well-drained alkaline soil. It reaches a height of ~75 cm and has a hairy stem, soft oval-shaped dark-green leaves, and tiny pinkish-white flowers. The leaves are typically harvested just after flower bud formation but before flowering. For blanching, harvested stems are hung in a dark, dry room ~7–10 days, and leaves are stripped from the stems and stored in an airtight container. *O. majorana* is an economically important species (Werker et al. 1993). Its EOs are used as flavoring in foods and beverages, as fragrances, and as fungicides or insecticides in pharmaceutical and industrial products (Deans and Svoboda 1990). *O. majorana* has strong antioxidant activity, primarily because of its high content of phenolic acids and flavonoids; this activity makes it useful in health supplements and food preservation (Vági et al. 2005). *O. majorana* contains up to 3 % volatile oils, comprising more than 40 distinct compounds. The major EOs, accounting for ~85 % of the total oil volume, are terpinen-4-ol, *cis*-sabinene hydrate, α-terpineol, and *trans*-sabinene hydrate (Banchio et al. 2008).

The effects of inoculation on plant development differed between *P. fluorescens* and *B. subtilis* (Table 13.1, Fig. 13.1). Some differences among treatments were observed even after 90 days of growth. Leaf number was 80 % higher in plants inoculated directly with *P. fluorescens* than in controls ($p < 0.05$) (Table 13.1). Shoot fresh weight and root dry weight were, respectively, 3.2-fold and 6-fold higher in *P. fluorescens*-inoculated plants than in controls ($p < 0.05$) (Table 13.1).

In terms of EO composition, PGPR inoculation caused increased production of certain terpenes (Fig. 13.2). The total EO yield in *P. fluorescens*-treated plants was ~24-fold higher than in controls ($p = 0.001$) (Fig. 13.1).

The EO components that were affected most notably by *P. fluorescens* inoculation (Fig. 13.2) were terpinen-4-ol, *cis*-sabinene hydrate, *trans*-sabinene hydrate, and α-terpineol. PGPR inoculation caused increases of not only EO synthesis but

Table 13.1 Effects of single inoculation with *P. fluorescens* and *B. subtilis* on growth of *O. majorana* plants

Treatment	Leaf number	Shoot fresh weight (mg)	Root dry weight (mg)
Control	$14.77 \pm 0.60a$	$0.17 \pm 0.01a$	$0.018 \pm 0.01a$
P. fluorescens	$25.70 \pm 2.10b$	$0.53 \pm 0.03b$	$0.120 \pm 0.02b$
B. subtilis	$13.00 \pm 0.59a$	$0.21 \pm 0.02a$	$0.016 \pm 0.02a$

Values followed by the same letter within a column are not significantly different according to Fisher's LSD test ($p < 0.05$)

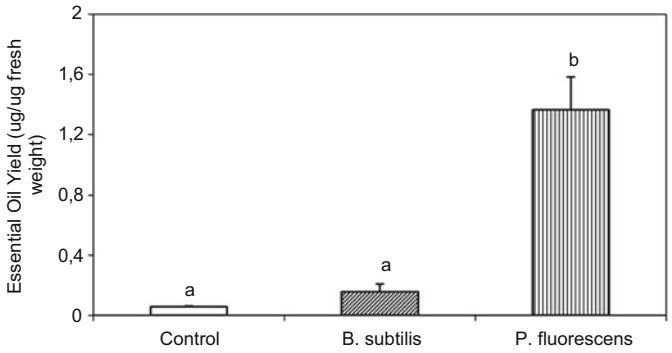

Fig. 13.1 Total EO concentrations in *O. majorana* inoculated with *B. subtilis* and *P. fluorescens*. *Letters above bars* indicate significant differences according to Fisher's LSD test

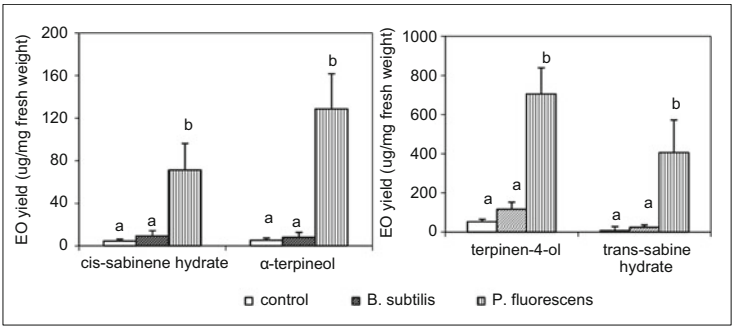

Fig. 13.2 Concentrations of major EO components in shoots of *O. majorana* inoculated with *B. subtilis* and *P. fluorescens*. *Letters above bars* indicate significant differences according to Fisher's LSD test

also relative percentages (R%) of the EO components. Terpinen-4-ol showed an increase of 66.65 % in *P. fluorescens*-treated plants as compared with 53.9 % in controls. Percent increases for *trans*-sabinene hydrate (17.33 %, 15.50 %) showed a similar trend.

13.3.2 *Italian Oregano (*Origanum x majoricum*)*

Oregano, a member of the family Lamiaceae, is used extensively in the food industry because of its aromatic and antioxidant properties (Petersen and Simmonds 2003). One economically important species is *Origanum x majoricum* Cambess. (Italian oregano), a hybrid of *O. majorana* L. *x O. vulgare* L. ssp. virens Ietswaart (Werker et al. 1993). *O. x majoricum* is a bushy, semiwoody subshrub with upright or spreading stems and branches. It grows in mats and spreads by rhizomes. The aromatic leaves are oval-shaped, ~3.8 cm long, and usually pubescent. The plant bears tiny purple tube-shaped flowers ~0.3 cm long throughout the summer. The flowers peek out from whorls of purplish-green leafy 2.5 cm long bracts that resemble tiny pinecones. The abundant EOs located in the leaf trichomes are lipophilic VOCs (mostly monoterpenes, sesquiterpenes, and phenylpropanoid metabolites) that are widely used as flavoring in foods and beverages, as fragrances, and as fungicides or insecticides in pharmaceutical and industrial products (Harrewijn et al. 2001). *O. x majoricum* contains up to 3 % volatile oils, comprising more than 35 different compounds (Tabanca et al. 2004). The major EOs, accounting for ~55 % of the total oil volume, are *cis*- and *trans*-sabinene hydrate, terpinene, carvacrol, and thymol (Banchio et al. 2010).

The effects of direct PGPR inoculation on *O. x majoricum* development differed for the three PGPR species examined (*B. subtilis*, *P. fluorescens*, *A. brasilense*) (Table 13.2, Fig. 13.3). Leaf numbers did not differ significantly ($p > 0.05$), but certain differences among the treatments were evident even after 90 days' growth. Shoot fresh weight in all inoculated plants was ~50 % higher than in controls (Table 13.2). This increase was due to a combination of increased leaf size and internode elongation. Root dry weight was promoted by all three treatments and was ~2-fold higher ($p < 0.05$) in *P. fluorescens*-treated and *A. brasilense*-treated plants than in controls (Table 13.2).

The total EO yield for *P. fluorescens*- and *A. brasilense*-treated plants was 3.57 and 3.41 μg/mg fresh weight, respectively, ~2.5-fold higher than for controls ($p = 0.001$) (Fig. 13.3). PGPR inoculation caused increased production of certain terpenes. No change of monoterpene production was observed in *B. subtilis*-treated plants.

Table 13.2 Effect of single inoculation with three PGPR on growth of *O. x majoricum* plants

Treatment	Leaf number	Shoot fresh weight (g)	Root dry weight (g)
Control	$19.77 \pm 0.60a$	$0.59 \pm 0.13a$	$0.10 \pm 0.01a$
P. fluorescens	$19.61 \pm 0.59a$	$0.89 \pm 0.04b$	$0.31 \pm 0.04b$
B. subtilis	$22.70 \pm 2.10a$	$0.97 \pm 0.08b$	$0.21 \pm 0.04b$
A. brasilense	$18.33 \pm 1.52a$	$0.83 \pm 0.09b$	$0.32 \pm 0.05b$

Values followed by the same letter within a column are not significantly different according to Fisher's LSD test ($p < 0.05$)

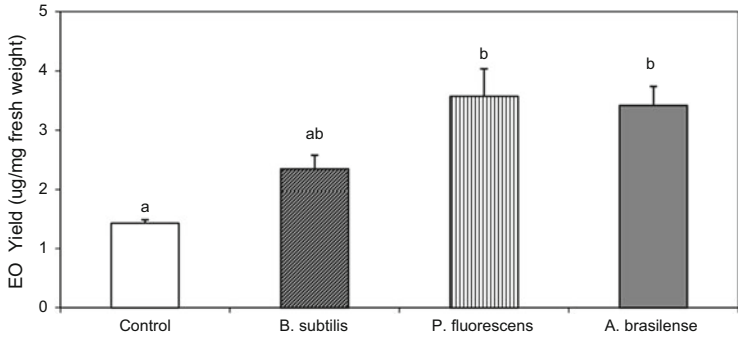

Fig. 13.3 Total EO concentrations in *O. x majoricum* inoculated with three PGPR. *Letters above bars* indicate significant differences according to Fisher's LSD test

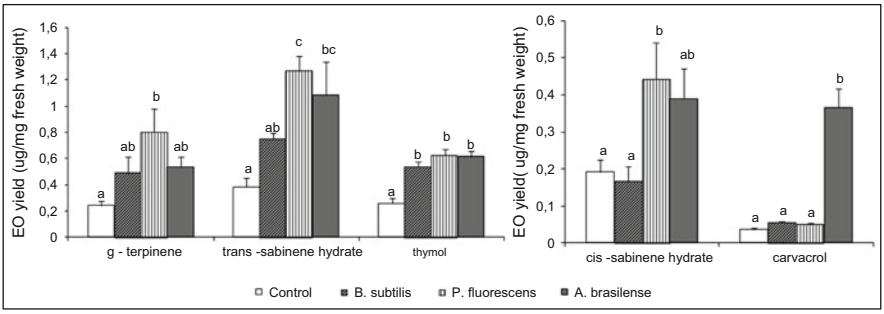

Fig. 13.4 Concentrations of major EO components in shoots of *O. x majoricum* inoculated with three PGPR. *Letters above bars* indicate significant differences according to Fisher's LSD test

Concentrations of γ-terpinene, *trans*-sabinene hydrate, *cis*-sabinene hydrate, and thymol were higher in PGPR-inoculated plants than in controls in most cases (Fig. 13.4). Concentrations of *trans*- and *cis*-sabinene hydrate, the major EO components, were ~3-fold and 2-fold higher, respectively, in *P. fluorescens*- and *A. brasilense*-treated plants than in controls. The thymol content was increased by all treatments. γ-terpinene showed a significant increase only in *P. fluorescens*-treated plants. Carvacrol showed a significant increase (~9-fold; $p < 0.05$) only in *A. brasilense*-treated plants (Banchio et al. 2010).

*13.3.3 Sweet Basil (*Ocimum basilicum*)*

Ocimum basilicum is an aromatic, annual herb, generally 0.3–0.5 m tall (as high as 1 m tall for certain cultivars). The leaves of some cultivars have leaves and stems with a deep purple color. The leaves are ovate, often puckered, the flowers are white or pink, and the fruits have four small nutlets that become mucilaginous when wet.

Table 13.3 Plant growth parameters of *O. basilicum* exposed to *B. subtilis* GB03 medium root media inoculation or GB03 VOCs

Treatment	Leaf number	Shoot fresh weight (g)	Root dry weight (g)
Root inoculation			
Control	$6.30 \pm 0.03a$	$0.25 \pm 0.04a$	$0.05 \pm 0.01a$
B. subtilis	$8.01 \pm 0.01b$	$0.65 \pm 0.11b$	$0.09 \pm 0.01b$
VOC exposure			
Control	$5.04 \pm 0.40a$	$0.88 \pm 0.04a$	$0.01 \pm 0.001a$
B. subtilis	$6.40 \pm 0.40b$	$1.72 \pm 0.09b$	$0.02 \pm 0.002a$

Values followed by the same letter within a column are not significantly different according to Fisher's LSD test ($p < 0.05$)

O. basilicum is used in perfumery, soapmaking, and flavoring liqueurs. The seeds are edible and become mucilaginous when soaked in water. The leaves are used to make an insecticide that protects stored crops from beetle damage. *O. basilicum* is rich in stored EOs and is commonly utilized in the spice industry (Werker et al. 1993). The abundant EOs located in leaf trichomes are lipophilic VOCs that consist mostly of monoterpenes, sesquiterpenes, and phenylpropanoid metabolites. *O. basilicum* EOs contain ~40 different metabolites. Two components, R-terpineol and eugenol, account for almost 60 % of the total VOC content (Simon et al. 1990; Zheljazkov et al. 2008).

O. basilicum was exposed to direct root inoculation with *B. subtilis* GB03 culture medium and to VOCs emitted by GB03 (Banchio et al. 2009). To investigate whether GB03 VOCs affected *O. basilicum* growth, the plants and bacteria were grown on the same dish with physical separation such that VOCs but not solutes from the bacteria could reach the plant. Leaf number was increased by both root inoculation and VOC exposure in comparison with controls ($p < 0.05$) (Table 13.3). Leaf area was increased 2-fold in plants exposed to GB03 VOCs. Fresh shoot weight was increased 3-fold and 2-fold by root inoculation and VOC exposure, respectively ($p < 0.05$). Root dry weight was increased only in root-inoculated plants (Table 13.3).

EO production was increased by both GB03 medium root inoculation and exposure to GB03 VOCs (Fig. 13.5). The total EO yield measured on a fresh weight basis was 2-fold less for root inoculation than for VOC exposure.

Increases in the major EO components were observed for both experimental treatments. Terpineol yield was increased ~2-fold for both treatments. Eugenol yield was increased ~8-fold for root inoculation and ~6-fold for VOC exposure (Fig. 13.6).

13.3.4 Wild Marigold (Tagetes minuta)

Wild marigold (*Tagetes minuta*) is an important member of the Asteraceae family. It has tiny involucres, toxic flowers, and a unique odor. *T. minuta* is native to the temperate grasslands and mountain regions of southern South America but is now

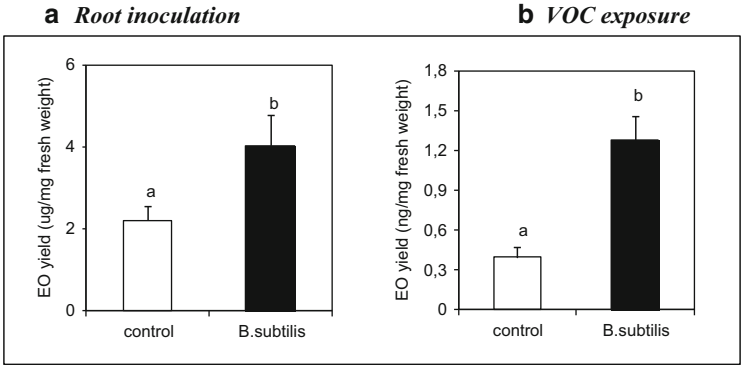

Fig. 13.5 EO concentration in *O. basilicum* exposed to *B. subtilis* GB03 medium root inoculation vs. GB03 VOCs. *Letters above bars* indicate significant differences according to Fisher's LSD test. (**a**) Root inoculation, (**b**) VOC exposure

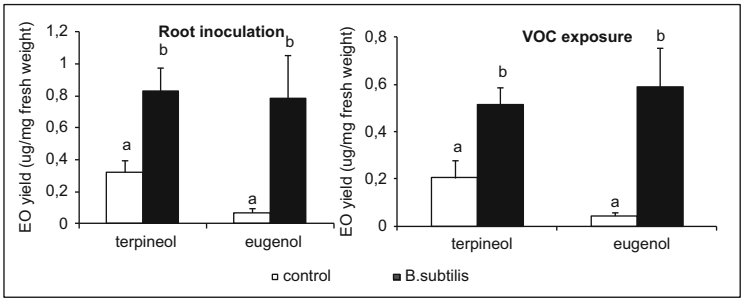

Fig. 13.6 EO concentrations in *O. basilicum* exposed to *B. subtilis* GB03 medium root inoculation or GB03 VOCs. *Letters above bars* indicate significant differences according to Fisher's LSD test

distributed worldwide; it is a "weed" with the ability to grow in environments ranging from extreme temperate to tropical (Singh and Singh 2003). *T. minuta* is an annual plant, 50–150 cm high, with a glabrous, erect, branched stem and opposite branches. The leaves are opposite and pinnately parted; the upper leaves are alternate. The leaves have a length of 4–8 cm, width of 3–4.5 cm, and margins that are acute and serrate. There are corymbiform dense inflorescences at the ends of branches. The phyllaries form a cylindrical tube that is naked at the base. There are three florets that are ligulate, dark brown, or lemon colored. Tubular florets are orange. The achene is dark brown and covered with appressed hairs. In tropical regions, *T. minuta* is grown for EO production (Shahzadi et al. 2010). The EO, known as "*Tagetes* oil" to retailers and end users, is a commercially valuable product (Singh and Singh 2003) used primarily in the preparation of high-grade perfumes (Kaul et al. 2000). Because of the high demand for *Tagetes* oil, there has been increasing cultivation of *T. minuta* for commercial production (Ghera and Leon 1999).

Table 13.4 Effects of single inoculation and co-inoculation with *P. fluorescens* and *A. brasilense* on *T. minuta* growth parameters and total phenol content

Treatment	Leaf number	Shoot fresh weight (g)	Root dry weight (g)	Total phenol content (Ac Gal/mg fresh weight)
Control	$12.44 \pm 0.40a$	$0.70 \pm 0.01a$	$0.14 \pm 0.02a$	$0.15 \pm 0.02a$
P. fluorescens	$16.18 \pm 0.54c$	$0.97 \pm 0.04b$	$0.20 \pm 0.03ab$	$0.27 \pm 0.03b$
A. brasilense	$14.94 \pm 0.45b$	$0.77 \pm 0.04a$	$0.26 \pm 0.04b$	$0.33 \pm 0.03b$
P. fluorescens + *A. brasilense*	$16.68 \pm 0.52c$	$1.01 \pm 0.06b$	$0.24 \pm 0.02b$	$0.30 \pm 0.03b$

Values followed by the same letter within a column are not significantly different according to Fisher's LSD test ($p < 0.05$)

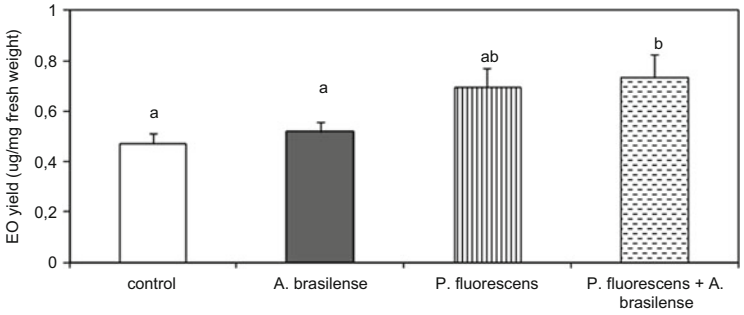

Fig. 13.7 Total EO concentrations in *O. basilicum* single inoculated or co-inoculated with *P. fluorescens* and *A. brasilense*. *Letters above bars* indicate significant differences according to Fisher's LSD test

The effects of PGPR inoculation on *T. minuta* growth and development varied depending on the inoculated strain (*P. fluorescens* WCS417r, *A. brasilense*, or their combination) (Table 13.4, Fig. 13.7). Most of the growth parameters evaluated were significantly ($p < 0.05$) increased by each of the three treatments (Table 13.4).

Leaf number, shoot fresh weight, and root dry weight were all increased significantly by *A. brasilense* treatment (Table 13.4); root dry weight was 80 % higher than in controls. The increase of root weight was due primarily to an increased number of lateral roots (data not shown). Shoot fresh weight was increased significantly (~50 %) by single inoculation of *P. fluorescens* or co-inoculation of *P. fluorescens* and *A. brasilense*. Leaf number showed a similar trend (Table 13.4). Leaf number was 33 % higher in *P. fluorescens*-inoculated and co-inoculated plants than in controls, as reflected by the increased shoot fresh weight. Root dry weight in these treated plants was significantly (~35 %) increased, partly because of an increase in root length.

The total phenol content was 2-fold higher ($p < 0.005$) in single-inoculated or co-inoculated plants than in controls (Table 13.4). The total EO yield was 50 % higher ($p = 0.02$) in *P. fluorescens* single-inoculated or co-inoculated plants than in

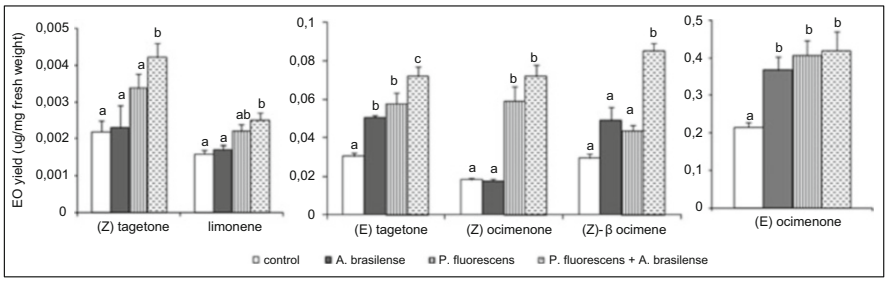

Fig. 13.8 Concentrations of major EO components in shoots of *T. minuta* plants single inoculated or co-inoculated with *A. brasilense* and *P. fluorescens*. *Letters above bars* indicate significant differences according to Fisher's LSD test

controls (Fig. 13.7). Single inoculation with *A. brasilense* did not significantly affect the total monoterpene content.

Levels of the major EO components analyzed, i.e., (*Z*)-(*E*)-tagetone, (*Z*)-(*E*)-ocimenone, (*Z*)-β-ocimene, and limonene (which together accounted for ~60 % of the total EO content), were usually different in inoculated plants than in controls (Fig. 13.8). (*E*)-ocimenone was by far the predominant component (accounting for ~50 % of the total EO content) and was increased affected by each of the experimental treatments. *A. brasilense* single inoculation increased the levels of (*E*)-ocimenone and (*E*)-tagetone by 71 and 66 %, respectively ($p < 0.005$) (Fig. 13.8). *P. fluorescens* single inoculation caused increases of each of the EO components except (*Z*)-β-ocimene. The effects of co-inoculation were similar to those of *P. fluorescens* single inoculation.

Single inoculation with *A. brasilense* or (to a greater degree) *P. fluorescens* affected plant growth and development. Co-inoculation caused greater increases in plant growth/development parameters and secondary metabolites, indicating a synergistic effect of the two PGPR. The population size of *P. fluorescens* increased from 10^5 CFU/ml at day 0 to 10^8 CFU/ml at day 7 and remained roughly constant thereafter ($p > 0.05$ for comparison between days 7 and 14). The population size of *A. brasilense* increased from 10^5 to 10^6 CFU/ml during the same period ($p < 0.05$ for comparison between days 7 and 14). Copresence of the two strains was observed throughout the co-inoculation experiments. *P. fluorescens* showed the same behavior in co-inoculation as in single inoculation (10^8 CFU/ml; $p > 0.05$). In contrast, *A. brasilense* in co-inoculation increased its population during days 0–7 and maintained its population thereafter (10^6 CFU/ml) (Cappellari et al. 2013).

13.3.5 Peppermint (Mentha x piperita)

The genus *Mentha*, which includes >25 species, is responsible for ~2,000 t of EO production worldwide, making it the second most important genus (after *Citrus*) in this regard (Mucciarelli et al. 2003). Peppermint, a naturally occurring hybrid of

Table 13.5 Effect of VOCs from three PGPR on *M. x piperita* growth parameters

Treatment	Leaf number	Shoot fresh weight (g)	Root dry weight (g)
Control	$23.48 \pm 2.20a$	$0.162 \pm 0.08a$	$0.005 \pm 0.001a$
P. fluorescens	$33.81 \pm 3.61a$	$0.278 \pm 0.04ab$	$0.014 \pm 0.004ab$
B. subtilis	$34.91 \pm 5.63a$	$0.319 \pm 0.03b$	$0.019 \pm 0.004b$
A. brasilense	$28.22 \pm 4.11a$	$0.21 \pm 0.04a$	$0.009 \pm 0.003a$

Values followed by the same letter within a column are not significantly different according to Fisher's LSD test ($p < 0.05$)

water mint (*Mentha aquatica*) and spearmint (*Mentha spicata*), was first cultivated in England in the late seventeenth century. It is an herbaceous rhizomatous perennial plant 30–90 cm tall, with smooth stems that are square in cross section. The rhizomes are wide-spreading, fleshy, and bare fibrous roots. The leaves are 4–9 cm long and 1.5–4 cm wide, dark green with reddish veins, with an acute apex and coarsely toothed margins. The leaves and stems are usually slightly fuzzy. The flowers are purple, 6–8 mm long, with a four-lobed corolla ~5 mm in diameter; they are produced in whorls around the stem, forming thick, blunt spikes. Flowering is from middle to late summer. *M. x piperita* is a fast-growing plant and spreads very quickly. Plants growing in vitro contain 3 % volatile oils, consisting of >50 different compounds. The EOs, which account for 60 % of the total oil volume, are (+) pulegone, (−) menthone, (−) menthol, and (+) menthofuran (Santoro et al. 2011).

To investigate the effect of VOCs from three PGPR on *M. x piperita* growth, plants and bacteria were grown in I-plates. The effect of VOC emission on plant development varied depending on the PGPR species (Table 13.5, Fig. 13.9). Clear differences among the treatments were detectable after 30 days' growth.

Exposure to *B. subtilis* VOCs caused a 2-fold increase ($p < 0.05$) in shoot fresh weight, and similar effects were observed for *P. fluorescens* treatment (Table 13.5). Root dry weight in *B. subtilis*-treated plants was 3.5-fold higher than in controls and significantly ($p < 0.05$) higher than in plants exposed to VOCs of *P. fluorescens* or *A. brasilense*. The increased shoot fresh weight of *B. subtilis*-treated plants was due to a 2-fold increase in leaf area in combination with internode elongation (data not shown). Leaf number was not changed significantly by any of the treatments (Table 13.5).

EO yields for *P. fluorescens*- and *A. brasilense*-treated plants were, respectively, 4.46 and 3.22 mg/mg fresh weight, ~2-fold higher than for controls (Fig. 13.9). Yields of the major EOs (+) pulegone, (−) menthone, (−) menthol, and (+) menthofuran were generally higher in treated plants than in controls (Fig. 13.10). Pulegone concentration was significantly increased (3.14-fold; $p < 0.05$) only by *P. fluorescens* treatment. Menthone was increased 15.4- and 13.5-fold ($p < 0.05$) in *P. fluorescens*- and *A. brasilense*-treated plants, respectively. Menthofuran was increased significantly in *P. fluorescens*-treated plants. The only decreases in EO yield (~5-fold) were observed for menthol and menthofuran in *A. brasilense*-treated plants. Exposure to PGPR VOCs led to changes in relative percentage (R%), as well

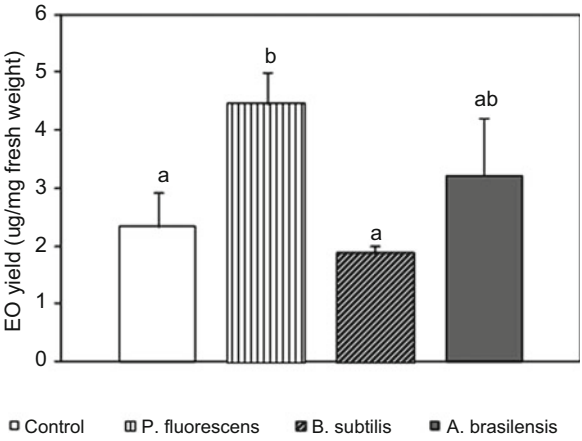

Fig. 13.9 EO concentrations in *M. x piperita* VOCs from three PGPR. *Letters above bars* indicate significant differences according to Fisher's LSD test

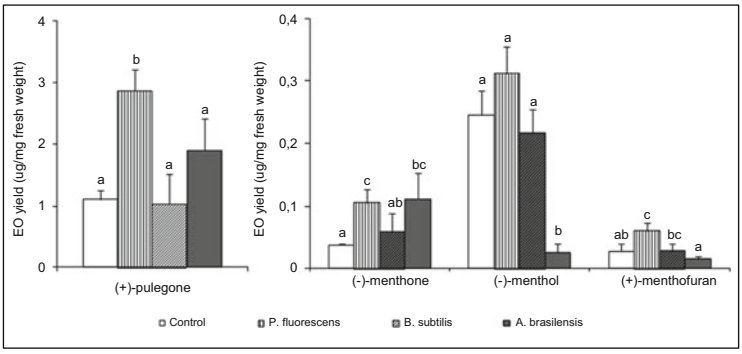

Fig. 13.10 Concentrations of major EO components in *M. x piperita* exposed to VOCs from three PGPR. *Letters above bars* indicate significant differences according to Fisher's LSD test

as yield, of EOs. R% for pulegone, the major EO component, increased to 59.9 % in *P. fluorescens*-treated plants, compared with 45.3 % in controls. R% for menthone increased in all cases. R% for menthol was lower in *P. fluorescens*- and *A. brasilense*-treated plants (6.1 %; 5.9 %) than in controls (9.6 %) but was higher in *B. subtilis*-treated plants (11.3 %). The only EO that showed a significant R% decrease in *A. brasilense*-treated plants was menthofuran.

13.4 Discussions

Enhanced growth and development following inoculation with PGPR has been reported for a number of plant species (Vessey 2003; Gray and Smith 2005; Van Loon 2007). The possible causes vary depending on the species and may include both direct and indirect mechanisms (Glick 1995; Gupta et al. 2002). Some examples of these mechanisms, which may be active simultaneously or sequentially at different stages of plant growth, are (1) increased mineral nutrient solubilization and nitrogen fixation, which make nutrients available for the plant; (2) suppression of soilborne pathogens (through production of hydrogen cyanide, siderophores, antibiotics, and/or competition for nutrients); (3) enhancement of plant tolerance to stress factors such as drought, salinity, and metal toxicity; and (4) production of phytohormones such as indole-3-acetic acid (IAA) (Gupta et al. 2002). Some PGPR have the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyzes ACC, the immediate precursor of ethylene in plants. By lowering ethylene concentration (and thereby the inhibitory effect of ethylene) in seedlings, these PGPR increase seedling root length (Glick 1995).

The effects of PGPR inoculation or VOC emission on the plant species (*O. majorana*, *O. x majoricum*, *O. basilicum*, *T. minuta*, *M. x piperita*) evaluated in this study varied depending on the inoculated strain (*P. fluorescens* WCS417r, *A. brasilense* Sp7, *Bacillus subtilis* GB03, or their combination). Previous studies have demonstrated host response specificity in plant species treated with PGPR (O'Neal et al. 2002) and diverse responses to PGPR inoculation.

In our study, the growth parameters evaluated were significantly modified in most cases by *P. fluorescens* single inoculation and by *P. fluorescens*/*A. brasilense* co-inoculation. *A. brasilense* single inoculation promoted all growth parameters in *O. x majoricum*, but only enhanced root dry weight in *T. minuta*.

B. subtilis inoculation caused significant increases in shoot fresh weight and root dry weight in *O. x majoricum* and *O. basilicum*, but had no significant effect on *O. majorana*.

Exposure of *O. basilicum* and *M. x piperita* to *B. subtilis* VOCs caused increases in shoot fresh weight whereas exposure of *M. x piperita* to *A. brasilense* VOCs had no such effect.

All plants in the study received Hoagland's nutrient solution and were grown on a sterilized, inert substrate in which nitrogen and other nutrients were available. The growth stimulatory effects observed were therefore not due to solubilization of phosphates, oxidation of sulfates, increased nitrate availability, extracellular production of antibiotics, or induction of plant systemic resistance (Kloepper 1993). Rather, the enhanced growth of the plant species observed following PGPR inoculation was presumably due to increased production of growth hormones and/or VOCs emitted by the PGPR.

Consistent with our findings, fluorescent pseudomonads were reported to promote overall growth of various crop species (Vikram 2007). *P. fluorescens*

enhanced plant growth through production of growth-promoting substances such as IAA and cytokinins (Vikram 2007; De Salamone et al. 2001). The role of auxins and cytokinins in enhancing plant cell division and root development is well documented (Arshad and Frankenberger 1993). IAA is involved in root initiation, cell division, and cell enlargement (Gray and Smith 2005) and increases root surface area and consequent access to soil nutrients. Cytokinins promote cell division, cell enlargement, and tissue expansion in certain plant parts (Gray and Smith 2005). *A. brasilense*, in addition to its nitrogen-fixing ability, secretes phytohormones such as auxins, cytokinins, and gibberellins. Auxins are quantitatively the most abundant phytohormones secreted by *Azospirillum*. Auxin production, rather than nitrogen fixation, is considered to be the major factor responsible for stimulation of rooting and enhancement of plant growth (Bloemberg and Lugtenberg 2001).

We found that the effects of PGPR inoculation and VOCs on the formation of plant secondary compounds are species specific. The total phenol content in *T. minuta* was increased by single inoculation or co-inoculation with *P. fluorescens* and *A. brasilense*. Phenolic compounds are a major class of plant secondary metabolites and one of the most common and widespread groups of plant components in general. They are essential for plant growth and reproduction. Some phenolic compounds are produced constitutively; others are induced as a plant defensive response. In contrast to basic metabolism, which refers to the anabolic and catabolic processes required for cell maintenance and proliferation, secondary metabolism refers to compounds present in specialized cells that are not directly essential for basic photosynthetic or respiratory metabolism, but are considered to be necessary for plant survival in the external physical environment (Lattanzio et al. 2006). There has been recent interest in phenolic acids because of their potential protective role, via ingestion of fruits and vegetables, against oxidative damage diseases (coronary heart disease, stroke, cancers). Recent studies have clearly demonstrated the important antioxidant activities of phenolic compounds and the advantages of their use as natural antioxidants in processed foods (Lattanzio et al. 2006). Phenolic compounds also act as defensive compounds (against herbivores, microbes, viruses, or competing plants) and as signaling compounds (to attract pollinating or seed-dispersing animals) and protect the plant from ultraviolet (Kutchan 2001).

EO yield was increased to varying degrees by *P. fluorescens* inoculation in *O. majorana*, *O. x majoricum*, and *T. minuta*. Monoterpene production was increased 2-fold in some plants and 24-fold in *O. majorana*. VOCs emitted by *P. fluorescens* had the same effect on *M. x piperita* as direct root inoculation, whereas the effects of *B. subtilis* VOCs vs. inoculation were different. *O. majorana* and *O. x majoricum* did not show changes in EO yield, whereas EO yield in *O. basilicum* was increased 2-fold. Similar results were observed for VOC exposure in *O. basilicum*. VOC exposure did not affect the total monoterpene accumulation in *M. x piperita*. An increase in the total EO yield by root inoculation with *A. brasilense* was observed in *O. x majoricum*, but not in *T. minuta*.

The enhanced EO accumulation response was not due to increased biomass. It may have resulted from increased terpene biosynthesis, although we did not measure this process. In addition to increased EO synthesis, relative percentages (R%) of EO components were changed significantly by inoculation in several cases.

Our findings indicate that effects of PGPR VOCs on plants are species specific; i.e., VOCs from a particular bacterial strain do not cause the same effects, or to the same degree, in all plant species. A particular plant–bacteria combination has its own characteristic responses. Possible explanations for this phenomenon are as follows: (1) different plants respond to different component(s) of VOC mixtures; (2) reactive sites are different; (3) plants differ in their ability to metabolize VOCs.

The concentration and composition of oils in plants serve important ecological roles. Increased EO synthesis provides a defensive response to colonization by microorganisms; several EOs have antimicrobial properties (Sangwan et al. 2001). Analogously, monoterpene synthesis is induced by herbivore feeding in *Minthostachys mollis* (Banchio et al. 2005) and other plant species, apparently to protect damaged leaves from further attack (Harrewijn et al. 2001).

There have been few attempts to elucidate the relative quantitative and qualitative contributions of rhizobacteria to formation of plant secondary compounds. Induction of secondary metabolite responses has been reported in other beneficial microbe–plant interactions involving arbuscular mycorrhizal (AM) fungi. Gupta et al. (2002) inoculated the AM fungus *Glomus fasciculatum* in cultivars of wild mint (*Mentha arvensis*) and observed increased plant height, shoot growth, and oil content. Khaosaad et al. (2006) observed changes of EO concentration (but not composition) following mycorrhizal inoculation of *Origanum* sp. Copetta et al. (2006) reported increases of glandular hair abundance and EO yield in inoculated *O. basilicum*. The increased EO yield was associated with a larger number of peltate glandular trichomes, the primary site of EO synthesis. Belowground AM fungi cause changes in leaf isoprenoid content that favor EO production, particularly under drought stress condition or following jasmonic acid (JA) application (Asensio et al. 2012). AM fungi increase plant growth and EO production because mycorrhization allows the root system to exploit a greater volume of soil by (1) extending the root zone, (2) reaching smaller soil pores not accessible by root hairs, and (3) acquiring organic phosphates through production of extracellular acid phosphatases (Bouwmeester et al. 2007).

Terpene compounds help the plant's photosynthetic apparatus recover from brief episodes of high temperature. Isoprene may physically stabilize thylakoid membranes at high temperature or quench reactive oxygen species (e.g., ozone) that cause membrane damage (Pichersky and Gershenzon 2002). Enhanced biosynthesis of secondary metabolites can be triggered by certain stress factors (Ramomoorthy et al. 2001). Nonpathogenic rhizobacteria have been shown to stimulate secondary metabolism in plants through a mechanism termed ISR (induced systemic resistance) (van Oosten et al. 2008; Pozo et al. 2008; Pieterse et al. 2009; Pineda et al. 2012). The occurrence of ISR has been demonstrated in various plants inoculated with various species of rhizobacteria (Pineda et al. 2013). ISR may be local or systemic (when it is expressed at sites not directly exposed to the inducing

agent). The inducing agent may be a chemical activator or an extract of cells of living organisms or microorganisms. ISR has been described as "activation of the host plant's physical or chemical defenses by an inducing agent" (Kloepper 1993). Interestingly, PGPR simultaneously induce an ISR response and promote plant growth (Kloepper et al. 2004; Yi et al. 2013).

Both direct and indirect defenses are under the control of a complex network of signal transduction pathways that are regulated by various phytohormones, of which JA is a central regulator (Snoeren et al. 2009; Kusnierczyk et al. 2011). JA exerts its protective effects by regulating a wide range of defense-related processes, including the synthesis of toxic secondary metabolites (Pauwels et al. 2009). JA also triggers the biosynthesis of mono- and sesquiterpenes (Arimura et al. 2000) that are presumed to act as master switches for plant responses stimulated by root-colonizing bacteria, leading to activation of distinct sets of defense genes responsible for terpenoid formation (Pineda et al. 2012).

Some of the roles of EO components are relatively straightforward; e.g., they play numerous generalized protective roles (antioxidant, free radical scavenging, UV light absorbing, antiproliferative, etc.) and defend the plant against microorganisms (bacteria, fungi, viruses). EO components also help modulate interplant relationships, acting as allelopathic defenders of the plant's growing space against competing plants. More complex roles include defining or modifying the plant's relationship with herbivores (Tahara 2007; Wink 2000). The primary role of EO components is often viewed as feeding deterrence; to this end, many phytochemicals are bitter and/or toxic to potential herbivores. The toxic effects often extend to direct interactions with the herbivore's central and/or peripheral nervous systems (Rattan 2010). Secondary metabolites often act as agonists or antagonists of neurotransmitter systems (Wink 2000; Rattan 2010) or form structural analogs of endogenous hormones (Miller and Heyland 2010).

Biosynthesis of terpenoids depends on primary metabolism (e.g., photosynthesis) and oxidative pathways for carbon and energy supply (Singh et al. 1990). Giri et al. (2003) found that net photosynthesis of PGPR host plants increases as a result of improved nutritional status. Factors that increase dry matter production may influence the interrelationship between primary and secondary metabolism, leading to increased biosynthesis of secondary products (Shukla et al. 1992). Increased plant biomass may result in greater availability of substrate for monoterpene biosynthesis (Harrewijn et al. 2001). The increased concentration of monoterpenes in inoculated plants may be caused by growth-promoting substances produced by the inoculated microorganism that affect plant metabolic processes. Because the plants in the present study were grown in enriched medium containing nitrogen and other nutrients, bacterial metabolites are the most likely growth-promoting substance.

Knowledge of the adaptive mechanisms of plants is of interest from an ecophysiological point of view. These mechanisms also provide an important (probably crucial) starting point for improvement of plant production, including optimization of secondary metabolite production. The use of fungal and bacterial inoculants is an efficient biotechnological alternative for stimulating secondary metabolism in plants. Studies of such inoculants will also clarify certain adaptive processes that are poorly understood at present.

13.5 Conclusions

The present findings show that inoculation of certain PGPR causes systemic induction of monoterpene pathways in various aromatic plants species, suggesting that PGPR inoculation can significantly increase productivity and reduce the amount of fertilizer required for economically viable aromatic crop production. The markets for medicinal plants, aromatic plants, and organic foods are steadily expanding (Adam 2005; Hartman Group 2006). As consumers become more concerned and knowledgeable about their own health and wellness, there is increasing demand for quality plant material, produced by sustainable methods and uncontaminated by synthetic pesticides or genetically modified organisms (Craker 2007).

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Chapter 14
Medicinal Plants and PGPR: A New Frontier for Phytochemicals

Dilfuza Egamberdieva and Jaime A. Teixeira da Silva

14.1 Introduction

Plant-derived medicines have been used worldwide in the treatment of numerous human diseases for centuries (Chiariandy et al. 1999). Herbal products have been an integral part of ancient traditional medicine systems that have enriched our modern knowledge of herbal medicine (Abu-Irmaileh and Afifi 2003; Sarker and Nahar 2007). Increasing awareness of hazards and toxicity associated with the indiscriminate use of synthetic drugs and antibiotics, as well as the use of medicinal plants for the treatment of various diseases, has became popular (Saganuwan 2010).

The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body (Edeoga et al. 2005). Numerous studies have validated the traditional use of medicinal plants by investigating numerous phytochemicals (including alkaloids, tannins, flavonoids, phenolic compounds, and terpenes) present in active extracts (Palombo 2006; Van Wyk and Wink 2004). Plant leaves, roots, rhizomes, stems, bark, flowers, fruits, grains, or seeds contain chemical components that are biologically active (Doughari et al. 2009). Plants synthesize a diverse array of secondary metabolites that are important for them to survive and flourish in their natural environment (Wu et al. 2007), where they also have protective actions in relation to abiotic stresses such as those associated with temperature, water status, and mineral nutrients (Kaufman et al. 1999).

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Plant-derived novel biological active compounds continue to be used worldwide and developed further for the treatments of various ailments, including asthma, gastrointestinal symptoms, skin disorders, respiratory and urinary problems, and hepatic and cardiovascular disease (Cousins and Huffman 2002; Saganuwan 2010). Plant secondary metabolites are a major source of bioactive natural products and are valuable pharma- and nutraceuticals; therefore, medicinal plants are commercially cultivated in many countries worldwide (Phillipson 2001). Successful cultivation of medicinal plants depends on biotic and abiotic factors which can modulate the secondary metabolites, essential oil composition, and yield (Juliani et al. 2006). It is important to avoid the use of chemical fertilizers and pesticides in the cultivation of plants since they are typically consumed without being further processed after harvest (Banchio et al. 2008).

Therefore, current research in drug discovery from medicinal plants involves innovative biotechnologies such as the introduction of biological fertilizers and biopesticides which increase the level of biologically active compounds in medicinal plants (Rajasekar and Elango 2011; Bharti et al. 2013; Teixeira da Silva and Egamberdieva 2013). Plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal (AM) fungi are able to promote plant growth, nutrient uptake, and phytochemical constituents, protect plants against various soilborne pathogens, and can help plants to adapt to a number of environmental stresses (Jeffries et al. 2003; Egamberdieva et al. 2013a; Egamberdieva and Lugtenberg 2014; Hameed et al. 2014).

In this review, we examine the plant-microbe interactions with medicinal plants and their functional characteristics. We also discuss the use of plant-associated beneficial microorganisms to enhance the levels of phytochemicals.

14.2 Phytochemical Constituents of Medicinal Plants

The primary focus of research to date on plants, which are reservoirs of biologically active compounds with therapeutic properties and have been used for curing various diseases, has been in the areas of phytochemistry and pharmacognosy (Briskin 2000). Biologically active compounds are primarily secondary metabolites and their derivatives such as alkaloids (Sarker and Nahar 2007), glycosides (Firn 2010), flavonoids (Kar 2007), phenolics (Puupponen-Pimiä et al. 2001), saponins (Sarker and Nahar 2007), tannins (Kar 2007), terpenes (Martinez et al. 2008), anthraquinones (Maurya et al. 2008), essential oils (Martinez et al. 2008), and steroids (Madziga et al. 2010). More than 12,000 alkaloids are known to exist in about 20 % of plant species, and only few have been exploited for medicinal purposes (Firn 2010), and over 4,000 flavonoids are known to exist with quercetin, kaempferol, and quercitrin being common flavonoids present in nearly 70 % of plants (Kar 2007). Glycosides are classified on the basis of type of sugar component, chemical nature of the aglycone, or pharmacological action (Sarker and Nahar 2007), and phenolics essentially represent a host of natural antioxidants (Kar 2007),

whereas saponins are shown to have hypolipidemic and anticancer activity. Essential oils are referred to as volatile oils or ethereal oils because they have a tendency to evaporate on exposure to air; chemically, a single volatile oil comprises more than 200 different chemical components (Martinez et al. 2008).

Plant secondary metabolites play protective roles as antioxidant, free radical-scavenging, and antiproliferative agents and defend the plant against herbivory and pathogen attack (Wink and Schimmer 1999; Briskin 2000), and it is likely that their ecological function may have potential medicinal effects for humans. According to Wink and Schimmer (1999), bioactive agents involved in plant defense through cytotoxicity toward microbial pathogens and/or against herbivores could have beneficial effects in humans.

Environmental factors such as soil type, nutrients, temperature, drought, salinity, as well as competition for nutrients among microorganisms are important variables affecting phytochemical production in medicinal plants (Perez-Balibrea et al. 2008; Egamberdieva et al. 2013b).

14.3 Plant Beneficial Microorganisms

The rhizosphere is colonized more intensively by microorganisms than other regions of the soil (Lugtenberg et al. 2001). Beneficial rhizosphere bacteria are of two general types, those forming a symbiotic relationship with the plant and those that are free living in the soil and root (Barriuso et al. 2005; Lugtenberg and Kamilova 2009; Berg et al. 2013). Beneficial rhizobacteria can improve seed germination, root and shoot growth, yield, nutrient uptake, and plant stress tolerance and are able to control various diseases (Çakmakcı et al. 2005; Egamberdieva and Islam 2008; Jabborova et al. 2013). Several root-associated bacteria showing plant growth-promoting activity belong to several genera, including *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Bradyrhizobium*, *Burkholderia*, *Cellulomonas*, *Clostridium*, *Enterobacter*, *Flavobacterium*, *Micrococcus*, *Paenibacillus*, *Pseudomonas*, *Rhizobium*, *Sinorhizobium*, and *Serratia* (Somers et al. 2004; Rajasekar and Elango 2011; Egamberdieva et al. 2011, 2013b). Several studies have reported that AM fungi also improve plant growth and development and supply mineral nutrients to plants, especially phosphorus, which is precipitated by ions such as Ca, Mg, and Zn (Al-Karaki et al. 2001; Hameed et al. 2014). They play a key role in alleviating toxicity induced by salt stress, thus normalizing the uptake mechanism in plants by supplying essential nutrients.

Moreover, the production of secondary metabolites such as total phenols, alkaloids, tannins, and lycopene and antioxidant activity on various plants was also stimulated after treatment with PGPR and AM fungi (Elango 2004). Mixed inoculation with PGPR and *Rhizobium* or AM fungi creates synergistic interactions that may result in a significant increase in growth, in symbiotic performance, and an enhancement in the uptake of mineral nutrients such as phosphorus, nitrogen, potassium, and other minerals (Adesemoye and Kloepper 2009; Egamberdieva

et al. 2010). Recent studies show that root-associated beneficial microorganisms play an important role in the improvement of plant growth of medicinally important plants and increase phytochemical constituents which are widely used for curing various diseases (Bharti et al. 2013; Teixeira da Silva and Egamberdieva 2013).

14.4 PGPR Improve Bioactive Phytochemical Levels in Plants

There are many reports on the beneficial effect of PGPR and AM fungi on plant growth, nutrient uptake, and secondary metabolite production, such as phenols, flavonoids, alkaloids, saponins, and tannins of medicinal plants, including *Catharanthus roseus* (Karthikeyan et al. 2009), *Origanum majorana* L. (Banchio et al. 2008), *Matricaria chamomilla* (Razmjoo et al. 2008), *Ocimum basilicum* (Banchio et al. 2009), *Salvia militiorrhiza* (Wu et al. 2007), *Mentha arvensis* (Gupta et al. 2002), and *Withania somnifera* (Rajasekar and Elango 2011). The improvement of secondary metabolites in medicinal plants by plant beneficial microorganisms is given in Table 14.1.

Ocimum basilicum L. (sweet basil) is rich in essential oils and contains approximately 40 different metabolites, and among them more than 60 % are terpineol and eugenol (Banchio et al. 2009). The content of those two essential oil components increased up to tenfold in plants exposed to *Bacillus subtilis* GB03 root inoculation or volatiles. In other studies, plant growth and the essential oil content of *Ocimum* spp. increased after plants were inoculated with *Glomus fasciculatum* and *Azotobacter chroococcum* (Vinutha 2005), *Pseudomonas putida* and *A. chroococcum* (Ordookhani et al. 2011), and the AM fungus, *Glomus mosseae* (Copetta et al. 2006).

Banchio et al. (2008) studied the effects of root colonization by PGPR on biomass and qualitative and quantitative composition of essential oils in the aromatic crop *Origanum majorana* L. (sweet marjoram). They found that plants inoculated with *P. fluorescens* or *Bradyrhizobium* increased total essential oil yield in plants and may have resulted from increased biosynthesis of terpenes. The main compounds affected by inoculation with *P. fluorescens* were terpinen-4-ol, *cis*-sabinene hydrate, *trans*-sabinene hydrate, and α-terpineol, and their concentrations increased by 1,000-fold compared to control plants.

Increased essential oil contents in the shoots of *Origanum* sp. (Khaosaad et al. 2006) and *Pelargonium* species (Venkateshwar Rao et al. 2002) by the AM fungus *Glomus mosseae* were also reported. Similar results were observed by Gupta et al. (2002) where inoculation of *Mentha arvensis* with the AM fungus *Glomus fasciculatum* increased plant height, shoot growth, and essential oil content. According to Cappellari et al. (2013), PGPR *Pseudomonas fluorescens* and *Azospirillum brasilense* increased the biosynthesis of the major EO components up to 70 % and total phenolic content in Mexican marigold (*Tagetes minuta*).

Table 14.1 The effect of plant beneficial microorganisms on phytochemical constituents of medicinal plants

PGPR	Plant	Phytochemicals	References
Glomus mosseae, *Trichoderma harzianum*	*Andrographis paniculata* Nees. (kalmegh)	Andrographolide	Arpana and Bagyaraj (2007)
Glomus macrocarpum, *Glomus fasciculatum*	*Anethum graveolens* L. (dill)	Limonene, α-phellandrene	Kapoor et al. (2002)
Pseudomonas putida	*Anethum graveolens* L. (dill)	Carvone, limonene	Tajpoor et al. (2013)
Glomus macrocarpum, *Glomus fasciculatum*	*Artemisia annua* L. (wormwood)	Artemisinin	Kapoor et al. (2007)
Glomus fasciculatum	*Coleus forskohlii* (Indian coleus)	Forskolin	Sailo and Bagyaraj (2005)
Glomus fasciculatum, *Pseudomonas monteilii*	*Coleus forskohlii* (Indian coleus)	Forskolin	Singh et al. (2012)
Azospirillum brasilense, *Pseudomonas fluorescens*	*Catharanthus roseus* L. (Madagascar periwinkle)	Terpenoid indole alkaloid (ajmalicine)	Karthikeyan et al. (2009)
Glomus lamellosum	*Geranium dissectum* L. (germanium)	Essential oil	Karagiannidis et al. (2012)
Glomus aggregatum, *Trichoderma harzianum*, *Bacillus coagulans*	*Glycyrrhiza glabra* L. (liquorice)	Phenols, *ortho*-dihydroxy phenols, tannins, flavonoids, alkaloids	Selvaraj and Sumithra (2011)
Glomus lamellosum	*Lavandula angustifolia* L. (lavender)	Essential oil	Karagiannidis et al. (2012)
Glomus intraradices, *Glomus etunicatum*	*Lonicera confuse* (honeysuckle)	Chlorogenic acid	Shi et al. (2013)
Glomus fasciculatum	*Mentha arvensis* (wild mint)	Essential oil	Gupta et al. (2002)
Glomus fasciculatum, *Azotobacter chroococcum*	*Ocimum* spp. (basil)	Essential oil	Vinutha (2005)
Pseudomonas putida, *Azotobacter chroococcum*	*Ocimum basilicum* (common basil)	Essential oil	Ordookhani et al. (2011)
Bacillus subtilis	*Ocimum basilicum* (common basil)	Terpineol, eugenol	Banchio et al. (2009)
Glomus mosseae	*Ocimum basilicum* (common basil)	Essential oil	Copetta et al. (2006)
Pseudomonas fluorescens *Bradyrhizobium* sp.	*Origanum majorana* L. (marjoram)	Terpinen-4-ol, *cis*-sabinene hydrate, *trans*-sabinene hydrate, α-terpineol	Banchio et al. (2008)

(continued)

Table 14.1 (continued)

PGPR	Plant	Phytochemicals	References
Glomus mosseae	*Origanum* sp. (oregano)	Essential oil	Khaosaad et al. (2006)
Glomus mosseae	*Pelargonium* sp. (germanium)	Essential oil	Venkateshwar Rao et al. (2002)
Bacillus cereus	*Salvia miltiorrhiza* Bunge (red sage)	Diterpenoid pigment, tanshinones	Wu et al. (2007)
Glomus intraradices	*Salvia officinalis* (common sage)	Essential oil, bornyl acetate, 1,8-cineole, α- and β-thujones	Geneva et al. (2010)
Glomus lamellosum	*Santolina chamaecyparissus* (cotton lavender)	Essential oil	Karagiannidis et al. (2012)
Glomus walkeri, *Bacillus subtilis*, *Trichoderma viride*	*Sphaeranthus amaranthoides* (L.) Burm (sivakaranthai)	Phenols, *ortho*-dihydroxy phenols, flavonoids, alkaloids, tannins	Sumithra and Selvaraj (2011)
Burkholderia gladioli, *Enterobacter aerogenes*, *Serratia marcescens*	*Stevia rebaudiana* Bert. (sweet leaf)	Stevioside, rebaudioside-A contents	Gupta et al. (2011)
Bacillus megaterium, *Azospirillum* sp. AM fungi	*Stevia rebaudiana* Bert. (sweet leaf)	Stevioside	Das and Dang (2010)
Pseudomonas fluorescens, *Azospirillum brasilense*	*Tagetes minuta* (Mexican marigold)	Essential oil, phenolic content	Cappellari et al. (2013)
Glomus mosseae, *Bacillus subtilis*	*Thymus daenensis* (thyme)	Essential oil	Bahadori et al. (2013)
Azospirillum, *Azotobacter chroococcum*, *Pseudomonas fluorescens*, *Bacillus megaterium*	*Withania somnifera* (Indian ginseng)	Withaferin A	Rajasekar and Elango (2011)

In other study the highest carvone content (63.22 %) and the lowest contents of limonene (25.16 %) in essential oil of *Anethum graveolens* L. were obtained after the treatment of *Pseudomonas putida* combined with vermicompost (Tajpoor et al. 2013).

Bahadori et al. (2013) reported that co-inoculation of *Thymus daenensis* with *G. mosseae* and *Bacillus subtilis* resulted in a 75 % increase in shoot/root dry weight and a 117 % increase in plant yield and stimulated essential oil yield by 93 % compared to uninoculated controls. Karagiannidis et al. (2012) observed the increase of essential oil content in plants such as *Santolina chamaecyparissus*, *Salvia officinalis*, *Lavandula angustifolia*, *Geranium dissectum*, and *Origanum dictamnus* by 28.75, 55.56, 56.95, 53.63, and 55.24 % when inoculated with AM fungus *Glomus lamellosum*. Similar results were observed by Geneva et al. (2010) where essential oil content, bornyl acetate, 1,8-cineole, and α- and β-thujones of

Salvia officinalis were increased by *Glomus intraradices* (Geneva et al. 2010). Inoculation of *Anethum graveolens* L. with AMF *Glomus macrocarpum* and *Glomus fasciculatum* significantly increased limonene and α-phellandrene content (Kapoor et al. 2002).

Salvia miltiorrhiza Bunge is a well-known herbal plant in Chinese medicine used for the treatment of menstrual disorders and cardiovascular disease and to prevent inflammation (Wang et al. 2007). Wu et al. (2007) studied the diterpenoid pigment derived from *S. miltiorrhiza* roots, which are generally known as tanshinones, and its content in root of *S. miltiorrhiza* was stimulated by more than 12-fold when the hairy root culture was inoculated with *Bacillus cereus*. *Withania somnifera* (Ashwagandha) is a plant used in the treatment of cancer and nervous disorders, and it contains withaferin A, a therapeutically active withanolide. The bacterial composition of *Azospirillum*, *Azotobacter chroococcum*, *Pseudomonas fluorescens*, and *Bacillus megaterium* significantly increased plant height, root length, and the alkaloid and withaferin-A content (Rajasekar and Elango 2011).

Coleus forskohlii Briq. (Lamiaceae) is widely used to relieve coughs, eczemas, skin infections, tumors, glaucoma, cardiac problems, and certain types of cancers (Kavitha et al. 2010) and contains a labdane diterpene compound forskolin (Seamon 1984). Forskolin content was significantly improved by as much as 25 % by inoculation with the AM fungus *Glomus fasciculatum* (Sailo and Bagyaraj 2005) and combined inoculation of *G. fasciculatum* and *Pseudomonas monteilii* (Singh et al. 2012). *Stevia rebaudiana* is a medicinal plant that serves as a source of natural sweeteners, steviol glycosides, which has been reported for hypotensive and heart tonic actions (Ferri et al. 2006). Gupta et al. (2011) observed that *S. rebaudiana* inoculated with a consortium of phosphorus-solubilizing bacteria (PSB) *Burkholderia gladioli* MTCC 10216, *B. gladioli* MTCC 10217, *Enterobacter aerogenes* MTCC 10208, and *Serratia marcescens* MTCC 10238 showed increased root and shoot biomass and stevioside and rebaudioside-A contents (291 and 575 %, respectively) on a whole-plant basis compared to control plants. The increased stevioside content of *S. rebaudiana* by the combined inoculation of *Bacillus megaterium*, *Azospirillum* sp., and AM fungi was also reported by Das and Dang (2010).

Artemisia annua L. (Asteraceae) or annual wormwood is an herbal plant in Chinese traditional medicine and has been used for the treatment of cerebral fever and malaria (Ram et al. 1997) and is a source of complex terpenoids, including artemisinin. Kapoor et al. (2007) observed increased plant growth and artemisinin production in *A. annua* by two AM fungi, *Glomus macrocarpum* and *Glomus fasciculatum*, which successfully colonized the roots.

Leaf-derived secondary metabolites such as total phenols, ortho-dihydroxy phenols, flavonoids, alkaloids, and tannins of *Sphaeranthus amaranthoides* (L.) Burm increased when plants were treated with *Glomus walkeri*, *Bacillus subtilis*, and *Trichoderma viride* (Sumithra and Selvaraj 2011). Karthikeyan et al. (2009) reported an increase in the production of terpenoid indole alkaloids (ajmalicine) in *Catharanthus roseus* inoculated with *Azospirillum brasilense* and *Pseudomonas fluorescens*. Arpana and Bagyaraj (2007) reported that *Glomus mosseae* and

Trichoderma harzianum increased plant root, shoot growth, dry weight, phosphorus uptake, and andrographolide (alkaloid) concentration in kalmegh (*Andrographis paniculata*) compared to uninoculated plants.

Glycyrrhizin is a very sweet ingredient of liquorice (*Glycyrrhiza glabra*) and has an anti-inflammatory effect which controls coughing (Patil et al. 2009). Selvaraj and Sumithra (2011) observed that the AM fungi *Glomus aggregatum*, *Trichoderma harzianum*, and *Bacillus coagulans* enhanced plant biomass and polyphenolic compound production, namely, total phenols, ortho-dihydroxy phenols, tannins, flavonoids, and alkaloids in liquorice. Shi et al. (2013) demonstrated increased growth and chlorogenic acid content in flowers of *Lonicera confusa*, a traditional Chinese medicine herb for treating cold, flu, and acute fever, by inoculation with *Glomus intraradices* rather than with *Glomus etunicatum*.

Those studies demonstrate the effectiveness of PGPR and AM fungi in improving the concentration of phytochemical constituents and essential oil concentrations in medicinally important plants.

14.5 The Role of Microbial Interactions in Nutrient Uptake of Medicinal Plants

The activity of soil organisms is very important for ensuring sufficient nutrient supply to a plant and plays a significant role in regulating the dynamics of organic matter decomposition and the availability of plant nutrients such as N, P, K, Mg, and other microelements (Egamberdieva 2011; Maheshwari et al. 2012). In earlier studies, several authors reported an increase in nutrient content such as P, K, Zn, Cu, and Fe due to mycorrhizal and PGPR (*Glomus mosseae*, *Bacillus coagulans*, and *Trichoderma harzianum*) inoculation for several medicinal plants including *Saraca asoca* (Roxb.) (Lakshmipathy et al. 2001), *Calamus thwaitesii* (Lakshmipathy et al. 2002), and *Begonia malabarica* Lam. (Selvaraj et al. 2008). The inoculation of annual wormwood (*Artemisia annua* L.) with AM fungi *Glomus macrocarpum* and *Glomus fasciculatum*, combined with P fertilizer, resulted in higher concentrations of Zn and Fe in shoots (Kapoor et al. 2007). Similar results were observed by Selvaraj and Sumithra (2011), in which the root phosphorus, potassium, zinc, copper, and iron contents increased after inoculation with a consortium of *Glomus aggregatum*, *Bacillus coagulans*, and *Trichoderma harzianum* in *Glycyrrhiza glabra*.

Prasad et al. (2012b) observed increased plant growth, alkaline phosphatase and acidic phosphatase activity, and phosphorus uptake in shoots and roots of *Chrysanthemum indicum* L. inoculated with *Glomus mosseae*, *Acaulospora laevis*, and phosphate-solubilizing *Pseudomonas fluorescens*. Similar results were observed by Singh et al. (2012) where N, P, and K uptake of *Coleus forskohlii* plant significantly (26, 60, and 43 %, respectively) increased following inoculation with *Pseudomonas monteilii* and *Glomus fasciculatum* under field experiments. PSB treatments with

Burkholderia gladioli, *Enterobacter aerogenes*, and *Serratia marcescens* combined with Mussoorie rock phosphate (MRP) showed an increase of 86–576 % in available P content of soil and 63.9–273 % P content in *Stevia rebaudiana* shoots than in control treatments (Gupta et al. 2011). A significant increase in N content of roots and shoots of *Galega orientalis* was also observed after co-inoculation of *Pseudomonas trivialis* with *Rhizobium galegae* which significantly increased the N content of the roots by 20 % and of the shoots by 52 % compared to *R. galegae* alone (Egamberdieva et al. 2010).

Marigold (*Calendula officinalis*) is known for its antioxidant, anti-inflammatory, and anticancer activities (Muley et al. 2009). The shoot and root growth, nitrogen, phosphorus, potassium, and photosynthetic pigment contents of *C. officinalis* were stimulated by PGPR strains *Azotobacter*, *Azospirillum*, *Pseudomonas*, and AM fungi (Hosseinzadah et al. 2011). Ordookhani et al. (2011) showed increased Fe, Mn, and Cu contents of *Ocimum basilicum* L. (sweet basil) by *Pseudomonas putida*, *Azotobacter chroococcum*, and *Azospirillum lipoferum*. According to Shi et al. (2013), concentrations of N, P, and K in leaves of *Lonicera confusa* increased significantly by AM fungi *G. intraradices* and *G. etunicatum* inoculation.

Sphaeranthes amaranthoides (L.) Burm is a common medicinal plant in India, and the plant juice is used in epilepsy, hepatopathy, gastropathy, diabetes, leprosy, fever, cough, hemorrhoids, and dyspepsia (Sumithra and Selvaraj 2011). The growth and nutrient uptake of phosphorus, potassium, zinc, copper, and iron content were increased in plants treated with *Glomus walkeri*, *Bacillus subtilis*, and *Trichoderma viride* (Sumithra and Selvaraj 2011).

Most P and K fertilizers are not readily available to a plant, and their use often causes an insignificant yield increase in plants (Chabot et al. 1996). Some rhizobacteria may convert insoluble rock P into soluble forms available for plant growth (Varsha and Patel 2000). Release of P by PSB from insoluble and fixed/adsorbed forms is an import aspect of P availability in soils (Khan et al. 2009). PSB, mainly *Enterobacter*, *Bacillus*, *Pseudomonas*, and *Arthrobacter*, are very effective for increasing the plant-available P in soil as well as plant growth (Egamberdieva and Hoflich 2004). Moreover, the higher N content in treatments may have resulted from the N_2-fixation ability of this bacterium, as reported in other studies (Çakmakcı et al. 2007).

14.6 Microbial Mediated Alleviation of Abiotic Stress in Medicinal Plants

Abiotic factors such as drought and salinity negatively affect plant growth of aromatic and medicinal plants and the production of biologic active compounds (Parida and Das 2005). Razmjoo et al. (2008) reported that increased salinity and drought stress caused a reduction in the fresh and dry flower weight and essential oil content of *Matricaria chamomilla*. Water stress caused a significant increase in the

concentrations of proline and soluble carbohydrate in the leaves of *Ocimum basilicum* L. (sweet basil) and decreased mineral uptake (Heidari et al. 2011). The content of chlorophyll, proline, and K uptake was significantly stimulated after inoculating basil with *Pseudomonas* sp., *Bacillus lentus*, and *Azospirillum brasilense* (Heidari et al. 2011). Similar results were observed for black henbane (*Hyoscyamus niger*), which is considered an important medicinal plant and a source of tropane alkaloids such as hyoscyamine (HYO) and scopolamine (SCO) (Pitta et al. 2000), in which water stress reduced plant growth and development. *Pseudomonas putida* and *Pseudomonas fluorescens* alleviated water stress and increased plant growth and tropane alkaloids such as hyoscyamine and scopolamine concentration in *H. niger* (Ghorbanpour et al. 2013).

Salinity decreased plant growth, development, and essential content of *Pelargonium* sp. Prasad et al. (2012a) studied the ameliorative effect of AM fungus, PSB, combined with P fertilizers on plant growth, nutrient uptake, and chemical composition of essential oil in *Pelargonium* sp. They observed that shoot growth, mineral element (P, K, Ca, Mg, Na, Fe, Cu, and Zn) uptake in shoot tissues, and essential oil content such as citronellol, geraniol, geranial, and a sesquiterpene (10-epi-γ-eudesmol) in shoot tissues of geranium were significantly increased by the co-inoculation with *Glomus intraradices* and PSB compared to the control. Similar results were observed by Golpayegani and Tilebeni (2011) in which PGPR strains *Pseudomonas* sp. and *Bacillus lentus* alleviated the effect of potentially toxic ions on the growth, antioxidant enzymes ascorbate peroxidase (APX) and glutathione reductase (GR), and mineral content (K, P, Ca, Na) in basil plants. *Galega officinalis* L. (goat's rue, French lilac) has been used for medicinal purposes (Atanasov and Spasov 2000; Pundarikakshudu et al. 2001). Plant growth and nitrogen content of co-inoculated plant roots with *P. extremorientalis* TSAU20 and *R. galegae* HAMBI 1141 increased significantly by on average 50 % under saline conditions (Egamberdieva et al. 2013b).

Bacopa monnieri (Indian pennywort), which is commonly used as a nootropic digestive aid, memory enhancer, and for improving respiratory functions (Russo and Borrelli 2005), has many active compounds including alkaloids, flavonoids, and saponins (bacoside A, bacoside B), but its synthesis is severely affected by abiotic factors such as drought and salinity (Tiwari et al. 2001). Bharti et al. (2013) studied the interaction of *B. monnieri* and PGPR under saline soil conditions. Salinity inhibited root and shoot growth of *B. monnieri* and bacoside-A content. Inoculation of plants with PGPR strains *E. oxidotolerans* and *Bacillus pumilus* alleviated salt stress, stimulated herb yield, and also recorded higher bacoside-A content under saline conditions. *E. oxidotolerans*-inoculated plants had 36 and 76 % higher bacoside-A content under primary and secondary salinity, respectively.

14.7 Biomechanisms Regulating Growth and Development

Mechanisms by which bacteria are able to stimulate plant growth, phytochemical constituents, and nutrient uptake and alleviate abiotic stresses include various enzymes (Lugtenberg and Kamilova 2009), mobilization of nutrients (Egamberdieva and Lugtenberg 2014), induction of systemic resistance (Van Loon 2007; Hameed et al. 2014), competition for nutrients and niches (Raaijmakers et al. 2009), production of phytohormones like indole-3-acetic acid (IAA), gibberellic acid, cytokinins (Mishra et al. 2010), production of ACC deaminase to reduce the level of ethylene in the roots of developing plants (Dey et al. 2004), and asymbiotic nitrogen fixation (Ardakani et al. 2010). For example, AM fungi increase plant growth and essential oil production by extending the root zone and acquisition of organic phosphates by production of extracellular acid phosphatases (Bouwmeester et al. 2007; Hameed et al. 2014). The increased level of artemisinin by AM fungi may be due to improved growth and nutrient status of the plants (Kapoor et al. 2007). PSB also play an important role in P nutrition of plants (Ekin 2010). Phosphorus is an important source for essential oil synthesis by plants, whereas isoprenoid biosynthesis requires acetyl coenzyme A, adenosine triphosphate (ATP), and nicotinamide adenine dinucleotide phosphate (NADPH) and is dependent on the concentration of inorganic P in the plant (Lichtenthaler 2009). Thus, increased P uptake mediated by PSB may stimulate essential oil synthesis in medicinal plants. However, there is another explanation for the increased oil concentration in plants: Sangwan et al. (2001) indicated that essential oil concentration and composition in medicinal plants serve important ecological roles in which the majority of oils have antimicrobial properties. Application of a microbial consortium to the root system of medicinal plants increased the synthesis of oils and can be considered as a defensive response of plants to colonization by microorganisms.

The colonization of a host plant's rhizosphere by plant beneficial microbes is an important factor for plant growth (Lugtenberg et al. 2001) because they deliver various plant growth-promoting metabolites (Berg et al. 2010; Egamberdieva 2009). Plant growth regulators such as auxins, gibberellins, and cytokinins produced by rhizobacteria can influence plant growth, including root development, all of which improve the uptake of essential nutrients and thus increase plant growth (Somers et al. 2004). Root-associated bacteria utilize root exudates that also contain tryptophan, a precursor of IAA, through which plants and bacteria may regulate IAA biosynthesis in the rhizosphere (Dakora and Phillips 2002). Plant cells take up some of the IAA that is secreted by the bacteria and, together with the endogenous plant IAA, can stimulate plant cell proliferation (Glick et al. 2007). This increase nutrient-absorbing surface may lead to greater rates of nutrient absorption through which plant growth will increase significantly (Egamberdieva 2012). Some root-associated rhizobacteria contain the enzyme ACC deaminase, which may decrease the level of ethylene in the root and enhance the stress tolerance of plants (Glick et al. 2007).

14.8 Conclusions and Future Prospects

This chapter highlights the role of plant-associated microbes in plant growth promotion and nutrient uptake under various climatic conditions. Most of the PGPR isolates and AM fungi showed a significant increase in root and shoot weight and nutrient uptake and improved the concentration of phytochemical constituents and essential soil concentrations in medicinally important plants. Knowledge of such interactions can provide direction as to which microbes might be selected for an increase in novel medicinal compounds that possess antimicrobial, antimalarial, antioxidant, and other biological activities. This microbial strategy offers an attractive way to replace the use of chemical fertilizers, pesticides, and other supplements for cultivation of herbal plants. Information from various studies available describes the mechanisms involved in the improvement of plant growth and stress tolerance in plants. However, our understanding of the ability of plant beneficial microbes to increase plant secondary metabolites remains scarce. Thus, more studies are needed to investigate the possible mechanisms by which bacteria increase phytochemical constituents in medicinal important plants at the tissue, cell, or molecular level.

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Chapter 15
Plant Growth Promoting Rhizobacteria for Value Addition: Mechanism of Action

H. Deka, S. Deka, and C.K. Baruah

15.1 Introduction

Application of microbes for improvement of plants has been carried out since ancient times even before the discovery of microscopic animals and microscope as well (Bhattacharyya and Jha 2012). The use of microorganisms with the aim of improving nutrients availability for plants is an important practice and necessary for agriculture (Freitas et al. 2007). During the past couple of decades, the use of plant growth promoting rhizobacteria (PGPR) for sustainable agriculture has increased tremendously in various parts of the world.

The soil zone in the vicinity of plant roots in which the chemistry and microbiology is influenced by their growth, respiration, and nutrient exchange is known as rhizosphere. In the rhizosphere, bacteria are the most abundant microbes besides other microbes like fungi, protozoa, algae, etc. Kloepper and Schroth (1978) introduced the term "rhizobacteria" to the soil bacterial community that competitively colonized plant roots and stimulated plant growth and reduces the incidence of plant diseases. In the rhizosphere, very important and intensive interactions take place between the soil, plant, microorganisms, and soil microfauna. In fact, biochemical interactions and exchanges of signal molecules between plants and soil microorganisms have been described and reviewed by various workers (Pinton et al. 2001; Werner 2001, 2004). These interactions can significantly influence plant growth and crop yields. The medicinal plants constitute a large segment of the

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flora, as the source of raw materials for pharmaceutical, cosmetic, and fragrance industries. A clear understanding and management of microbe species associated with the medicinal plants is utmost important to improve their yield and quality medicinal products (Karthikeyan et al. 2008).

15.1.1 What Are Plant Growth Promoting Rhizobacteria?

The species of bacteria that are associated with the plant rhizosphere that have beneficial effect on plant's growth and crop yield are collectively called as PGPR. Kloepper and Schroth (1978) defined PGPR for the first time and since then several definitions have been proposed by various workers. The PGPR are soil bacteria that colonize the roots of plants and on inoculation with seed enhance the growth of plant. It has been reported that about 2–5 % of rhizobacteria after reintroducing through plant inoculation in a soil containing competitive microflora shows a beneficial effect on plant growth (Kloepper et al. 1989). The PGPR are also termed as plant health promoting rhizobacteria (PHPR) or nodule promoting rhizobacteria (NPR) and are associated with the rhizosphere which is an important soil ecological environment for plant–microbe interactions (Hayat et al. 2010; Burr and Caesar 1984). The PGPR includes both free-living and plant tissue invading bacteria that cause unapparent and asymptomatic infections in plant root systems (Sturz and Nowak 2000). The latter groups are also known as endophytes as they inhabit within the plant tissue system. According to the original definition, rhizobacteria are free-living bacteria that colonize the root zone. They differ from the nitrogen-fixing *Rhizobia* and *Frankia* that forms symbiotic associations with plants and cannot be considered as PGPR (Antoun and Prevost 2005). However, some other workers divided PGPR into two groups according to their relationship and residing sites in the plants, i.e., iPGPR (i.e., symbiotic bacteria), which live inside the plant cells, produce nodules, and are localized inside the specialized structures, and ePGPR (i.e., free-living rhizobacteria), which live outside the plant cells and do not produce nodules, but still prompt plant growth (Gray and Smith 2005). The best-known iPGPR are the species of Rhizobia, which produce nodules in leguminous plants. A number of bacterial species have been used as soil inoculants intended to improve the supply of nutrients to crop plants. The bacteria such as *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, and *Sinorhizobium* have been successfully used worldwide to permit an effective establishment of the nitrogen-fixing symbiosis with leguminous crop plants (Bottomley and Maggard 1990; Bottomley and Dughri 1989). On the other hand, non-symbiotic nitrogen-fixing bacteria such as species of *Azotobacter*, *Azospirillum*, *Bacillus*, and *Klebsiella* are also used to inoculate a large area of arable land in the world with the aim of enhancing plant productivity (Lynch 1983). Besides these, phosphate solubilizing bacteria such as species of *Bacillus* and *Paenibacillus* (formerly *Bacillus*) have been applied specifically to enhance the phosphorus status of soil for plants (Brown 1974). Even more recently, the

application of PGPR has also been extended to remediate contaminated soils in association with plants (Zhuang et al. 2007).

15.1.2 Why Is PGPR for Value Addition?

Sustainable soil health and crop production are key global issues today. Both plant health and soil fertility are to be incorporated for better production and mitigating the growing demand of food products. It is the interaction between beneficial microbes and plants in the rhizosphere zone that primarily determines the plant health and soil fertility (Klyuchnikov and Kozherin 1990). Hence, at present, greater emphasis has been laid on application of beneficial microbes or PGPR instead of inorganic input in the crop field. Continuous uses of synthetic fertilizers have been reported to be deleterious for both chemical and biological components of the soil. The long-term use of inorganic fertilizer without organic supplements damages the physical, chemical, and biological properties of soil and causes environmental pollution (Albiach et al. 2000). Therefore, in order to develop a strategy for the sustainable soil health, it is necessary to apply the organic products. Organic fertilizers such as PGPR are microbial inoculants consisting of living cells of bacteria which help in increasing crop productivity. Organic fertilizers as against the chemical fertilizers have lower nutrient content but they are more effective for longer periods of use and maintain soil fertility intake due to their slow release of nutrients. The use of biofertilizer containing strains of plant growth promoting rhizobacteria instead of synthetic chemicals serves as an effective alternative and environmental friendly practice to improve plant growth through the supply of plant nutrients and soil productivity. Moreover, exploiting PGPR strains for the growth promotion could reduce the need of chemical fertilizers and cost of cultivation (Rajasekar and Elango 2011). However, survivability of PGPR in field condition, application dose, adaptability, etc. are the limiting factors which are yet to be addressed properly.

15.2 Role of PGPR in Improvement of Medicinal Plants and Its Products

The present world relies on natural products that have no adverse effect on whole biota. Therefore, exploitation of medicinal plants and extraction of its products in health sector is increasing day by day. Demand for medicinal plants has increased in both developing and developed nations due to growing recognition of natural products, which are non-toxic, having no side effects and can be obtained in affordable prices (Sekar and Kandavel 2010). The World Health Organization (WHO) estimated that 80 % of the population of developing countries relies on

traditional medicines, mostly plant base, for their primary health care (Farnsworth 1990). Moreover, it has been reported that modern pharmacopoeia contains at least 25 % drugs that are derived directly from plants (Sekar and Kandavel 2010).

Herbal products are the first choice in self-treatment to prevent immediate development of certain diseases in developing countries like India, China, South Africa, etc. It has been reported that traditional healers often prescribe mixtures of medicinal plants in raw form for the treatment of several diseases like common cold, malaria, arthritis, ulcers, hepatitis, and diabetes (Obiajunwa et al. 2002; Sarma and Sarma 2008; Sarma et al. 2008). Even, in the country like Ethiopia, more than 85 % of the population depends on herbal plants for primary health care (Meena et al. 2010). Medicinal plants are particularly important in developing countries because such plants are also dietary components and are essential for health (Maiga et al. 2005; Cantarelli et al. 2010). Moreover, since 1992, the average use of medicinal and aromatic plants in European countries has increased by 21 % in traditional as well as processed forms (Bernath 2002).

Considering the Indian scenario, it is estimated that about 2,000 drugs of plant origin are used which even leads to extinction/endangerment of 20–25 % plant species from their natural habitat (Sarma 2011; Laloo et al. 2006). Even, the occurrence and distribution of medicinal plants is now under great pressure in India because excessive amounts of them are collected from wild habitats and are exploited for use in medicine (Sarma 2011). Nevertheless, threats to the medicinal plants are not only because of over exploitation but rather because of the indiscriminate use of pesticides, insecticides, fertilizers, etc. which ultimately lead to degradation of the quality of the environment.

Various research reports are available regarding use of PGPRs for quality improvement of medicinal plant products. A brief reference of the use of PGPR for improvement of medicinal plants products has been listed in Table 15.1.

15.3 Mechanism of Action of PGPR

15.3.1 An Overview

Rhizosphere manipulation involves a very complex mechanism. In order to achieve maximum benefit from plant–microbe interaction, it is necessary to understand the PGPR action mechanisms for manipulating the rhizosphere. Traditionally, PGPR action mechanisms can be divided into two groups, viz., direct and indirect mechanisms. In case of indirect mechanisms, action occurs outside the plant, whereas direct mechanisms are those that occur inside the plant and directly affect the plant's metabolism. Nevertheless, the differences between these two types of mechanisms are not always obvious. A schematic illustration of some important mechanism of PGPRs has been presented in Fig. 15.1.

Table 15.1 Use of PGPR for production of compounds from medicinal plants

Name of the PGPR strains	Plants compound/plants name	References
Pseudomonas fluorescens	Ajmalicine; *Catharanthus roseus* L.	Jaleel et al. (2007)
Pseudomonas monteilii	Forskolin; *Coleus forskohlii*	Singh et al. (2013)
Azotobacter	Essential oil content	Sefidkon (2012)
Azospirillum	Anethol, methyl chavicol	
Pseudomonas	*Pimpinella anisum* L.	
Azotobacter chroococcum	Essential oil content, chamazulene	Salehi et al. (2012)
Azospirillum lipoferum	*Matricaria chamomilla* L	
Pseudomonas flouresence		
Pseudomonas fluorescens	Essential oil	Banchio et al. (2008)
Bacillus subtilis, *Sinorhizobium meliloti*, *Bradyrhizobium* sp.	*Origanum majorana* L.	
Pseudomonas fluorescens elicitors (PF elicitors)	Ajmalicine, catharanthine, tabersonine, serpentine, Vindoline, *Catharanthus roseus* (L.) G. Don	Jaleel et al. (2009)
Azotobacter, Bacillus, Pseudomonas	Overall plant growth and alkaloids *Catharanthus roseus* (L.) G. Don	Karthikeyan et al. (2010)
Bacillus pumilus (STR2)	Bacoside-A	Bharti et al. (2013)
Exiguobacterium oxidotolerans (STR 36)	*Bacopa monnieri* (L.)	
Bacillus cereus	Tanshinone	Zhao et al. (2010)
	Salvia miltiorrhiza	
Azospirillum lipoferum	Yield and essential oil	Dastborhan et al. (2010)
Azotobacter chrocooccum	*Matricaria chamomilla* L.)	
Species of *Pseudomonas*	*Valeriana officinalis*	Ghodsalavi et al. (2013)
Klebsiella, *Xanthomonas*, *Bacillus*, *Erwinia*, *Agrobacterium*, and *Arthrobacter*	Production of IAA, HCN, Lipase, and protease	
Azotobacter	*Thymus vulgaris* L. Thyme	Naseri and Sharafzadeh (2013)
Pseudomonas fluorescens, Azospirillum brasilense	Essential oil and phenolic content	Cappellari et al. (2013)
Bacillus coagulans	*Tagetes minuta*	
Begonia malabarica Lam.	Luteolin, quercetin, and β-sitosterol	

Good numbers of literatures are available to describe the plant growth promotion by PGPR through direct or indirect modes of action (Kloepper 1993; Van Loon and Glick 2004; Van Loon 2007). In broader sense, direct mechanisms include the production of stimulatory bacterial volatiles and phytohormones, lowering of the ethylene level in plant, improvement of the plant nutrient status (liberation of phosphates and micronutrients from insoluble sources; non-symbiotic nitrogen fixation), and stimulation of disease-resistance mechanisms (induced systemic

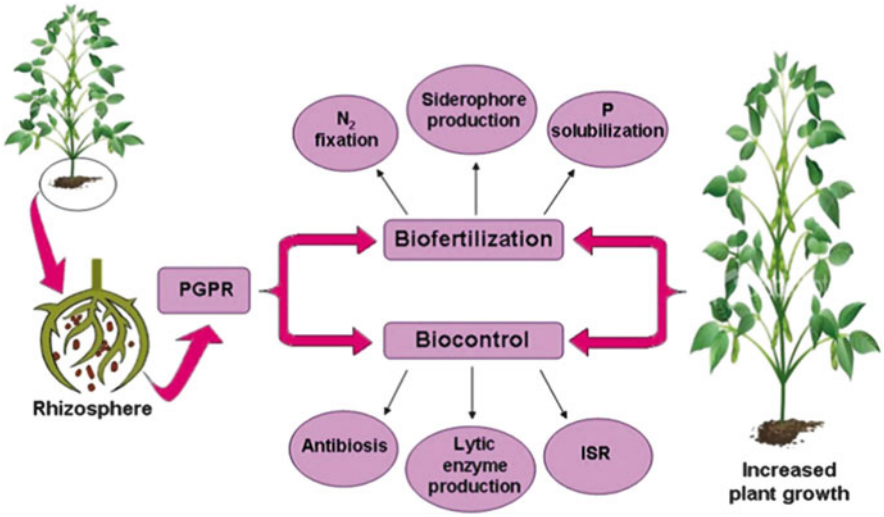

Fig. 15.1 Schematic illustration of important mechanisms known for plant growth promotion by PGPR. Different mechanisms can be broadly studied under (1) Biofertilization and (2) Biocontrol of pathogens. Biofertilization encompasses: (**a**) N_2 Fixation, (**b**) Siderophore production, (**c**) Phosphate solubilization by rhizobacteria. Biocontrol involves: (**a**) Antibiosis, (**b**) Secretion of enzymes, and (**c**) Induction of Systemic Resistance (ISR) of host plant by PGPR (Adopted from Kumar et al. 2011)

resistance). Indirect mechanism of PGPR originates when it acts like biocontrol agents reducing diseases, when they stimulate other beneficial symbioses, or when they protect the plant by degrading xenobiotics in inhibitory contaminated soils (Jacobsen 1997; Jacobsen et al. 2004). PGPR strains such as *Pseudomonas fluoresces* and *Bacillus subtilis* are well studied (Damayanti et al. 2007). Depending on the activities of the PGPR, some workers like Somers et al. (2004) classified them as biofertilizer (increasing the availability of nutrients to plant), phytostimulators (plant growth promoting, usually by the production of phytohormones), rhizoremediators (degrading organic pollutants), and biopesticides (controlling diseases, mainly by the production of antibiotics and antifungal metabolites). However, Dey et al. (2004) reported that the exact mechanisms of PGPR-mediated enhancement of plant growth and yield for many crops are not known. According to them the possible mechanism includes:

1. The ability to produce a vital enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, to reduce the level of ethylene in the root of developing plants thereby increasing the root length and growth (Li et al. 2000, 2005)
2. The ability to produce hormones like auxin, i.e., indole acetic acid (IAA) (Patten and Glick 2002), abscisic acid (ABA) (Dangar and Basu 1987; Dobbelaere et al. 2003), gibberellic acid (GA), and cytokinins (Dey et al. 2004)
3. A symbiotic nitrogen fixation (Kennedy et al. 1997, 2004)

4. Antagonism against phytophatogenic bacteria by producing siderophores, β-1,3-glucanase, chitinases, antibiotic, fluorescent pigment, and cyanide (Cattelan et al. 1999; Glick and Pasternak 2003)
5. Solubilization and mineralization of nutrients, particularly mineral phosphates (De Freitas et al. 1997; Richardson 2001; Banerjee and Yasmin 2002);
6. Enhanced resistance to drought (Alvarez et al. 1996), salinity, waterlogging (Saleem et al. 2007), and oxidative stress (Stajner et al. 1995, 1997)
7. Production of water-soluble B group vitamins niacin, pantothenic acid, thiamine, riboflavine, and biotin (Martinez-Toledo et al. 1996; Sierra et al. 1999; Revillas et al. 2000).

15.3.2 Direct Mechanism

The direct mechanism of PGPR involves production of stimulatory bacterial volatiles and phytohormones, lowering of the ethylene level in plant, improvement of the plant nutrient status (liberation of phosphates and micronutrients from insoluble sources; non-symbiotic nitrogen fixation), and stimulation of the disease-resistance mechanisms (induced systemic resistance). A list of direct mechanism has been presented in Table 15.2. Among these more emphasis has been given in production of phytohormones and their regulation (ethylene), volatile organic compounds (VOCs), and their stimulatory effects. Hence, a brief mechanism of PGPR pertaining to this has been addressed below.

Table 15.2 Direct PGPR action mechanisms (Adopted from Solano et al. 2008; Chanway 1997)

Mechanism	Effect	References
Plant growth regulator production	Biomass (aerial part and root)	Gutierrez Manero et al. (1996)
Flowering		Gutierrez Manero et al. (2001)
Ethylene synthesis inhibition	Root length	Glick et al. (1994)
Induction of systemic resistance	Health	Van Loon et al. (1998)
Root permeability increase	Biomass and nutrient absorption	Sumner (1990)
Organic matter mineralization (nitrogen, sulfur, phosphorus)	Biomass and nutrient content	Liu et al. (1995)
Mycorrhizal fungus association	Biomass and phosphorus content	Germida and Walley (1996)
		Toro et al. (1998)
Insect pest control	Health	Zehnder et al. (1997)

15.3.2.1 PGPR in Production of Plant Growth Regulators/Hormones and Their Regulation

PGPRs are reported to be associated with the production of plant growth regulators. Plant growth regulators are the substances that regulate the growth, development, and physiology of the plants. The principal plant growth regulators are auxin (IAA), gibberellins (GBs), ethylene, cytokinins, and absisic acid (ABA). Out of these, the production of auxin and ethylene is very common trait among PGPR (Solano et al. 2008). Production of auxin and ethylene has been also enumerated by other workers (Mishra et al. 2010; Saleem et al. 2007). Similarly, production of gibberellins has been documented in several PGPR belonging to *Achromobacter xylosoxidans*, *Acinetobacter calcoaceticus*, *Azospirillum* spp., *Azotobacter* spp., *Bacillus* spp., *Herbaspirillum seropedicae*, *Gluconobacter diazotrophicus*, and rhizobia (Gutierrez Manero et al. 2001; Bottini et al. 2004; Dodd et al. 2010). Again, although production of absisic acid (ABA) by bacteria is infrequent, several workers documented about the involvement of PGPR during its production (Dodd et al. 2010; De Smet et al. 2006). It has been reported that inoculation of *Azospirillum brasilense* Sp245 has increased the ABA content in *Arabidopsis*, especially when grown under osmotic stress (Cohen et al. 2008). According to an estimate about 80 % of bacteria isolated from the rhizosphere can produce plant growth regulator IAA (Hayat et al. 2010). Moreover, production of cytokinins by PGPRs is also well documented and correlated with plant growth. Castro et al. (2008) reported about the role of PGPR in production of cytokinins. A recent report has provided important information on the role played by cytokinin receptors in plant growth promotion by *Bacillus megaterium* rhizobacteria. *B. megaterium* UMCV1 strain isolated from the rhizosphere of bean (*Phaseolus vulgaris* L.) plants and on inoculation of this bacterium was found to promote biomass production of *Arabidopsis thaliana* and bean plants both in laboratory as well as field condition (Ortiz-Castro et al. 2009; Lopez-Bucio et al. 2007). According to them, the effect was related to altered root system architecture in inoculated plants, with an inhibition in primary root growth followed by an increase in lateral root formation and root hair length. Further, the effects of bacterial inoculation on plant growth and development were found to be independent of auxin and ethylene signaling as revealed by normal responses of auxin resistant mutants *aux1-7*, *axr4-1,* and *eir-1* and ethylene-response mutants *etr-1* and *ein-2*, and the failure to activate the expression of auxin-reporter markers (Lopez-Bucio et al. 2007). Similarly, Narula et al. (2006) reported about the gibberellins productions which are limited to a few species of *Bacillus* (Solano et al. 2008).

PGPR plays an important role in reduction of ethylene level which is necessary for growth and development of plant as at higher concentration it induces defoliation and other cellular processes that show negative effect on plant's health (Bhattacharyya and Jha 2012). PGPR have the capacity to divert the ethylene biosynthesis pathway particularly in the root system by using the amino cyclopropane carboxylic acid (ACC) deaminase activity. The work has been well illustrated

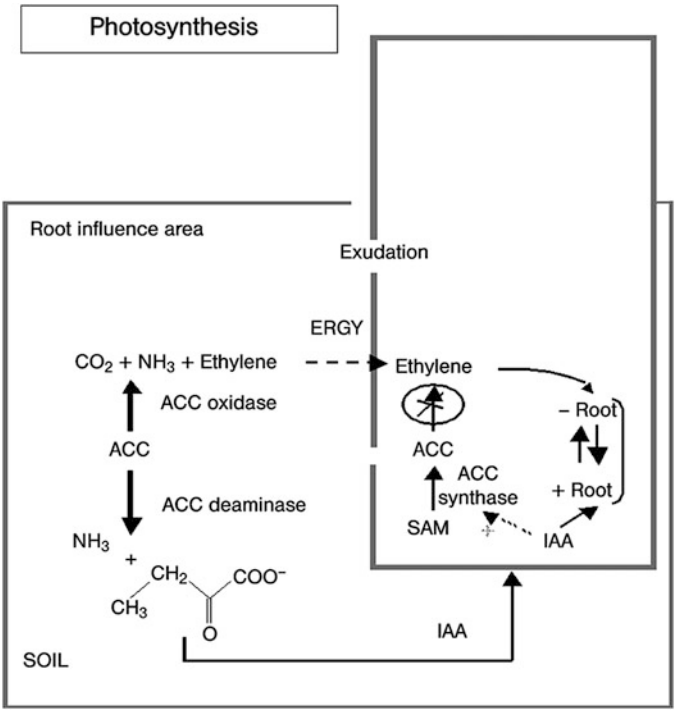

Fig. 15.2 Ethylene regulation by PGPR, a proposed model (Adopted from Glick et al. 1998)

by Desbrosses et al. (2009) in case of *Arabidopsis thaliana* plant. Glick et al. (1998) reported in detail about the mechanism of ethylene regulation in plant by PGPR which is primarily based on the ability of some bacteria to degrade ACC, the direct precursor of ethylene. The degradation of this compound generates ACC concentration gradient between the interior and the exterior of the plant, favoring its exudation, which causes a reduction of the internal ethylene level. This, in combination with auxin that may be produced by the same microorganism, causes a considerable effect on important physiological processes such as root system development (Fig. 15.2). The bacterial ACC deaminase competes with the plant's ACC oxidase. This enzyme has been isolated and identified in several PGPR, all having the ability to use ACC as the sole nitrogen source. Even, this model has been widely confirmed using various mutants (Solano et al. 2008).

15.3.2.2 Production of Volatile Organic Compounds

The volatile organic compounds (VOCs) produced by some PGPR also plays important role in plant growth. Volatile organic compounds (VOCs) are defined as compounds that have high enough vapor pressures under normal conditions to

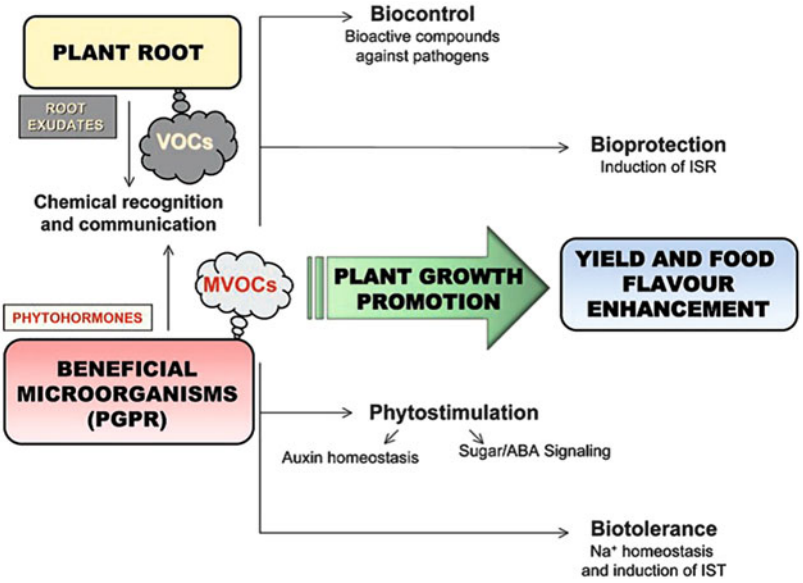

Fig 15.3 Mechanisms involved in volatile organic compound modulation of plant growth. Microorganisms produce VOCs, which can be sensed by plants to alter morphogenesis or activate defense and stress-related responses (Adopted from Ortiz-Castro et al. 2009)

significantly vaporize and enter the atmosphere. In most of the mechanisms that PGPR use to interact with plants, VOC emission has a crucial participation (Fig. 15.3). The mechanism that has received most attention in the last decade is the role of VOCs on antibiosis and the biocontrol of plant pathogens. The discovery of rhizobacteria that produce volatile organic compounds (VOCs) constitutes an important mechanism for the elicitation of plant growth by rhizobacteria (Bhattacharyya and Jha 2012). There are numerous reports showing volatiles produced by bacteria such as ammonia, HCN, phenazine-1-carboxylic acid, alcohols, etc.

The production of bioactive VOCs by PGPR is a strain specific phenomenon. For example, PGPR strains namely *Bacillus subtilis* GB03, *B. amyloliquefaciens* IN937a, and *Enterobacter cloacae* JM22 release a blend of volatile components, particularly, 2,3-butanediol and acetoin that has been found to stimulate the growth of *Arabidopsis thaliana* plant (Ryu et al. 2003). Forlani et al. (1999) also reported acetoin-forming enzymes in certain crops like tobacco, carrot, maize, and rice, but their possible functions in plants were not properly established. Now, it has been established that the VOCs production by the rhizobacterial strains can act as signaling molecule to mediate plant–microbe interactions. This is possible because volatiles produced by PGPR colonizing roots are generated at sufficient concentrations to trigger the plant responses (Ryu et al. 2003). Farmer (2001) identified low-molecular weight plant volatiles such as terpenes, jasmonates, and green leaf components as potent signal molecules for living organisms in different trophic

levels. Nevertheless, to understand the details of molecular and physiological mechanisms and how volatile organic compounds signal plants and serve in plant defense systems, detailed investigations are still needed.

15.3.3 Indirect Mechanism

The list of indirect action of PGPR includes various activities and is not conclusive as the indirect mechanisms mentioned in this chapter do not cover all the activities of PGPRs. Besides, it is also to be mentioned that several mechanisms are not fully understood; hence, it is very difficult to have a comprehensive view regarding indirect mechanism of action of rhizobacteria or PGPRs. As mentioned above, indirect mechanisms are related to biocontrol, including antibiotic production, chelation of available Fe in the rhizosphere, synthesis of extracellular enzymes that hydrolyze the fungal cellular wall, and competition for niches within the rhizosphere (Zahir et al. 2004). Some important indirect actions along with the associated PGPRs are mentioned below (Table 15.3).

Table 15.3 Some indirect mechanism of PGPR

Mechanism	Associated PGPRs	References
Nitrogen fixation	*Azoarcus* sp. *Beijerinckia* sp. *Klebsiella pneumoniae* *Pantoea agglomerans Rhizobium* sp. *Bacillus polymyxa* *Azotobacter* sp. *Rhizobia* spp. *Azospirillum* sp., etc.	Riggs et al. (2001) Bhattacharyya and Jha (2012) Hayat et al. (2010) Solano et al. (2008)
Production of siderophores	*Rhizobium meliloti* *Pseudomonas* sp. *Pseudomonas fluorescens*	Arora et al. (2001) Solano et al. (2008)
Phosphate solubilization	Species of *Azospirillum* *Azotobacter* *Bacillus* *Beijerinckia* *Burkholderia* *Enterobacter* *Erwinia* *Flavobacterium* *Microbacterium* *Pseudomonas* *Rhizobium* *Serratia*	Sturz and Nowak (2000) Sudhakar et al. (2000) Mehnaz and Lazarovits (2006)
Hydrolysis of molecules released by pathogens	*Pseudomonas solanacearum* *Pseudomonas cepacia*	Toyoda and Utsumi (1991)
Synthesis of cyanhydric acid	*Pseudomonas* sp.	Voisard et al. (1989)

15.4 Conclusions

PGPRs are the microbial inoculants. They can enhance plant growth as well as quality of plants by various ways. They are ecofriendly, cost-effective, and nonhazardous. Also they keep the soil health for sustainable use. It is revealed from the literatures that PGPRs have an important role in improvement of medicinal properties of the plants. No doubt, application of inorganic fertilizers can help the growth and development of the plants, but not the quality of the plants. Intensive use of inorganic fertilizers may cause noticeable damage to our environment as well as soil health. It also leads to deposit heavy metals in the soil. These heavy metals can transmit into different parts of the plant and accumulated there. The medicinal properties of the plants may vary or deteriorate as a result of accumulation of such hazardous metals. So, application of PGPRs is important to maintain the quality of the medicinal values of the plants. Not only that, it will particularly help to protect our precious soil resource and environment as a whole. Moreover, molecular and physiological mechanisms of growth and development of the plants or value addition in it after application of PGPRs have not studied adequately. Comprehensive research in this field is needed to understand the detailed mechanisms of action.

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Chapter 16
Rhizosphere Microflora in Advocacy of Heavy Metal Tolerance in Plants

Shivangi Upadhyay, Monika Koul, and Rupam Kapoor

16.1 Introduction

The term "heavy metal" is used for metallic elements with a specific mass higher than 5 g cm^{-3} and form sulfides (Adriano 1986). Heavy metals (HMs) are present in background concentrations in the Earth's crust, but over the years their concentrations have increased owing to anthropogenic activities, posing as a major abiotic stress. Superfluous levels of HMs can result in decadence of soil quality, subsequent crop yield attrition, and substandard agricultural products, thus pose as a preeminent health hazard. Some HMs have a tendency to get bioaccumulated, they enter the food chains through uptake by producers and get magnified at consumer level (Nagajyoti et al. 2010). Plants have evolved various ubiquitous and specific metal-resistant and metal-tolerant mechanisms to maintain ionic homeostasis at the advent of HM stress (Milner and Kochian 2008).

Rhizosphere microflora and their metabolic processes profoundly influence plant growth and yield as they have an enormous potential to improve soil quality and degrade and immobilize the toxic compounds (Gadd 1990). The composition of soil microbial population is complex. Arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) have been confirmed to enhance (Joner and Leyval 1997; Sheng and Xia 2006) or reduce (Heggo et al. 1990; Rajkumar et al. 2006) the uptake of HMs by plants.

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AMF offer plant a number of benefits in lieu of carbon products from the plant and are important in capturing nutrients that have low availability and mobility in soils (Parniske 2008). The basis of symbiosis is a bidirectional exchange of nutrients, where the fungus provides phosphorous to plant and in return the plant provides carbon products to it. The hyphal network prevents nutrient leakage, thereby providing enhanced nutrition and greater water uptake. It has been connoted that AMF provide tolerance against biotic as well as abiotic stress to a variety of plant species (Kapoor et al. 2013).

Another group of symbiotic and free-living soil microbes known as PGPR influence the plant growth by improving plant nutritional status and synthesizing plant growth-promoting compounds and phytohormones (Glick et al. 1998). A number of PGPR species have been noted to increase HM stress tolerance in plants by aiding plant growth in HM-contaminated soils (Burd et al. 2000).

Drugs are bioactive constituents or secondary metabolites released by plants often in response to stress. Several HMs directly affect biochemical and physiological processes such as altered production of bioactive compounds and reduced resistance to abiotic stress (Verpoorte et al. 2002). For over two decades HMs in medicinal plants have been reported from Asia, Europe, and the United States (Olujohungbe et al. 1994; Kakosy et al. 1996). Though it is well studied that HM accumulation in medicinal plants exposes humans to a number of health risks (Dwivedi and Dey 2002), still the utilization of AMF and PGPR in the same respect has not been hard lined much. The review provides an insight into the role of AMF and PGPR in HM stress alleviation in plants. The various mechanisms involved in the purpose have also been discussed briefly (Fig. 16.1).

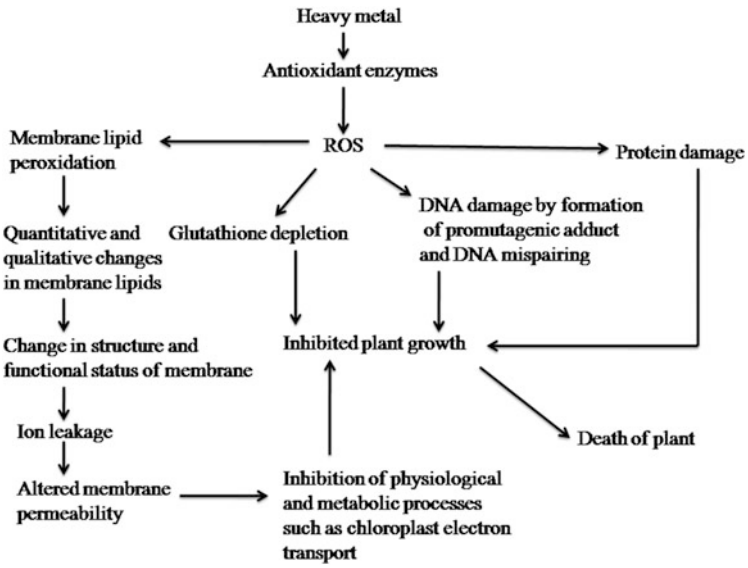

Fig. 16.1 Events describing HM-induced oxidative stress in plants

16.2 Bioavailability of HMs in Soil and Their Site of Accumulation in Plants

Metals exist in two forms: bioavailable and non-bioavailable (Sposito 2000). Levels of HMs in plants vary extensively as the bioavailability of elements is influenced by a multitude of factors (Nagajyoti et al. 2010). Soil physicochemical properties, metal and plant species; competition between metals in rhizosphere; plant growth conditions; plant root density and volume; and characteristics of the association between plant and soil microbes affect the accumulation of HMs in soil (Leyval et al. 1997). Transfer of a metal from soil to plant can be delineated quantitatively by the soil–plant transfer factor (TF) that can be described as the ratio of contaminant concentration in plant parts to concentration in dry soil (Rodriguez et al. 2002).

Accumulation of HMs in plant parts depends on the site of contact with metal and its subsequent translocation. Ultracellular studies have depicted the metals in the intercellular spaces and in the cell wall of root tissues (Marques et al. 2007). It has been observed that the accumulation of HMs in plant parts increases in a concentration-time-dependent manner (Khan et al. 2007). On the basis of ability to accumulate metals differently, plants can be distinguished into metal excluders, metal indicators, and metal accumulators (Baker and Walker 1990).

16.3 Rhizosphere Microflora Mediating HM Acquisition in Plants

Interaction of plant roots with rhizosphere microflora influences the bioavailability and uptake of HM ions through secretion of protons, organic acids, phytochelatins (PCs), amino acids, and enzymes (Yang et al. 2005). Soil bacteria and AMF have been characterized to catalyze redox transformations leading to an altered soil metal bioavailability (Lasat 2002).

In anaerobic respiration, many microorganisms that catalyze redox reactions use metals as terminal electron acceptors and are known as dissimilatory metal-reducing bacteria. These bacteria are not only phylogenetically (Lonergan et al. 1996) but also physiologically (Lovley et al. 1997) disparate, though most of these administer Fe^{3+} and S^0 as terminal electron acceptors. Rhizobacteria produce metal-chelating agents called siderophores that have a conspicuous role in the acquisition and speciation of several HMs (Burd et al. 2000). Fungal symbiotic associations enhance the root absorption area and stimulate the remuneration of certain HM ions such as Cu and Zn (Smith and Read 2008). Low molecular weight organic acids (LMWOA) can influence metal release from absorbed metal in the soil and increase its solubility through formation of

complexes like release of Cd by formation of Cd-LMWOA complex (Krishnamurti et al. 1997). The hyphal mass produced by AMF can bind HMs beyond the plant rhizosphere by releasing an insoluble glycoprotein commonly known as glomalin (Gohre and Paszkowski 2006).

16.4 Implications of HM Stress on Plants

Over the years many studies have connoted the effects of HMs on medicinal plants and these can be abridged as follows:

16.4.1 Growth and Development

Reduced growth and development has been reported in medicinal plants upon exposure to HMs (Jiang et al. 2001). However, certain positive effects such as yield enhancement have been observed in *Matricaria chamomilla*, *Mentha arvensis*, and *Stevia rebaudiana* upon exposure to Zn, Co, Pb, and Ni (Misra 1992; Kartosentono et al. 2002; Das et al. 2005; Grejtovsky et al. 2006).

16.4.2 Disturbed Mineral Nutrition

Most HM ions compete with other metal ions for uptake, transport, and utilization by plants and consequently result in various element deficiencies, for example, arsenic competes with phosphorous (Meharg and Macnair 1992).

16.4.3 Membrane Disruption

HMs affect transport of solutes across plasma membrane by resulting in cellular alterations such as plasma membrane disruption and chloroplast thylakoid swelling (Valcho et al. 2008).

16.4.4 Effects on Physiological and Metabolic Processes

Certain HMs like Cu and Zn either serve as cofactors/activators in enzyme reactions or exert a catalytic property as prosthetic group in metalloproteins (Nagajyoti

et al. 2010). These HMs interact with nonspecific protein sites and displace other metals from their characteristic binding sites. Reduction in seed germination has been observed in medicinal plants such as *Catharanthus roseus* L., *Eucomis autumnalis*, and *Bowiea volubilis* (Pandey et al. 2007; Street et al. 2007).

16.4.5 Oxidative Stress

Metal ions are essential cofactors of enzymes involved in antioxidant network; for example, all isoforms of superoxide dismutase (SOD) contain bound HM ions like Cu/Zn-SOD associated with chloroplast and Mn-SOD with glyoxysomes. Metals are involved in direct or indirect generation of free radicals (FR) and reactive oxygen species (ROS) in the following ways: (1) direct transfer of electron in single electron reduction, (2) disturb the metabolic pathways and ensue an increase in the rate of FR and ROS formation, (3) inactivation and downregulation of the enzymes of the antioxidative defense system, and (4) depletion of low molecular weight antioxidants (Aust et al. 1985).

16.4.6 Effect on DNA

Metal binding to the cell nucleus causes promutagenic damage including DNA base modifications, inter- and intramolecular cross-linkage of DNA and proteins, DNA strand breaks, rearrangements, and de-purination (Kasprzak 1995). Metal-mediated production of ROS in DNA vicinity generates a promutagenic adduct 8-oxoG (7,8-dihydro-8-oxoguanine) that could miss pair with adenine in the absence of DNA repair, resulting in C to T transversion mutations (Cunningham 1997). Cell treatment with Ni can cause chromatin condensation, leading to silencing of putative anti-oncogenic gene expression, thus driving treated cells to a carcinogenic state (Lee et al. 1995). Concentration and time-dependant Cd, Cu, and Ni clastogenic effects have been observed in *Helianthus annuus* (Chakravarty and Srivastava 1992).

16.4.7 Effects on Secondary Metabolite Production

The chemical composition of plants under HM stress may be altered, and as a result the quality and potency of the natural products from medicinal plants may be seriously affected (Zhu and Cullen 1995). The reduction in biosynthesis of active constituents may be a result of loss or inactivation of specific essential enzymes involved in the production of secondary metabolites, for example, on exposure to Ni, the capacity of *Hypericum perforatum* to produce and accumulate hyperforin is

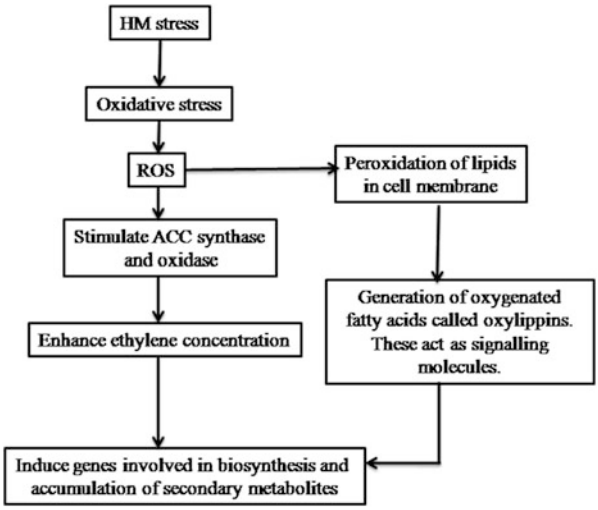

Fig. 16.2 Effect of HMs on secondary metabolite production

ceased completely (Murch et al. 2003). Probably, to mitigate the phytotoxic effects of HMs, alteration in secondary metabolism may be one of the plant's strategies (Cobbett and Goldsbrough 2000) (Fig. 16.2).

On the other hand, HMs in small concentrations may also act as abiotic elicitors and improve the biosynthesis of specific compounds in certain medicinal plants such as Cd enhanced the production of ajmalicine in *Catharanthus roseus* (Zheng and Wu 2004), phyllanthin and hypophyllanthin production in *Phyllanthus amarus* (Rai et al. 2005), and tropane alkaloids in *Atropa belladonna* (Lee et al. 1998); and Pb enhanced sitosterol in *Costus speciosus* (Kartosentono et al. 2002).

16.4.7.1 Mechanism Involved in the Alteration of Secondary Metabolite Production

The defensive processes in plants get activated in response to induced stress, ensuing a change in the transcription of genes coding for enzymes that are involved in the biosynthesis of secondary metabolites (Kasparova and Siatka 2004). Under HM-induced oxidative stress, the ROS may elicit secondary plant metabolism to result in structurally similar or even identical compounds (Mithöfer et al. 2004). Besides this, in HM-exposed plants, upon stimulation of ACC (1-aminocyclopropane-1-carboxylic acid) synthase and oxidase, ethylene has been known to regulate a pathway that accounts for the production of tropane alkaloids, scopolamine, and hyoscyamine (Maksymiec 2007; Nasim and Dhir 2010). In *Brugmansia candida* there is an increase in scopolamine in response to Ag, whereas hyoscyamine production is diminished (Pitta-Alvarez et al. 2000). Ag probably acts as ethylene blocking agent which downregulates hyoscyamine-

6-β-hydroxylase (H6H); this enzyme is responsible for converting hyoscyamine to scopolamine.

16.5 Effect of HM Stress on Rhizosphere Microflora

Elevated concentrations of HMs have multifarious effects on the soil microbial communities like altered community structure of microbes (Gray and Smith 2005), decreased numbers of specific populations (Chaudri et al. 2000), and reduced total microbial biomass (Giller et al. 1998). Certain HMs like Hg, Pb, Zn, Cd, Cu, Ni, and As inhibit various microbial metabolic activities by DNA damage, transcription inhibition, protein denaturation, and inhibition of enzyme activity (Khan et al. 2009). A few rhizospheric microorganisms can develop resistance or tolerance that may be inherited or induced (Giller et al. 1998), for example, *Bradyrhizobium* sp. RM8 provides IAA and siderophores to plants even in the presence of Zn and Ni stress (Wani et al. 2007). Similarly, HM-tolerant AMF species have also been reported; for example, *Glomus intraradices* is tolerant to Pb (Malcova et al. 2003).

To tolerate HM stress, PGPR have evolved several mechanisms (Nies 1999) that can be enlisted as: (1) exclusion, physiologically active sites are kept away from the metal ions; (2) extrusion, the metals are made to exit the cells through chromosomal/plasmid mediated events; (3) accommodation, metals associate with the metal-binding proteins forming complexes such as metal–phytochelatin (PC) and metal–metallothionein (MT) complexes; (4) biotransformation, reduction of a toxic metal to a lesser toxic form; and (5) methylation and demethylation of DNA. These mechanisms could be inducible or constitutive under HM stress (Khan et al. 2009). In generic terms, the resistance mechanisms in bacteria are encoded on plasmids and transposons. Resistance against As, Cr, and Cd in some PGPR has been reported through plasmid-encoded energy-dependent metal efflux systems involving ATPases and chemiosmotic ion/proton pumps (Roane and Pepper 2000).

At elevated concentration HMs in soil reduce or entirely inhibit AMF colonization and henceforth forbid the beneficial effects of the mycorrhizal association (Chen et al. 2005). Studies cast light on the morphogenetic changes in extraradical hyphae of *G. intraradices* in response to Cu, Cd, Pb, and Zn concentrations (Bago et al. 2004; Ferrol et al. 2009). At raised concentration the growth of the extraradical mycelium is localized and limited to changes like loss of apical dominance, cytoplasmic protrusions, extrametrical coils, and abatement of sporulation (Gonzàlez-Guerrero et al. 2005). These morphological alterations in fungus indicate adaptive changes as (1) growth revocation can be a strategy aimed to avert toxic-metal-contaminated areas (Gadd 2007), (2) augmentation of extramatrical coils allows the fungus to produce steep local concentrations of extracellular products, like metal chelators (this would depreciate metal availability in vicinity of hyphae creating a stress-free zone), and (3) exaggerated hyphal elongation at lower HM concentration portrays a strategy to grasp relatively less-contaminated pockets of the soil to escape local metal enriched microenvironments (Fomina

et al. 2003). Studies propose a potential adaptation of the indigenous AMF populations in metal-contaminated sites; therefore, these are seen to have potential for reclamation of degraded soils (Gildon and Tinker 1981).

16.6 AMF and PGPR at Plant's Rescue

Experimental evidences connote positive effects of co-inoculation with AMF and PGPR on removal of HMs from soils (Barea et al. 2005). Some of the mechanisms by which soil microbes can alleviate HM stress include:

16.6.1 Dilution Effect

AMF can enhance plant growth and establishment against the high levels of HMs in soil, owing to better nutrition, water uptake and availability, and soil aggregation properties (Hildebrandt et al. 2007). An extensive range of PGPR is able to mitigate the HM stress by advocating plant growth like *Rhizobium*, *Pseudomonas*, *Agrobacterium*, *Burkholderia*, *Azospirillum*, *Bacillus*, *Azotobacter*, *Serratia*, *Alcaligenes* (*Ralstonia*), *Arthrobacter*, and *Brevibacillus* (Glick 2003; Vivas et al. 2006). The coalesced effects of AMF and soil bacteria can augment HM tolerance in plants by promoting plant growth and by production of growth regulators such as indoleacetic acid (IAA) (Vivas et al. 2006).

16.6.2 Chelation

Extraradical mycelium of AMF is important for metabolism-independent binding of HMs to cell walls and metabolism-dependent intracellular uptake and transport of HMs (Leyval et al. 1997). Phytostabilization of HMs in the rhizosphere can occur by production of compounds that precipitate them in soil and by chelation of these in the cell wall and cellular structures of AMF (Gaur and Adholeya 2004; Gohre and Paszkowski 2006). Constituents of hyphal cell wall like chitin (Zhou 1999) and production of insoluble glycoprotein, glomalin, by the fungal hyphae influence the absorption of HMs (Gonzàlez-Chavez et al. 2004). Also, AMF stimulates plant roots to produce elevated levels of compounds that are able to chelate HMs, such as cysteine and glutathione (Galli et al. 1995). PGPR produce siderophores and acids for mobilizing metals in soils such as Fe. Siderophores mitigate deficiency of Fe caused due to Ni toxicity (Burd et al. 2000).

16.6.3 Compartmentalization

Immobilization of metals in the fungal biomass or in mycorrhizosphere is an important mechanism of AMF to alleviate HM stress. In the intracellular compartmentalization strategy operated by AMF, the excess of HM such as Cu is translocated to subcellular compartments (vacuoles) where it is stored in specific fungal structures (extraradical spores and intraradical vesicles) that have restricted core metabolic functions (Ferrol et al. 2009).

16.6.4 Biotic Sequestration

Metal dissolution by AMF may occur by proton-promoted or ligand-promoted mechanisms and by organic acids as they provide both protons for solubilization and metal-chelating anions to complex with metal cations (Finlay 2008). Interestingly, AMF associated with metal-tolerant plants accumulate HMs in plant roots in nontoxic forms, for example, AMF in *Voila calaminaria*, an important medicinal plant, efficiently sequester metals in the roots (Tonin et al. 2001). Certain PGPR have also been attributed to have similar effects, such as *Ochrobactrum bacillus* that lowers the toxicity of chromium by reducing Cr (VI) to Cr (III) (Faisal and Hasnain 2006).

16.6.5 Molecular Mechanisms

In the roots of mycorrhizal plants, HM content is significantly altered, evincing that the expression of genes involved in HM tolerance is altered at transcriptional and translation levels (Ouziad et al. 2005). It has been connoted that AMF colonization in roots significantly affects the expression of multifarious plant genes involved in HM tolerance and detoxification (Rivera-Becerril et al. 2005). The expression of an MT gene of *Gigaspora margarita* (BEG 34) is upregulated in the presence of Cu (Lanfranco et al. 2002). Another gene encoding MT involved in metal chelation and ROS scavenging has been classified in AMF (Ferrol et al. 2009). Expression of certain transporter genes also gets affected under HM stress, like on exposure to Zn there is enhanced transcript level of a Zn transporter gene *GintZnT1* in *G. intraradices* mycelium indicating its plausible role in protection against Zn stress (González-Guerrero et al. 2005). Another transporter gene *GintABC1* in *G. intraradices* is upregulated in response to Cd and Cu. It encodes for a polypeptide bearing homology to the N-terminal region of the multidrug-resistance-protein (MRP) subfamily of ABC transporters. This connotes that *GintABC1* may be involved in the detoxification of Cd and Cu (Gonzàlez-Guerrero et al. 2006). The products of such HM responsive genes may act in a rather localized manner,

conceivably restricted to fungal structures like the arbuscules. The concomitant upregulation of stress-related AMF genes evince that in mycorrhizal plants increased HM tolerance could be attributed to effective fungal HM tolerance mechanisms (Hildebrandt et al. 2007).

A few genes encoding proteins plausibly involved in ROS homeostasis have also been identified and delineated in AMF, viz., three genes coding for SODs (González-Guerrero et al. 2005) and ten genes allegedly encoding glutathione S-transferases (GSTs) (Waschke et al. 2006); in the hyphae of *G. intraradices*, expression of four genes that result in the production of GST is observed under Zn stress (Hildebrandt et al. 2007). The enzyme GST acts as a catalyzer in combination with glutathione and some electron receivers ensuing alleviation of oxidative stress (Moons 2003).

16.6.6 Effect of AMF and PGPR on Secondary Metabolite Production in Medicinal Plants

A number of studies have revealed the potential of AMF in enhancing plant growth and altering secondary metabolite production (Kapoor et al. 2002a, b; Copetta et al. 2006), for example, castanospermine (an alkaloid of the indolizidine type) was found to increase with AMF colonization in *Castanospermum australe*, an important medicinal plant (Abu-Zeyad et al. 1999). *Gigaspora rosea* increases biomass as well as the total amount of essential oil in *Ocimum basilicum*. Similar roles of PGPR have been reported in medicinal plants, for example, PGPR increase ajmalicine in *Catharanthus roseus* (Karthikeyan et al. 2010) and withaferin A in *Withania somnifera* (Khalid et al. 2004). Attempts have been made to study the synergism of AMF and PGPR in context of plant growth and secondary metabolite production in medicinal plants, as in *Begonia malabarica*, upon co-inoculation of PGPR and *G. mosseae* increase in plant growth, and enhancement of secondary metabolite production has been noted (Thangavel et al. 2008).

16.7 Conclusions

The fact that HMs enhance the production of bioactive compounds in certain medicinal plants presents a pragmatic aspect of utilizing contaminated sites for cultivation of such plants. However, the applicability of this strategy will primarily depend on the part of the plant used for medicinal purposes and the ability of that medicinal plant species to exclude or accumulate HMs. The knowledge of which plant part has medicinal value and its usage, viz., direct use such as dried powdered form or a processed extract from it, is important. Direct consumption of medicinal plant parts from HM accumulators poses a huge risk of exposure to HMs and their

accumulation in humans over a period, leading to chronic health disorders. Also, the medicinal extracts from the plants growing in such soils should undergo strict quality check. Leverages of this strategy include the utilization of an otherwise wasteland for achieving enhanced production of bioactive compounds, and at the same time cultivation of contaminated food crops can be avoided.

To such sites application of rhizosphere microflora that enhances HM uptake can further benefit. As the response of AMF and PGPR to HM toxicity is variable, there are gaps in the understanding of pathways involved in metal transport and regulatory mechanisms. There is a need to evaluate rhizosphere microflora-induced changes in HM speciation and whether these changes can affect the extent of accumulation and site of distribution of HMs in medicinal plants. For the success of this artifice, knowledge of a superior suitable strain of AMF and PGPR for every medicinal plant species being used here is imperative. Assimilation of pertinent features of microbial–plant–HM interaction in rhizosphere and understanding the regulation of metal homeostasis along with metal forbearance strategies form the very base of this strategy.

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Part V
PGPR: Diversity and Characterization

Chapter 17
Diverse Endophytic Microflora of Medicinal Plants

Pranay Jain and Ram Kumar Pundir

17.1 Introduction

The term endophyte (Gr. *endon*, within; *phyton*, plant) was first coined by De Bary (1866), and an endophyte is a bacterial or fungal microorganism, which spends the whole or part of its life cycle colonizing inter- and/or intracellularly inside the healthy tissues of the host plant, typically causing no apparent symptoms of disease (Sturz et al. 2000; Wilson 1995). All vascular plants harbor endophytic organisms (Zhang et al. 2006). These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites (Carroll and Carroll 1978; Azevedo et al. 2000; Strobel 2003).

Endophytes are now considered as an important component of biodiversity. The distribution of endophytic microflora differs with the host. Medicinal plants are known to harbor endophytic microorganisms that are believed to be associated with the production of pharmaceutical products (Zhang et al. 2006). Therefore, it is important to explore endophytic microflora in the medicinal plants. Endophytes are mostly an unexplored group of microorganisms, but a few studies show them as a huge source of medicinal compounds. Approximately 300,000 plant species growing in an unexplored area on the Earth are host to one or more endophytes, and the presence of biodiverse endophytes in huge number plays an important role on ecosystems with the greatest biodiversity (Souza et al. 2004).

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Considering that only a small amount of endophytes have been studied, recently, several research groups have been motivated to evaluate and elucidate the potential of these microorganisms applied on biotechnological processes focusing on the production of bioactive compounds. Endophytes provide a broad variety of bioactive secondary metabolites with unique structure, including alkaloids, benzopyranones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones, and others (Tan and Zou 2001). Such bioactive metabolites find wide-ranging application as agrochemicals, antibiotics, immunosuppressants, antiparasitics, antioxidants, and anticancer agents (Strobel 2003).

Endophytic microorganisms are a significant reservoir of genetic diversity and an important source in the discovery of novel bioactive secondary metabolites. These group of strains can produce high or multiple kinds of antibiotics including terpenoids, alkaloids, aromatic compounds, and polypeptides (Gao et al. 2010) which are similar to host plant chemicals, thus triggering the expectations that endophytes can serve as an alternative source (Priti et al. 2009). So, plants with beneficial ethnobotanical history are also likely a candidate for study, since the medicinal uses to which the plant may have selected relate more to its population of endophytes than to the plant biochemistry itself. Endophytic organisms are found in all the types of plant tissues such as stems, roots, leaves, fruits, ovules, seeds, tubers, rachis, and bark. Probably, hundreds of endophytic species from a single plant are also possible, and among them, at least one generally shows host specificity (Tan and Zou 2001).

Medicinal plants harbor a distinctive microbiome due to their unique and structurally divergent bioactive secondary metabolites that are most likely responsible for the high specificity of the associated microorganisms (Qi et al. 2012). Plants contain numerous different biologically active compounds, and plant-derived medicines have been part of traditional healthcare in most parts of the world for thousands of years. In general, natural products play a highly considerable role in the drug discovery and development process, as about 26 % of the new chemical entities introduced into the market worldwide from 1981 to 2010 were either natural products or those derived directly there from, reaching a high of 50 % in 2010 (Newman and Cragg 2012). In regard to the alarming incidence of antibiotic resistance in bacteria with medical relevance, medicinal plants with antibacterial properties are of central importance as bioresources for novel active metabolites (Palombo and Semple 2001).

Likewise, there is an increasing need for more and better antimycotics to treat those with weakened immune systems who are more prone to developing fungal infections, such as from the AIDS epidemic, cancer therapy, or organ transplants (Strobel and Daisy 2003; Strobel et al. 2004). For centuries, several phytotherapeutics have also been known for their antiphlogistic features, yet despite the progress within medical research, chronic inflammatory diseases such as asthma, arthritis, and rheumatism remain one of the world's leading health problems (Li et al. 2003). Hypertension is another critical issue for human health and is a primary risk factor for stroke, heart disease, and renal failure. Many herbal remedies as well as foods, however, are known and effective folk medicines in the prevention and/or treatment of high blood pressure (Abdel-Aziz et al. 2011). Hence,

nature must still harbor plenty of currently unknown active agents that may serve as leads and scaffolds for the development of desperately needed efficacious drugs for a multitude of diseases (Newman and Cragg 2012). Today, globalization has also had an impact on the use of medicinal plants and has proven beneficial in allowing greater access to these medicines for people all across the globe.

17.2 Biodiversity of Endophytes

Of the myriad of ecosystems on Earth, those having the greatest biodiversity also have endophytes with the greatest number and the most biodiverse microorganisms. Almost all vascular plant species examined to date were found to anchor endophytic bacteria and/or fungi (Sturz et al. 2000; Arnold et al. 2000). Moreover, the colonization of endophytes in marine algae [Smith et al. 1989; mosses and ferns (Petrini et al. 1992; Raviraja et al. 1996)] had also been detected. Based on fact, endophytes are important components of microbial biodiversity. Commonly, numerous endophyte species can be isolated from a single plant, and among them, at least one species shows host specificity. The environmental conditions under which the host is growing also affect the endophyte population (Hata et al. 1998), and the endophyte profile may be more diversified in tropical areas. Tropical and temperate rain forests are the most biologically diverse terrestrial ecosystems on Earth. The most threatened of these spots cover only 1.44 % of the land's surface, yet they harbor more than 60 % of the world's terrestrial biodiversity (Mittermeier et al. 1999). 418 endophyte morphospecies (estimated 347 genetically distinct taxa) were isolated from 83 healthy leaves of *Heisteria concinna* and *Ouratea lucens* in a lowland tropical forest of central Panama and proposed that tropical endophytes themselves could be hyperdiverse with host preference and spatial heterogeneity (Arnold et al. 2000). Various species of endophytic fungi as *Cladosporium cladosporoides*, *Phoma* spp., *Phomopsis* spp., and *Xylaria* spp. had been reported in four types of tropical forests: dry thornforest, dry deciduous forest, moist deciduous forest, and semi-evergreen forest (Suryanarayanan et al. 2002).

As such, one expects that areas of high plant endemicity also possess specific endophytes that may have evolved with the endemic plant species. Ultimately, biological diversity implies chemical diversity because of the constant chemical innovation that exists in ecosystems where the evolutionary race to survive is the most active. Tropical rain forests are a remarkable example of such type of environment. Competition is great, resources are limited, and selection pressure is at its peak. This gives rise to a high probability that rain forests are a source of novel molecular structures and biologically active compounds (Redell and Gordon 2000). A metabolic distinction was described between tropical and temperate endophytes through statistical data which compared the number of bioactive natural products isolated from endophytes of tropical regions to the number of those isolated from endophytes of temperate origin. Not only did they find that tropical endophytes provide more active natural products than temperate endophytes, but they also distinguished that a significantly higher number of tropical endophytes produced

a larger number of active secondary metabolites than did fungi from other temperate substrata (Bills et al. 2002). This observation suggests the importance of the host plant in influencing the general metabolism of endophytic microbes. Moreover, genotypic diversity too has been observed in single endophyte species originating from conifers, birch, and grasses (Reddy et al. 1998). Accordingly, fungal endophytes have the ability to produce novel metabolites with novel structures than soil isolates (Schulz et al. 2002) and are presumably ubiquitous in the plant kingdom with the population being dependent on host species and location.

17.3 Biodiversity of Fungal Endophytes

A variety of relationships exist between fungal endophytes and their host plants, ranging from mutualistic or symbiotic to antagonistic or slightly pathogenic (Arnold 2007; Schulz and Boyle 2005). Endophytes may produce overabundance of substances of potential use to agriculture, industry, and modern medicine such as novel antibiotics, antimycotics, immunosuppressant, and anticancer compounds (Mitchell et al. 2008). In addition, the studies of endophytic fungi and their relationships with host plants will shed light on the ecology and evolution of both the endophytes and their hosts: the evolution of endophyte plant symbioses and the ecological factors that influence the direction and strength of the endophyte host plant interaction (Saikkonen et al. 1998). Since natural products are likely adapted to a specific function in nature, so search for novel secondary metabolites should concentrate on organisms that inhabit novel biotopes (Schulz et al. 2002).

A study was undertaken by Das et al. (2012) to investigate the influence of plant probiotic fungus *Piriformospora indica* on the medicinal plant *Coleus forskohlii*. Interaction of the *C. forskohlii* with the root endophyte *P. indica* under field conditions results in an overall increase in aerial biomass, chlorophyll contents, and phosphorus acquisition. The fungus also promoted inflorescence development, consequently the amount of *p*-cymene in the inflorescence increased. Growth of the root thickness was reduced in *P. indica*-treated plants as they became fibrous, but developed more lateral roots. Because of the smaller root biomass, the content of forskolin was decreased. The symbiotic interaction of *C. forskohlii* with *P. indica* under field conditions promoted biomass production of the aerial parts of the plant including flower development. The plant aerial parts are an important source of metabolites for medicinal application. Therefore it was suggested that the use of the root endophyte fungus *P. indica* in sustainable agriculture will enhance the medicinally important chemical production.

Azadirachta indica A. Juss. (neem), native to India, is well known worldwide for its insecticidal and ethanopharmacological properties. A variety of procedures and a number of different media were used to isolate the maximum number of endophytic fungi from unripe fruits and roots by Verma et al. (2011). A total of 272 isolates of 29 filamentous fungal taxa were isolated at a rate of 68.0 % from 400 samples of three different individual trees (at locations Az1, Az2, Az3).

Mycological agar (MCA) medium yielded the highest number of isolates (95, with a 14.50 % isolation rate) with the greatest species richness. Mycelia Sterilia accounted for 11.06 % and coelomycetes 7.25 %, while hyphomycetes showed the maximum number of representative isolates (81.69 %). Mycelia Sterilia, based on their 5.8S ITS1, ITS2, and partial 18S and 28S rDNA sequences, were identified as *Fusarium solani* (99 %), *Chaetomium globosum* (93 %). *Humicola*, *Drechslera*, *Colletotrichum*, and *Scytalidium* sp. respectively were some of the peculiar fungal endophytes recovered from this plant.

Schulz et al. (2002) isolated about 6500 endophytic fungi from herbaceous plants and trees and screened them for biologically active compounds. They found a correlation between biological activity and biotope. They also got a higher proportion of the fungal endophytes, in contrast to the soil isolates, suppressed at least one of the test organisms for anti-algal and herbicidal activities. Medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites of pharmaceutical importance (Kumar et al. 2005; Strobel et al. 2004; Wiyakrutta et al. 2004). The various natural products produced by endophytic fungi possess unique structures and great bioactivities, representing a huge reservoir which offers an enormous potential for exploitation for medicinal, agricultural, and industrial uses (Tan and Zou 2001; Zhang et al. 2006).

Most fungal endophytes isolated to date have been ascomycetes and their anamorphs; however, Rungjindamai et al. (2008) reported that several endophytes may also belong to basidiomycetes. However, their colonization rate and the isolation rate of endophytic fungi from plants varied greatly. Some medicinal plants harbored more endophytic fungi than others. Some of the common endophytes not only existed in more plant hosts but also had higher relative frequencies within each of the hosts. In contrast, some other endophytic fungi were detected in only one given plant host (Arnold et al. 2001; Bettucci et al. 2004). Most of the researches on endophytes have been carried out using hosts from temperate countries, specifically from the Northern Hemisphere and New Zealand. The update data available from tropical regions are scarce. However, these data are showing that tropical plant hosts contain a great diversity of endophytic microorganisms, and many of them are not yet classified and possibly belonging to new genera and species. In fact potentially, they are of biotechnological importance as new pharmaceutical active compounds, secondary metabolites, biological control agents, and other useful characteristics could be found by further exploration of tropical endophytes. A better understanding of plant–endophyte relationships in tropical conditions can be achieved from these studies.

Enumeration of the endophytic fungi from the red listed, critically endangered medicinal plant, *Coscinium fenestratum* was investigated for the first time in India by Goveas et al. (2011). The ubiquitous presence of 41 endophytic fungi belonging to 16 different taxa was identified from 195 samples of healthy leaves and stem using traditional morphological methods. The overall colonization rate of endophytes in both the leaf and the stem was found to be 21.02 %. The stem showed low percentage frequency of colonization of the endophytic fungi when compared to

leaf segments. Among the endophytic flora, *Phomopsis jacquiniana* was found to be the core-group fungus with a colonization frequency of 4.6 %.

A study by Dhanalakshmi et al. (2013) at Salem, Tamil Nadu, India, was undertaken to isolate and identify the potential endophytic fungi from *Moringa oleifera*, a traditional medicinal plant. A total of 24 segments each 12 from the leaf and stem were collected, surface sterilized, and inoculated on to Sabouraud dextrose agar (SDA) plates. Based on the macroscopic and microscopic features, the fungal isolates were identified as *Alternaria* spp., *Aspergillus* spp., *Bipolaris* spp., *Exophiala* spp., *Nigrospora* spp., and *Penicillium* spp. Many unidentified sterile mycelia forms were also found which were grouped under the class Mycelia Sterilia. The colonization frequency (CF) and endophytic infection rate (EIR) were observed as 91.66 % and 45.83 %, respectively. The results of this study suggest that traditional medicinal plants are a rich and reliable source of novel endophytic fungi.

Endophytic fungi residing in medicinal plants have not been systematically characterized. In a study carried out by, they classified 1,160 fungal isolates from 29 medicinal plant species using traditional morphological methods. The colonization rate, isolation rate, and relative frequency of these endophytes were investigated. The relationship between the composition of endophytic fungi and chemical constituents of host plants was also explored for the first time. The results showed that endophytic fungi from these medicinal plants exhibited high biodiversity, host recurrence, tissue specificity, and spatial heterogeneity. The taxa of *Alternaria*, *Colletotrichum*, *Phoma*, *Phomopsis*, *Xylariales*, and Mycelia Sterilia were the dominant fungal endophytes. Some phenolic compounds were found to more likely coexist with certain endophytic fungi in the same plants. Their systematic investigation revealed that traditional medicinal plants are a rich and reliable source of novel endophytic fungi.

Qadri et al. (2013) conducted a study to characterize and explore the endophytic fungi of selected medicinal plants from the Western Himalayas for their bioactive potential. A total of 72 strains of endophytic fungi were isolated and characterized morphologically as well as on the basis of ITS1-5.8S-ITS2 ribosomal gene sequence acquisition and analyses. The fungi represented 27 genera of which two belonged to *Basidiomycota*, each representing a single isolate, while the rest of the isolates comprised of ascomycetous fungi. Among the isolated strains, ten isolates could not be assigned to a genus as they displayed a maximum sequence similarity of 95 % or less with taxonomically characterized organisms. Among the host plants, the conifers, *Cedrus deodara*, *Pinus roxburgii*, and *Abies pindrow*, harbored the most diverse fungi, belonging to 13 different genera, which represented almost half of the total genera isolated. Several extracts prepared from the fermented broth of these fungi demonstrated strong bioactivity against *E. coli* and *S. aureus* with the lowest IC50 of 18 μg/ml obtained with the extract of *Trichophaea abundans* inhabiting *Pinus* sp. In comparison, extracts from only three endophytes were significantly inhibitory to *Candida albicans*, an important fungal pathogen. Further, 24 endophytes inhibited three or more phytopathogens by at least 50 % in coculture, among a panel of seven test organisms. Extracts from 17 fungi possessed

immunomodulatory activities with five of them showing significant immune suppression as demonstrated by the in vitro lymphocyte proliferation assay. This study was an important step towards tapping the endophytic fungal diversity from the Western Himalayas and assessing their bioactive potential. Further studies on the selected endophytes may lead to the isolation of novel natural products for use in medicine, industry, and agriculture.

Calotropis gigantea (L.) R.Br., a widely used medicinal plant in India, was exploited for endophytes as a possible source of bioactive secondary metabolites by Selvanathan et al. (2011). About 700 segments from 10 plants of *Calotropis gigantea*, collected from different locations of Guindy Campus, University of Madras during the year 2009–2010, were processed for the presence of endophytic fungi. A total of 13 fungal species, viz., *Aspergillus niger*, *Aspergillus flavipes*, *Alternaria porri*, *Curvularia lunata*, *Fusarium oxysporum*, *Nigrospora sphaerica*, *Colletotrichum falcatum*, *Pestalotiopsis sydowiana*, *Phoma exigua*, *Phomopsis archeri*, *Leptosphaerulina chartarum*, and *Mycelia Sterilia*, were isolated and identified based on the morphology of the fungal culture and characteristics of the spores.

The genus *Acacia* comprises over 1,300 species of which nearly 1,000 are found in Australia. *Acacia* species are used widely as food (e.g., seeds are ground into flour and the gum is edible), and the wood has been traditionally made into clubs, spears, boomerangs, and shields. Several species are used as narcotics and painkillers, to treat headaches, cold, and fevers, and as antiseptics and bactericides, to treat skin disorders by the indigenous people of Australia. While there is some information available about the medicinal properties of *Acacia*, there is no information about the endophytic microorganisms of these plants. With increased need for new bioactive compounds with medical, industrial, or biotechnological applications, Tran et al. (2010) investigated the bioactive properties of fungal endophytes of *Acacia* species. Specifically, they isolated endophytic fungi from the phyllodes of *Acacia baileyana*, *Acacia podalyriifolia*, and *Acacia floribunda*. These were classified as *Aureobasidium*, *Chaetomium*, and *Sordariomycetes* through genetic analysis of ribosomal RNA genes. The bioactivity of the fungal endophytes was examined, and a number of isolates exhibited antibacterial and antifungal properties. Other isolates also exhibited amylase activity and were thus able to hydrolyze starch. This study showed that fungal endophytes are readily isolated from the phyllodes of *Acacia* species and that these exhibit promising bioactive properties. Thus, endophytes from Australian native plants may be a useful source of novel bioactive compounds.

Glycine max (L.) Merr, a widely used agricultural and pharmaceutical plant in India, was exploited for endophytes as a possible source of bioactive secondary metabolites by Tenguria and Firodiya (2013). All isolates were identified based on colony morphology and examination of spores and fruiting bodies using stereo and light microscopes. Total 118 endophytic fungi of nine genera were isolated from 200 segments of fresh *Glycine max* (L.) leaves, collected from central region of Madhya Pradesh, India. The endophytic fungi recovered belong to ascomycetes (4.26 %), coelomycetes (18.64 %), hyphomycetes (65.23 %), and sterile mycelium

(11.86 %) each. The most dominant endophytes were *Alternaria* (25.42 %), *Phoma* sp. (18.64 %), *Fusarium* sp. (15.24 %), and *Penicillium* sp. (12.71 %).

First complete information on occurrence, distribution, and diversity of endophytic fungi associated with organs of *Butea monosperma* was presented by Tuppad and Shishupala (2013). Seventy-three endophytic fungal isolates belonging to genera *Aspergillus*, *Cladosporium*, *Curvularia*, *Fusarium*, *Pithomyces*, *Scopulariopsis*, *Colletotrichum*, *Chaetomium*, *Papulaspora*, and *Sclerotium* and three different morphotypes were found in different tissues. *Colletotrichum* sp. was dominant in most of the plant parts with relative frequency of 21.9 %. Isolates belonging to Sclerotium sp. had relative frequency of 13.6 %. Endophytic fungal diversity appeared maximum in stem and lamina samples. Frequency of occurrence of endophytic fungi differed greatly in different plant parts. Extent of similarity in endophytic fungal colonization was maximum between stem and lamina as indicated by Jaccards coefficient. Differential distribution of fungi in various tissues of *B. monosperma* was evident.

Tropical and subtropical plants are rich in endophytic community diversity. Endophytes, mainly fungi and bacteria, inhabit the healthy plant tissues without causing any damage to the hosts. These fungi can be useful for biological control of pathogens and plant growth promotion. Some plants of the genus *Piper* are hosts of endophytic microorganisms; however, there is little information about endophytes on *Piper hispidum*, a medicinal shrub used as an insecticide, astringent, diuretic, stimulant, liver treatment, and for stopping hemorrhages. Orlandelli et al. (2012) isolated the fungal endophyte community associated with *P. hispidum* leaves from plants in a Brazilian forest remnant. The endophytic diversity was examined based on the sequencing of the ITS1-5.8S-ITS2 region of rDNA. A high colonization frequency was obtained, as expected for tropical angiosperms. Isolated endophytes were divided into 66 morphogroups, demonstrating considerable diversity. They identified 21 isolates, belonging to 11 genera (*Alternaria*, *Bipolaris*, *Colletotrichum*, *Glomerella*, *Guignardia*, *Lasiodiplodia*, *Marasmius*, *Phlebia*, *Phoma*, *Phomopsis*, and *Schizophyllum*); one isolate was identified only to the order level (*Diaporthales*). *Bipolaris* was the most frequent genus among the identified endophytes. Phylogenetic analysis confirmed the molecular identification of some isolates to genus level, while for others it was confirmed at the species level.

Endophytic fungi from medicinal plants are important due to their ability to produce a variety of novel bioactive compounds possibly those produced by their host plant. In a study carried out by Agarkar (2013), the endophytic fungus isolated from the medicinal plant *Ocimum sanctum* Linn. was identified as *Colletotrichum* species based on its morphological characteristics. Further, antimicrobial activity of the ethyl acetate extract of endophytic *Colletotrichum* sp. was tested against five different human pathogenic bacteria and a fungus *Candida guilliermondii*. The extract was effective against all test pathogens, and significant activity was observed against *Salmonella typhi* and *Candida guilliermondii*. In case of *C. guilliermondii*, the combined effect of extract and standard antibiotic was

enhanced greatly showing synergistic activity. Thus, endophytic *Colletotrichum* sp. is a promising fungus for obtaining novel antimicrobial agent.

Dendrobium spp. are traditional Chinese medicinal plants, and the main effective ingredients (polysaccharides and alkaloids) have pharmacological effects on gastritis infection, cancer, and antiaging. Previously, we confirmed endophytic xylariaceous fungi as the dominant fungi in several *Dendrobium* species of tropical regions from China. In the study carried out by Chen et al. (2013), the diversity, taxonomy, and distribution of culturable endophytic xylariaceous fungi associated with seven medicinal species of *Dendrobium* (*Orchidaceae*) were investigated. Among the 961 endophytes newly isolated, 217 xylariaceous fungi (morphotaxa) were identified using morphological and molecular methods. The phylogenetic tree constructed using nuclear ribosomal internal transcribed spacer (ITS), large subunit of ribosomal DNA (LSU), and beta-tubulin sequences divided these anamorphic xylariaceous isolates into at least 18 operational taxonomic units (OTUs). The diversity of the endophytic xylariaceous fungi in these seven *Dendrobium* species was estimated using Shannon and evenness indices, with the results indicating that the dominant *Xylariaceae* taxa in each *Dendrobium* species were greatly different, though common xylariaceous fungi were found in several *Dendrobium* species. These findings implied that different host plants in the same habitats exhibit a preference and selectivity for their fungal partners. Using culture-dependent approaches, these xylariaceous isolates may be important sources for the future screening of new natural products and drug discovery.

In a study carried out by Paul et al. (2006), endophytic fungi were isolated from healthy leaf and root samples of *Taraxacum coreanum*. Of the 72 isolates recovered, 39 were from leaves and 33 from roots with an isolation frequency of 54 % and 46 %, respectively. Based on ITS sequence analysis, 72 isolates were classified into 19 genera of which 17 were under the phylum *Ascomycota* and 2 were under *Basidiomycota*. Diverse genera were found and *Alternaria*, *Cladosporium*, *Fusarium*, and *Phoma* were dominant. Out of 19 genera, *Apodus*, *Ceriporia*, *Dothideales*, *Leptodontidium*, *Nemania*, *Neoplaconema*, *Phaeosphaeria*, *Plectosphaerella*, and *Terfezia* were new to Korea. Seventy-two isolates were screened for antifungal activity, of which 10 isolates (14 %) were found active at least against one of the tested fungi. Isolate 050603 had the widest antifungal spectra of activity, and isolates 050592 and 050611 were active against three plant pathogenic fungi.

Jatropha curcas L., a perennial plant grown in the tropics and subtropics is popularly known for its potential as biofuel. The plant is reported to survive under varying environmental conditions having tolerance to stress and an ability to manage pest and diseases. The plant was explored for its endophytic fungi for use in crop protection by Kumar and Kaushik (2013). Endophytic fungi were isolated from the leaf of *Jatropha curcas*, collected from New Delhi, India. Four isolates were identified as *Colletotrichum truncatum*, and other isolates were identified as *Nigrospora oryzae*, *Fusarium proliferatum*, *Guignardia cammillae*, *Alternaria destruens*, and *Chaetomium* sp. Dual plate culture bioassays and bioactivity assays of solvent extracts of fungal mycelia showed that isolates of *Colletotrichum truncatum* were effective against plant pathogenic fungi *Fusarium oxysporum* and

Sclerotinia sclerotiorum. Isolate EF13 had the highest activity against *S. sclerotiorum*. Extracts of active endophytic fungi were prepared and tested against *S. sclerotiorum*. Ethyl acetate and methanol extract of *C. truncatum* EF10 showed 71.7 % and 70 % growth inhibition, respectively. Hexane extracts of *C. truncatum* isolates EF9, EF10, and EF13 yielded oil, and the oil from EF10 was similar to the oil of the host plant, i.e., *J. curcas*.

17.4 Biodiversity of Endophytic Bacteria and *Actinomycetes*

A total of 18 endophytic bacteria and 32 phyllosphere bacteria were isolated from the herbal plants of *Citrus* sp., *Pluchea indica*, *Curcuma longa*, *Nothopanax scutellarium*, *Piper crocatum*, and *Andrographis paniculata* by Soka et al. (2012) from Indonesia. About 72 % of endophytic bacteria isolates have proteolytic activity, and about 11 % have lipolytic activity. On the other hand, about 59 % of phyllosphere bacteria isolates have proteolytic activity, and about 19 % have lipolytic activity. Phylogenetic diversity analysis was conducted by using the amplified ribosomal DNA restriction analysis (ARDRA) method, and the sequence of 16S rDNA was digested with endonuclease restriction enzymes: MspI, RsaI, and Sau961. The diversity of endophytic and phyllosphere bacterium from the samples of herbal plants was high. Bacteria isolated from the same herbal plant do not always have a close genetic relationship except for the bacteria isolated from the *P. indica* plant which showed a close genetic relationship with each other.

The study carried out by Baker et al. (2012) uncapped the bacterial endophytes inhabiting the stems and roots of *Mimosa pudica* L. located in the southern parts of India. The screening resulted in isolation of 141 myriad bacterial endophytes with different morphological characteristics. The endophytes isolated in the study could be exploited for pharmaceutical research.

In traditional medicine, *Tridax procumbens* Linn. is used in the treatment of injuries and wounds. The bacterial endophytes of medicinal plants could produce medicinally important metabolites found in their hosts, and hence, the involvement of bacterial endophytes in conferring wound healing properties to *T. procumbens* cannot be ruled out. But, we do not know which types of bacterial endophytes are associated with *T. procumbens*. The aim of study carried out by Praveena and Bhore (2013) was to investigate the fast growing and cultivable bacterial endophytes associated with *T. procumbens*.

Leaves and stems of healthy *T. procumbens* plants were collected and cultivables. Bacterial endophytes were isolated from surface-sterilized leaf and stem tissue samples using Luria–Bertani (LB) agar (medium) at standard conditions. A polymerase chain reaction was employed to amplify 16S rRNA-coding gene fragments from the isolates. Cultivable endophytic bacterial isolates were identified using 16S rRNA gene nucleotide sequence similarity-based method of bacterial identification.

Altogether, 50 culturable endophytic bacterial isolates were isolated. 16S rRNA gene nucleotide sequence analysis using the Basic Local Alignment Search Tool (BLAST) revealed identities of the endophytic bacterial isolates. Analysis revealed that cultivable *Bacillus* spp., *Cronobacter sakazakii*, *Enterobacter* spp., *Lysinibacillus sphaericus*, *Pantoea* spp., *Pseudomonas* spp., and *Terribacillus saccharophilus* are associated with *T. procumbens*. Based on the results, we conclude that 24 different types of culturable BEs are associated with traditionally used medicinal plant, *T. procumbens*, and require further study.

The diversity and beneficial characteristics of endophytic microorganisms have been studied in several host plants. However, information regarding naturally occurring seed-associated endophytes and vertical transmission among different life-history stages of hosts is limited. Endophytic bacteria were isolated from seeds and seedlings of 10 *Eucalyptus* species and two hybrids by Ferreira et al. (2008). The results showed that endophytic bacteria, such as *Bacillus*, *Enterococcus*, *Paenibacillus*, *and Methylobacterium*, are vertically transferred from seeds to seedlings. In addition, the endophytic bacterium *Pantoea agglomerans* was tagged with the gfp gene, inoculated into seeds, and further reisolated from seedlings. These results suggested a novel approach to change the profile of the plants, where the bacterium is a delivery vehicle for desired traits. This is the first report of an endophytic bacterial community residing in *Eucalyptus* seeds and the transmission of these bacteria from seeds to seedlings. The bacterial species reported in this work have been described as providing benefits to host plants. Therefore, we suggest that endophytic bacteria can be transmitted vertically from seeds to seedlings, assuring the support of the bacterial community in the host plant.

The association of endophytic bacteria with their plant hosts has a beneficial effect for many different plant species. Taghavi et al. (2009) identified endophytic bacteria that improve the biomass production and the carbon sequestration potential of poplar trees (*Populus* spp.) when grown in marginal soil and to gain an insight in the mechanisms underlying plant growth promotion. Members of the *Gammaproteobacteria* dominated a collection of 78 bacterial endophytes isolated from poplar and willow trees. As representatives for the dominant genera of endophytic *Gammaproteobacteria*, we selected *Enterobacter* sp. strain 638, *Stenotrophomonas maltophilia* R551-3, *Pseudomonas putida* W619, and *Serratia proteamaculans* 568 for genome sequencing and analysis of their plant growth-promoting effects, including root development. Derivatives of these endophytes, labeled with *gfp*, were also used to study the colonization of their poplar hosts. In greenhouse studies, poplar cuttings (*Populus deltoides* × *Populus nigra* DN-34) inoculated with *Enterobacter* sp. strain 638 repeatedly showed the highest increase in biomass production compared to cuttings of noninoculated control plants. Sequence data combined with the analysis of their metabolic properties resulted in the identification of many putative mechanisms, including carbon source utilization, that help these endophytes to thrive within a plant environment and to potentially affect the growth and development of their plant hosts. Understanding the interactions between endophytic bacteria and their host plants should ultimately result in the

design of strategies for improved poplar biomass production on marginal soils as a feedstock for biofuels.

Wang et al. (2010) isolated four new *p*-aminoacetophenonic acid antibiotic from endophytic *Streptomyces* sp. HK10552 of the mangrove plant *Aegiceras corniculatum*. *Streptomyces albidoflavus* isolated from mangrove plants were able to produce antimycin A18 which showed broad spectrum of activity against pathogenic microorganisms (Yan et al. 2010). Vollmar et al. (2009) isolated a *Streptomyces* sp. *GS DV232* from traditional Chinese medicinal plants which was reported to produce an alkaloid, 4-methyl-2-quinazolinamine, and exhibited antiproliferative bioactivity. *Streptomyces aureofaciens* CMYAc130 isolated from *Zingiber officinale* Rose by Taechowisan et al. (2005) reportedly produced antifungal and antitumour compound 4-arylcoumarins (5,7-dimethoxy-4-*p*-methoxylphenylcoumarin (1), 5,7-dimethoxy-4-phenylcoumarin (2)). These compounds were found to exhibit inhibitory effect on s.c. transplanted Lewis lung carcinoma (LLC) BDF-1 mice by intraperitoneal administration. The T/C value of 5,7-dimethoxy-4-phenylcoumarin was found to be 80.8 and 50.0 % at the doses of 1 and 10 mg kg^{-1}. These two antitumor compounds exhibited low toxicity in human cell lines and are potentially active in malignant cell lines, *Streptomyces* sp. A35-1 (NRRL 30566) isolated from *Grevillea pteridifolia* by Castillo et al. (2003) produced broad-spectrum antibiotic kakadumycins. This quinoxaline compound-related antibiotic was found to be more effective against plant pathogenic fungi including *Botrytis* sp., *Alternaria* sp., *Helminthosporium* sp., and *Pythium ultimum*. They were found to be potentially active against various drug-resistant pathogenic bacteria and inhibitory against malaria parasite *Plasmodium falciparum* with an IC50 of 4.5 ng ml^{-1}.

Castillo et al. (2002) isolated *Streptomyces* NRRL 30562 from *Kennedia nigricans* which reportedly produced broad-spectrum active munumbicins A, B, C, and D. These all were highly active against *Bacillus anthracis*, multidrug-resistant *Mycobacterium tuberculosis*, methicillin-resistant *Staphylococcus aureus*, and plant pathogenic fungal pathogens.

A novel actinomycete strain, designated YIM 61105T, was isolated from a leaf of *Maytenus austroyunnanensis* from the tropical rain forest in Xishuangbanna, Yunnan Province, southwest China, by Qin et al. (2009b). A 16S rRNA gene sequence analysis revealed that the organism belonged to the phylogenetic cluster of the genus *Nonomuraea* and was most closely related to *Nonomuraea candida* HMC10T (98.2 %), *Nonomuraea aegyptia* S136 (97.9 %), *Nonomuraea kuesteri* GW 14-1925T (97.5 %), and *Nonomuraea turkmeniaca* DSM 43926T (97.4 %). The 16S rRNA gene sequence similarities to other *Nonomuraea* species were less than 97.4 %. The main chemotaxonomic properties of strain YIM 61105T, such as the principal amino acid of the peptidoglycan, the predominant menaquinone, and the polar lipid profile, supported its classification within the genus *Nonomuraea*. Strain YIM 61105T was also readily differentiated from closely related species on the basis of a broad range of phenotypic properties and DNA–DNA hybridization values. Thus, this isolate was considered to represent a novel species of the genus

Nonomuraea, for which the name *Nonomuraea antimicrobica* sp. nov. was proposed.

Endophytic Actinobacteria are relatively unexplored as potential sources of novel species and novel natural products for medical and commercial exploitation. Xishuangbanna is recognized throughout the world for its diverse flora, especially the rain forest plants, many of which have indigenous pharmaceutical histories. However, little is known about the endophytic Actinobacteria of this tropical area. In a study carried out by Qin et al. (2009a), they studied the diversity of Actinobacteria isolated from medicinal plants collected from tropical rain forests in Xishuangbanna. By the use of different selective isolation media and methods, a total of 2,174 Actinobacteria were isolated. Forty-six isolates were selected on the basis of their morphologies on different media and were further characterized by 16S rRNA gene sequencing. The results showed an unexpected level of diversity, with 32 different genera. This was the first report describing the isolation of *Saccharopolyspora*, *Dietzia*, *Blastococcus*, *Dactylosporangium*, *Promicromonospora*, *Oerskovia*, *Actinocorallia*, and *Jiangella* species from endophytic environments. At least 19 isolates are considered novel taxa. In addition, all 46 isolates were tested for antimicrobial activity and were screened for the presence of genes encoding polyketide synthetases and nonribosomal peptide synthetases. The results confirmed that the medicinal plants of Xishuangbanna represent an extremely rich reservoir for the isolation of a significant diversity of Actinobacteria, including novel species, which are potential sources for the discovery of biologically active compounds.

17.5 Future Prospectives

Endophytes, found ubiquitous in all plant species in the world, contribute to their host plants by producing plenty of substances that provide protection and ultimately survival value to the plant. Many researches have proven that endophyte is a new and potential source of novel natural products for exploitation in modern medicine, agriculture, and industry. So far, a great number of novel natural products possessing antimicrobial, antioxidant, immunosuppressant, and anticancer activities have been isolated from endophytes. It is believed that screening for bioactive compounds from endophytes is a promising way to overcome the increasing threat of drug-resistant strains of human and plant pathogen. The bioactive substances isolated from endophytes belong to diverse structural classes, including alkaloids, peptides, steroids, terpenoids, phenols, quinones, and flavonoids. These achievements would provide the opportunity to utilize endophytes as a new source for production of new drugs from the medicinal plants globally.

17.6 Conclusions

Endophytes are ubiquitous with rich biodiversity, which have been found in every plant species estimated to date. In this view of the special colonization in certain hosts, it is estimated that there may be as many as one million different endophyte species. However, only a handful of them have been described, which means that the opportunity to find new and targeting natural products from interesting endophytic microorganisms among myriads of plants in different niches and ecosystem is great. Some of the endophytes are the chemical synthesizers inside the plants. It is noteworthy that, of the nearly 300,000 plant species that exist, each individual plant is host to one or more endophytes. Only a few of these plants have been completely studied for their endophytic biology. Accordingly, an opportunity to find new and beneficial endophytic microorganisms among the diversity of plants in different ecosystems is considerable. Currently, endophytes looked upon as a prominent source of bioactive natural products. It appears that these biotypical factors can be important in plant selection, since they may govern the novelty and biological activity of the products associated with endophytic microbes. Research on endophytes is burgeoning immense importance since recent years with almost all plants harboring untold number of microorganisms as endophytes. Endophytic plethoras are reported to secrete unique novel metabolites bearing therapeutic properties which are being constantly exploited. The emergence of antibiotic-resistant microorganisms calls for inventive research and development strategies. Inhibition of these pathogenic microorganisms may be a promising therapeutic approach. The screening of antimicrobial compounds from endophytes is a promising way to meet the increasing threat of drug-resistant strains of human and plant pathogens.

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Chapter 18
Molecular Approach to Study Soil Bacterial Diversity

Satwant Kaur Gosal and Amita Mehta

18.1 Introduction

The biosphere is dominated by microorganisms which sustain $4–6 \times 10^{30}$ prokaryotic cells (Whitman et al. 1998). The large number of microorganisms is an essential component of the earth's biota which represents unexplored reservoir of genetic diversity. The microorganisms present in soil represent bacteria, fungi, actinomycetes, protozoans, and viruses. These microorganisms play key role in maintaining biogeochemical cycles, soil structure formation, decomposition of organic matter, and the microbiological characteristics of soils and act as indicator of soil health because of the relationship between microbial diversity, soil and plant quality, and ecosystem sustainability. The information theory defines diversity as the amount and distribution of information in an assemblage of community (Torsvik et al. 1998). In other words, microbial diversity refers to biological diversity within species, species number, and community diversity (Harpole 2010). Diversities at different levels of resolution have been distinguished as α, β, and γ-diversity, where α-diversity represents diversity within a local habitat, β-diversity represents the changes of species composition along a gradient, and γ-diversity represents microbial diversity over a region comprising many different habitats. Diversity index provides us a measure of overall diversity in the biological systems. Primary indices give the numbers of taxa in a community, whereas secondary or composite indices are based on two components, i.e., richness and evenness. The richness component describes the number of taxa in a community and the evenness component describes how evenly distributed the individuals are among the taxa.

As soil bacteria are responsible for the majority of biogeochemical processes in soil, the assessment of soil microbial community structure and function through the

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analysis of DNA/RNA molecules extracted from soil is of fundamental importance so as to understand the soil environment as an ecological system. The culturable bacteria represent a minor fraction of the total bacterial population (Giovannoni et al. 1990). So, the work on both the culturable as well as the non-culturable bacteria from different environments needs to be continued. The lack of adequate knowledge about the extant and extinct bacteria is another reason for the study of microbial diversity. Our knowledge of diversity existing within natural microbial communities is partially limited by the inability to study microorganisms as a very low percentage of bacteria can be cultured by standard laboratory methods (Kirk et al. 2004). Bacteria can exchange and acquire genes from distantly related organisms by horizontal gene transfer (HGT), consequently increasing rates of speciation. There is no consensus on how many species exist in the world, the potential usefulness of most of them, or the rate at which they are disappearing or emerging. The capability of an ecosystem to resist extreme perturbations or stress conditions can be dependent on the diversity that exists within the system. Diversity analyses are important in order to:

- Comprehend the distribution of organisms and the diversity of genetic resources
- Increase the knowledge of the functional role of diversity
- Identify differences in diversity associated with management disturbing
- Infer the regulation of biodiversity

As indicated, the cultivation-dependent methods will only reveal information about the soil bacteria that are able to grow under the conditions used. Direct microscopic studies circumvent the biases of culturing and provide a more accurate measure of the microbial diversity in soil, in terms of the numbers of organisms present. Due to the non-culturability of the major fraction of bacteria from natural microbial communities, the overall structure of the community has been difficult to interpret (Dokić et al. 2010). Moreover, the information contained in the nucleic acids can be used to address diversity at different levels from the community to within species level. The information contained in nucleic acids can be used to address diversity at different levels. The molecular-phylogenetic perspective is a reference framework within which microbial diversity is described; the sequences of genes can be used to identify organisms (Amann et al. 1995). The collection of all genomes of bacteria in a soil sample can be considered to represent one large soil microbial community genome, a microbial "metagenome." The genomes of the organisms in a soil community contain all information about the diversity in that community. Recent improvements in techniques that allow us to survey the diversity of microbial communities have revolutionized in our understanding of microbial diversity. For many decades, microbiologists had grossly underestimated microbial diversity levels by relying on cultivation-based techniques, which capture only a few microbial taxa, which could grow under artificial laboratory conditions (Pace 1997; Rappé and Giovannoni 2003). With few obvious morphological differences delineating most microbial taxa, direct microscopic analyses of environmental samples are of little use for quantifying microbial diversity (Fierer and Lennon 2011). The use of high-throughput nucleic-acid-based analyses of

microbial communities enables researchers to study the dynamics of microbial diversity in specific habitats, the spatial and temporal variability in the levels of microbial diversity, and the factors driving this variability (Christen 2008; Hamady and Knight 2009; Hirsch et al. 2010).

18.2 Factors Governing Microbial Diversity

Ecologists have been busy in describing and systemizing the biodiversity on Earth. Despite best efforts, we still lack sturdy estimates of species richness for the majority of taxa present in the ecosystems. It is often challenging to determine the factors that affect the patterns of species diversity in time and space (Pennisi 2005). Many different organisms may perform the same processes and probably be found in the same niches in a bacterial community (Zhao et al. 2012). The factors affecting microbial diversity can be classified into two groups, i.e., abiotic factors and biotic factors. Abiotic factors include both physical and chemical factors such as soil texture, salinity, oxic/anoxic conditions, temperature, pH, pressure, available NPK, heavy metals, pesticides, antibiotics, and water availability (Bååth et al. 1998). Ammoniacal and nitrate nitrogen contents of soils are important factors which influence the diazotrophic count (Gosal et al. 2011). In general, all environmental variations affect bacteria in different ways and to different extent, resulting in a shift in the diversity profile. Biotic factors include plasmids, phages, and transposons that are types of accessory DNA that influence the genetic properties and, in most cases, the phenotypes of their host and, thus, have a great influence on microbial diversity (Zhao et al. 2012).

Furthermore, many basic questions remain unanswered, including why some habitats have more species than other habitats, and what are the abiotic, biotic, ecological, and evolutionary forces that determine how many species can be found in a given set of environmental conditions? This is particularly true for many microbial taxa as we often lack even a rudimentary understanding of their diversity patterns.

The vast majority of bacterial communities in nature have not been cultured in the laboratory. Therefore, the primary source of information for these uncultured but viable organisms is their biomolecules such as nucleic acids, lipids, and proteins. Culture-independent nucleic acid approaches include analyses of whole genomes or selected genes such as 16S and 18S ribosomal RNA (rRNA) for prokaryotes and eukaryotes, respectively. Based on the comparative analyses of these rRNA signatures, cellular life has been classified into three primary domains: one eukaryotic (Eukarya) and two prokaryotic (Bacteria and Archaea) (Hugenholtz 2002). Over the last few decades, microbial ecology has seen tremendous progress, and a wide variety of molecular techniques have been developed for describing and characterizing the phylogenetic and functional diversity of microorganisms. Broadly, these techniques have been classified into two major categories depending on their capability of revealing the microbial diversity structure and function:

(1) partial community analysis methods and (2) whole community analysis methods.

Partial community analysis methods generally include polymerase chain reaction (PCR)-based methods where total DNA/RNA extracted from an environmental sample is used as a template for the characterization of microorganisms. The PCR product thus generated reflects a mixture of bacterial gene signatures from all organisms present in a sample, including the VBNC fraction. PCR amplification of conserved genes such as 16S rRNA from an environmental sample has been used extensively in bacterial ecology primarily because these genes (1) are ubiquitous, i.e., present in all prokaryotes, (2) are structurally and functionally conserved, and (3) contain variable and highly conserved regions (Hugenholtz 2002). Sequence analysis of 16S rRNA genes is commonly used in most microbial ecological surveys. However, being a highly conserved molecule, the 16S rRNA gene does not provide sufficient resolution at species and strain level (Konstantinidis et al. 2006). The libraries of PCR-amplified 16S rRNA genes do not always represent complete picture of bacterial community (Dokić et al. 2010). The whole community analysis offers a more comprehensive view of genetic, metabolic, and phylogenetic diversity stored in soil metagenome as compared to PCR-based molecular approaches that target only a single or few genes. These techniques attempt to analyze all the genetic information present in total DNA extracted from a soil sample.

18.3 Genetic Fingerprinting Techniques

Genetic fingerprinting generates a profile of microbial communities based on direct analysis of PCR products amplified from environmental DNA (Muyzer 1999). These techniques include DGGE/TTGE, SSCP, RAPD, ARDRA, T-RFLP, LH-PCR, RISA, and RAPD and produce a community fingerprint based on either sequence polymorphism or length polymorphism. In general, genetic fingerprinting techniques are rapid and allow simultaneous analyses of multiple samples. Fingerprinting approaches have been devised to demonstrate an effect on bacterial communities or differences between microbial communities and do not provide direct taxonomic identities. The "fingerprints" from different samples are compared using computer assisted cluster analysis by software packages such as GelCompar, and community relationships are inferred. Community fingerprints are scored as present or absent, and the similarities among samples are determined using Jaccards' coefficient.

The general principle of most molecular techniques relies on the electrophoretic separation of a pool of PCR products amplified from DNA or RNA directly extracted from soil. The difference in the sequences of amplified gene can be used for separation based on:

- Different melting behavior of the double-stranded PCR products due to differences in the primary structures of the target gene fragments using denaturing gradient gel electrophoresis or temperature gradient gel electrophoresis
- Different localization of restriction endonuclease digestion sites along the investigated gene using terminal restriction length polymorphism, restriction fragment length polymorphism, or amplified ribosomal DNA restriction analysis
- Different electrophoretic mobilities of single DNA strands in non-denaturing gels using single strand conformational polymorphism analysis
- Length polymorphism of entire gene fragments using length heterogeneity PCR or ribosomal intergenic spacer analysis

18.4 Assessment of Soil Microbial Diversity Using Molecular Methods

The assessment of soil microbial diversity can be divided into three categories (Fig. 18.1) such as:

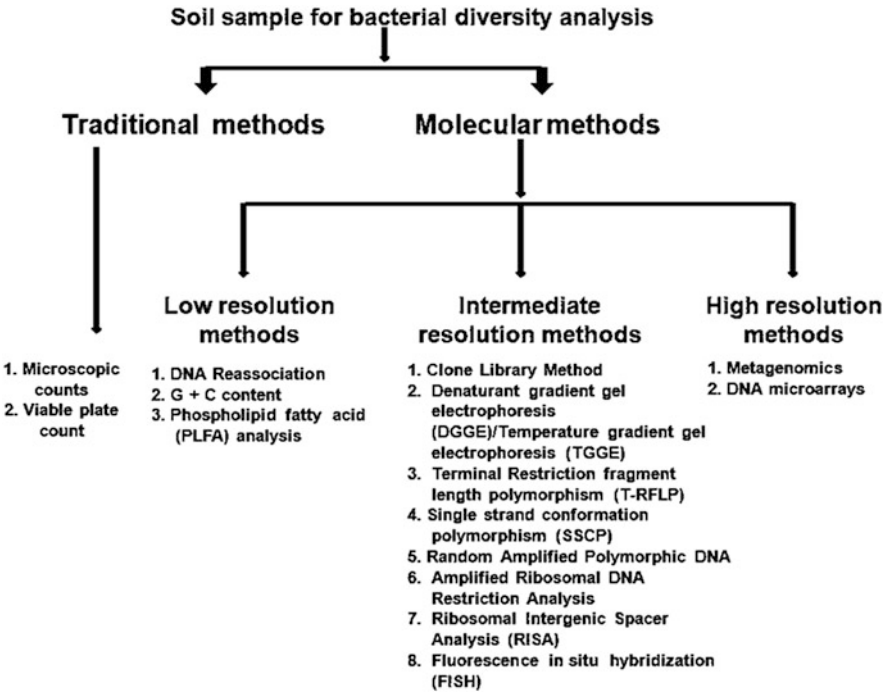

Fig. 18.1 Different methods for assessing bacterial diversity

1. Broad scale low resolution methods
2. Intermediate resolution methods
3. High resolution methods

18.4.1 Broad Scale, Low Resolution Methods

These include methods based on nucleic acids or some biochemical markers (fatty acids) as DNA reassociation or PLFA and LPS, respectively.

DNA Reassociation: In this method, total DNA is extracted from environmental samples, purified, denatured, and allowed to reanneal. DNA extracted from a microbial community is a mixture of DNA from different microbial taxa that are present in different proportions. The rate of hybridization or reassociation will depend on the similarity of sequences present. As the complexity or diversity of DNA sequences increases, the rate at which DNA reassociates will decrease (Theron and Cloete 2000). In other words, the kinetics of DNA reassociation in a sample reflects the variety of sequences present in the environment, thus reflecting the diversity of the microbial community of that environment. DNA reassociation estimates diversity by measuring the genetic complexity of the microbial community (Torsvik et al. 1996). The DNA reassociation rate can be used to calculate the genome size or genome complexity. The parameter controlling the reassociation reaction is concentration of DNA product (Co) and time of incubation (t), usually described as the half association value, $Cot_{1/2}$ (the time needed for half of the DNA to reassociate). Under specific conditions, $Cot_{1/2}$ can be used as a diversity index, as it takes into account both the amount and distribution of DNA re-association (Torsvik et al. 1998). Alternatively, the similarity between communities of two different samples can be studied by measuring the degree of similarity of DNA through hybridization kinetics (Griffiths et al. 1999). The community diversity comprises of the total amount of genetic information in the community (richness) and the distribution of this information among the different genetic types (evenness). Thus, the DNA reassociation method provides an estimate of the extent of diversity in prokaryotic communities.

G + C content: The diversity in soil bacterial communities can be assessed by studying the difference in guanine + cytosine content of DNA (Nüsslein and Tiedje 1999). Different groups of microorganisms differ in their G + C content and the taxonomically related groups of microorganisms differ by 3–5 % in their G + C content. The technique provides us with a fractionated profile of entire community that indicates relative abundance of DNA as a function of G + C content. The total DNA is separated into different fractions which can be analyzed by additional molecular techniques as DGGE and ARDRA to assess total community diversity. The technique is advantageous as it includes all the DNA extracted from soil but requires large amount of DNA, i.e., 50 μg (Tiedje et al. 1999).

Phospholipid Fatty Acid (PLFA) Analysis: PLFA analysis has been used as a culture-independent method for assessing the structure of soil microbial communities. PLFAs are important components of cell membranes and potentially useful

signature molecules. They constitute a significant proportion of organism biomass under natural conditions (Kozdroj and van Elsas 2001). Phospholipids are found exclusively in microbial cell membranes and not in other parts of the cell as storage products. Cell membranes, consisting of phospholipids, are rapidly degraded following cell death. Consequently, phospholipids can serve as important indicators of active microbial biomass as opposed to nonliving microbial biomass (Drenovsky et al. 2004). An essential consideration in the use of PLFA molecules to describe microbial communities is that unique fatty acids are indicative of specific groups of organisms. The changes in PLFA patterns under environmental stress conditions are useful biomarker tool to describe the community and physiological state of certain microbial taxa (Misko and Germida 2002). Our knowledge of such signature molecules comes from the use of fatty acid analysis for bacterial taxonomy, in which specific fatty acid methyl esters (FAMEs) have been used as an accepted taxonomic discriminator for species identification. Furthermore, PLFAs are easily extracted from microbial cells in soil (Tunlid and White 1992; Zelles and Bai 1993), allowing access to a greater proportion of the microbial community resident in soil than would otherwise be accessed during culture-dependent methods of analysis. Although direct extraction of PLFA from soil does not permit delineation down to species level, it is an efficient means by which gross changes in microbial community structure can be profiled (Nannipieri et al. 2003). The method allows the direct analysis of soil samples suitable to monitor changes in microbial community composition and can be used to assess specific microbial groups by measuring signature fatty acids. Despite the usefulness of this method, there are some important limitations (Haack et al. 1994).

Limitations

1. Appropriate signature molecules are not known for all organisms in a soil sample and, in a number of cases, a specific fatty acid present in a soil sample cannot be linked with a specific microorganisms or group of microorganisms. In general, the method cannot be used to characterize microorganisms to species.
2. Since the method relies heavily on signature fatty acids to determine gross community structure, any variation in these signatures would give rise to false community estimates created by artifacts in the methods.
3. Bacteria produce widely different amounts of PLFA and the types of fatty acids vary with growth conditions and environmental stresses. Although signature PLFAs can be correlated with the presence of some groups of organisms, they may not necessarily be unique to only those groups under all conditions. Consequently, this could give rise to false community signatures.

18.4.2 Intermediate Resolution Methods

Clone library and community finger printing techniques based on differences in conserved genes like rRNA gene are considered as intermediate resolution

methods. The 16S rRNA gene can be amplified from community DNA extracted from soil, cloned, and sequenced to determine genetic diversity within microbial community. They can be separated based on length, restriction pattern, or denaturing conformational properties. Hybridization using phylogenetic probes offers the possibility to perform specific in situ measurements. The probes are used to target specific rDNA sequences in community DNA or to probe colonies or single cells. Whole cell fluorescence hybridization or fluorescence in situ hybridization (FISH) using phylogenetic probes in combination with fluorescence microscope allows the simultaneous detection and quantification of single cells of different phylogenetic groups in same sample.

Clone Library Method: The most widely used method to analyze PCR products amplified from an environmental sample is to clone and then sequence the individual gene fragments (DeSantis et al. 2007). Sequencing of the clone library generated from environmental DNA has advantages over fingerprint-based methods, such as denaturing gradient gel electrophoresis, as it provides precise identification and quantification of the phylotypes present in samples (Hur and Chun 2004). The sequences are compared to known sequences in a database such as GenBank, Ribosomal Database Project (RDP), and Greengenes. Cloned sequences are assigned to phylum, class, order, family, subfamily, or species at sequence similarity cutoff values of 80, 85, 90, 92, 94, or 97 %, respectively (Rastogi et al. 2010). While clone libraries of 16S rRNA genes permit an initial survey of diversity and identify novel taxa, studies have shown that environmental samples like soil may require over 40,000 clones to document 50 % of the richness (Dunbar et al. 2002). However, typical clone libraries of 16S rRNA genes contain fewer than 1,000 sequences and therefore reveal only a small portion of the microbial diversity present in a sample. A cloning and sequencing method was used to decipher the microbial community composition in mining-impacted deep subsurface soils of the former Homestake gold mine of South Dakota, USA (Rastogi et al. 2009). Phylogenetic analysis of 230 clone sequences could reveal only a partial view of phylogenetic breadth present in soil samples. Rarefaction analyses of clone libraries generated non-asymptotic plots, which indicated that diversity was not exhaustively sampled due to insufficient clone sequencing, a common problem when assessing environmental microbial diversity using cloning approaches. Despite its limitations (e.g., labor-intensive, time-consuming, and cost factor), clone libraries are still considered the "gold standard" for preliminary microbial diversity surveys (DeSantis et al. 2007). With the advent of newer and inexpensive sequencing methods, great progress is expected in this method of microbial diversity analysis.

Denaturant Gradient Gel Electrophoresis (*DGGE*)/*Temperature Gradient Gel Electrophoresis* (*TGGE*): In denaturing gradient gel electrophoresis, DNA is extracted from environmental samples and amplified using primers for specific molecular markers such as 16S rRNA sequences. The PCR products are electrophoresed on a polyacrylamide gel containing a linear gradient of DNA denaturant such as a mixture of urea and formamide (Muyzer et al. 1993). Temperature gradient gel electrophoresis uses the same principle as DGGE except that a temperature gradient is provided rather than using chemical denaturants. DNA

fragments of same length but with different base pair sequences can be separated using DGGE or TGGE. The separation of different DNA molecules is based on the difference in mobility of partially melted DNA molecules in polyacrylamide gels which have a gradient of DNA denaturants. The DNA fragments will migrate according to their melting behavior under different denaturing conditions as chemical denaturants or temperature. The variation in sequences within the DNA fragments causes a difference in melting behavior and therefore amplicons or DNA fragments with different sequences stop migrating at different positions in the gel. The melting of the products occurs in different melting domains, which are stretches of nucleotides with identical melting temperatures (Mühling et al. 2008). In a linearly increasing denaturing gradient, DNA fragments migrating under the influence of an electric current remain double-stranded until they reach the denaturing conditions that cause melting of their lower temperature melting domains. As a result of this melting branching of the molecules occurs, which results in decreased mobility of molecules in the gel. The electrophoresis of mixed amplicons from a complex community results in fingerprinting consisting of bands at different migration distances in the gel. Both DGGE and TTGE involve the use of a GC clamped (30–50 nucleotides) forward primer during the PCR step. This is essential to prevent the two DNA strands from complete dissociation into single strands during electrophoresis. DNA sequences having a difference in only one base pair can be separated by DGGE (Miller et al. 1999). The DGGE/TGGE fingerprints are reliable, reproducible, rapid, and less expensive. They can be used to determine the phylogenetic identities by reamplification of the bands excised from the gel and blotting them onto nylon membranes and hybridizing them to molecular probes specific for different taxonomic groups. DGGE profiles have successfully been used to determine the genetic diversity of microbial communities inhabiting different temperature regions in a microbial community (Ferris et al. 1996), and to study the distribution of sulfate reducing bacteria in a stratified water column (Teske et al. 1996).

Despite certain advantages, the DGGE/TGGE holds some limitations as PCR biases (Wintzingerode et al. 1997), laborious sample handling (Muyzer 1999), and variable DNA extraction efficiency (Theron and Cloete 2000). DGGE can only detect 1–2 % of the microbial population representing dominant species present in an environmental sample (MacNaughton et al. 1996). DNA fragments of different sequences may have similar mobility characteristics in the polyacrylamide gel. Therefore, one band may not necessarily represent one species (Gelsomino et al. 1999) and one bacterial species may also give rise to multiple bands because of multiple 16S rRNA genes with slightly different sequences (Maarit-Niemi et al. 2001). DGGE/TGGE analysis of microbial communities produces a complex profile which can be quite sensitive to spatial and temporal sampling variation (Murray et al. 1998).

Terminal Restriction Fragment Length Polymorphism (*T-RFLP*): T-RFLP is an extension of RFLP/ARDRA analysis which provides an alternate method for rapid analysis of bacterial community diversity in various environments. It is based on the same principle as RFLP except that one PCR primer is labeled with a fluorescent

dye, such as TET (4,7,2′,7′-tetrachloro-6-carboxyfluorescein) or 6-FAM (phosphoramiditefluorochrome 5-carboxyfluorescein) during the PCR reaction. The PCR products are digested with restriction enzyme(s), and the fluorescently labeled terminal restriction fragments (T-RFs) are separated on an automated DNA sequencer (Thies 2007). The size, number, and peak height of T-RFs are used to estimate community diversity. Each unique fragment length can be counted as an Operational Taxonomic Unit (OTU) or ribotype and the frequency of each OTU can be calculated. The banding pattern can be used to measure species richness and evenness as well as similarities between samples (Liu et al. 1997). The technique helps in the analysis of complex bacterial communities as only fluorescently labeled terminal fragments are detected, thus simplifying the banding pattern (Marsh 1999). T-RFLP has also been thought to be an excellent tool to compare the relationship between different samples (Dunbar et al. 2000). T-RFLP has been used to measure spatial and temporal changes in bacterial communities (Lukow et al. 2000), to study complex bacterial communities (Moeseneder et al. 1999), and to detect and monitor populations (Tiedje et al. 1999). The recent developments in bioinformatics have provided us with several web-based programs to analyze T-RFLP patterns, which enable us to rapidly assign putative identities based on a database of fragments produced by known 16S rDNA sequences.

Despite the usefulness of T-RFLP in bacterial diversity analysis, it has some limitations. T-RFLP may underestimate true diversity as only numerically dominant species are detected due to large quantity of available DNA (Liu et al. 1997). Another limitation of T-RFLP is the choice of the universal primers. None of the presently available universal primers can amplify all sequences from eukaryote, bacterial, and archaeal domains. These primers are based on existing 16S rRNA, 18S rRNA, or Internal Transcribed Spacer (ITS) databases, which until recently contained mainly sequences from culturable microorganisms, and therefore may not be representative of the true bacterial diversity in a sample (Rudi et al. 2007). Different enzymes will produce different community fingerprints (Dunbar et al. 2000). The method underestimates community diversity because only a limited number of bands per gel (generally <100) can be resolved, and different bacterial species can share the same T-RF length. However, the method does provide a robust index of community diversity, and T-RFLP results are generally very well correlated with the results from clone libraries (Fierer and Jackson 2006).

18.4.2.1 Procedure for RFLP Analysis

Reagents

1. Lambda DNA (Hind III digest)
2. 100 bp ladder
3. 5× TBE buffer: 54 g Tris base, 20 ml of 0.5 M EDTA, pH 8.0, and 27.5 g of boric acid dissolved in 1 l of water
4. Ethidium bromide (5 mg ml^{-1})

5. Loading dye (6×) (0.25 % Bromophenol blue in 40 % sucrose w/v)
6. Restriction endonucleases (*Alu* 1, *Bsu*R 1, Msp 1, and *Rsa* 1) along with the buffer (10×)

Procedure

1. The reaction mixture for restriction analysis should be prepared as follows:

PCR product	12.3 µL
Restriction endonuclease (3U)	0.3 µL
Recommended buffer (for restriction enzyme)	1.4 µL
Total volume	14.0 µL

2. Keep the tubes in water bath maintained at 37 °C FOR 2 h.
3. Analyze the restricted DNA by horizontal electrophoresis in 3 % agarose gel. Carry out the electrophoresis at 100 V for 2 h. 30 min with the standard gels (11 × 14 cm).
4. Visualize the gels under UV and record the observations.

Single Strand Conformation Polymorphism (*SSCP*): SSCP also relies on electrophoretic separation based on differences in DNA sequences and allows differentiation of DNA molecules having the same length but different nucleotide sequences. This technique was originally developed to detect known or novel polymorphisms or point mutations in DNA (Peters et al. 2000). In SSCP, the environmental PCR products are denatured followed by electrophoretic separation of single-stranded DNA fragments on a nondenaturing polyacrylamide gel (Schwieger and Tebbe 1998). As formation of folded secondary structure or heteroduplex and hence mobility are dependent on the DNA sequences, this method reproduces an insight of the genetic diversity in a bacterial community. All the limitations of DGGE are also equally applicable for SSCP. Again, some single-stranded DNA can exist in more than one stable conformation. As a result, same DNA sequence can produce multiple bands on the gel (Tiedje et al. 1999). However, it does not require a GC clamp or the construction of gradient gels and has been used to study bacterial or fungal community diversity (Stach et al. 2001). Similar to DGGE, the DNA bands can be excised from the gel, reamplified, and sequenced. However, SSCP is well suited only for small fragments (between 150 and 400 bp) (Muyzer 1999). A major limitation of the SSCP method is the high rate of reannealing of DNA strands after an initial denaturation during electrophoresis, which can be overcome using a phosphorylated primer during PCR, followed by specific digestion of the phosphorylated strand with lambda exonuclease. SSCP has been used to measure succession of bacterial communities (Peters et al. 2000), rhizosphere communities (Schmalenberger et al. 2001), bacterial population changes in an anaerobic bioreactor (Zumstein et al. 2000), and AMF species in roots (Kjoller and Rosendahl 2000).

Random-Amplified Polymorphic DNA Fingerprinting: Random-amplified polymorphic DNA (RAPD) and DNA amplification fingerprinting (DAF) techniques

utilize PCR amplification with a short (usually ten nucleotides) primer, which anneals randomly at multiple sites on the genomic DNA under low annealing temperature, typically £35 °C (Franklin et al. 1999). These methods generate PCR amplicons of various lengths in a single reaction that are separated on agarose or polyacrylamide gel depending on the genetic complexity of the bacterial communities. Because of the high speed and ease of use, RAPD/DAF has been used extensively in fingerprinting overall bacterial community structure and closely related bacterial species and strains (Franklin et al. 1999). Both RAPD and DAF are highly sensitive to experimental conditions (e.g., annealing temperature, $MgCl_2$ concentration) and quality and quantity of template DNA and primers. Thus, several primers and reaction conditions need to be evaluated to compare the relatedness between bacterial communities and obtain the most discriminating patterns between species or strains. A RAPD profiling study was used with 14 random primers to assess changes in bacterial diversity in soil samples that were treated with pesticides (triazolone) and chemical fertilizers (ammonium bicarbonate) (Yang et al. 2000). RAPD fragment richness data demonstrated that pesticide-treated soil maintained an almost identical level of diversity at the DNA level as the control soil (i.e., without contamination). In contrast, chemical fertilizer caused a decrease in the DNA diversity compared to control soil.

Amplified Ribosomal DNA Restriction Analysis: Amplified ribosomal DNA restriction analysis (ARDRA) is based on DNA sequence variations present in PCR-amplified 16S rRNA genes (Smit et al. 1997). The PCR product amplified from environmental DNA is generally digested with tetracutter restriction endonucleases (e.g., *Alu*I, *Hae*III), and restricted fragments are resolved on agarose or polyacrylamide gels (Liu et al. 1997). Divergence of a community rRNA restriction pattern on a gel is highly influenced by the type of restriction enzyme used (Gich et al. 2000). Although ARDRA provides little or no information about the type of bacteria present in the sample, the method is still useful for rapid monitoring of bacterial communities over time, or to compare bacterial diversity in response to changing environmental conditions. ARDRA is also used for identifying the unique clones and estimating OTUs in environmental clone libraries based on restriction profiles of clones (Smit et al. 1997). The major limitation of ARDRA is that restriction profiles generated from complex bacterial communities are sometimes too difficult to resolve by agarose/PAGE (Kirk et al. 2004). Optimization is required to produce fingerprinting profiles characteristic of the bacterial community (Spiegelman et al. 2005). The ARDRA technique was applied for assessing the effect of copper contamination on the bacterial communities in soil. Whole community ARDRA profiles showed a lower diversity in copper-contaminated soil compared with control soil with no contamination (Smit et al. 1997).

Ribosomal Intergenic Spacer Analysis: RISA requires the extraction of genomic DNA of the total bacterial population from the soil sample. The method involves the PCR amplification of the selected DNA fragments with universal primers and subsequent electrophoresis on a polyacrylamide gel. RISA profiles can be generated from most of the dominant bacteria present in a sample by using primers for conserved regions in the 16S and 23S rRNA genes. It is useful for differentiating

between bacterial strains and closely related species because of heterogeneity of the IGS length and sequence (Fisher and Triplett 1999). RISA provides a community-specific profile, with each band corresponding to at least one organism in the original community.

The RISA technique has been enhanced by the addition of an automated component to the technique by using an automated genetic analyzer. The automated ribosomal intergenic spacer analysis (ARISA) method is an effective, rapid, and fairly inexpensive process that can be used to estimate the diversity and composition of bacterial communities without demonstrating a bias towards fast-growing or dominant species. This is especially useful in ecological studies, where a large number of samples need to be processed and diversity needs to be determined at a spatial and temporal level. It involves use of a fluorescence-labeled *****forward primer, and ISR fragments are detected automatically by a laser detector. ARISA allows simultaneous analysis of many samples; however, the technique has been shown to overestimate bacterial richness and diversity (Fisher and Triplett 1999). Ranjard et al. (2001) evaluated ARISA to characterize the bacterial communities from four types of soil differing in geographic origins, vegetation cover, and physicochemical properties. ARISA profiles generated from these soils were distinct and contained several diagnostic peaks with respect to size and intensity. Their results demonstrated that ARISA is a very effective and sensitive method for detecting differences between complex bacterial communities at various spatial scales (between- and within-site variability). Limitations of RISA include requirement of large quantities of DNA, relatively longer time requirement, insensitivity of silver staining in some cases, and low resolution (Fisher and Triplett 1999). ARISA has increased sensitivity than RISA and is less time consuming, but traditional limitations of PCR also applies for ARISA (Fisher and Triplett 1999). RISA has been used to compare bacterial diversity in soil (Borneman and Triplett 1997), in the rhizosphere of plants (Borneman and Triplett 1997), in contaminated soil (Ranjard et al. 2000), and in response to inoculation (Yu and Mohn 2001).

Length Heterogeneity (*LH*) *PCR*: LH-PCR analysis is similar to the commonly used T-RFLP method. The difference between these two methods is that the T-RFLP method identifies PCR fragment length variations based on restriction site variability, whereas LH-PCR analysis distinguishes different organisms based on natural variations in the length of 16S ribosomal DNA sequences (Ritchie et al. 2000). LH-PCR differentiates microorganisms on the basis of natural length polymorphisms which occur due to mutation within genes (Mills et al. 2007). Amplicon LH-PCR interrogates the hypervariable regions present in 16S rRNA genes and produces a characteristic profile. LH-PCR utilizes a fluorescent dye-labeled forward primer, and a fluorescent standard is run with each sample to measure the amplicon lengths in base pairs. The height or area under the peak in the electropherogram is proportional to the relative abundance of that particular amplicon. The advantage of using LH-PCR over the T-RFLP is that the former does not require any restriction digestion and therefore PCR products can be directly analyzed by a fluorescent detector. The limitations of LH-PCR technique include inability to resolve complex amplicon peaks and underestimation of

diversity, as phylogenetically distinct taxa may produce same-length amplicons (Mills et al. 2007). LH-PCR was used in combination with FAME analysis to investigate the bacterial communities in soil samples that differed in terms of type and/or crop management practices (Ritchie et al. 2000). LH-PCR results strongly correlated with FAME analysis and were highly reproducible and successfully discriminated different soil samples.

Fluorescence in situ Hybridization (*FISH*): FISH has been used for identification and quantification of microorganisms within their natural habitat (Amann et al. 1995; Kenzaka et al. 1998). Bacterial cells are hybridized with fluorescently labeled taxon-specific oligonucleotide probes and the cells are viewed by scanning confocal laser microscopy (Sanz and Kochling 2007). Hybridization with rRNA-targeted probes enhances the characterization of uncultured bacteria and also facilitates the description of complex bacterial communities (Edgeomb et al. 1999). The intensity of fluorescence is correlated to rRNA content of the cells and their growth rate, thus, provides information regarding metabolic state of the cells. FISH has certain advantages over immunofluorescence techniques as it can detect bacteria at all phylogenetic levels and it is more sensitive as nonspecific binding to soil does not take place (Amann et al. 1995). The fluorescing bacteria can be differentiated from autofluorescing soil particles and plant debris by using distinct florescent dyes (Macnaughton et al. 1996). For the analysis of mixed bacterial populations, FISH can be combined with flow cytometry. FISH use does not provide any insight to metabolic function of microorganisms. However, it can be coupled with other techniques such as microautoradiography to describe functional properties of microorganisms in their natural environment (Wagner et al. 2006). Many improvements have been made in FISH analysis to solve the problems associated with it: Bright fluorochromes, hybridization with the probes carrying multiple fluorochromes, treatment with chloramphenicol to increase the RNA content of the cells (Rogers et al. 2007), or addition of nutrients to stimulate bacterial activity (Hahn et al. 1992). The low signal intensity, target inaccessibility, and background fluorescence are the common problems associated with FISH analysis. The soil microorganisms should be in a metabolically active stage and their cell wall should be permeable to allow the penetration of probes (Christensen and Poulson 1994).

18.4.3 High Resolution Methods

The method with the highest level of resolution is based on sequencing of the entire soil metagenome followed by careful analysis of the functional genes. Soil metagenomic clone libraries can be used in combination with fingerprinting, hybridization, and sequencing techniques to assess the diversity of particular genes.

Metagenomics: Nowadays, one of the most widely used strategies for studying bacterial diversity is the metagenomic research. A metagenome is the entire genetic composition of bacterial communities of soil which is based on direct isolation of

total DNA in soil samples, construction of libraries, the amplification of 16S rRNA genes and functional genes to study the total diversity, physiology, ecology, and phylogeny of bacteria that cannot be cultivated in the laboratory (Lorenz and Schleper 2002; Rondon et al. 2000; Voget et al. 2003; Steele and Streit 2005; Streit and Schmitz 2004). Such investigations aim to reveal and understand the relationship between community composition and functional diversity in natural bacterial ecosystems.

Metagenomics is the investigation of collective bacterial genomes retrieved directly from environmental samples and does not rely on cultivation or prior knowledge of the bacterial communities (Riesenfeld et al. 2004). It is also known as environmental genomics or community genomics, or bacterial ecogenomics. Metagenomic research is useful to exploit the unknown bacterial diversity in different environments; it can be used to discover novel genes and to increase our knowledge on bacterial ecology and physiology (Cowan et al. 2005). The 16S rRNA gene accounts for a minor fraction of the average prokaryotic genome (Rodríguez-Valera 2002) and 16S rRNA gene sequences have been used as a phylogenetic marker to characterize uncultivated prokaryotes and can help to discover metabolic functions, enhancing our knowledge about bacterial ecology and phylogeny (Oremland et al. 2005; Riesenfeld et al. 2004; Tringe et al. 2005). We can use metagenomic sequences to help understand how complex bacterial communities function and how bacteria interact within these niches. Metagenomics aims at identifying novel genes and increasing our understanding of bacterial ecology.

Essentially, metagenomics does not include methods that interrogate only PCR-amplified selected genes (e.g., genetic fingerprinting techniques) as they do not provide information on genetic diversity beyond the genes that are being amplified. In principle, metagenomic techniques are based on the concept that the entire genetic composition of environmental bacterial communities could be sequenced and analyzed in the same way as sequencing a whole genome of a pure bacterial culture. Metagenomic investigations have been conducted in several environments such as soil, the phyllosphere, the ocean, and acid mine drainage and have provided access to phylogenetic and functional diversity of uncultured microorganisms (Handelsman 2004). Metagenomics is crucial for understanding the biochemical roles of uncultured microorganisms and their interaction with other biotic and abiotic factors. Environmental metagenomic libraries have proved to be great resources for new bacterial enzymes and antibiotics with potential applications in biotechnology, medicine, and industry (Riesenfeld et al. 2004; Rondon et al. 2000).

The construction of metagenomic library involves the following steps:

1. Isolation of total DNA from an environmental sample
2. Shotgun cloning of random DNA fragments into a suitable vector
3. Transforming the clones into a host bacterium and screening for positive clones

Metagenomic libraries containing small DNA fragments in the range of 2–3 kb provide better coverage of the metagenome of an environment than those with

larger fragments. It has been estimated that to retrieve the genomes from rare members of bacterial communities, at least 1,011 genomic clones would be required (Riesenfeld et al. 2004). Small-insert DNA libraries are also useful to screen for phenotypes that are encoded by single genes and for reconstructing the metagenomes for genotypic analysis. Large-fragment metagenomic libraries (100–200 kb) are desirable while investigating multigene biochemical pathways. Metagenomic libraries could be screened either by sequence-driven metagenomic analysis that involves massive high-throughput sequencing or by functional screening of expressed phenotypes. Sequence-driven massive whole-genome metagenomic sequencing sheds light on many important genomic features such as redundancy of functions in a community, genomic organizations, and traits that are acquired from distinctly related taxa through HGTs (Handelsman 2004). In function-driven metagenomic analysis (functional metagenomics), libraries are screened based on the expression of a selected phenotype on a specific medium.

DNA microarrays: DNA microarrays have been used primarily to provide a high-throughput and comprehensive view of bacterial communities in environmental samples. The PCR products amplified from total environmental DNA is directly hybridized to known molecular probes, which are attached on the microarrays (Gentry et al. 2006). After the fluorescently labeled PCR amplicons are hybridized to the probes, positive signals are scored by the use of confocal laser scanning microscopy. The microarray technique allows samples to be rapidly evaluated with replication, which is a significant advantage in bacterial community analyses. In general, the hybridization signal intensity on microarrays is directly proportional to the abundance of the target organism. DNA microarrays used in bacterial ecology could be classified into two major categories depending on the probes as 16S rRNA gene microarrays and functional gene arrays (FGA). 16S rRNA gene Microarrays (PhyloChip) contain 30,000 probes of 16S rRNA gene targeted to several cultured bacterial species and "candidate divisions" (DeSantis et al. 2007). PhyloChip technology has been used for rapid profiling of environmental bacterial communities during bioterrorism surveillance, bioremediation, climate change, and source tracking of pathogen contamination (Brodie et al. 2007; DeSantis et al. 2007). PhyloChips had been used to investigate the indigenous soil bacterial communities in two abandoned uranium mine sites, the Edgemont and the North Cave Hills in South Dakota (Rastogi et al. 2010). PhyloChip analysis revealed greater diversity than corresponding clone libraries at each taxonomic level and indicated the existence of 1,300–1,700 bacterial species in uranium mine soil samples. Most of these species were members of the phylum Proteobacteria and contained lineages that were capable of performing uranium immobilization and metal reduction. FGA are used to detect specific metabolic groups of bacteria. FGA contains more than 24,000 probes from all known metabolic genes involved in ammonia oxidation and nitrogen fixation (He et al. 2007).

DNA–DNA hybridization has been used together with DNA microarrays to detect and identify bacterial species (Cho and Tiedje 2001) or to assess bacterial diversity (Greene and Voordouw 2003). This tool could be valuable in bacterial diversity studies since a single array can contain thousands of DNA sequences

(DeSantis et al. 2007) with high specificity. Specific target genes coding for enzymes such as nitrogenase, nitrate reductase, and naphthalene dioxygenase can be used in microarray to elucidate functional diversity information of a community. Sample of environmental "standards" (DNA fragments with less than 70 % hybridization) representing different species likely to be found in any environment can also be used in microarray (Greene and Voordouw 2003).

Another DNA microarray-based technique for analyzing bacterial community is Reverse Sample Genome Probing (RSGP). This method uses genome microarrays to analyze bacterial community composition of the most dominant culturable species in an environment (Greene and Voordouw 2003). RSGP has four steps:

(1) Isolation of genomic DNA from pure cultures
(2) Cross-hybridization testing to obtain DNA fragments with less than 70 % cross-hybridization (DNA fragments with greater than 70 % cross-hybridization are considered to be of the same species)
(3) Preparation of genome arrays onto a solid support
(4) Random labeling of a defined mixture of total community DNA and internal standard

This method has been used to analyze bacterial communities in oil fields and in contaminated soils (Greene et al. 2000). Like DNA–DNA hybridization, RSGP and microarrays have the advantages that these are not confounded by PCR biases. Microarrays can contain thousands of target gene sequences but it only detects the most abundant species. Using genes or DNA fragments instead of genomes on the microarray offers the advantages of eliminating the need to keep cultures of live organisms, as genes can be cloned into plasmids or PCR can continuously be used to amplify the DNA fragments (Gentry et al. 2006). In addition, fragments would increase the specificity of hybridization over the use of genomes, and functional genes in the community could be assessed (Greene and Voordouw 2003).

Cross-hybridization is a major limitation of microarray technology. In addition, the microarray is not useful in identifying and detecting novel prokaryotic taxa. The ecological importance of a genus could be completely ignored if the genus does not have a corresponding probe on the microarray.

18.5 Conclusions

All of the approaches that are available today have advantages and limitations, though none of them provide complete access to the extremely important and complex bacterial world. These new techniques, which are in constant development, have provided powerful and important conformation of previous phenotypic and genotypic studies of bacteria. The combination of different methods is still the most suitable way of having a better understanding about diversity, phylogeny, ecology, evolution, and taxonomy of the largest group of living organisms on Earth, the Prokaryotes. Several questions remain to be resolved and the collaboration of

taxonomists, microbiologists, and molecular biologists is essential and very important for the integration of the different research methods to allow for a proper assessment of bacterial diversity and its real potential. Several important questions such as "How many bacterial species are there on the Earth?", "What is the extent of metabolic diversity in natural bacterial communities?", and "How bacterial communities are governed by biological, chemical, and physical factors?" remain to be understood. An interdisciplinary systems approach embracing several "omics" technologies to reveal the interactions between genes, proteins, and environmental factors will be needed to provide new insights into environmental microbiology. Development of multi-"omics" approaches will be a high-priority area of research in the coming years.

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

Anjali Chauhan, C.K. Shirkot, Rajesh Kaushal, and D.L.N. Rao

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 1.2×10^8 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.

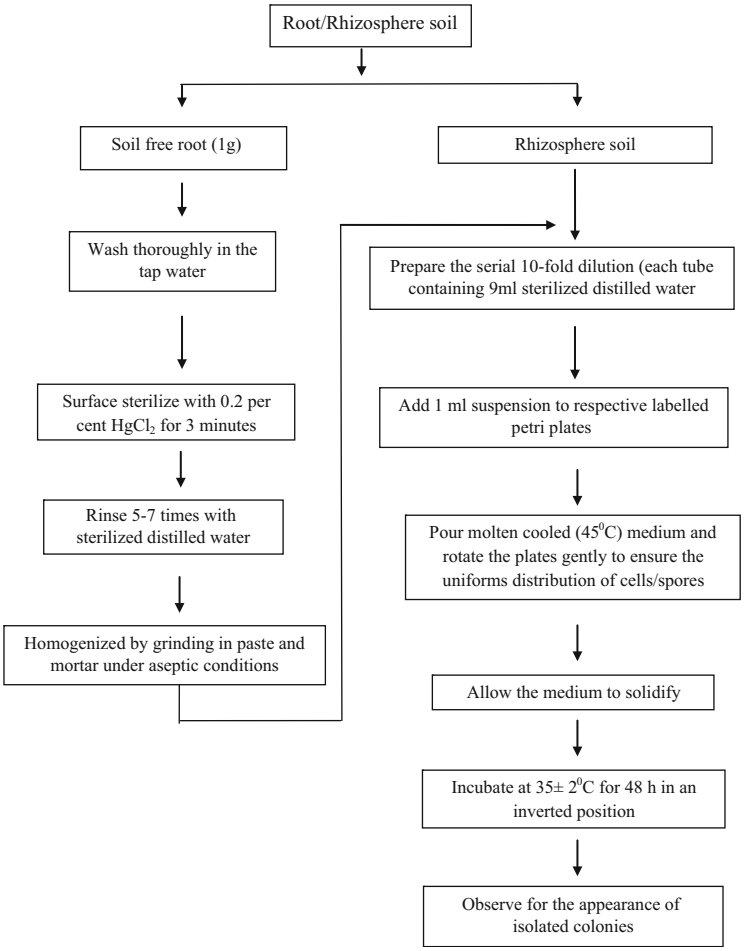

Fig. 19.1 Flow sheet for the isolation of rhizospheric and endophytic rhizobacteria

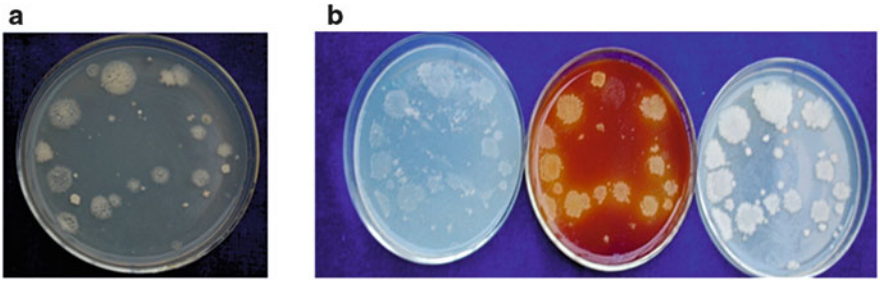

Fig. 19.2 Master plate for isolation of PGPR of *Picrorhiza kurroa* (**a**); replica plating on different media (**b**): NA, PVK, N-free medium

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

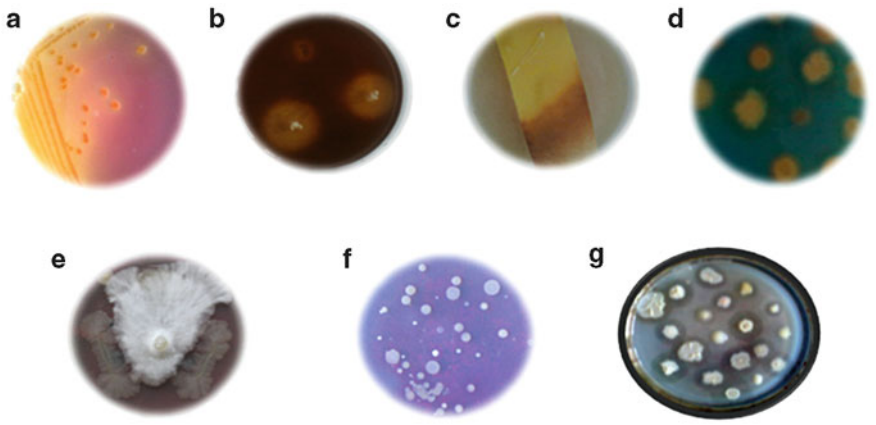

Fig. 19.3 Multifarious plant growth-promoting traits of P-solubilizing bacterial isolates: P-solubilization (**a**), chitinase activity (**b**), HCN production (**c**), siderophore production (**d**), antifungal activity against *Dematophora necatrix* (**e**), growth on nitrogen-free medium (f) and proteolytic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

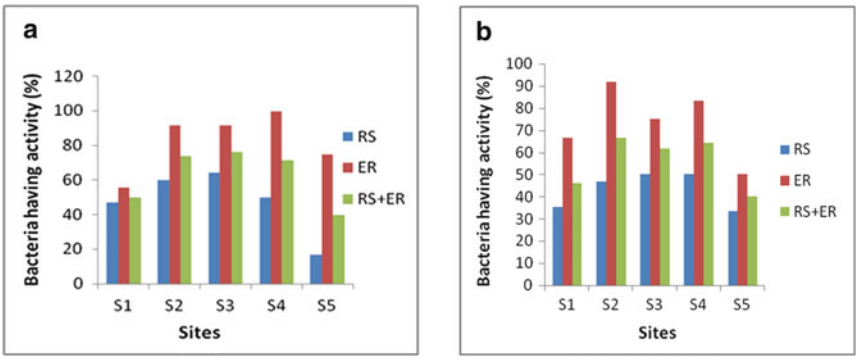

Fig. 19.4 Graphical representation of percentages of rhizosphere and endophytic bacterial isolates of five sites for PGP traits: (**a**) phosphate solubilization and (**b**) siderophore production (AINP on Biofertilizer laboratory, Solan Centre, UHF, Nauni)

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

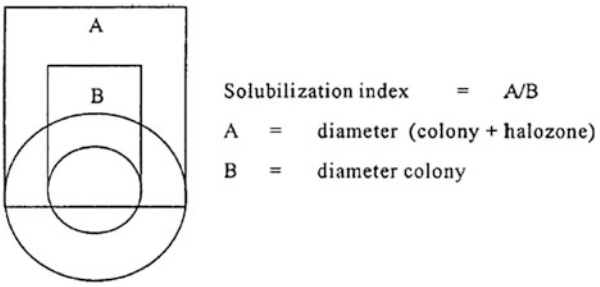

Fig. 19.5 Figure showing formula for calculating phosphate solubilization index (PSI)

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

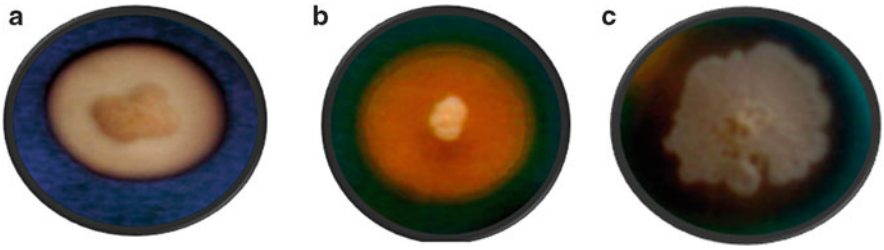

Fig. 19.6 Plate assay for detection of type of siderophore: (**a**) catecholate, (**b**) hydroxamate and (**c**) carboxylate

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 ± 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).

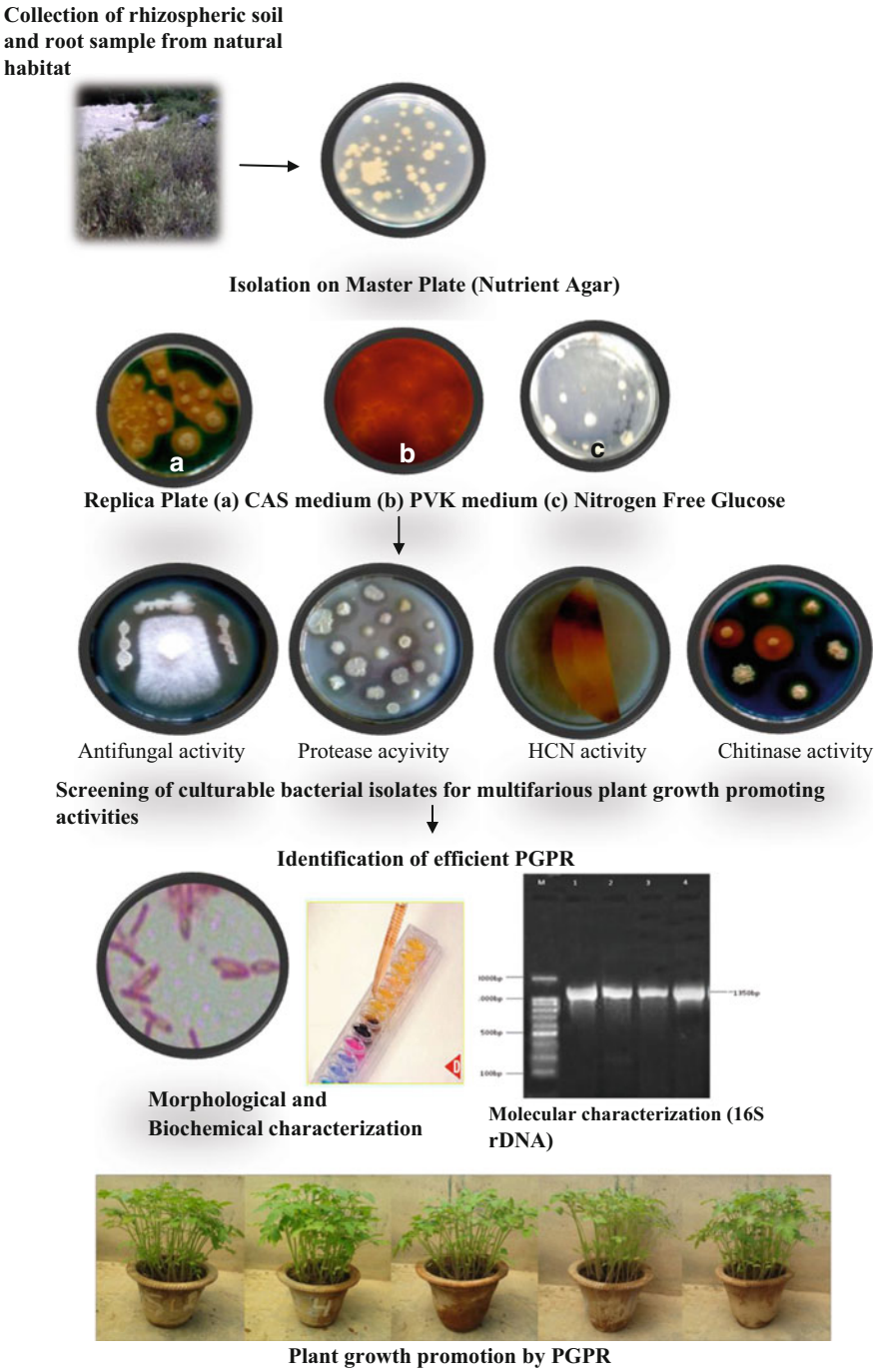

Fig. 19.7 Stepwise schematic representation of steps for the isolation, identification and characterization of plant growth-promoting rhizobacteria

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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Chapter 20
Biocontrol Activity of Medicinal Plants from Argentina

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20.1 Introduction

Crops are easily infected by phytopathogenic fungi around the world, and fungal diseases are hard to control without the use of synthetic fungicides. However, the application of large quantities of chemicals in agriculture has the potential to exert toxic effects on humans and wildlife as well as to cause environmental pollution (Nguyen et al. 2009).

The intensive use of fungicides has resulted in major problems such as the induction of resistance, altering the dynamic equilibrium of terrestrial and aquatic ecosystems, the accumulation of toxic waste, elimination of natural enemies, the death of humans and animals, household poisoning caused by exposure to toxic substances or by eating foods with waste, pollution of virtually all components of the biosphere, the emergence of new diseases, and the increase in production costs (Alcalá de Marcano et al. 2005; Bajpai et al. 2007).

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For that reasons, the discovery of new antifungal agents against fungal plant pathogen with less toxic effects is desirable. Natural products obtained from plants are an attractive alternative for disease control in agricultural crops since they can be degraded by one or other organism.

The use of plants with therapeutic properties is as ancient as human civilization, and for a long time, they were the main sources of drugs. In recent years, there has been a growing interest in alternative therapies especially those derived from plants (Rates 2001).

Often plant products used for the treatment of endemic infections served as a starting point for researchers to find treatments for these diseases (Aqil et al. 2006).

The plant kingdom is extremely rich in biologically active compounds, and only 10–15 % of higher plant species have been studied to clarify, compare, and classify their properties or determine the chemical structures of their active ingredients. The latter are products or substances exerting a pharmacological action on the living body and are found in low concentration in medicinal plants (Bisht et al. 2006; Wilkinson 2006).

However, these substances have the potential to exert toxic effects on humans and wildlife as well as to cause environmental pollution. Within this context, natural products from plants seem to be a good alternative since numerous plants have the potential to control phytopathogenic fungi and have much prospect to be used as a fungicide. Additionally, natural products are generally easily biodegradable. In many countries there are now available in the market pesticides based on plant for the biological control of plant diseases. One example of those commercial products is developed with neem (*Azadirachta indica*) (Dubey et al. 2009).

20.2 Use of Plants as Pesticides

The use of plant extracts for the management of plant diseases has gained importance in recent decades. They are relatively easy to obtain, are safe for the environment and populations, and are easily broken down into agricultural systems. Currently it is possible to extract substances from plants grown under natural conditions or cultured in the laboratory to evaluate their insect antifeedant or antimicrobial properties against fungi, bacteria, and viruses (Zarins et al. 2009).

Natural fungicides from plants are presented as an alternative for the control of crop diseases. The use of extracts of several plant species is investigated in order to explore their biological activities. In some cases, these extracts are able to safely replace, completely or partially to conventional chemical fungicides (Meepagala et al. 2005; Park et al. 2005; Aliero et al. 2006; Gulluce et al. 2007; Tabanca et al. 2007).

The active principles of plants are usually secondary metabolites, which are relatively complex chemical structures, restricted and characteristic distribution of the different vegetables. The functions of these metabolites include biochemical defense to repel the aggression of herbivorous animals, fungi, and other microorganisms, attract pollinators, and adapt to situations such as water stress or lack of light (Lira-Saldívar 2003).

A large number of secondary metabolites in plants are phenolic compounds that can come from two biosynthetic pathways: polyacetates route, which originates quinones, xanthones, orcinoles, and the shikimic acid pathway, where from the synthesis of aromatic amino acids cinnamic acid synthesized as simple phenols and derivatives, phenolic acids, coumarins, flavonoids, tannins, quinones, and lignans (Lira-Saldívar 2003).

Over a hundred different lignans have been described in different parts of plants, including heartwood, bark, stems, roots, rhizomes, flowers, leaves, fruits, and seeds. Furthermore, lignans can be secreted by the plant in the form of resin. Lignans play an important role in the defense of plants against pathogens and predators. They were reported as chemicals with effects on bacteria, fungi and viruses (Ríos et al. 2002).

In species of the Asteraceae and Piperaceae families has been isolated the lignan sesamolin, which has inhibitory activity of the monooxygenase enzyme. This enzyme is present in insects, such as *Ostrinia nubilalis*, which attack crops of economic importance in Europe (Bernard et al. 1989).

Other lignans, nordihydroguaiaretic acid (NDGA) and methyl NDGA isolated from leaves of *Larrea tridentata*, inhibit the growth of *Aspergillus flavus* and *Aspergillus parasiticus* in a concentration of 500 mg ml^{-1} (Vargas-Arispuro et al. 2005). In another study, these two compounds at concentrations of 10 and 25 μM completely inhibited β-1,3-glucanase enzyme that plays an important physiological role in the development process and fungal differentiation (Vargas-Arispuro et al. 2009).

The lignan 8,8′-bis-(methylenedioxy) cinnamic acid has been reported to be a powerful competitive inhibitor of the enzyme lignin peroxidase of the fungus *Phanerochaete chrysosporium* and *Phlebia radiata*. The accumulation of some lignans in the trunks of the trees is a chemical defense strategy of the plant to inhibit fungal enzymes involved in the degradation of wood (Frías et al. 1995).

NDGA also presents allelopathic activity and is responsible for inhibiting the growth of other species around *Larrea tridentata*. A study conducted in vitro lignan found that this dramatically reduces root growth of seedlings of barnyard grass, green foxtail, perennial ryegrass, annual ryegrass, red millet, pigweed, lettuce, and alfalfa (Elakovich and Stevens 1985).

20.3 Medicinal Plants of Argentina

The central region of Argentina has a rich and varied flora, little studied in the search for antifungal compounds of plant application. Of the wide range of plants, seven species were selected as features that stand out places to study their activity against phytopathogenic fungi. Different extracts of *Achyrocline satureioides*, *Aspidosperma quebracho blanco*, *Larrea cuneifolia*, *Larrea divaricata*, *Maytenus vitis-idaea*, *Minthostachys verticillata*, and *Verbascum thapsus* were studied in vitro and in vivo on phytopathogenic growth affecting crops of regional importance and were also evaluated for their safety in seedlings.

20.3.1 Botanical Description and History of the Species Under Study

20.3.1.1 Genus *Achyrocline*

This genus includes 32 species distributed in tropical regions of South America and Africa. The main native species are *A. satureioides*, *A. alata*, *A. flaccida*, and *A. tormentosa*. Members of this genus are herbs or shrubs, with a dense undercoat, erect stems, often branched. The leaves are simple, alternate, and longer than the wide (linear) sheet.

Achyrocline satureioides, popularly known as "marcela" or "marcela del campo" is a sub-bush that belongs to the family Asteraceae and is widely used in South America (Rivera et al. 2004). Experimental studies have shown hepatoprotection (Kadarian et al. 2002), antioxidant (Desmarchelier et al. 1998), antitumor and cytotoxic (Ruffa et al. 2002), antiviral (Zanon et al. 1999), and immunomodulatory properties (Cosentino et al. 2008). In spite of the widespread biological activities investigated for *A. satureioides* aerial part, there is no report of the activity on fungal plant pathogens growth.

20.3.1.2 Genus *Larrea*

This genus is composed of species of woody evergreen shrubs with a wide geographical distribution in the large hot deserts of America, covering large arid and semiarid regions of Argentina, Chile, Bolivia, Peru, Mexico, and the southwestern United States. It contains five species (*Larrea ameghinoi*, *L. cuneifolia*, *L. divaricata*, *L. nitida* and *L. tridentata*), of which the first four are found in Argentina (Sakakibara et al. 1976).

A common feature of all members of the genus is that they have resin blades. This resin has been of interest because it represents 10–15 % of the dry weight of the leaves. The composite material is approximately 50 % by NDGA and the remaining 50 % of flavonoids plus waxy substances (Horn and Gisvold 1945; Waller and Gisvold 1945; Gonnet and Jay 1972; Sakakibara et al. 1976).

In *L. cuneifolia*, Valesi et al. (1972) identify the following structure flavonoids: quercetin 3,7,3′,4′-tetramethyl ether, quercetin 3,7,3′-trimethyl ether, quercetin 3,7,4′-trimethyl ether, quercetin 3,7-dimethyl ether, quercetin 3,3′-dimethyl ether, quercetin 7,3′-dimethyl ether, quercetin 3-methyl ether, kaempferol 3,7-dimethyl ether, kaempferol 3-methyl ether, apigenin 7-methyl ether, and apigenin.

Other studies in this specie report the presence of proteins (Trione and Ruiz Leal 1972), essential oil composed of monoterpenes, phenylpropanoids, sesquiterpenes (Bohnstedt and Mabry 1979), and flavonoids in leaves (Timmermann 1979).

Larrea divaricata (jarilla) is a perennial woody shrub with a wide distribution in Argentina and has long been used for its medicinal and aromatic properties. It is frequently used in traditional medicine as anti-inflammatory, antirheumatic,

febrifuge, and as a pest control agent and is a plant with reports of traditional antifungal use (Goleniowski et al. 2006; Svetaz et al. 2010). Recently, the water extract of *L. divaricata* was found to decrease proliferation and induced apoptosis of lymphoma cell line (Davicino et al. 2010), and the alcoholic extract has been reported to exert antibacterial activity (Zampini et al. 2007) and antifungal activity against yeast and filamentous dermatophytes (Svetaz et al. 2010). Phytochemical studies had reported the presence of lignans, essential oils, flavonoids, and glycosides (Mabry et al. 1977).

20.3.1.3 Genus *Verbascum*

This genus is native to Europe and Asia and is composed of about 250 species. They are biennial or perennial, and rarely annuals or subshrubs plants will reach 0.5–3 m high. The leaves are arranged spirally and they have a lot of hairs. The flowers have five symmetrical petals: yellow or white, orange, brown, red, purple, and blue. The fruit is a capsule containing numerous seeds.

Verbascum thapsus is a medicinal plant popularly known as "common mullein." It has been used for the treatment of inflammatory diseases, asthma, spasmodic coughs, diarrhea, and other pulmonary problems. Although it is native from Europe and Asia, it was introduced in America several times (Turker and Camper 2002). This plant is reported to be active against influenza virus (Mehrotra et al, 1989), bovine herpesvirus type 1 (Mc Cutcheon et al. 1995), bacteria (Turker and Camper 2002), fungi (Mc Cutcheon et al. 1994), and against mosquito larvae (Gross and Werner 1978). In spite of the numerous studies made of mullein, antifungal activity against plant pathogens has not been investigated.

20.3.1.4 Genus *Aspidosperma*

The genus Aspidosperma is from South America and comprises about 80 species, distributed in tropical and subtropical regions. It consists of large or medium trees, with simple leaves, alternate, opposite, or whorled. The most important species in Argentina is *A. quebracho blanco*, dwelling from the north to the Midwest. It is used in carpentry for being trees with very good quality wood as firewood, fencing poles, and rods. It is traditionally used as febrifuge, antiasthmatic, in cardiac dyspnea and an appetizer. Leaves and shoots were cited as abortion, contraception, hepatoprotective, blood purifier, and against colics. The database shows that the genus *Aspidosperma* is known to be a rich source of indole alkaloid compounds and tannins (Deutsch et al. 1994; Landau et al. 2000).

In the case of *A. quebracho blanco*, studies to evaluate their bioactivities are scarce, reporting only antimalarial activity (Bourdy et al. 2004).

20.3.1.5 Genus *Maytenus*

Maytenus is a genus of trees which has about 200 species of which 11 are in Argentina. Species of the genus grow in a variety of climates from tropical to subpolar. It is widely distributed in America, Africa, and South Asia (Alonso and Desmarchelier 2007).

Las hojas y tallos preparados en infusión son empleados en caso de úlceras sangrantes, hipertensión arterial, dolores articulares, como depurativo, contra el asma y como antitumoral; la raíz es utilizada como diurético (Bueno et al. 2009).

The specie *M. vitis-idaea* is known as "colquiyuyo," "ibirá-Yuqui," "salt of the Indian," "fat meat," and "salty logging," among others. In traditional medicine it is used as an astringent, ophthalmic, and antiasthmatic contraceptive. This species is very important in maintaining the ecological balance, primarily for the restoration of degraded forest areas in the original because of its rapid turnover of organic matter (Bueno et al. 2009).

One study reports that compounds isolated from the root bark of *M. vitis-idaea* have insecticidal effects, antifeedant and growth regulator on larvae of the codling moth. Aqueous extracts from fresh and dried leaves showed the presence of non-hydrolizable or condensed tannins. These are responsible for the astringency of the leaves and they may be used as antiviral and antioxidant compounds (Vonka and Chifa 2008).

20.3.1.6 Genus *Minthostachys*

Plants of the genus are aromatic shrubs and climbers up to 3 m high. Some species are used in South American countries as a condiment to flavor foods and in the treatment of respiratory diseases. Moreover, infusions or oils of this genus have been used as digestive, sedative, and as a topical antifungal and antiparasitic. Decoctions have also been used to protect stored potatoes against insects (Schmidt-Lebuhn 2008).

This plant lives in the central and northern Argentina. The leaves, stems and flowers are used in many preparations, from teas to spirits. The applications range from peppermint tea to liquor. In addition, *M. verticillata* is a commercially important source of essential oil. Stimulant, digestive, carminative, vulnerary, antispasmodic, and antirheumatic are attributed to this plant (Schmidt-Lebuhn 2008).

Some studies report on antibacterial and antiviral properties (De Feo et al. 1998) and that the oil is the most active fraction against bacteria (Primo et al. 2000). Other authors reported antiviral activity against herpesvirus type I (Zanon et al. 1999) and immunomodulatory and antiallergic properties in human cell lines (González Pereyra et al. 2005).

20.4 Obtention of Plant Extracts and Antifungal Activity

Plants of different species under study were collected manually, following the instructions of the World Health Organization for the collection of medicinal plants.

The aerial part was left dried, powdered, and successively extracted for 48 h at room temperature in n-hexane (HE), methanol (ME), and chloroform (CE). Warm aqueous extract (WAE) was obtained when plant material was extracted with water at 70 °C for 48 h. Extracts were concentrated to dryness and dissolved in dimethyl sulfoxide (DMSO) to give a concentration of 100 mg ml^{-1}.

The microorganisms used for the antifungal evaluation were the following plant pathogens of economic importance in agriculture: *Fusarium graminearum*, *Fusarium solani*, *Fusarium verticillioides*, *Macrophomina phaseolina*, and *Sclerotium rolfsii*.

20.4.1 Agar Dilution Method

The extracts were added to molten potato dextrose agar (PDA) to obtain a final concentration of 1,000 μg ml^{-1} and then pour in to the Petri dishes (9.0 cm in diameter). A 4 mm diameter plug of actively growing fungi, taken from PDA plates, was placed onto the center of Petri dishes; treatments were incubated at 30 °C.

Each treatment was tested in triplicate and experiment was repeated three times. Parallel negative controls were included by mixing DMSO with PDA medium. Sensitivity of each fungal species to each tested extract was calculated as percentage of mycelia growth inhibition, according to Pandey et al. (1982).

The chloroform extracts of *L. cuneifolia* and *L. divaricata* inhibited the growth of the microorganisms tested between 54.6 and 98.9 %. At the concentration of 1,000 mg ml^{-1}, the *L. divaricata* extract showed a high inhibitory effect on the radial growth of all pathogens. The effect of *L. cuneifolia* extract was also high, inhibiting fungal growth: *S. rolfsii* (98.9 %), *M. phaseolina* (91.6 %), *F. verticillioides* (81.8 %), *F. graminearum* (65.1 %), and *F. solani* (58.0 %). Due to the high inhibitory activities shown by these extracts, they were again tested at lower concentrations (500, 300, 100, and 50 μg ml^{-1}). *Macrophomina phaseolina* was the most sensitive fungus.

Other extracts showed high antifungal activity. Thus, the methanol extract of *L. cuneifolia* significantly reduced the growth of *F. graminearum*, *F. verticillioides*, *S. rolfsii*, and *M. phaseolina*; the hexane extract of *L. cuneifolia* inhibited *F. verticillioides*, *M. phaseolina*, and *S. rolfsii*. The hexane extract of *A. satureioides* significantly decreased the growth of *S. rolfsii*, *M. phaseolina*, and *F. verticillioides*, while the chloroform extract of this species inhibited *F. graminearum*, *M. phaseolina*, and *S. rolfsii*. The hexane extract of *M. verticillata* exerted strong inhibition on the growth of *M. phaseolina*.

Among the extracts that showed moderate inhibitory activity against at least one of the tested fungi is the chloroform extract of *A. quebracho blanco*, which only inhibited *M. phaseolina*.

Several investigators have used the same methodology for the study of plant fractions on the growth of phytopathogenic fungi. This is how Tegegne et al. (2008) evaluated in assays in vitro activity of different extracts of the plant species *Agapanthus africanus*. In their results, the growth of *S. rolfsii* was inhibited 100 % by the methanol extract of the roots, leaves, and flowers to the concentration of 1,000 mg ml^{-1}. Also evaluated is the antifungal effect of ethanol extracts of *L. divaricata* and *L. cuneifolia*, and results were similar to those found by our group (Table 20.1).

20.4.2 Broth Dilution Method

Potato dextrose broth (PDB) was prepared for estimation of *M. phaseolina* mycelial yield at 1,000, 500, and 100 μg ml^{-1} of HE, ME, CE, and WAE. Flasks containing 20 ml of PDB (potato dextrose broth) with appropriate volume of extracts were inoculated with three agar blocks (each of 2 mm diameter) taken from a PDA plate of actively growing fungi and were incubated at 30 °C for 3 days. Thereafter, cultures were filtered through pre-weighed Whatman filter paper No. 1. Mycelial yield was determined after drying the mycelial at 75 °C for 5 days. Percent loss/gain in mycelial dry weight was calculated according to Dubey et al. (2009).

In this test, the majority of the tested extracts inhibited the growth of *M. phaseolina and S. rolfsii* in liquid medium. Among the extracts that exerted inhibition greater than 20 % at the concentration of 100 mg ml^{-1}, the methanol extract of *L. cuneifolia* stands decreased the growth of *M. phaseolina* and *S. rolfsii*.

There are no previous reports of the use of this methodology with extracts of the plant species we studied. Dubey et al. (2009) found a high inhibition in the production of mycelium from the aqueous extract of the bark and leaves of *Azadirachta indica* (neem) at 10 % concentration in Czapek Dox broth (Table 20.2).

20.5 Phytotoxicity Assay

A bioassay based on germination, radicle, and epicotil growth of *Lycopersicon esculentum* Mill. (tomato) and *Triticum aestivum* (wheat) was used to study the allelopathic effects of extracts when applied at a concentration of 1,000 μg ml^{-1}. Seeds were surface disinfected and then placed on Petri dishes (20–40 seeds per dish) containing a layer of Whatman filter paper on cotton, which had previously been impregnated with 20 ml of extract solution dissolved in distilled water or 1 % DMSO (control). Dishes were then incubated at 25 °C for 3 days for *T. aestivum* and

Table 20.1 Effect of plant extracts against plant pathogenic fungi growth

Plant species and extracts	Concentration (μg ml^{-1})	Inhibition (%)				
		Fusarium graminearum	*Fusarium solani*	*Fusarium verticillioides*	*Macrophomina phaseolina*	*Sclerotium rolfsii*
Achyrocline satureioides						
HE	1,000	43.0 ± 5.9^{a}	20.6 ± 1.1^{a}	52.9 ± 2.4^{a}	57.2 ± 6.8^{a}	49.9 ± 6.7^{a}
CE	1,000	46.8 ± 7.9^{a}	9.4 ± 1.3^{c}	39.0 ± 4.0^{b}	62.6 ± 7.2^{a}	47.1 ± 6.9^{a}
WAE	1,000	17.4 ± 7.7^{b}	13.2 ± 2.9^{b}	8.9 ± 1.6^{c}	39.3 ± 9.3^{b}	17.8 ± 6.9^{b}
Aspidosperma quebracho blanco						
HE	1,000	8.0 ± 2.2^{c}	3.1 ± 1.5^{b}	10.4 ± 3.3^{b}	13.0 ± 2.5^{c}	4.1 ± 2.8^{a}
CE	1,000	14.7 ± 4.3^{ab}	6.3 ± 0.6^{a}	21.7 ± 4.3^{a}	43.9 ± 5.5^{a}	12.1 ± 5.3^{a}
ME	1,000	7.2 ± 2.9^{c}	N.I.	8.6 ± 5.1^{b}	24.3 ± 4.0^{b}	6.3 ± 2.3^{a}
WAE	1,000	19.0 ± 2.7^{a}	1.4 ± 3.8^{b}	9.3 ± 2.7^{b}	N.I.	11.7 ± 0.9^{a}
Larrea cuneifolia						
HE	1,000	35.2 ± 4.6^{b}	27.9 ± 1.2^{b}	57.1 ± 0.6^{c}	65.0 ± 3.9^{c}	72.1 ± 5.9^{b}
CE	1,000	65.1 ± 6.5^{a}	58.0 ± 4.2^{a}	81.8 ± 0.7^{a}	91.6 ± 3.5^{a}	98.9 ± 3.2^{a}
	500	49.3 ± 1.8^{b}	30.9 ± 1.1^{b}	73.1 ± 0.1^{b}	71.6 ± 3.2^{c}	
	300	44.1 ± 1.4^{b}	19.5 ± 0.3^{c}	69.4 ± 0.8^{b}	59.6 ± 1.8^{d}	
ME	1,000	62.8 ± 6.7^{a}	31.4 ± 5.7^{b}	69.4 ± 3.7^{b}	76.8 ± 5.8^{b}	76.0 ± 6.3^{b}
WAE	1,000	13.3 ± 3.6^{c}	9.4 ± 1.0^{d}	33.7 ± 4.9^{d}	14.2 ± 4.2^{e}	36.7 ± 6.5^{c}
Larrea divaricata						
HE	1,000	5.3 ± 3.7^{d}	5.1 ± 3.5^{d}	13.8 ± 4.9^{d}	13.3 ± 4.2^{c}	7.1 ± 6.1^{c}
CE	1,000	71.0 ± 5.4^{a}	54.6 ± 6.5^{a}	80.6 ± 1.3^{a}	91.1 ± 5.8^{a}	96.8 ± 4.7^{a}
	500	63.3 ± 7.7^{a}	41.2 ± 2.2^{b}	65.9 ± 4.2^{b}	87.2 ± 8.0^{a}	85.1 ± 9.0^{a}
	300	56.2 ± 2.5^{b}	26.6 ± 6.3^{c}	48.2 ± 2.8^{b}	81.8 ± 1.7^{a}	48.3 ± 6.2^{b}
ME	1,000	25.2 ± 3.8^{c}	3.0 ± 2.1^{d}	23.3 ± 6.4^{c}	25.2 ± 6.1^{b}	8.3 ± 6.5^{b}
Maytenus vitis-idaea						
CE	1,000	NI	2.9 ± 2.0	1.7 ± 1.0	NI	NI

(continued)

Table 20.1 (continued)

Plant species and extracts	Concentration (μg ml^{-1})	Inhibition (%)				
		Fusarium graminearum	*Fusarium solani*	*Fusarium verticillioides*	*Macrophomina phaseolina*	*Sclerotium rolfsii*
ME	1,000	NI	NI	5.4 ± 2.1	NI	NI
Minthostachys verticillata						
HE	1,000	61.9 ± 6.3[a]	14.7 ± 3.4[a]	50.5 ± 4.9[b]	43.1 ± 6.4[a]	36.7 ± 7.2[b]
CE	1,000	63.2 ± 6.6[a]	15.1 ± 3.1[a]	69.2 ± 6.5[a]	39.5 ± 4.9[a]	57.6 ± 6.1[a]
ME	1,000	33.4 ± 3.9[b]	0.9 ± 1.9[b]	17.3 ± 4.3[c]	16.2 ± 6.5[b]	6.1 ± 6.5[d]
WAE	1,000	25.2 ± 4.0[c]	0.5 ± 4.7[b]	10.5 ± 1.8[d]	13.0 ± 7.6[b]	15.1 ± 6.4[cd]
Verbascum thapsus						
HE	1,000	34.5 ± 2.1[c]	NI	17.7 ± 1.6[b]	21.0 ± 1.5[b]	20.4 ± 0.7[b]
CE	1,000	44.0 ± 2.9[b]	NI	14.3 ± 3.8[bc]	26.3 ± 2.8[b]	14.9 ± 3.3[b]
ME	1,000	56.3 ± 4.1[a]	29.6 ± 6.9[a]	36.6 ± 6.5[a]	56.8 ± 1.8[a]	49.1 ± 6.8[a]
WAE	1,000	8.1 ± 2.7[d]	0.9 ± 5.0[b]	2.2 ± 5.7[d]	NI	NI
Control captan	643	88.3 ± 0.7	91.1 ± 4.8	94.4 ± 1.9	100.0 ± 0.0	100.0 ± 0.0

Agar dilution method

[a]*HE* hexanic extract, *CE* chloroformic extract, *ME* methanolic extract, *WAE* warm aqueous extract, *NI* no inhibition

[b]Values within the same column, and for each plant, followed by the same letter do not differ significantly ($p < 0.05$) according to the Turkey test

Table 20.2 Effect of plant extracts against plant pathogenic fungi growth

Plant species and extracts	Concentration (μg ml^{-1})	Inhibition (%)	
		Macrophomina phaseolina	*Sclerotium rolfsii*
Achyrocline satureioides			
HE	1,000	96.6 ± 0.3^{a}	66.0 ± 15.1^{a}
	500	59.0 ± 2.9^{b}	35.6 ± 10.2^{b}
	100	53.2 ± 7.0^{b}	NI
CE	1,000	94.3 ± 1.0^{a}	57.7 ± 18.2^{a}
	500	49.6 ± 6.3^{b}	NI
	100	26.2 ± 20.0^{c}	NI
WAE	1,000	8.5 ± 12.0^{d}	18.6 ± 9.4^{b}
Aspidosperma quebracho blanco			
HE	1,000	NI	NI
CE	1,000	42.0 ± 5.6^{a}	30.1 ± 4.2^{a}
ME	1,000	48.3 ± 6.0^{a}	35.8 ± 7.1^{a}
WAE	1,000	NI	1.9 ± 4.6^{b}
Larrea cuneifolia			
CE	1,000	99.2 ± 0.4^{a}	98.1 ± 0.5^{a}
	500	98.3 ± 0.4^{a}	
	100	98.0 ± 0.6^{a}	
	50	61.0 ± 0.9^{b}	
	25	43.8 ± 5.0^{c}	
ME	1,000	99.7 ± 0.1^{a}	96.4 ± 2.3^{a}
	500	86.1 ± 7.8^{a}	95.3 ± 1.5^{a}
	100	21.9 ± 9.3^{c}	54.6 ± 10.2^{b}
	50	NI	30.0 ± 10.8^{b}
Larrea cuneifolia			
WAE	1,000	96.0 ± 1.0^{a}	11.7 ± 4.5
	500	25.6 ± 8.0^{b}	NI
Larrea divaricata			
HE	1,000	8.5 ± 1.8^{d}	NI
CE	1,000	97.8 ± 2.7^{a}	97.6 ± 0.2^{a}
	500	92.8 ± 2.2^{a}	96.1 ± 1.1^{a}
	100	83.1 ± 5.8^{a}	79.6 ± 1.2^{ab}
	50	35.0 ± 2.6^{b}	69.0 ± 3.8^{b}
	25	26.8 ± 3.7^{c}	45.8 ± 10.3^{b}
ME	1,000	83.3 ± 8.7^{a}	88.4 ± 2.5^{a}
	500	48.2 ± 4.6^{b}	19.9 ± 10.8^{c}
	100	19.3 ± 8.0^{c}	NI
Maytenus vitis-idaea			
CE	1,000	NI	NI
ME	1,000	NI	NI
Minthostachys verticillata			

(continued)

Table 20.2 (continued)

Plant species and extracts	Concentration (μg ml^{-1})	Inhibition (%)	
		Macrophomina phaseolina	*Sclerotium rolfsii*
HE	1,000	99.5 ± 0.5^{a}	98.0 ± 0.3^{a}
	500	64.0 ± 9.8^{b}	97.8 ± 0.9^{a}
	100	7.2 ± 9.0^{c}	9.3 ± 10.0^{b}
CE	1,000	97.6 ± 2.2^{a}	96.1 ± 2.9^{a}
	500	61.6 ± 8.7^{b}	93.2 ± 4.3^{a}
ME	1,000	0.2 ± 7.2^{c}	NI
WAE	1,000	NI	20.5 ± 14.3^{b}
Verbascum thapsus			
HE	1,000	40.1 ± 6.8^{b}	NI
CE	1,000	82.5 ± 8.4^{a}	38.4 ± 7.3^{b}
	500	25.6 ± 1.3^{c}	N.I.
ME	1,000	66.2 ± 8.2^{a}	66.6 ± 9.8^{a}
	500		25.0 ± 9.3^{b}
WAE	1,000	NI	25.4 ± 4.5^{b}
Control captan	643	97.3 ± 1.4^{a}	99.0 ± 0.1^{a}
	64.3	97.2 ± 0.8^{a}	88.7 ± 10.0^{a}
	6.43	52.6 ± 8.7^{b}	39.2 ± 10.7^{b}

Broth dilution method

[a]*HE* hexanic extract, *CE* chloroformic extract, *ME* methanolic extract, *WAE* warm aqueous extract, *NI* no inhibition

[b]Values within the same column, and for each plant, followed by the same letter do not differ significantly ($p < 0.05$) according to the Turkey test

7 days for *L. esculentum*. Three replicates were carried out for each assay. The number of germinated seeds was determined according to the 2 mm radicle extrusion criterion. Radical and epicotyl growth were measured in twenty germinated seeds.

The possible toxic effect of the extracts was evaluated on some plant species, as suggested Macias et al. (1999). These authors recommend conducting such trials on *L. esculentum*, *T. aestivum*, *Lactuca sativa* L., *Daucus carota* L., *Lepidium sativum* L., *Allium cepa* L., *Hordeum vulgare* L., and *Zea mays* L. since they have low coefficient of variation in growth medium and high values in the parameters of epicotyl and root length. All these species are called together by the authors as standard species or "standard target species" for the development of bioassays in allelopathic phytotoxicity studies.

Plant extracts variously affect germination of *L. esculentum* and growth parameters evaluated. Some of the extracts increased epicotyl length only, as with the hot aqueous extract of *A. satureioides*, others stimulated root growth, as the methanol extract of *M. verticillata* and hot aqueous extract of *V. thapsus*, while in other cases both parameters were positively affected, as with the hexane extract of *V. thapsus* and aqueous extracts of *L. cuneifolia* and *A. quebracho blanco*.

Among the extracts that significantly reduced some of the evaluated parameters are the hot aqueous extract of *L. cuneifolia*, the methanol extract of *M. vitis-idaea*, the aqueous extract of *A. satureioides*, the hexane and methanol extracts of *A. quebracho blanco*, the methanol extracts of *V. thapsus* and *L. cuneifolia*, the hexane extract of *L. divaricata*, and the hot aqueous extract of *M. verticillata*.

Some extracts showed high toxicity. These include the hexane extract of *A. satureioides*, the aqueous extract of *A. quebracho blanco*, the chloroform and methanol extracts of *L. cuneifolia*, the methanol extract of *L. divaricata*, and the hexane and chloroform extracts of *M. vitis-idaea* and *M. verticillata*.

The results obtained by germinating seeds of *T. aestivum* with plant extracts showed, as in *L. esculentum*, various effects. So, the hot aqueous extracts of *A. satureioides*, *A. quebracho blanco*, and *L. cuneifolia* and the hexane and chloroform extracts of *M. vitis-idaea* and the hexane extract of *V. thapsus* increased root length and epicotyl.

At the other end, the chloroform and methanolic extracts of *L. cuneifolia*, the chloroform extract of *L. divaricata*, and the hexane and methanolic extracts of *M. verticillata* negatively affected all parameters, showing a considerable toxicity at the concentrations tested.

As the chloroform extract of *L. divaricata* was one that showed higher antifungal activity and at the same time proved toxic to *L. esculentum* and *T. aestivum*, it was evaluated at lower concentrations in order to find a concentration that maintains the antifungal capacity without toxic effect on the germination of the crop. The results showed that a concentration of 100 mg ml^{-1} was the appropriate (Vogt 2011).

Dicot specie *L. esculentum* was more sensitive than *T. aestivum* in certain parameters on germination and seedling growth. This result is similar to that obtained by other authors (Gonçalves et al. 2009).

20.6 Isolation and Structural Identification of Secondary Metabolites of *Larrea divaricata*

The chloroformic extract was subjected to flash chromatography on silica gel, eluting with *n*-hexane, *n*-hexane-EtOAc with increasing polarity mixtures, and EtOAc–MeOH (97:3) to afford 36 fractions. The n-hexane-EtOAc (7:3) fraction was purified by column chromatography on Sephadex LH-20 eluting with MeOH to give 23 fractions. Each fraction obtained from Sephadex column was monitored by TLC (C_6H_6–dioxane-AcOH 30:5:1), and fractions 6–7 were separated and purified by TLC (C_6H_6–AcOH 8.5:1.5) to furnish compound 1 (11 mg). Fractions 10–11 were separated and purified by TLC (C_6H_6–AcOH 8.5:1.5) to furnish compound 2 (10 mg). Fractions 12–20 were separated and purified by TLC (C6H6–AcOH 8.5:1.5) to furnish compound 3 (9 mg). Their structures were determinated by spectroscopic methods and comparison with authentic samples (NMR spectra: Bruker-Avance-200 instrument, 1H NMR: 200 MHz, 13C NMR: 50 MHz, $CDCl_3$

as solvent. Mass spectra: EIMS, ionization energy 70 eV, Finnigan-Mat-GCQ ion tramp instrument).

Results of the in vitro evaluation indicate that the chloroform extract was active against all the fungi tested, and *F. graminearum* and *M. phaseolina* were the most sensitive species. The *n*-hexane extract was inactive against the fungi tested and methanol extract inhibited *M. phaseolina* only (Vogt et al. 2013).

The differences in the inhibition effect of the extracts may be due to the lignans compounds present in *L. divaricata*, which had similarity to the chloroform solvent. In relation with the n-hexane extract, lower inhibition activity indicates that there were interactions among nonpolar inactive structures (Jasso de Rodríguez et al. 2011).

Previously antifungal activity was described in *L. divaricata*. Svetaz et al. (2010) studied *L. divaricat*a ethanolic extract against dermatophytes of high incidence in superficial infections. Author's results were similar to the inhibitions obtained with chloroform extract by our group.

In the chloroform extract from *L. divaricate*, we detected the presence of flavonoids and lignans. Three compounds were isolated using chromatographic methods and identified by spectroscopic methods in this extract: Apigenine-7-methylether, nordihydroguaiaretic acid, and 3,4-dihydroxy-3,4-dimethoxy-6,7-cyclolignan. The latter compound is described for the first time in the species and it was the most active against *F. graminearum* on in vitro tests (Fig. 20.1).

On infected pots, this compound was more effective than *L. divaricata* chloroformic extract reducing damping-off preemergence in 14 % (5 day after emergence) and postemergence in 11 % (15 days postemergence). Disease developed extensively in roots and subcrown internodes and less in leaf sheaths of 15 days wheat plants. Data showed that treatment with this compound significantly reduced severity of symptoms of *F. graminearum* crown rot as compared with the non-treated controls.

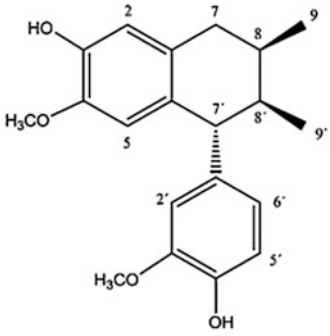

Fig. 20.1 Chemical structure of 3,4-dihydroxy-3,4-dimethoxy-6,7-cyclolignan isolated from *Larrea divaricata* Cav

20.7 Conclusions

The use of plant products for the management of plant diseases has achieved greater significance in recent years due to its readily available nature, antimicrobial activity, easy biodegradability, and lower phytotoxicity, besides inducing resistance in host. The species studied are part of the traditional flora of the central region of Argentina, and the results help to characterize and extend the available information on the biological activities of the same. It is important to continue investigating the biological properties of these species and identify the active compounds that are present in the extracts.

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