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Plant Growth and Health Promoting Bacteria

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Preface

Plants provide an excellent ecosystem for microorganisms that interact with plant cells and tissues with differing degrees of dependence. Investigation on the relationship between roots and microbiota are essential to achieve innovations in agriculture and biotechnology. Similar to other industries, one such system is adoption of biological agents in the form of Plant Growth Promoting Bacteria (PGPB). These groups of bacteria are as effective as pure chemical on plant growth enhancement and disease control besides managing abiotic and other stresses in plants. Such organisms are now alternative paradigms for commercialization. Seeing the importance of these bacteria in the protection of plant health, new biotechnological approaches are employed regulating to develop newer and much better microbial agents for management of the phytopathogens.

This volume of the Microbiology Monograph series has 18 chapters that cover various facets of current scientific knowledge on PGPB that colonize the root and rhizosphere. *Bacillus*- and *Paenibacillus*-based bioinoculant formulations have met with great success in improving plant growth. A large number of PGPB genera on one hand and rhizobia and few endophytes on the other promise benefit to crop ecosystem for sustainable agriculture. A due account is provided with respect to basic concept on plant–bacteria interaction, mineral–nutrient exchange, biofilm formation, and bacteria inhabiting in harsh and cold tropical environment and their role in ethylene regulation via ACC deaminase, as well as the mechanisms of action of PGPB-mediated antifungals. In relation to plant health, the exploitation of such beneficial bacteria may improve agriculture system with economically sound production of human food and animal feed.

This book will be useful not only for students, teachers, and researchers but also for those interested in agriculture microbiology, plant pathology, ecology, environmental science, and agronomy.

I would like to express my sincere thanks to all the contributors for their much needed cooperation, authoritative and up to date information organized in a befitting manner. I acknowledge with thanks the assistance rendered by my research students Abhinav, Rajat, Pankaj, and Dr. Sandeep. I am also thankful to Council of

Scientific and Industrial Research (CSIR), New Delhi, and Director, Uttarakhand Council of Science and Technology (UCOST), Dehradun, India, for their support in execution of my research projects on PGPB that served as a prelude to lay foundation for compilation of the volume like this. I owe my special thanks to Prof. Alexander Steinbüchel, series editor, 'Microbiology Monographs,' University of Münster, Germany, for his professional advice from time to time in multifarious manner. I extend my sincere thanks to Drs. Christina Eckey and Jutta Lindenborn from the publisher Springer for their valuable support to facilitate completion of this volume.

Haridwar, India Dinesh K. Maheshwari

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Benefits of Plant Growth-Promoting Rhizobacteria and Rhizobia in Agriculture

Marta S. Dardanelli, S.M. Carletti, N.S. Paulucci, D.B. Medeot, E.A. Rodriguez Cáceres, F.A. Vita, M. Bueno, M.V. Fumero, and M.B. Garcia

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Abstract The rhizosphere is the volume of soil under the influence of plants roots, where very important and intensive microbe–plant interactions take place. These interactions can both significantly influence plant growth and crop yields and have biotechnological applications. The rhizosphere harbors a diverse community of microorganisms that interact and compete with each other and with the plant root. The activity of some of the members of this community affects the growth and the physiology of the others, as well as the physical and chemical properties of the soil. Among all these interactions, those resulting in symbiotic and non-symbiotic nitrogen fixation are considerably important. In recent years, the use of bacteria

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(rhizobacteria) to promote plant growth has increased in several regions of the world and has acquired relevant importance in developing countries that are the producers of raw materials for food. Rhizobacteria can affect plant growth by producing and releasing secondary metabolites, which either decrease or prevent the deleterious effects of phytopathogenic organisms in the rhizosphere, and/or by facilitating the availability and uptake of certain nutrients from the root environment. Significant increases in the growth and yield of agriculturally important crops in response to inoculation with rhizobacteria have been reported. This practical application of plant growth-promoting rhizobacteria is the main focus of this chapter.

1 Introduction

The roles of microbiology and biotechnology in agriculture are very important because plant sources satisfy up to 80% of humans dietary needs. The Earth's population increases by 1.4% annually and is expected to reach 8.3 billion by 2025; therefore, unprecedented increases in crop production will be needed if the current levels of N (nitrogen) are to be maintained (11 g of N per person per day) (Mannion 1998; Graham and Vance 2000). The needs for N of most crop plants are second only to their photosynthetic requirement. Because soil N deficiency is common in many areas of crop production and land areas now considered marginal, N supply, N management, and N-use efficiency are significant factors in crop production, and are important as to the availability of fossil fuel reserves for future fertilizer N production (Graham and Vance 2000). On the other hand, farmers and breeders have long known that it is often the simultaneous occurrence of several abiotic stresses, rather than a particular stress condition, that is most lethal to crops. Tolerance to a combination of different stress conditions, particularly those that mimic the field environment, should be the focus of future research programmes aimed at developing transgenic crops and plants with enhanced tolerance to naturally occurring environmental conditions (Mittler 2006).

Arable land resources are limited. Thus, meeting food needs in some regions has already led to the adoption of agricultural practices that can degrade the soil, and to the use of land that is marginal for crop production. Nutrient depletion and soil acidification are only two of the common consequences of inadequate soil management (Hungria and Vargas 2000). In this context, the presence of microorganisms in the soil is critical to the maintenance of soil function, in both natural and managed agricultural soils, because of their involvement in key processes such as soil structure formation, decomposition of organic matter, toxin removal, and the cycling of carbon, nitrogen, phosphorus, and sulphur (van Elsas and Trevors 1997). In addition, microorganisms play key roles in suppressing soil-borne plant diseases and in promoting plant growth and changes in vegetation (Doran et al. 1996). Future exploitation of interactions will be as dependent on a better understanding of the biology of plant–microbe interaction as on developments in

biotechnology (Beringer 1986). The beneficial use of rhizobacteria in agriculture is discussed in this chapter.

2 Rhizosphere in Action

The associations that occur between plant roots and soil microorganisms have been known for many decades. Considerable efforts have been devoted to study ectomycorrhizal fungi, nitrogen-fixing bacteria, soil-borne pathogenic fungi, and other microorganisms. As a consequence of the many investigations of the variable response of plants to different soils, an awareness of the complexity of the interactions between roots and soil microbes has been developed (Atkinson and Watson 2000). When seeds germinate and roots grow through the soil, the loss of organic material provides the driving force for the development of active microbial populations around the root. This effect is known as "the rhizosphere effect" (Whipps 1990). The term "rhizosphere" was first defined by Lorenz Hiltner in 1904 as "the soil compartment influenced by the root" (Hiltner 1904).

Although bacteria were not proven to exist until von Leeuwenhoek in 1683 discovered microscopic "animals" under the lens of his microscope, their use to stimulate plant growth in agriculture has been exploited since ancient times. Theophrastus (372–287 BC) suggested the mixing of different soils as a means of "remedying defects and adding heart to the soil" (Tisdale and Nelson 1975). The rhizosphere of plants is a zone of intense microbial activity, and some bacteria from this zone, termed rhizobacteria, exhibit different functions. The rhizosphere contains an increased microbial biomass and activity compared with nonrhizosphere soil: the number of microorganisms in the rhizosphere is 19–32 times larger than in root-free soil (Bodelier et al. 1997). Rhizobacteria that exert beneficial effects on plant development are referred to as plant growth-promoting rhizobacteria (PGPR) because their application is often associated with increased rates of plant growth (Kloepper and Schroth 1978). The well-known PGPR include members of the genera *Arthrobacter, Azoarcus, Azospirillum*, *Bacillus*, *Burkholderia, Enterobacter*, *Gluconacetobacter, Herbaspirillum, Klebsiella, Paenibacillus*, *Pseudomonas*, and *Serratia*, among others. PGPR can affect plant growth either directly (by providing plants with a compound synthesized by the bacterium or by facilitating the uptake of certain nutrients from the environment) or indirectly (by decreasing or preventing the deleterious effects of one or more phytopathogenic organisms) (Glick 1995). In order to exert their function, PGPR must colonize the rhizosphere around the roots, the rhizoplane (root surface) or the root itself (within root tissues) (Glick 1995).

Non-pathogenic rhizobacteria can induce a systemic resistance in plants that is phenotypically similar to the pathogen-induced systemic acquired resistance (SAR). Rhizobacteria-mediated induced systemic resistance (ISR) has been demonstrated against fungi, bacteria, and viruses in bean, carnation, cucumber, radish, tobacco, and tomato under conditions in which the inducing bacteria and the

challenging pathogen remained spatially separated (van Loon et al. 1998). ISR elicited by PGPR has suppressed plant diseases caused by a range of pathogens in both greenhouse and field conditions. However, fewer reports have been published on PGPR as elicitors of tolerance to abiotic stresses, such as drought, salt and nutrient deficiency or excess. Recently, Yang et al. (2009) have proposed the term "induced systemic tolerance" (IST) for PGPR-induced physical and chemical changes in plants that result in enhanced tolerance to abiotic stresses.

Beneficial bacteria that are able to establish a nitrogen-fixing symbiotic relationship with leguminous plants (collectively called rhizobia) are usually not considered as PGPR. Endosymbiotic interactions between legume plants and the genera *Azorhizobium, Bradyrhizobium, Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* have been intensively studied (Vance 1998; Graham and Vance 2000; Perret et al. 2000). Rhizobia infect legumes and have a global distribution, ranging from high latitudes in Europe and North America to the equator, to tropics in Australia and South America. In equatorial and tropical areas, legumes are particularly important and are used in sylvopastoral and agroforestry systems (Dommergues and Subba Rao 2000). Intricate signaling between the host and rhizobial symbiont is required for successful symbiotic interactions, which result in the reduction of atmospheric N_2 to ammonia by the bacteroids. Recently, some of these bacteria have been shown to be plant-growth promoting on nonlegumes, through mechanisms different from nitrogen fixation. Nevertheless, these will not be further considered, as the mechanisms involved are not different from those of the well-known and better-documented PGPR (Spaepen et al. 2009). Thus, in the broadest sense, PGPR include the N_2-fixing rhizobacteria that colonize the rhizosphere and provide N to plants.

Rhizosphere interactions are based on complex exchanges that take place round plant roots. Beneficial, detrimental, and neutral relationships between plant roots and microorganisms are all regulated by complex molecular signaling. It is clear that all the biological community, rather than only the immediate micro-flora, plays a role in the interaction of the rhizosphere. The existence of both microbial responses to plants and plant responses to the presence of microbes suggests a degree of coevolution between two partners. Two of the best-studied interactions between plant hosts and bacteria include the root nodule inhabiting *Rhizobium* spp. and tumor-forming *Agrobacterium* spp. The study of these systems has led to the discovery that plants and bacteria communicate by using chemical signals, which are involved in a successful interaction (Peters et al. 1986; Bolton et al. 1986; Fisher and Long 1992; Dardanelli et al. 2008a, b, 2009).

Chemical signaling between plant roots and other soil organisms, including the roots of neighboring plants, is often based on root-derived chemicals. Forty to ninety percent of the carbon transferred to the roots is lost and is called rhizodeposition (Kennedy 1998). In the rhizosphere environment, rhizodeposition includes different fractions: root exudates, lysates, mucilage, secretions, and dead cell material (Lynch and Whipps 1990). A substantial portion of the root exudates consists of carbon and energy sources readily available for microbial growth development and the physiology of microbial cell populations (Sommers et al. 2004).

Different organic compounds, such as amino acids, sugars, vitamins, organic acids, auxins, and flavonoids, which are rapidly utilized by microorganisms, have been identified in root exudates (Sommers et al. 2004; Dardanelli et al. 2008a,b, 2009; Raaijmakers et al. 2009). The signal components largely responsible for specific host–microbe relationships belong to a class of compounds termed flavonoids (Peters et al. 1986). More than 4,000 different flavonoids have been identified in vascular plants, and a particular subset of them is involved in mediating host specificity in legumes (Perret et al. 2000). Isoflavonoids are found only in members of the legume family, and despite the great importance of chemical molecules, some problems may arise because the same chemical signals may elicit dissimilar responses from different recipients. The chemical components of root exudates may deter one organism and attract another, or two very different organisms that may cause different consequences to the plant may be attracted. For example, the secretion of isoflavones by soybean roots is able to attract both a mutualist (*Bradyrhizobium japonicum*) and a pathogen (*Phytopthora sojae*) (Morris et al. 1998).

The attraction and subsequent migration toward plant roots is probably a key factor for the initiation of several plant–bacterial interactions. Motility may increase the probability that the microbe and the plant meet in the soil environment. By means of directed movement, bacteria are able to move toward plant roots, where they can benefit from a wide range of exudate-derived nutrients, enabling them to survive in and subsequently colonize the rhizosphere (Sommers et al. 2004). A variety of compounds, such as surface proteins and polysaccharides, have been implicated in the adherence of several rhizobacteria to plant roots (Dardanelli et al. 2003; Rodríguez-Navarro et al. 2007). The importance of bacterial attachment in PGPR–plant interactions has been intensively studied in *Azospirillum* and *Pseudomonas*. It is generally believed that the main mechanism by which *Azospirillum* enhances plant growth is by the production of plant hormones (Steenhoudt and Vanderleyden 2000). These growth-promoting substances stimulate the density and length of root hairs and root surface area, improving the utilization of water and mineral nutrients. Zhu et al. (2002) have shown that *Azospirillum irakense* cells are mainly associated with rice root hairs, whereas *Azospirillum brasilense* are mainly located on root surfaces. These differences in spatial distribution are the reason why these two species do not compete for root colonization.

Plant-associated *Pseudomonas* bacteria live as saprophytes but also as pathogenic parasites on plant surfaces and inside plant tissues. In addition, some *Pseudomonas* species show plant growth-promoting activity by suppressing the growth (biocontrol) of other phytopathogenic microorganisms, synthesizing growth-stimulating plant hormones and promoting plant mechanisms involved in disease resistance. Initial attachment to biotic or abiotic surfaces leads to a global change in gene expression in *Pseudomonas putida* (Rodríguez-Navarro et al. 2007). The isolation of genes involved in the adhesion to abiotic surfaces and the attachment to plant roots suggests that initial colonization of both biotic and abiotic surfaces proceeds via similar pathways (Sauer and Camper 2001). Although agglutinin plays a major role in the adherence and colonization abilities of *P. putida* strain Corvallis to bean and cucumber, the role of agglutinins is not general for all biocontrol

strains. No agglutination-dependent adherence or root colonization has been demonstrated for 30 different *Pseudomonas* isolates on tomato, potato, and grasses (Lugtenberg and Dekkers 1999).

3 Role of PGPR in Agriculture

The plant growth-promoting capacity has been related to different physiological activities that may have a profound effect on the growth and/or health of plants. Although some chapters in this book comment on different functions of PGPR and rhizobia in agriculture, in this chapter we will briefly discuss some of them.

In most agricultural ecosystems, soil-borne plant pathogens can be a major limitation in the production of marketable yields. They are also more recalcitrant to management and control as compared to pathogens that attack the above-ground portions of the plant (Bruehl 1987). In addition, they are adapted to growing and surviving in the bulk soil, but the rhizosphere is the infection court where they encounter the plant and establish a parasitic relationship (Raaijmakers et al. 2009). Estimating crop loss caused by pathogens is difficult and there are only a few well-documented studies. From 2001–2003, an average of 7–15% of major world crops (wheat, rice, potato, maize, and soybean) was lost because of fungi and bacteria (Oerke 2005). From 1996 to 1998, these pathogens caused a loss of 9.9%, although the potential loss without controls could have been 14.9% (Oerke and Dehne 2004). Losses caused by soil-borne pathogens are even more difficult to estimate, because of the difficulty of diagnosis. Some estimate that soil-borne pathogens cause 50% of the crop loss in the United States (Lewis and Papavizas 1991).

The increased use of chemical inputs causes several negative effects, i.e., development of pathogen resistance to the applied agents and their non-target environmental impacts (Gerhardson 2002). Furthermore, the growing cost of pesticides, particularly in less affluent regions of the world, and the growing consumer demand for pesticide-free food, have led to a search for substitutes for these products. There are also a number of fastidious diseases for which chemical solutions are few, ineffective, or nonexistent (Gerhardson 2002). Biological control is thus being considered as an alternative or supplemental way of reducing the use of chemicals in agriculture (Whipps 2001; Gerhardson 2002). For several years, a great diversity of rhizobacteria have been described, characterized, and tested as biocontrol agents of diseases caused by soil-borne plant pathogens. Different biocontrol activities of PGPR are mediated by the synthesis of bacterial allochemicals, including iron-chelating siderophores, antibiotics, biocidal volatiles, lytic enzymes, and detoxication enzymes, among others (Glick 1995; Compant et al. 2005). In the last few years, some studies have been carried out on bacteria applied; studies of bacteria applied to seeds and roots for the purpose of controlling bacterial diseases. One example is the application of non-pathogenic strains of *Streptomyces* to control scab of potato (*Solanum tuberosum* L.) caused by *S. scabies* (Ryan and

Kinkel 1997). Here, the biocontrol may operate through antibiosis or competition for space or nutrients in the rhizosphere.

The global market for phytosanitary products used worldwide to ensure crop yield was estimated at US$ 26.7 billion in 2005 (Thakore 2006). Synthetic pesticides dominate this market. However, irrational selection and use has led to environmental toxicity of their residues, decrease or loss of efficacy because of adaptation of pathogens, or undesirable effects on non-target organisms sharing the ecosystem (Ongena and Jacques 2007). The use of beneficial microorganisms as biopesticides is considered one of the most promising methods for more rational and safe crop-management practices. Among all biopesticides, microorganism-based products represent 30% of total sales and new products are regularly brought to the market (Thakore 2006). Biopesticides are used in field crops and greenhouses to reduce diseases on various cereals, legumes, fruits, flowers, and ornamentals caused by soil-borne, foliar, or postharvest pathogens. Most of the bacterial strains exploited as biopesticides belong to the genera *Agrobacterium*, *Bacillus*, and *Pseudomonas* (Fravel 2005). *Bacillus thuringiensis*, specifically used for insect pest control, accounts for >70% of total sales (Ongena and Jacques 2007; Sanchis and Bourguet 2008). As for the rest, *Bacillus*-based products, such as *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus pumilus,* represent about half of the commercially available bacterial biocontrol agents (Ongena and Jacques 2007).

The *Bacillus* genus produces a wide range of biologically active molecules that are potentially inhibitory for phytopathogen growth. Among these antimicrobial compounds, cyclic lipopeptides (LPs) of the surfactin, iturin, and fengycin (or plipastatin) families have well-recognized potential uses in biotechnology and biopharmaceutical applications because of their surfactant properties (Ongena and Jacques 2007). Recent investigations indicate that these lipopeptides can also influence the ecological fitness of the producing strain in terms of root colonization (and thereby persistence in the rhizosphere) and that they have a key role in the beneficial interaction of *Bacillus* species with plants by stimulating host defence mechanisms (Ongena and Jacques 2007). The production of LPs has been demonstrated in *Bacillus* populations growing on roots, leaves, and fruits (Touré et al. 2004; Romero et al. 2007). In the rhizosphere, LPs are difficult to be estimated because of the small amounts excreted as compared to the other organic compounds present in the environment, their difficult extraction from the complex soil matrix, and the possibility that the low quantities produced are restricted from diffusing freely and can be rapidly embedded in the membrane structure of the target organism (Ongena and Jacques 2007).

Pseudomonas fluorescens strains, have been reported to control diseases caused by soil-borne pathogens and are known to survive in both rhizosphere and phyllosphere (Weller, 1988; Wilson et al. 1991). Several studies have indicated that foliar diseases could be controlled by the application of *P. fluorescens* as seed, soil, or root treatments, and it is presumed that they may produce ISR and thus protect the leaves (Wei et al. 1991). The ability of rhizosphere-associated fluorescent pseudomonads to inhibit the growth of plant pathogenic fungi has generated increased interest in their use as crop protectants (Schippers et al. 1987; Lam and Gaffney 1993;

Weller 1988; Fravel 2005). A formulation of *P. fluorescens* strains, for instance, has been reported to control the foliar pathogen *Pyricularia oryzae* that causes blast disease in rice in field trials (Vidhyasekaran et al. 1997).

4 Potential Uses of PGPR and Rhizobia

Although there are several works on the role of specific strains of PGPR and rhizobia in plant-growth promotion, N_2 fixation, biofertilizer activities, and biological control, there is a need for more attention with regard to the negative effects of environmental stresses, diseases on rhizobacteria–plant interactions (Barea et al. 1998; Kloepper et al. 1999; Jetiyanon et al. 2003; Vessey 2003; Bashan et al. 2004; Morrissey et al. 2004). For example, rhizobia are sensitive to drought stress, resulting in a significant decrease of N_2 fixation when faced with low soil-water content. In a study under drought stress, coinoculation of bean (*Phaseolus vulgaris* L.) with *Rhizobium tropici* and two strains of *Paenibacillus polymyxa* resulted in increased plant height, shoot dry weight, and nodule number (Figueiredo et al. 2008). Interestingly, the effect on IST and the increased nodule number was greater when the two strains of *P. polymyxa* were applied than individual strain, suggesting some synergistic effects from themixed strains. Recently, Dardanelli et al. (2009) have shown how biotic and abiotic stresses can alter the pattern of flavonoids exuded by Osumi soybean roots. In that work, in the presence of *Chryseobacterium balustinum* Aur9, soybean roots did not exude quercetin and naringenin, and under salt stress (50 mM NaCl), flavonoids daidzein and naringenin could not be detected. Soybean root exudates obtained under saline conditions showed a diminished capacity to induce the expression of the *nodA* gene in comparison to the exudates obtained in the absence of salt. In addition, lipochitooligosaccharides (LCOs) were either not detected or weakly detected when *Sinorhizobium fredii* SMH12 was grown in the exudates obtained under salt stress conditions or under salt stress in the presence of *C. balustinum* Aur9, respectively.

Another abiotic stress that plants face is the obtaining of adequate soil nutrients. Although soil fertilization is typically required for agricultural production, it can cause nitrate and phosphate accumulation that eventually contaminates surface and ground waters. The use of fertilizers, including chemical fertilizers and manures, to enhance soil fertility and crop productivity has often negatively affected the complex system of the biogeochemical cycles (Perrott et al. 1992; Steinshamn et al. 2004). The use of fertilizers has caused leaching and run-off of nutrients, especially N and phosphorus (P), leading to environmental degradation (Tilman 1998; Gyaneshwar et al. 2002). Important reasons for these problems are the low use efficiency of fertilizers and the continuous long-term use. Despite the negative environmental effects, the total amount of fertilizers used worldwide is projected to increase with the growing world population because of the need to produce more food through intensive agriculture (Vitousek et al. 1997; Frink et al. 1999).

The challenge, therefore, is to continue agricultural productivity in a way that minimizes harmful environmental effects of fertilizers.

Research activities aimed at achieving a better use efficiency of fertilizers, including the use of PGPR and/or arbuscular mycorrhizal fungi as supplements to fertilizers, have steadily increased in the last two decades. However, it is important to emphasize those agro-environmental problems which are not limited to the use of chemical fertilizers but also occur with manures and compost (Mitchell and Tu 2006). Both animal waste and chemical fertilizers have the potential of environmental pollution (McLaughlin and Mineau 1995; Jarecki et al. 2008). Release of greenhouse gases (Flessa et al. 2002; Jarecki et al. 2008), ozone layer depletion (Ma et al. 2007), global warming, and acid rain are reported as negative impacts of fertilizers (Vitousek et al. 1997; Frink et al. 1999). Microbial inoculants, such as PGPR, are promising components for integrated solutions to agro-environmental problems because inoculants possess the capacity to promote plant growth, enhance nutrient availability and uptake, and support the health of plants (Barea et al. 1998; Dobbelaere et al. 2001; Hodge et al. 2001; Bonfante 2003; Vessey 2003; Kloepper et al. 2004; Han and Lee 2005; Weller 2007; Adesemoye et al. 2008).

On the basis of the beneficial effects of PGPR and rhizobia, studies using inoculant mixtures are very promising (Berg 2009). Benefits to plants from plant–PGPR interactions have been shown to include increase in seed germination, root growth, yield, leaf area, chlorophyll content, nutrient uptake, protein content, hydraulic activity, tolerance to abiotic stress, shoot and root weights, biocontrol, and delayed senescence (Mahaffee and Kloepper 1994; Raaijmakers et al. 1997; Bashan et al. 2004; Mantelin and Touraine 2004; Bakker et al. 2007; Yang et al. 2009).

Amir et al. (2005) reported enhanced uptake of N and P in oil palm seedlings, following PGPR inoculation in the field nursery. Aseri et al. (2008), on the other hand, conducted field experiments in India and assessed the effectiveness of PGPR (*Azotobacter chroococcum* and *A. brasilense*) and arbuscular mycorrhizal fungi (*Glomus mosseae* and *Glomus fasciculatum*) on the growth, nutrient uptake, and biomass production of pomegranate (*Punica granatum* L.). Strains were applied individually or in combinations and the results showed that dual inoculation of PGPR and arbuscular mycorrhizal fungi led to higher biomass production and increase in the uptake of N, as well as of P, K, Ca, and Mg, in pomegranate seedling. The increase in N and P uptake was suggested to result from improved symbiotic N_2 fixation and improved phosphatase activity.

The study by Adesemoye et al. (2008) confirmed that inoculation with mixed strains was more efficient than single-strain inoculations. A proposal made by Adesemoye et al. (2009) toward solving the agro-environmental problems mentioned is integrated nutrient management (INM), which does not aim to remove fertilizer totally in the short run but to reduce the negative impacts of the overuse of fertilizers containing N, P, and other elements. The INM system promotes low chemical input but improved nutrient-use efficiency by combining natural and man-made sources of plant nutrients in an efficient and environmentally prudent manner. This will not sacrifice high crop productivity in the short term nor endanger sustainability in the long term (Gruhn et al. 2000; Adesemoye et al. 2008).

Recently, it has been demonstrated that PGPR-elicited plant-growth promotion results in enhanced N uptake by plant roots (Adesemoye et al. 2008).

Owing to the broad-range metabolic activities found in many PGPR, another interesting topic is the potential use of PGPR in rhizoremediation (microbial degradation of hazardous compounds in the rhizosphere) in contaminated zones in order to obtain a dual effect: first, the remediation of the soil and then the promoting of plant growth for agriculture purposes. It is well known that bacteria of the *Burkholderia cepacia* complex (Bcc), which include nine species or genomovars (Mahenthiralingam et al. 2005), may be found in soils (including polluted soils), rhizospheres of crop plants, water, various animal species, humans, and hospital environments (Coenye and Vandamme 2003). More recently, Caballero-Mellado et al. (2007) reported the occurrence of nitrogen-fixing *Burkholderia* species associated with tomato (*Lycopersicon esculentum*) cultivated in different locations in Mexico. These authors found that the rhizosphere of tomato is a reservoir of different known and unknown diazotrophic *Burkholderia* species that, in vitro, are able to exhibit some activities involved in bioremediation, plant-growth promotion, and biological control. Similarly, Perin et al. (2006) reported that the isolation of *Burkholderia unamae* from field-grown sugarcane in Brazil and Mexico, as well as the isolation of *Burkholderia tropica* from maize cultivated in Mexico, find probably novel diazotrophic species (the *Burkholderia* NAR group) in rhizospheric and endophytic association with both maize and sugarcane in Brazil. Manipulating biotic interactions to provide desired services and thus reduce or eliminate the need for external inputs is fundamental to the practice of ecologically sound agriculture. The challenge is how to encourage positive interactions and reduce negative ones. Shennan (2008) indicates that the potential for a greater use of ecological management approaches is high; however, owing to the nature of ecosystems as medium number systems, there is some inherent unpredictability about the responses to different management interventions, which needs to be accommodated in the development of recommendations for farm management. This requires an increased emphasis on the effective synthesis of complex and often apparently contradictory information and a greater emphasis on field-based adaptive research that includes monitoring performance as adaptations are made, along with social learning mediated by farmer/researcher collaborations.

5 PGPR Studies in Argentina

PGPR have been studied by Argentinean scientists from universities and other research laboratories for the last three decades, with the aim to assess the beneficial effects on plant growth and yield of many crops of agronomic importance. In this section, we present some of the most relevant data on this issue in Argentina.

Bacteria of the genus *Azospirillum* are free-living nitrogen-fixing rhizobacteria that are found in close association with plant roots of a large number of plants,

including forage and cereal crops (Okon 1994). Early studies with these bacteria in Argentina aimed to isolate local strains. By using an improved culture medium with Congo red, the colonies were typical red scarlett and easy to isolate (Rodríguez Cáceres 1982). Several local strains were selected in greenhouse conditions by their capacity of inducing changes in root systems and were preserved at the collection of Agricultural Microbiology and Zoology Institute of INTA, Castelar, Buenos Aires. To investigate the practical use of *Azospirillum* as a plant biofertilizer, wheat field trials were carried out at different locations of country. The local isolate *A. brasilense* strain Az39, which was obtained from wheat roots in the province of Córdoba, showed a consistent positive effect on the yield of different cultivars, from 13.4 to 33% increase over the control in three growing cycles tested (Rodríguez Cáceres et al. 1996). In addition, it is known that increases in crop yield derived from *Azospirillum* inoculation are consistently obtained when water is deficient (Fig. 1) and soil nutrients are limiting (low organic matter) (Fig. 2) (Rodríguez Cáceres et al. 2008a).

Inoculation trials of corn and wheat carried out with a liquid formulation of *A. brasilense* Az39 in 2002–2003 and 2006–2007 at 110 different sites showed an average yield increase of 6%. In most of the sites, the inoculation with this liquid formulation increased root and shoot early growth and grain number of the crops (Diaz-Zorita et al. 2004). Similar increases of forage yield were obtained in foxtail millet (*Setaria italica*) inoculated with these rhizospheric bacteria (Di Ciocco and Rodríguez Cáceres 1994). All these positive results motivated the agro-industry to produce new inoculants based on *Azospirillum* and other PGPR. Coinoculation studies with PGPR and *Rhizobium* spp. have been shown to increase root and

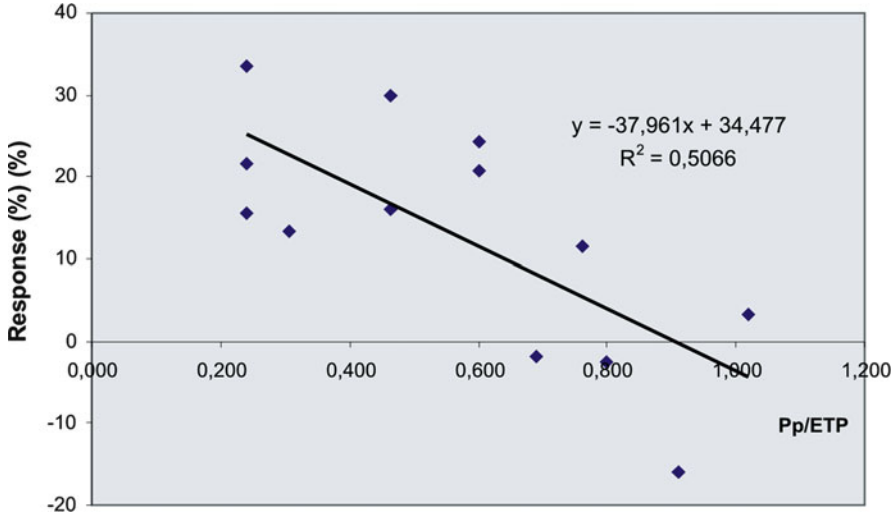

Fig. 1 Yield responses of wheat to inoculation with *Azospirillum brasilense* Az 39 conditioned to hidric conditions

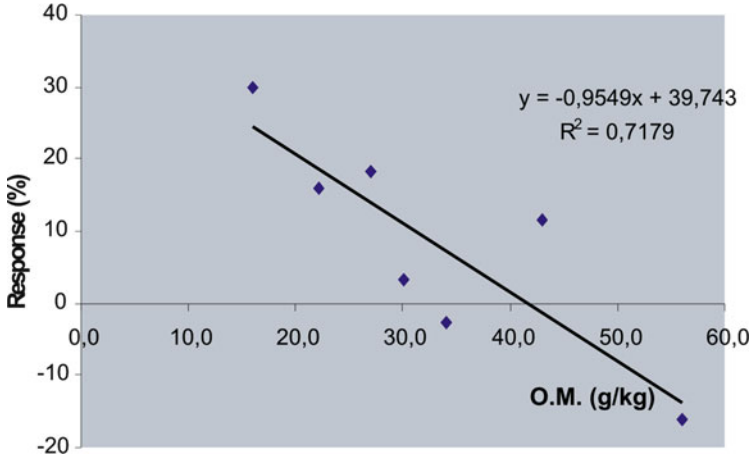

Fig. 2 Yield responses of wheat to inoculation with *Azospirillum brasilense* Az 39 conditioned to soil organic matter

shoot dry weight, plant vigour, nodulation, and nitrogen fixation in various legumes (Saxena et al. 2006). It is still a matter of controversy whether the stimulation of plant growth and N fixation by coinoculation is due either to an increase in root surface by hormonal effects or to nodulation and nutrient uptake. In relation with physiological and biochemical changes, Groppa et al. (1998) found that coinoculation with *B. japonicum* and *A. brasilense* on soybean plants showed a significantly higher proportion of nodules attached to the main root and located in the upper 3 cm of the root system. Although no significant differences were detected in total dry matter production, nitrogen content of coinoculated plants was significantly increased (23% as compared with plants inoculated only with *B. japonicum*). Accordingly, a strong stimulation of acetylene reduction activity and a significant increase (39%) in leghemoglobin content were observed using this treatment.

In another study, Dardanelli et al. (2008) have worked with a combination of *A. brasilense* strain Cd, and *R. tropici* CIAT899 and *Rhizobium etli* ISP42 in *P. vulgaris*. These authors observed that *A. brasilense* promoted root branching and increased secretion of nodgene-inducing flavonoid species. Similar results, and changes in flavonoids and sugar composition, were obtained when *Arachis hypogaea* was inoculated with *Bradyrhizobium* SEMIA6144-*A. brasilense* Cd (Dardanelli unpublished results).

It was also shown that *A. brasilense* Az39 and *B. japonicum* E109, singly or in combination, had the capacity to promote seed germination, nodule formation, and early development of soybean seedlings. Both strains were able to excrete plant-regulating substances into the culture medium, at a concentration sufficient to produce morphological and physiological changes in young seed tissues (Cassán et al. 2009). In order to obtain a positive effect on growth and nodulation of

Table 1 Effect of *Azospirillum brasilense* Az39 inoculum concentration on nodulation and acetylene reduction activity (ARA) of pouch-grown plants of soybean

Treatment	CFU ml^{-1}	Nodule number (per plant)	Nodule dry weight (mg)	ARA nmol C_2H_4 h^{-1} $plant^{-1}$
Control	10^9	144 a	0.14 a	20.75
Azospirillum	10^3	148 a	0.15 a	20.11
	10^5	226 b	0.21 b	28.00
	10^8	139 a	0.15 a	24.10

All treatments were inoculated with 1 ml (1×10^9 CFU ml^{-1}) of *B. japonicum*. Values followed by the same letter were not significantly different ($P = 0.05$). *CFU* Colony-forming unit, *ARA* acetylene reduction activity

coinoculated leguminous plants, it is necessary to establish optimal cell concentration of each biological component (Rodríguez Cáceres, unpublished data) (Table 1).

Barassi et al. (2006) also demonstrated growth-promoting effects of *A. brasilense* strain 245 on lettuce in saline conditions. These authors observed that *Azospirillum*-inoculated lettuce seeds had a better germination and vegetative growth than non-inoculated controls after exposure to NaCl. Plants grown from inoculated seeds and irrigated with saline medium displayed higher total fresh and dry weights and biomass partition to the aerial portion than non-inoculated controls.

Since in the semiarid pampas of Argentina the phosphorus distribution available is not uniform, Rosas et al. (2006) studied the possible action of phosphate-solubilizing bacteria on the leguminous–rhizobia symbiosis. The strains used were *Sinorhizobium meliloti* 3DOh13, a good solubilizer of iron and phosphorus for alfalfa, *B. japonicum* TIIIB for soybean, and two phosphorus-solubilizing strains of *P. putida* for growth-promotion treatments. Modification in the dry weights of the shoot and root systems were observed in soybean, but not in alfalfa, in the presence of the *Pseudomonas* strains (Rosas et al. 2006).

6 New Studies and Applications of PGPR

Rhizospheric bacteria such as *Azospirillum and Azotobacter* are also being applied for the induction of rooting in micropropagated plants (jojoba, photinia, ornamental grasses) (Carletti et al. 1998). It seems that PGPR can replace all or at least some synthetic plant hormones commonly used in in vitro cultures of plants (Carletti et al. 2006).

In a study by Larraburu et al. (2007), bacterial inoculation was able to induce earlier rooting of photinia (*Photinia* × *fraseri* Dress) shoots. *A. brasilense* Cd with an indole-3-butyric acid pulse showed a significant increase in root fresh and dry weight, root surface area, and shoot fresh and dry weight, *A. brasilense* Sp7 enhanced root fresh weight and root surface area, but no significant differences were detected with *A. chroococcum* inoculation.

On the other hand, *Bacillus* spp. and *P. polymyxa* have attracted considerable interest because of their great biotechnological potential in different industrial processes and sustainable agriculture (Lal and Tabacchioni 2009). In Argentina, for example, two sporulating bacterial strains were isolated from the rhizosphere of the legume *Cicer arietinum*. The results of DNA–DNA hybridization showed that these strains constitute a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus rhizosphaerae* sp. nov. was proposed (Rivas et al. 2005).

Correa et al. (2009) showed the ability of *Bacillus amyloliquefaciens* BNM122 strain to colonize seeds and roots when applied as a coating on soybean seeds. This bacterium is a potential microbial biocontrol agent able to control the damping-off caused by *Rhizoctonia solani*, both in a plant-growth chamber and in a greenhouse. Correa et al. (2009) also observed that it had a lesser effect on soil microbial community than fungicides, because of the less environmental persistence and toxic effects of the strain.

7 Perspectives and Conclusion

The rhizosphere represents one of the most complex ecosystems on Earth with almost every root on the planet expected to have a chemically, physically, and biologically unique rhizosphere. Despite its intrinsic complexity, understanding the rhizosphere is vital if we are to solve some of the world's most impending environmental crises, such as sustainable food, fibre and energy production, preservation of water resources and biodiversity, and mitigation against climate change (Jones and Hinsinger 2008). The secretion of rhizodeposition is an important way for plants to respond to and alter their environment. Over the last several years, research and technical advances have provided a better understanding of how root exudates mediate communication between plants and other organisms. These advances could be applied to agricultural systems to enhance production by increasing defence responses against soil-borne pathogens and/or favoring the association with beneficial soil microbes. An improvement in plant–microbe symbioses should involve the reorganizations of the integrated genetic systems due to coordinated modifications in the plant and microbial genotypes. Sustainable agriculture should switch from growing plants to the cultivation of plant–microbial communities, which can reach a high productivity under minimal energy and chemical investments and with minimal pressures on the environment. However, we are only at the beginning of this process and much more efforts and cooperation between experts on plant and microbial genetics, molecular biology and ecology are required to be successful in attaining sustainable microbial-based agrotechnologies.

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Plant Growth Promoting Rhizobacteria: Fundamentals and Applications

Márcia do Vale Barreto Figueiredo, Lucy Seldin, Fabio Fernando de Araujo, and Rosa de Lima Ramos Mariano

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Abstract Plant growth promoting rhizobacteria (PGPR) have gained worldwide importance and acceptance for agricultural benefits. This is due to the emerging demand for dependence diminishing of synthetic chemical products, to the growing

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necessity of sustainable agriculture within a holistic vision of development and to focalize environmental protection. Scientific researches involve multidisciplinary approaches to understand adaptation of PGPR, effects on plant physiology and growth, induced systemic resistance, biocontrol of plant pathogens, biofertilization, and potential green alternative for plant productivity, viability of coinoculating, plant microorganism interactions, and mechanisms of root colonization. By virtue of their rapid rhizosphere colonization and stimulation of plant growth, there is currently considerable interest in exploiting these rhizosphere bacteria to improve crop production. The main groups of PGPR can be found along with the phyla Cyanobacteria, Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. Therefore, the examples coming up next are related to these microorganisms. Although taxonomic affiliation of validated genera containing PGPR strains described in literature is vast, phenotypic and genotypic approaches are now available to characterize these different rhizobacteria. The progress to date in using PGPR in a variety of applications is summarized and discussed here.

1 Introduction

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. Significant increases in growth and yield of agronomically important crops in response to inoculation with PGPR have been repeatedly reported (Kloepper et al. 1980; Seldin et al. 1984; Chen et al. 1994; Zhang et al. 1996; Amara and Dahdoh 1997; Chanway 1998; Pan et al. 1999; Bin et al. 2000; Gupta et al. 2000; Biswas et al. 2000; Mariano and Kloepper 2000; Asghar et al. 2002; Vessey 2003; Gray and Smith 2005; Silva et al. 2006; Figueiredo et al. 2008; Araújo 2008). Studies have also shown that the growth-promoting ability of some bacteria may be highly specific to certain plant species, cultivar and genotype (Bashan 1998; Gupta et al. 2000; Lucy et al. 2004).

PGPR can affect plant growth by different direct and indirect mechanisms (Glick 1995; Gupta et al. 2000). Some examples of these mechanisms, which can probably be active simultaneously or sequentially at different stages of plant growth, are (1) increased mineral nutrient solubilization and nitrogen fixation, making nutrients available for the plant; (2) repression of soilborne pathogens (by the production of hydrogen cyanide, siderophores, antibiotics, and/or competition for nutrients); (3) improving plant stress tolerance to drought, salinity, and metal toxicity; and (4) production of phytohormones such as indole-3-acetic acid (IAA) (Gupta et al. 2000). Moreover, some PGPR have the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyses ACC, the immediate precursor of ethylene in plants (Glick et al. 1995). By lowering ethylene concentration in seedlings and thus its inhibitory effect, these PGPR stimulate seedlings root length (Glick et al. 1999).

The bacteria presenting one or more of these characteristics are known as plant growth promoting rhizobacteria – PGPR (Kloepper and Schroth 1978).

Bashan and Holguin (1998) proposed the division of PGPR into two classes: biocontrol-PGPB (plant growth promoting bacteria) and PGPB. This classification may include beneficial bacteria that are not rhizosphere bacteria but it does not seem to have been widely accepted. According to Vessey (2003), numerous species of soil bacteria which flourish in the rhizosphere of plants, but which may grow in, on, or around plant tissues, and stimulate plant growth by a plethora of mechanisms are collectively known as PGPR. 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 (ePGPR), existing in the rhizosphere, on the rhizoplane, or in the spaces between cells of the root cortex, and intracellular (iPGPR), which exist inside root cells, generally in specialized nodular structures.

There are several PGPR inoculants currently commercialized that seem to promote growth through at least one mechanism: suppression of plant disease (bioprotectants), improved nutrients acquisition (biofertilizers), or phytohormone production (biostimulants). Bacteria in the genera *Bacillus*, *Streptomyces*, *Pseudomonas*, *Burkholderia*, and *Agrobacterium* are the biological control agents predominantly studied and increasingly marketed. They suppress plant disease through at least one mechanism, production of antibiotics or siderophores and induction of systemic resistance (Tenuta 2003).

Biofertlilizers are also available for increasing crop nutrient uptake of nitrogen from nitrogen-fixing bacteria associated with roots (Bashan and Holguin 1997), iron uptake from siderophore-producing bacteria (Scher and Baker 1982), sulfur uptake from sulfur-oxidizing bacteria (Stamford et al. 2008), and phosphorus uptake from phosphate-mineral solubilizing bacteria (Chabot et al. 1996). Biofertlilizers, that can cater different needs of growing plant, act as a consortium along with other microorganisms in the rhizosphere. Understanding the interaction between consortium of microbial inoculants and plant systems will pave way to harness more benefits from microbial inoculants for improving plant growth and yield (Raja et al. 2006).

2 Coinoculation of PGPR and Rhizobia: Improving Nodulation

Coinoculation studies with PGPR and Rhizobia have shown increased plant nodulation and N fixation (Li and Alexander 1988; Araújo and Hungria 1999; Vessey and Buss 2002; Silva et al. 2006; Figueiredo et al. 2007). Coinoculation of some *Bacillus* strains with effective *Bradyrhizobium* resulted in enhanced nodulation and plant growth of green gram (*Vigna radiata* L.) (Sindhu et al. 2002). A variety of rhizosphere microorganisms, including *Bacillus* and *Pseudomonas* species, are commonly found in the rhizosphere of leguminous and nonleguminous crops (Li and Alexander 1988). By virtue of their rapid colonization of the rhizosphere and stimulation of plant growth, there is currently considerable interest in exploiting

these rhizosphere bacteria to improve crop production. Application of *Bacillus* and/or *Paenibacillus* species to seeds or roots has been shown to cause alteration in the composition of rhizosphere leading to increase in growth and yield of different crops (Li and Alexander 1988; Vessey and Buss 2002). Disease suppression of alfalfa by *B. cereus* enhanced nodulation and seedling emergence in common bean (Camacho et al. 2001; Figueiredo et al. 2007), soybean (Araújo and Hungria 1999; Vessey and Buss 2002), cowpea (Silva et al. 2006, 2007), and pea (Cooper and Long 1994) have been demonstrated as beneficial effects on plants. Bacilli are also very attractive as potential inoculants in agriculture, as they produce very hardy spores that can survive for prolonged periods in soil and in storage containers (Nelson 2004).

Araújo and Hungria (1999) demonstrated the viability of coinoculating soybean seeds with crude or formulated metabolites, or with cells of *Bacillus subtilis*, to increase the contribution of the biological nitrogen fixation process.

PGPR, in combination with efficient rhizobia, could improve the growth and nitrogen fixation by inducing the occupancy of introduced rhizobia in the nodules of the legume (Tilak et al. 2006). According to Saravana-Kumar and Samiyappan (2007), *Bradyrhizobium* promoted the nodulation and growth of legumes in combination with active ACC deaminase containing PGPR. It has also been established that certain rhizobacteria possess an enzyme ACC-deaminase that hydrolyses ACC into ammonia and α-ketobutyrate (Mayak et al. 1999). ACC-deaminase activity in PGPR plays an important role in the host nodulation response (Remans et al. 2007). PGPR containing ACC-deaminase could suppress accelerated endogenous ethylene synthesis and thus may facilitate root elongation a nodulation and improve growth and yield of plant (Zafar-ul-Hye 2008).

3 Identification and Characterization of Beneficial Bacterial Strains for Agriculture

Identification and characterization of beneficial bacteria involves morphological, physiological and molecular characteristics based on fatty acid analysis, mol (%), G + C contents, DNA–DNA hybridization, and 16S rRNA sequencing. These characteristics help in defining the taxonomy and nomenclature of PGPR.

3.1 Taxonomy of PGPR

Taxonomy is defined as the science dedicated to the study of relationships among organisms and has to do with their classification, nomenclature, and identification (Mayr and Ashlock 1991; Coenye et al. 2005). The accurate comparison of organisms depends on a reliable taxonomic system. Although many new characterization methods have been developed over the last 30 years, the principle of identification

remains the same. Current schemes for identifying different bacterial strains may be roughly divided into four categories effectively based upon (1) traditional biochemical, morphological, and physiological characters, (2) miniaturized versions of traditional biochemical tests (e.g., API kits, VITEK cards, and Biolog plates), (3) chemotaxonomic characters (such as polyacrylamide gel electrophoresis [PAGE], and fatty acid methyl ester [FAME] profiles), and (4) genomic characters (16S rRNA gene sequencing, and DNA–DNA relatedness, and other techniques). Since the fifties, it was becoming clear that no one phenotypic technique would be suitable for identifying all bacterial species. Therefore, the potentials of chemotaxonomic analyses and studies of nucleic acids have been investigated. However, it is impossible to set up standardized conditions to accommodate the growth of all bacterial strains of all species for chemotaxonomic work, and a polyphasic approach is now imperative for a confident classification study. Polyphasic approach refers to the integration of genotypic, chemotypic, and phenotypic information of a microbe in order to perform reliable grouping of the organism (Colwell 1970). Some of the features used for polyphasic characterization of rhizobacteria are presented below. For overviews of modern taxonomy, recent papers can be referred, such as Vandamme et al. (1996), Prakash et al. (2007), Rodríguez-Díaz et al. (2008), and Logan et al. (2009).

3.2 Phenotypic Features

Phenotype includes morphological, physiological, and biochemical properties of the microorganism (de Vos et al. 2009). Traditional phenotypic tests used comprise colony morphology (color, dimensions, form) and microscopic appearance of the cells (shape, endospore, flagella, inclusion bodies), characteristics of the organism on different growth substrates, growth range of microorganisms on different conditions of salt, pH, and temperature, and susceptibility toward different kinds of antimicrobial agents, etc. Even if cell wall composition is analyzed, the Gram reaction is still a valuable diagnostic character. Biochemical tests in bacterial identification include the relationship with oxygen, fermentation reactions, and nitrogen metabolism. Other tests may be performed as appropriate, depending on the bacterial strains studied (Heritage et al. 1996; Rodríguez-Díaz et al. 2008). However, reproducibility of results from phenotypic tests between different laboratories is a great problem, and only standardized procedure should be used during execution of experiment. Other major disadvantage with phenotypic methods is the conditional nature of gene expression wherein the same organism might show different phenotypic characters in different environmental conditions. Therefore, phenotypic data must be compared with similar set of data from type strain of closely related organism(s).

Miniaturized versions of traditional biochemical tests are available for taxonomical studies and mostly contain a battery of dehydrated reagents. Addition of a standardized inoculum initiates the reaction (growth, production of enzymatic activity, etc.). The results are interpreted as recommended by the manufacturer and are readily

accessible with a minimal input of time. The phenotypic fingerprinting systems API 50CH – composed of 49 different carbohydrates and one negative control – have been used to identify *Bacillus* (Logan and Berkeley 1984) and *Paenibacillus* strains (Seldin and Penido 1986), while the API 20NE system has yielded the highest rate of correct identification of *Pseudomonas* species (Barr et al. 1989). In the same way, Biolog assay is considered a much less laborious system for bacterial identification (Miller and Rhoden 1991). This technique is based on the differential utilization of 95 carbon sources and a redox dye, tetrazolium violet, permits colorimetric determination of the increased respiration that occurs when cells are oxidizing a carbon source. The Biolog system was very useful for the identification of PGPR strains belonging to the species *P. azotofixans* (Pires and Seldin 1997).

3.3 Chemotaxonomic Characters

Some chemotaxonomic fingerprinting techniques applied to PGPR identification include FAME profiling, PAGE analysis of whole-cell proteins, polar lipid analysis, quinone content, cell wall diamino acid content, pyrolysis mass spectrometry, Fourier transform infrared spectroscopy, Raman spectroscopy, and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Fatty acids are the major constituents of lipids and lipopolysaccharides and have been used extensively for taxonomic purposes. FAME analysis is presently the only chemotaxonomic technique that is linked to a commercial database for identification purposes. Fatty acid profiles showing variability in chain length, double-bond position, and substituent groups are perfectly suitable for taxon description and also for comparative analyses of profiles that have been obtained under identical growth conditions (Suzuki et al. 1993).

Sodium dodecyl sulfate-PAGE of whole-cell proteins requires standardized conditions of growth, combined with a rigorously standardized procedure for analysis, and normalization of the data for computer-assisted comparison of the results. Nevertheless, it has made important contributions to polyphasic taxonomic studies among the aerobic endospore formers (Logan et al. 2009).

Determination of the cell wall composition has traditionally been important in Gram-positive bacteria which contain various peptidoglycan types. The peptidoglycan type of Gram-negative bacteria is rather uniform and provides little information. Preparation of cell wall samples and determination of peptidoglycan structure is usually carried out using the methods described by Schleifer and Kandler (1972).

Isoprenoid quinones occur in the cytoplasmic membranes of most prokaryotes and play important roles in electron transport, oxidative phosphorylation, and, possibly, active transport (Collins and Jones 1981). There are two major structural groups, the naphthoquinones (subdivided into two types: the phylloquinones and the menaquinones) and the benzoquinones. The large variability of the side chains (differences in length, saturation, and hydrogenation) can be used to characterize bacteria at different taxonomic levels (Collins and Jones 1981).

The taxonomic importance of polar lipids has now been demonstrated for some novel genera among the *Bacillaceae*, although many polar lipids detected have not yet been structurally characterized. Likewise, quinones (MK-7, MK-8, and MK-9) have so far been reported for representatives of *Bacillaceae* (Logan et al. 2009).

Finally, pyrolysis mass spectrometry, Fourier transform infrared spectroscopy, and UV resonance Raman spectroscopy are sophisticated analytical techniques which examine the total chemical composition of bacterial cells. These methods have been used for taxonomic studies of particular groups of bacteria, including the members of the family *Bacillaceae* (Vandamme et al. 1996; Logan et al. 2009).

3.4 Genetic Approaches

Genotypic methods are those that are directed toward DNA or RNA molecules. Undoubtedly, these methods have revolutionized the bacterial identification system and taxonomy. Different techniques are now available to subtype bacteria up to strain level, such as restriction fragment length polymorphism (RFLP), plasmid profiling, ribotyping, amplified ribosomal DNA restriction analysis (ARDRA), pulsed field gel electrophoresis (PFGE), and randomly amplified polymorphic DNA (RAPD). Different PGPR have already been characterized by one or more of these methods (Oliveira et al. 2000; von der Weid et al. 2000; Depret and Laguerre 2008; Monteiro et al. 2009; and many others). For a detailed description of these methods, the reviews by Vandamme et al. (1996), Prakash et al. (2007), Rodríguez-Díaz et al. (2008), and Logan et al. (2009) can be referred.

For the description of bacterial taxa, other methods are essentially used. Determination of the moles percent guanosine plus cytosine is one of the classical genotypic methods. Generally, the range observed is not more than 3% within a well-defined species and not more than 10% within a well-defined genus (Stackebrandt and Goebel 1994).

DNA–DNA hybridization or DNA–DNA reassociation technique is based on the fact that at high temperatures DNA can be denatured, but the molecule can be brought back to its native state by lowering down the temperature (reassociation). This technique considers the comparison between whole genome of two bacterial species (Stackebrandt and Liesack 1993). A bacterial species, generally, would include the strain with 70% or greater DNA–DNA hybridization values with 5°C or less ΔTm values, and both the values must be considered. There are many different methods for DNA–DNA hybridization [presented and compared by Mora (2006)], but it is important to state that this technique gives the relative % of similarity but not the actual sequence identity.

DNA microarray is a method which was lined up to overcome the shortcomings of DNA–DNA hybridization. Although DNA microarray also involves hybridization of DNA, it uses fragmented DNA instead of whole genomic DNA. Numerous DNA fragments can be hybridized on a single microarray and gives resolution up to strain level. However, it is still an expensive methodology.

Indeed, taxonomy was revolutionized when the gene sequences of rRNA molecules were introduced to compare evolutionary similarities among strains (phylogenetic comparisons). All the three kinds of rRNA molecules, i.e., 5S, 16S, and 23S and spacers between these can be used for phylogenetic analyses, but 16S rRNA gene (1,650 bp) is the most commonly used marker. It has a universal distribution, highly conserved nature, fundamental role of ribosome in protein synthesis, no horizontal transfer, and its rate of evolution which represents an appropriate level of variation between organisms (Stackebrandt and Goebel 1994). The 16S rRNA molecule comprises of variable and conserved regions, and universal primers for the amplification of full 16S rRNA gene are usually chosen from conserved region while the variable region is used for comparative taxonomy. The 16S rRNA gene sequence is deposited in databases such as Ribosomal Database Project II (http://rdp.cme.msu.edu/) and GenBank (http://www.ncbi.nlm.nih.gov/). Sequences of related species for comparative phylogenetic analysis can also be retrieved from these databases. Thereafter, sequence comparing software packages such as BLAST and CLUSTAL X are used for alignment of 16S rRNA gene sequence. The extent of relatedness between bacterial species can be scrutinized by the construction of phylogenetic trees or dendrograms. The phylogenetic tree ascertains the genus to which the strain belongs and its closest neighbors, i.e., those sharing the clade or showing >97% 16S rRNA gene sequence similarity, are obtained from various culture collections to perform further genotypic, chemotaxonomic, and phenotypic analysis. At present, by correlation with experimental data obtained in the comparison of total genomic DNA (DNA–DNA hybridization), it is proposed that a similarity below 98.7–99% on the 16S rRNA gene sequences of two bacterial strains is sufficient to consider them as belonging to different species. On the other hand, two strains showing similarities above the 98.7% threshold may represent two different species. In these cases, total genome DNA–DNA hybridization must be performed and those strains for which similarities are below 70% are considered to belong to different species (Stackebrandt and Liesack 1993; Stackebrandt and Goebel 1994).

Finally, sequences of other highly conserved housekeeping or other protein-encoding genes, such as *rpoB*, *gyrB*, *recA*, have also great potential for taxonomic analysis at the species level. For example, Mota et al. (2005) obtained clustering patterns for *Paenibacillus* based upon *rpoB* sequence comparisons that were similar to those obtained with 16S rRNA gene sequences. Moreover, Wang et al. (2007) included *gyrB* sequence comparisons in the studies of the *B. subtilis* group and Cerritos et al. (2008) included *recA* sequence comparisons in the work that led to the proposal of a new *Bacillus* species.

4 Prospective Biocontrol Agents of Plant Diseases

Since 1987 in China, PGPR, called yield increasing bacteria (YIB) have been largely applied in 48 different crops over 3.35 millions of hectares (Wenhua and Hetong 1997). In that country, productivity gains as high as 23.1% and 22.5% were

obtained, respectively, in sweet potatoes and potatoes. Additionally, remarkable 85.5% and 80.3% reduction levels of diseases caused by *Xanthomonas oryzae* pv. *oryzae* and *Glomerella cingulata*, respectively, were recorded (Zhang et al. 1996).

Rhizobacteria are effective competitors in the rhizosphere which can establish and persist on roots of agronomically grown plants (Kloepper and Mariano 2000). PGPR may promote plant growth directly on healthy plants or indirectly when controlling phytopathogens or pests in different crops (Kloepper 1993; Medeiros et al. 2005; Zhender et al. 1997; Keel and Maurhofer 2009). They can be isolated from any other plant part besides the roots as well as from the plant surface or interior. According to Hallman et al. (1997), the endophytic bacteria involved in biological control showed advantages of having the same ecological niche of the pathogen and could be protected from diverse abiotic influences.

The PGPR mechanisms for plant growth improvement were already discussed in this chapter. PGPR also exhibit several mechanisms of biological disease control, most of which involve competition and production of metabolites which affect the pathogen directly. Examples of such metabolites include antibiotics, cell wall-degrading enzymes, siderophores, and HCN (Enebak et al. 1998; Kloepper 1993; Weller 1988). It is noteworthy to state that different mechanisms may be found in a single strain and act simultaneously. Some PGPR do not produce metabolites against the pathogens and are spatially separated from them. These two traits suggest that alteration of host defense mechanisms account for the observed disease protection. Induced systemic resistance (ISR) or systemic acquired resistance (SAR) is defined as the activation of chemical and physical defenses of the plant host by an inducer which could be a chemical or a microorganism, leading to the control of several pathogens (Kloepper et al. 1992). Several PGPR strains can act as inducers of ISR (Kloepper et al. 1992), and PGPR-mediated ISR may be an alternative to the use of chemical inducers or pathogens for inducing SAR. This mechanism is discussed separately in this chapter.

Two cases of study will be discussed here: black rot of crucifers, a foliar disease, and Fusarium wilt of banana, a vascular disease. Black rot caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*) causes severe economic losses in all developmental crucifer stages (Mariano et al. 2001). *Bacillus* spp. isolated from healthy cabbage, kale, and radish had reduced black rot incidence in kale and cabbage in greenhouse and field experiments (Assis et al. 1996). Monteiro et al. (2005) showed that four of these *Bacillus* strains produced lipopeptides active against *Xcc* during its late growth phase. These peptide antibiotics are amphiphilic compounds with surfactant activity (Zuber et al. 1993). Recently, it was demonstrated that lipopeptides can stimulate ISR in plants, probably by interacting with plant cell membranes and inducing temporary alterations in the plasma membrane which could raise plant defenses (Ongena et al. 2009).

Fusarium wilt of banana caused by *Fusarium oxysporum* f. sp. *cubense* is a very destructive disease in Brazil and other parts of the world. The rhizomes and pseudostems of infected plants used for propagation are the principal sources of inoculum and disease dispersion. Therefore, micropropagated health plantlets are used to prevent or delay the introduction of this pathogen in soils. However, these plantlets

are more susceptible to this and other soilborne pathogens and should be protected before transplanting. PGPR are an alternative for improving this system. In greenhouse studies, endophytic and epiphytic bacteria applied, isolated or in mixtures, as root and substrate treatments, significantly increased the growth of micropropagated banana plantlets and controlled fusarium wilt (Mariano et al. 2004) (Fig. 1). According to Nowak and Shulaev (2003), the production of high-quality propagules with disease resistance may be achieved among others methods by their "in vitro" and "ex vitro" biopriming (priming with beneficial microorganisms).

Commonly, control is based on the use of single biocontrol agents. This strategy must be changed because, from the ecological point of view, the disease is part of a complex agroecosystem. As reported by Fravel (2007), a holistic view of this system can help take correct decisions about management. Therefore, a special approach for improving the PGPR efficiency is the use of mixtures containing different genera or species that presents additive or synergistic effects such as nitrogen-fixing bacteria and mycorrhiza helper bacteria (MHB). Another strategy is to use PGPR, mixed or alternated with fungicides, integrating biological and chemical control.

MHB are those which either assist mycorrhiza formation or promote the functioning of their symbiosis. They exist in arbuscular and ectomycorrhizal systems. MHB present three significant functions: nutrient mobilization from soil minerals, fixation of atmospheric nitrogen, and plant protection against root pathogens (Frey-Klett et al. 2007). According to these authors, PGPR induced increases in mycorrhizal root colonization from 1.1 to 17.5 fold in different interactions. Some of the MHB cited were

Fig. 1 Biocontrol of Fusarium wilt in micropropagated banana plantlet cv. Pacovan treated with *Bacillus pumilus* ENF24 (*right*) compared with plantlet not treated (*left*). Plantlets were vertically sliced to show rhizome discoloration, an internal disease symptom

Pseudomonas fluorescens, *P. monteilii*, *Bacillus coagulans*, *B. subtilis*, *Paenibacillus brasilensis*, *Rhizobium leguminosarum*, and *Bradyrrhizobium japonicum*.

Wheat seeds treated with different mixtures of *Paenibacillus macerans* and difenoconazole showed significant reduced incidences of pathogens (Luz 2003a), and in field all treatments promoted germination and grain yield except for difenoconazole alone that increased only yield. Similar results were obtained when corn seeds were bacterized with the same bioprotector + fludioxonil + metalaxyl M (Luz 2003b). Also *Bacillus*-based treatments have been successfully combined with traditional chemical seed treatments (Bugg et al. 2009). Therefore, the use of such mixtures may lead to a substantial reduction of pesticide use in several crops.

It is also important to focus on the critical stages of commercialization of biocontrol agents. Screening for new agents should consider the biology and ecology of the pathosystem, as well as agricultural practices associated with the crop (Fravel 2007). This knowledge will help prevent variation in field performance which is responsible for lack of wider adoption of biocontrol for disease management. The formulation stage aim is to deliver the biocontrol agent in a physiologically active state to provide the needed control. The formulation must be economical and present good shelf-life and a suitable form for shipping, storage, and application. Risk assessment to human health and to the environment are needed before releasing the new product, and early in the screening; even microorganisms with good biocontrol potential but capable of growing at human body temperature should be eliminated (Fravel 2007). In the United States, organisms currently registered for biocontrol and active compounds isolated from plants or other organisms are listed at http://www.epa.gov/oppbppd1/biopesticides/ingredients/index.htm. A few examples of PGPR and biocontrol products are: *Agrobacterium radiobacter* K1026 (Nogall®), *Bacillus pumilus* QST 2808 (Sonata® TM), *B. pumilus* GB34 (YieldShield®), *B. subtilis* GBO3(Kodiak®), *Pantoea agglomerans* C9-1 (BlightBan C9-1®), *P. agglomerans* E325 (Bloomtime®), *Pseudomonas aureofaciens* Tx-1(Spot-Less®T), *P. syringae* ESC-10 and ESC-11 (Bio-save®), *P. fluorescens* A506 (BlightBan®), *P. chlororaphis* MA 342 (Cedomon®), *Streptomyces griseoviridis* K61 (Mycostop®), and *S. lydicus* WYEC 108 (Actinovate®).

5 Induced Systemic Resistance as a Mechanism of Disease Suppression by Rhizobacteria

The increased level of resistance using external agents, without modifying the genome of the plant, is known as induced or acquired resistance. The expression of induced resistance can be local or systemic when it is expressed at sites not directly exposed to the inducers agent (Stadnik 2000). This agent may be a chemical activator, extracts of cells of living organisms or microorganisms (Romeiro 2000). The event of ISR has been demonstrated in various plants inoculated with different species of rhizobacteria (Liu et al. 1995; Raj et al. 2003; Halfeld-Vieira et al. 2006). This type of induced resistance can occur under

controlled conditions and in the field, and shows advantages such as: effectiveness against various pathogens; stability due to the action of different mechanisms of resistance, systemicity, energy economy; and metabolic utilization of genetic potential for resistance in all susceptible plants (Bonaldo et al. 2005).

The ISR occurs when plants previously exposed to biotic and abiotic agents are induced to defense against pathogens, which are spatially separated from the inducer agent (Pieterse and Van Loon 1999; Stadnik 2000). PGPR that inhabit the soil and are often isolated from the rhizosphere of several plants have been studied as potential biotic agents of ISR (Mariano and Kloepper 2000). *Bacillus* and *Pseudomonas* are among the most studied genera of PGPR.

It is known that susceptible plants have genetic information for efficient mechanisms of resistance to diseases and that these mechanisms can be systematically expressed for long periods of time by prior inoculation with avirulents pathogens, microbial components, and chemical substances (Kuc 1995). The ISR is persistent and presents complex components in different locations which are responsible for the activity of various defense compounds. Consequently, it is more stable when compared with the few pathways arising from the use of chemical pesticides.

Despite the many studies in this area, only in 1961 the induced resistance was first analyzed, by preinoculation of tobacco plants with tobacco mosaic virus (Ross 1961). This procedure protected the plant against other viruses and resulted in the conception of "Systemic Acquired Resistance" (SAR). The activation of defense mechanisms induced by fungi, bacteria, viruses, and nematodes can be achieved by different routes, which may occur alone or concomitantly (Bonaldo et al. 2005).

Problems of variability in the effectiveness of induced resistance to diseases in plants in different soil and climatic conditions may occur, similar to that found in biological control (Kuc 1995). In agriculture, the use of biological products on the induction of resistance in plants has one more benefit that can be added to the already known to reinforce the plant growth promotion. Induction of resistance by the application of chemical inducers has been used in some crops in the integrated management of diseases and pests. The use of biological inducers may be an option in the management of diseases in plants. The positive effects of PGPR on plants usually are included in two categories: promotion of growth and biological control (Mariano and Kloepper 2000). In practice, these effects are often induced by the same strain of PGPR; therefore, some PGPR selected to promote growth also are able to control diseases and vice versa. The presence of the PGPR in the rhizosphere makes the entire plant, including the shoot, more resistant to pathogens.

Induction of resistance promoted by PGPR is active and signaling in the route of salicylic acid with induction of PR-proteins (proteins related to the pathogenesis) or route of the jasmonic acid and ethylene (Hoffland et al. 1995; Pieterse et al. 1998). When the PGPR colonize the root system, constituents of bacterial cell molecules or synthesized by elicitors act as a biochemical signal. This time, the genes that encode for the synthesis of components of the dynamic resistance are activated and ISR is expressed (Romeiro 2000). Wei et al. (1991) working with cucumber and anthracnose caused by *Colletotrichum orbiculare* showed that this plant could be used as a model for ISR.

In addition to the PR-proteins, the plants produce other enzymes of the defense, including peroxidases, phenylalanine ammonia-lyase (PAL), and polyphenol-oxidase (PPO). Peroxidase and PPO are catalysts in the formation of lignin. PAL and other enzymes are involved in the formation of phytoalexins. Chen et al. (2000) reported that ISR mediated by PGPR against *Pythium aphanidermatum* in cucumber was associated with an increase of peroxidases, PPO and PAL. Metabolic changes involved in the defense mechanism of plants are correlated with changes in activity of key enzymes in primary and secondary metabolism. The production of enzymes related to pathogenesis (PR-proteins) by strains of rhizobacteria is considered the largest property of the antagonistic strains (Saikia et al. 2004). Among these enzymes can be highlighted chitinases, lipoxygenases, peroxidases, and glucanases. Plants express the activity of peroxidase during pathogen–host interaction (Saikia et al. 2006), where this enzyme has been implicated in the oxidation of phenols (Schmid and Feucht 1980), lignification (Saparrat and Guillen 2005), plant protection (Hammerschmidt et al. 1982), and elongation of plant cells (Goldberg et al. 1986). Increased activity of peroxidase has been correlated with resistance in many plant species, including rice and wheat (Young et al. 1995). The action of lipoxygenase products contributes to the defense reactions involving the inhibition of growth of the pathogen and induction of phytoalexins (Li et al. 1991). The phytoalexins are secondary metabolites, antibiotics, low molecular weight produced by plants in response to physical stress, chemical, or biological. They are able to prevent or reduce the activity of pathogens, the rate of production dependent on the genotypes of host and/or pathogen (Daniel and Purkayastha 1995). The phytoalexin compounds are biocides and are directly related to the defense mechanisms of plants.

In several studies, the quantification of activity of enzymes involved in the induction of resistance has been used as a parameter to assess the induction mechanism (biotic or abiotic) involved (Knorzera et al. 1999; Campos et al. 2004; Nakkeeran et al. 2006; Silva et al. 2004; Halfeld-Vieira et al. 2006; Saikia et al. 2006). The increase in activity and accumulation of these enzymes depend mainly on the inducing agent but also the genotype of the plant, physiological conditions, and the pathogen (Tuzun 2001). Depending of pathosystem studied, a variety of substances are produced by rhizobacteria and has been linked to activation of mechanisms of disease suppression in plants which reduce the damage caused by phytopathogens. Thus, the application of PGPR in agriculture via soil or seed inoculation can be characterized as a beneficial component in the integrated management of diseases.

6 Bacterial Biofertilizers

Before initiating a review of PGPR as biofertilizers, it is necessary to define the term biofertilizer. It is proposed frequently here that biofertilizers designate the biological products which contain microorganisms providing direct and indirect gains in yield from crops. Vessey (2003) defines biofertilizers as a substance which

contains living microorganisms which, when applied to seed, plant surfaces, or soil colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients the host plant. Rhizobacteria, associated with rhizosphere, can fix nitrogen, and solubilizing phosphorus has been used as inoculum in nonleguminous species such as maize, rice, wheat, and sugar cane (Dobereiner 1997). Biofertilizers have been an alternative to mineral fertilizers to increase the yield and plant growth in sustainable agriculture (Canbolat et al. 2006).

The mechanisms by which PGPR promote plant growth are not fully understood but include among others: ability to produce or change the concentration of plant hormones (Mordukhova et al. 1991); asymbiotic N_2 fixation (Boddey and Dobereiner 1995); and solubilization of mineral phosphate and other nutrients (De Freitas et al. 1997). The production of hormones in PGPR in numerous studies reports the importance of indolacetic acid (IAA) in the roots development (Aloni et al. 2006). The effect of exogenous IAA in the plant can stimulate or inhibit growth and is often a function of hormones concentration available; in addition, the sensitivity of plant tissue changes according to hormones concentration (Persello-Cartieux et al. 2003). It was reported that isolates of *Pseudomonas* (fluorescent) produced exudates in roots of maize in response to IAA (Pan et al. 1999). Gibberellins were detected in several cultures of *B. subtilis*, but were not detected in the presence of auxin (Broadbent et al. 1971). Analyzing the sources of IAA with bacterial origin, Loper and Schroth (1986) found two strains of *Pseudomonas* spp. producing high concentrations of IAA (5–10 mg/ml), which reduced roots elongation and increased shoot/root proportion in sugar beet plants (*Beta vulgaris*) when applied as seed inoculant in this culture. Araújo et al. (2005) detected auxin production in two strains of *B. subtilis* which provided benefits in growth of soybean, in addition to be antagonists of phytopathogenic fungi in culture. Araújo and Hungria (1999) found that *B. subtilis* (AP-3) or its metabolites provided increase in nodulation and yield of soybean in the field.

Gains in nutrition in plants inoculated with rhizobacteria have also been demonstrated as a benefit of the presence of this group of microorganisms in the rhizosphere. In relation to nitrogen for several years has been discovered the potential of bacteria from the genus *Azospirillum*; fixing nitrogen when in free-living (Boddey and Dobereiner 1995), which when associated with the rhizosphere may contribute to nitrogen nutrition of plants. Concerning phosphate nutrition, Rodriguez and Fraga (1999) mention that strain from the genus *Pseudomonas*, *Bacillus* and *Rhizobium* are among the bacteria with the greatest potential of solubilization of phosphorus in the soil.

The solubilization of insoluble phosphates mediated by microorganisms is associated with the detachment of organic acids which are often combined with other metabolites, as found in vitro, that the potential for P solubilization by microorganisms is directly related to production of siderophores, lytic enzymes, and phytohormones (Vassilev et al. 2006). With the increased availability of nutrients in the soil by the action of *B. subtilis*, was shown higher absorption of nutrients such as phosphorus and nitrogen in plants inoculated with rhizobacteria

on seeds (Araújo 2008). Richardson (2000) reported that most soils are poor in available phosphorus and phosphate fertilizer represents a high cost to the farmer; therefore, it is interesting to take advantage of soil microorganisms used as inoculum for the mobilization of phosphorus in poor soils. In addition to phosphorus solubilization, other mechanisms are also related to the microbial metabolism in soil, such as enzymes production (nitrogenase, chitinases, and glucanases) (Cattelan et al. 1999).

Some failures derived from the use of biofertilizers containing PGPR may be related to interspecific genetic interaction by the rhizobacteria and the host plant. Previous studies have documented phenotypic variation within cultivars with respect to health and nutrition of plants from microbial inoculation (Remans et al. 2008). Different cultures and species or cultivars may produce different types of root exudates, which may support the activity of the inoculum or serve as substrate for the formation of biologically active substances by the inoculum (Khalid et al. 2004). Dalmastri et al. (1999) reported that different maize cultivars could provide variation in the rhizosphere colonization by *Burkholderia*. Phenotypic variation among cultivars may be partly due to genetic variation and suggested that the breeding of the host was performed in conjunction with PGPR in biofertilizers (Remans et al. 2008). Another strategy to reduce the effects of phenotypic variation can be the use of biofertilizers with more than two isolates in their composition. Studies conducted for 2 years with the application of biofertilizers originating from a mixture of isolates of *Bacillus* showed increase in plant growth and productivity (Adesemoye et al. 2008).

A major problem for massive use of PGPR has been formulated for its commercial use. These include production in the scale of fermentation microorganisms with management of the quality, stability, and effectiveness of the product. *B. subtilis* has been assessed as of great potential for use in agriculture and has been used in the formulation of commercial products for agricultural use in several countries (Lazzareti and Bettiol 1997). Several substances have been used in experimental formulations such as lactose, peptone, gum arabic and xanthan, cellulose, and others (Schisler et al. 2004). This formulation may require a significant value to determine the effectiveness of the final product based on rhizobacteria such as the *B. subtilis*.

Development of formulations with a potential PGP to ensure survival and activity in the field and compatibility with chemical treatment of seeds has been the focus of researches with application of PGPR in agriculture. The research among other things optimizes growth conditions before the formulation, development of vehicles, and appropriate technology for application (Date 2001). In registration and marketing of products with PGPR, a large number of constraints are found (Mathre et al. 1999).

The U.S. market based on the information of the committee of biological products from the American Phytopatology Society (APS) in 2005 has registered the following products: ten products based on the *Bacillus* (*BioYield*, *Companion*, *EcoGuard*, *HiStick N/T*, *Kodiak* , *Mepplus*, *Serenade*, *Sonata*, *Subtilex*, *YieldShield*), two products with *Burkholderia cepacia* (*Deny* and *Intercept*), and six

products based on *Pseudomonas* (*AtEze*, *Bio-save*, *BlightBan*, *Frostban*, *Spot-Less*). Most of these products has been disposed in powder solubleformulate. Different genera of bacteria have been studied as PGPR; however, investments in research and development of bioproducts have been higher in projects on *Pseudomonas* and *Bacillus*. Works on *Pseudomonas* have been focused on alternatives to improve the survival of this species of bacteria in commercial formulations. Furthermore, bacteria from the genus *Bacillus*, which are tolerant to desiccation and heat, have a longer life in commercial formulations; this explains the greater availability of commercial products based on *Bacillus*.

Currently, biofertilizers with PGPR are still not a reality of extensive commercialization – unlike the agricultural use of legume inoculants using rhizobia already a reality for almost a century – except for *Azospirillum* inoculants that are available for a variety of crops in Europe and Africa (Vessey 2003). There is no doubt that the lack of consistent responses in different host cultivars (Remans et al. 2008) and different field sites (Hilali et al. 2001) are reasons that limit expansion of the marketing of biofertilizers with PGPR. For these, it would be necessary to carry out more studies on ecology and colonization of microorganisms in the rhizosphere at different situations, since the biofertilizers with PGPR are restrictive for certain cultivars, climate, and soil conditions.

7 Concluding Remarks

PGPRs are the potential tools for sustainable agriculture and trend for the future. For this reason, there is an urgent need for research to clear definition of what bacterial traits are useful and necessary for different environmental conditions and plants, so that optimal bacterial strains can either be selected and/or improved. Combinations of beneficial bacterial strains that interact synergistically are currently being devised and numerous recent studies show a promising trend in the field of inoculation technology.

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Potential of PGPR in Agricultural Innovations

Haluk Caglar Kaymak

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Abstract Plant growth promoting rhizobacteria (PGPR) are a group of free-living bacteria that colonize the rhizosphere and benefit the root growth in plants. Bacteria of diverse genera such as *Azospirillium*, *Bacillus*, *Burkholderia*, *Klebsiella*, *Pseudomonas*, etc., were identified as PGPR. These PGPR exert a direct effect on plant growth by inducing the production of phytohormones, supplying biologically fixed nitrogen, and increasing the phosphorous uptake by the solubilization of inorganic phosphates. These bacteria affect plant growth by indirect mechanisms that involve suppression of bacterial, fungal, viral, and nematode pathogens. A lot of study showed that inoculation with PGPR resulted in significant yield increases in different crops, rooting of hardwood and semi-hardwood cuttings, increased germination and enhanced emergence of seeds under different conditions, promoted nutrient uptake of roots, total biomass of the plants, increased seed weight, induced early flowering, etc. In this review, the importance of PGPR is discussed for

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agricultural innovations with special references that utilises direct and indirect plant growth promotion.

1 Introduction

The rhizosphere, volume of soil surrounding roots influenced chemically, physically, and biologically by the plant root, is a highly favorable habitat for the reproduction of microorganisms, which exerts a potential impact on plant health and soil fertility (Sorensen 1997; Antoun and Prevost 2005; Podile and Kishore 2006). This environment is relatively rich in nutrients released by the plant roots, and its microbial communities are different from those that are not influenced by the roots (Alexander 1977; Burdman et al. 2000).

In the rhizosphere, very important and intensive interactions occur among the plant, soil, microorganisms, and soil microfauna (Antoun and Prevost 2005). These interactions can significantly influence plant growth and crop yields. In the rhizosphere, bacteria are the most abundant microorganisms. Rhizobacteria are rhizosphere-competent bacteria that aggressively colonize plant roots, could be free-living, parasitic, or saprophytic, and their diversity remains dynamic with a frequent shift in community structure and species abundance (Kunc and Macura 1988). These microbial communities are beneficial for plant growth, yield, and crop quality, and they have been called "plant growth promoting rhizobacteria (PGPR)" (Kloepper and Schroth 1978) including numerous strains of the genera *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Arthrobacter*, *Azospirillium*, *Azotobacter*, *Azoarcus*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Clostridium*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Gluconacetobacter*, *Klebsiella*, *Pseudomonas*, *Serratia*, *Rhizobium*, etc. (Burdman et al. 2000; Sudhakar et al. 2000; Hamaoui et al. 2001; Bertrand et al. 2001; Mirza et al. 2001; Bonaterra et al. 2003; Esitken et al. 2003a; Murphy et al. 2003; Raj et al. 2004; Joo et al. 2004; Esitken et al. 2006; Podile and Kishore 2006; Saleem et al. 2007).

PGPR can be divided into two groups according to their relationship with the plants: symbiotic bacteria and free-living rhizobacteria (Khan 2005). A lot of work have been done to study about the mechanisms and principles of the PGPR–plant relationship, which was widely accepted as the rhizosphere effect (Zhuang et al. 2007). Glick (1995) reported that PGPR function in three different ways: synthesizing particular compounds for the plants, facilitating the uptake of certain nutrients from the environment (Cakmakci et al. 2006; Garcia et al. 2004a, b; Siddiqui and Mahmood 2001), and preventing the plants from diseases (Guo et al. 2004; Jetiyanon and Kloepper 2002; Raj et al. 2003a, b).

In other words, these mentioned bacteria can directly cause plant growth, seed emergence, or improvement in crop yields by producing and secreting plant growth regulators such as auxins, gibberellins (GAs), and cytokinins. They elicit the root metabolic activities, supply biologically fixed nitrogen, and increase the phosphorous uptake by solubilization of inorganic phosphates (Burdman et al. 2000;

Podile and Kishore 2006). The near direct effect of PGPR is that these bacteria affect plant growth by indirect mechanisms that involve suppression of bacterial, fungal, viral, and nematode pathogens (Burdman et al. 2000; Kirankumar et al. 2008).

In this review, the importance of PGPR is discussed for agriculture innovations with special reference to their utilization in direct plant growth promotion such as seed emergence, secretion of plant growth regulators, and indirect plant growth promotion such as suppression of pest and disease.

2 Direct Plant Growth Promotion

PGPR influence direct growth promotion of plants by fixing atmospheric nitrogen, solubilizing insoluble phosphates, secreting hormones such as IAA, GAs, and Kinetins besides ACC deaminase production, which helps in regulation of ethylene.

2.1 Biological Nitrogen Fixation

Nitrogen is a well-known and essential key nutrient for plant growth and development. However, the global nitrogen cycle pollutes groundwater and increases the risk of chemical spills. The production of chemical fertilizers is a highly energy-intensive process using large amounts of fossil energy. High-input farming practices achieving high yields have created environmental problems and degradation in natural resources (Şahin et al. 2004). Thus, Figueiredo et al. (2008) reported that during the past couple of decades, the use of PGPR for sustainable and environment friendly agriculture has been increased tremendously in various parts of the world. Increasing and extending the role of bio-fertilizing with PGPR would reduce the need for chemical fertilizers and decrease their adverse environmental effects. Microorganisms are gaining importance in agriculture to promote the circulation of plant nutrients and reduce the need for chemical fertilizers (Şahin et al. 2004; Orhan et al. 2006).

Rhizosphere associated N-fixing bacteria have increasingly been used in nonlegume crop species such as sugar beet, sugar cane, rice, maize, and wheat (Döbereiner 1997; Hecht-Buchholz 1998; Şahin et al. 2004). For example, experiments with *Bacillus* species indicated yield increases in cereals (Belimov et al. 1995; Cakmakci et al. 2001; Öztürk et al. 2003) and maize (Pal 1998).

N-fixation is the first mechanism suggested to promote the growth of plants by *Azospirillum*. The majority of evidence collected during the last 3 decades concerning this mechanism has generated controversy (Bashan et al. 2004). At the same time, *Azospirillum* lead the list of PGPR assessed in worldwide experiments (Burdman et al. 2000; Dobbelaere et al. 2003; Vessey 2003; Lucy et al. 2004; Ramirez and Mellado 2005). *Pseudomonas* and *Bacillus* species (Alam et al. 2001; Cakmakci et al. 2001; Glick et al. 1994; Kokalis-Burelle et al. 2002), and the other PGPR and endophytic

bacteria, such as *Enterobacter*, *Klebsiella*, *Burkholderia*, and *Stenotrophomonas*, have been gaining attention in the recent years, because of their association with important crops and potential to enhance the plant growth (Chelius and Triplett 2000; Sturz et al. 2001; Verma et al. 2001; Dong et al. 2003; Ramirez and Mellado 2005).

Some greenhouse and field experiments have shown repeatedly that the transfer of nitrogen fixed by *Azospirillum* spp. to the plant is not enough (Bashan and Holguin 1997; Kennedy et al. 1997; Kennedy and Chellapillai 1998; Bashan et al. 2004). Yet other studies showed that the bacteria cannot fulfil all of the nitrogen requirements of the plants; nevertheless, it can contribute only significant amounts of nitrogen. For example, seed inoculation of chickpea with *Rhizobium*, N-fixing *Bacillus subtilis* (OSU-142) significantly increased N percentage compared with the control treatment and may substitute costly N fertilizers in chickpea production even in cold highland areas (Elkoca et al. 2008).

Similarly, N-fixing bacterial strains *Pseudomonas putida* RC06, *Paenibacillus polymyxa* RC05 and RC14, and *Bacillus* OSU-142 have great potential, and as formulations, they are used as biofertilizers for better yield and the quality of wheat, sugar beet, and spinach growth (Cakmakci et al. 2007; Cakmakci et al. 2006). The N-fixing *Bacillus* strains and *A. brasilense* sp246 have a potential on plant growth activity of spring wheat and barley cultivation in organic and low-N input agriculture (Öztürk et al. 2003; Canbolat et al. 2006). Inoculation with the *Rhizobium leguminosarum* E11 and *Azotobacter* sp. S8, strain E11 increased root dry weight, root length, and growth in cotton (Hafeez et al. 2004). Significant positive effects on growth, nodule number, and yield of soybean were obtained after inoculation with *Bradyrhizobium* spp strains S62 and S63 (Egamberdiyeva et al. 2004).

Furthermore, inoculation commonly and significantly reduced the required doses of nitrogen fertilization in numerous greenhouse and field experiments in a lot of plant species (Bashan and Levanony 1990; Bashan and Holguin 1997; Bashan et al. 2004).

The strain(s), soil types, climate, and the development of appropriate formulations as well as strategies of field experimentations should be considered for a successful application of PGPR when using as fertilizers.

2.2 Solubilization of Phosphates

Phosphorous (P), next to nitrogen, is one of the major and key nutrients limiting plant growth (Kumar and Narula 1999; Sundara et al. 2002; Podile and Kishore 2006). Even in phosphorous rich soil, most of the P is unavailable for the plants, as large amount of soil P is found in its insoluble form. Phosphate solubilizing bacteria (PSB) are common in the rhizosphere and can be used to overcome this problem (Vessey 2003).

PSB secretes organic acids and phosphatases that converts the insoluble phosphates into soluble monobasic and dibasic ions and may also solubilize inorganic phosphate and makes soil phosphorus, which otherwise remain fixed, available to

the plants (Kumar and Narula 1999; Whitelaw 2000; Gyaneshwar et al. 2002). In other words, phosphate solubilizing microorganisms convert insoluble phosphates into soluble forms through the process of acidification, chelation, exchange reactions, and production of gluconic acid (Rodriguez et al. 2004; Chung et al. 2005; Hameeda et al. 2008).

PSB are ubiquitous (Gyaneshwar et al. 2002), and *Bacillus*, *Enterobacter*, *Erwinia*, and *Pseudomonas* spp. are among the most potent strains (Podile and Kishore 2006). PSB is common in rhizospheres of crop plants, and few examples of beneficial association with phosphate solubilizing PGPR and plants include *B. megaterium* (M-3) and chickpea (Elkoca et al. 2008), *B. licheniformis* RC08 and *B. megaterium* RC07, and wheat and spinach (Cakmakci et al. 2007), *Enterobacter agglomerans* and tomato (Kim et al. 1998), *P. chlororaphis*, *P. putida*, and soybean (Cattelan et al. 1999), *Avena sativa* and PGPR strains isolated from the rhizosphere of forage (WenXing et al. 2008), *Serratia marcescens* EB 67, *Pseudomonas* sp. CDB 35, and maize (Hameeda et al. 2008).

In the controlled environment and in the field trials, single and dual N-fixing *B. subtilis* (OSU-142) and P-solubilizing *B. megaterium* (M-3) inoculations significantly increased all the parameters investigated in chickpea (plant height, shoot, root and nodule dry weight, N%, chlorophyll content, pod number, seed yield, total biomass yield, and seed protein content) compared with the control treatment, equal to or higher than N, P, and NP treatments (Elkoca et al. 2008).

In another research, Orhan et al. (2006) reported that plant growth promoting effects of two *Bacillus* strains OSU-142 (N-fixing) and M3 (N-fixing and phosphate solubilizing) were tested alone or in combinations of organically grown primocane fruiting raspberry (cv. Heritage) plants and a significant increase in yield (33.9 and 74.9%), cane length (13.6 and 15.0%), number of cluster per cane (25.4 and 28.7%), and number of berries per cane (25.1 and 36.0%) were observed when compared with that of the control.

Hameeda et al. (2008) reported that plant biomass increased with *Serratia marcescens* EB 67 and *Pseudomonas* sp CDB 35 under both glasshouse and field conditions. And also, seed treatment with EB 67 and CDB 35 increased the grain yield of field-grown maize by 85 and 64% compared with the uninoculated control.

Furthermore, four strains namely, *Arthrobacter aureofaciens*, *Phyllobacterium myrsinacearum*, *Rhodococcus erythropolis*, and *Delftia* sp. are being reported for the first time as PSB after confirming their capacity to solubilize considerable amount of tricalcium phosphate in the medium by secreting organic acids (Chen et al. 2006). Peix et al. (2001) notified that *Mesorhizobium mediterraneum* strain PECA21 was able to mobilize phosphorous efficiently in barley and chickpea when tricalcium phosphate was added to the soil. Also, treating with insoluble phosphates and inoculating with strain PECA21, the phosphorous content, dry matter, nitrogen, potassium, calcium, and magnesium content in both plants were significantly increased.

It was known that natural phosphate rocks have been identified as an alternative for P fertilizers. For example, there are almost 40 million tons of phosphatic rock deposits in India (Rodríguez and Fraga 1999), and this material should provide a

cheap source of phosphate fertilizer for crop production (Halder et al. 1990); especially, should be considered in organic production of horticulture and the other crops.

2.3 Plant Growth Regulators

Several stages of plant growth and development such as cell elongation, cell division, tissue differentiation, and apical dominance are controlled by the plant hormones, especially auxins and cytokinins. The biosynthesis and the underlying mechanism of auxins and cytokinins action are subjects of intense investigation. Auxins and cytokinins can be synthesized by both the plants and the microorganisms. Although the role of phytohormone biosynthesis by microorganisms is not fully explained, it is stated that direct mechanisms of plant growth by PGPR include production of plant hormones such as auxins, cytokinins, GAs, and lowering of plant ethylene levels (Glick 1995; Costacurta and Vanderleyden 1995; Lucy et al. 2004). A list of examples of plant growth stimulating phytohormones produced by PGPR is given in Table 1.

Auxin, indole-3-acetic acid (IAA), is a quantitatively important phytohormone produced by a member of PGPR, and treatment with auxin-producing rhizobacteria increased the plant growth (Vessey 2003; Erturk et al. 2008). On the one hand, most beneficial bacteria such as *Rhizobium*, *Bradyrhizobium*, and *Azospirillum* synthesize IAA via the Indole-3-pyruvic acid (IPyA) pathway (Manulis et al. 1991; Costacurta and Vanderleyden 1995; Patten and Glick 1996; Burdman et al. 2000). On the other hand, some pathogenic bacteria such as *Pseudomonas syringae*, *Agrobacterium tumefaciens*, and *Erwinia herbicola* synthesize IAA predominantly via the indole-3-acetamide (IAM) pathway (Dobbelaere et al. 2003).

The role of IAA in the observed plant growth promotion was obtained by attempting to mimic the effect of the bacterium for the root growth by the direct application of IAA on the roots. Inoculation with *Bacillus* RC23, *Paenibacillus polymyxa* RC05, *B. subtilis* OSU142, *Bacillus* RC03, *Comamonas acidovorans* RC41, *B. megaterium* RC01, and *B. simplex* RC19 with tea (*Camellia sinensis)* cuttings enhanced rooting percentages when compared with control because of IAA production of bacteria. Similarly, treatments of hardwood stem cuttings of kiwifruit cv. Hayward, stem cuttings of two rose selections (ERS 14, *Rosa canina*, and ERS 15, *Rosa dumalis*), sour cherry (*Prunus cerasus*) softwood and semi-hardwood cuttings and *Pistacia vera* cuttings with *Agrobacterium rubi* (A1, A16, and A18) and *Bacillus subtilis* OSU142 promoted rooting ratio and increased the numbers of lateral roots (Ercisli et al. 2000; Ercisli et al. 2003; Esitken et al. 2003b; Ercisli et al. 2004; Orhan et al. 2007).

In addition, *Azospirillum* is not only capable of nitrogen fixation but also codes for plant growth hormone auxins (Elmerich 1984). Strains of *Azospirillum* showed that production depended on the type of culture media and availability of tryptophan as a precursor. *A. brasilense* Cd produced the highest level of IAA among the

Table 1 Examples of plant growth stimulating phytohormones produced by PGPR

Phytohormones	PGPR	References
Gibberellin	*Acetobacter diazotropicus*	
	Herbospirillum seropedicae	Bastian et al. (1998)
	Bacillus lichenifirmis	
	Bacillus pumilus	Gutierrez-Manero et al. (2001)
	Bacillus cereus MJ-1	
	Bacillus macroides CJ-29	Joo et al. (2004)
	Bacillus pumilus CJ-69	
IAA	*Agrobacterium* sp.	
	Alcaligenes piechaudii	Barazani and Friedman (1999)
	Comamonas acidovorans	
	Azospirillum brasilense	Kaushik et al. (2000)
	Aeromonas veronii	
	Enterobacter cloacae	Mehnaz et al. (2001)
	Enterobacter sp.	Mirza et al. (2001)
	Comamonas acidovorans RC41	
	Paenibacillus polymyxa RC05	
	Bacillus RC23	Erturk et al. (2008)
	Bacillus simplex RC19	
	Bacillus RC03	
	Bacillus megaterium RC01	
Cytokinin	*Paenibacillus polymyxa*	Timmusk et al. (1999)
	Pseudomonas fluorescens	de Salamone et al. (2001)
		Bent et al. (2001)
ACC deaminase	*Pseudomonas putida*	Mayak et al. (1999)
	Pseudomonas cepacia	Cattelan et al. (1999)
	Enterobacter cloacae	Saleh and Glick (2001)
	Pseudomonas brassicacearum Am3	Belimov et al. (2007)
	Variovorax paradoxus 5C-2	Belimov et al. (2009)
	Pseudomonas putida Biovar B	Rodriguez et al. (2008)
	Pseudomonas putida N21	
	Pseudomonas aeruginosa N39	Zahir et al. (2009)
	Serratia proteamaculans M35	

Azospirillum strains tested (El-Khawas and Adachi 1999; Radwan 1998; Bashan et al. 2004).

The isolation and quantification of cytokinins in nonpathogenic soil bacteria in general and diazotrophic bacteria in particular has received a little attention. Cytokinins are a diverse group of labile compounds that are usually presented in small amounts in biological samples and are often difficult to identify and quantify (Dobbelaere et al. 2003).

Cytokinins are produced by bacteria such as *Azospirillum* and *Pseudomonas* spp. (Gaudin et al. 1994). Moreover, a few PGPR strains were reported to produce cytokinins, such as *Rhizobium leguminosarum*, *Paenibacillus polymyxa*, and *Pseudomonas fluorescens* (Noel et al. 1996; Timmusk et al. 1999; de Salamone et al. 2001; Bent et al. 2001; Vessey 2003). These studies sufficiently cloud the production of cytokinins, compared with IAA or GAs, in PGPR. Also, it appears that more

work is necessary before proving for the role of PGPR-produced cytokinins in plant growth promotion.

Also in the case of GAs, the bacterial genetic determinants have not been identified so far. Therefore, no mutants are available to demonstrate the role of this phytohormone in plant growth promotion (Dobbelaere et al. 2003). Also the evidence of GA production by PGPR is rare (Vessey 2003). On the other hand, PGPR such as *R. phaseoli*, *A. lipoferum*, *Azotospirillum brasilense*, *Acetobacter diazotropicus*, *Herbospirillum seropedicae*, *Bacillus licheniformis*, *B. pumilus*, *Bacillus cereus* MJ-1, *Bacillus macroides* CJ-29 were reported to produce GAs (Atzhorn et al. 1988; Bottini et al. 1989; Janzen et al. 1992; Bastian et al. 1998; Gutierrez-Manero et al. 2001; Joo et al. 2004 and Table 1). However, this is not a strong evidence of GA production in a common method of growth promotion by PGPR.

Nevertheless, in recent studies, Gutierrez-Manero et al. (2001) provide an evidence that four different forms of GAs are produced by *B. pumilus* and *Bacillus licheniformis*. Inoculation of alder (*Alnus glutinosa*) with these PGPR could effectively reverse a chemically induced inhibition of stem growth. In addition to this research, Joo et al. (2004) reported that the growth of red pepper plug seedlings was increased by *Bacillus cereus* MJ-1, *B. macroides* CJ-29, and *B. pumilus* CJ-69, though the number of leaves and stem diameter were not significantly changed. The greatest increase is in the height and the root fresh weight of the seedlings was by *B. pumilus*, which could increase the height by 12% and the root fresh weight by 20%.

In the last few years, a new mechanism of plant growth promotion involving ethylene has been proposed (Burdman et al. 2000). Showing that some soil bacteria contain 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Klee et al. 1991) and Glick et al. (1998) put forward the theory that the mode of action of some PGPR was the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme that could cleave ACC, the immediate precursor to ethylene in the biosynthetic pathway for ethylene in plants. They submitted that ACC deaminase activity would decrease ethylene production in the roots of host plants and results in root lengthening. In some cases, the growth promotion effects of ACC deaminase-producing PGPR is the best expressed in stress conditions including drought (Zahir et al. 2008) and salt (Nadeem et al. 2007; Zahir et al. 2009) stress.

PGPR (containing ACC deaminase) boost plant growth particularly under stressed conditions by the regulation of accelerated ethylene production in response to a multitude of abiotic and biotic stresses such as salinity, drought, waterlogging, temperature, pathogenicity, and contaminants (Saleem et al. 2007). For example, under salinity stress, 1-aminocyclopropane-1-carboxylic acid-deaminase activity of *P. putida* (N21), *P. aeruginosa* (N39) and *Serratia proteamaculans* (M35) might have caused reduction in the synthesis of stress (salt)-induced inhibitory levels of ethylene (Zahir et al. 2009). Similarly, inoculation with *Variovorax paradoxus* 5C-2 improved growth, yield, and water-use efficiency of droughty peas (Belimov et al. 2009). It is reported that inoculation with *P. fluorescens* was found to be more effective in promoting root growth than that with *P. putida* as it caused up to 46% increase in root elongation and up to 94% increase in root weight of pea over the respective uninoculated drought stressed control (Arshad et al. 2008).

In addition to stress factors, recent studies indicated that canola plants inoculated with the *P. putida* strain HS-2 produced an increase in plant biomass (Rodriguez et al. 2008). The ACC-utilizing PGPR *Pseudomonas brassicacearum* strain Am3 increased in-vitro root elongation and root biomass of soil-grown tomato cv. Ailsa Craig at low bacterial concentrations but had negative effects on in-vitro root elongation at higher bacterial concentrations (Belimov et al. 2007).

2.4 *Effects on Plant Growth*

Since the last few decades, the response of agriculturally important crops to inoculation with PGPR was investigated in numerous field and greenhouse experiments carried out in various countries. On the basis of the given data, it was concluded that inoculation with PGPR resulted in significant yield increases in different crops, enhanced rooting of hardwood and semi-hardwood cuttings, seed germination and emergence under different conditions. In other words, they can affect plant growth and yield in a number of ways and enhancement of vegetative and reproductive growth is documented in a range of crops such as cereals or vegetables. Treatments with PGPR increase germination percentage, seedling vigor, emergence, plant stand, root and shoot growth, total biomass of the plants, seed weight, early flowering, grains, fodder and fruit yields, etc., (van Loon et al. 1998; Ramamoorthy et al. 2001). Applications of PGPR in relation to the plant growth in different subjects are described later with recent studies.

2.4.1 Yield and Yield Components

In crop production, there is a continuous demand of increasing crop productivity and quality. There are lot of agricultural practices applied for increasing the yield and the yield components. Recently, one of them is applications of PGPR for increasing yield and environment friendly crop production.

Floral and foliar applications of PGPR strains *Pseudomonas* BA-8 and *Bacillus* OSU-142 on apple trees significantly increased yield per trunk cross-section area (13.3–118.5%), fruit weight (4.2–7.5%), shoot length (20.8–30.1%), and shoot diameter (9.0–19.8%) in "Starkrimson" and yield per trunk cross-sectional area (TCSA; 14.9%) and fruit weight (6.5–8.7%) in "Granny Smith" compared with the control (Pırlak et al. 2007). Karlıdağ et al. (2007) reported similar results in apple. Thus, *Bacillus* M3 and/or OSU-142 and/or *Microbacterium* FS01 in combination have the potential to increase the yield and growth of apple trees.

In addition, Esitken et al. (2003a, 2005, 2006) and Orhan et al. (2006) reported that *Pseudomonas* BA-8, *Bacillus* OSU-142 and M3 increased the shoot length, crop yield and improved fruit quality of apricot, sweet cherry, and raspberry.

In another research, Cakmakci et al. (2006) suggested that in the greenhouse, inoculations with PGPR increased sugar beet root weight by 2.8–46.7% depending

on the species. Leaf, root, and sugar yield were increased by the bacterial inoculation by 15.5–20.8%, 12.3–16.1%, and 9.8–14.7%, respectively. Effective *Bacillus* species, such as OSU-142, RC07 and M-13, *Paenibacillus polymyxa* RC05, *P. putida* RC06, and *Rhodobacter capsulatus* RC04 may be used in organic and sustainable sugar beet agriculture.

The average weight of tomato fruit per plant treated with *Rhodopseudomonas* sp KL9 strain (82.7 g) was higher than those of others including the uninoculated control. The content of lycopene in the ripe tomato fruit increased by 48.3% with the application of *Rhodopseudomonas* sp. KL9, but *Rhodopseudomonas* sp BL6 did not show any effect on lycopene content although the lycopene content in the cells of *Rhodopseudomonas* sp BL6 were 1.12 mg/g (Lee et al. 2008a).

Dursun et al. (2008) reported that the highest rocket yield, average leaf weight, leaf length, leaf stem diameter, leaf area and root weight were obtained from *Pseudomonas* BA-7 applications when compared with *P. putidae* BA-8, *B. subtilis* OSU-142 and MFD-5, *B. megatorium* M3, *A. rubi* A-1, A-16, and A-18. The highest leaf number (8.23), leaf dry matter (6.70%), and root dry matter (11.85%) were determined in A-18, OSU-142 and MFD-5 applications, respectively, and especially *Burkholderia gladii* BA-7, *Pseudomonas* BA-8, and *Bacillus* OSU-142 have a great potential to increase the parameters of plant growth of rocket.

Although the examples of relations between the yield and PGPR applications can be increased, other recent studies such as de Freitas (2000), Herman et al. (2008), and Yıldırım et al. (2008) clearly demonstrated the potential of PGPR in increasing the plant growth and yield.

2.4.2 Seed Germination and Emergence

Sivritepe and Dourado (1995) reported that priming (osmoconditioning) is one of the physiological methods, which improves seed performance and provides faster and synchronized germination in vegetables. However, bio-priming with different genera, especially PGPR, have a great potential over other priming methods.

Nelson (2004) noted that PGPR were able to exert a beneficial effect upon plant growth such as increase in seed germination rate and percentage. Rodriguez et al. (2001) reported that using *Azospirillum* spp. gave better germination in both tomato and pepper seeds. Also, Vargas et al. (2001) mentioned that *Hafnia alvei* strain P3 increased germination by 36.5% when compared with the control in lettuce and inoculation of the soybean plants either with *Pseudomonas* strain PMZ2 or with *B. japonicum* increased seed emergence (Zaidi 2003). Similarly, Basavaraju et al. (2002) reported that inoculation of *Azotobacter chroococcum* strain C2 significantly increased the germination percentage in radish. The greenhouse inoculation experiment with pepper and maize pointed out that *Azotobacter* sp. strains 17 and 20 promoted pepper germination, while the *Azospirillum* strains 1 and 23 promoted maize germination (Reyes et al. 2008). Although studies were mentioned about the effect of bacterial strains on germination of different vegetable species that were conducted out under optimum conditions, Kaymak et al. (2009) suggested that bio-priming with *A. rubi* strain A16, *Burkholderia gladii* strain BA7, *P. putida* strain BA8,

B. subtilis strain BA142, *B. megaterium* strain M3 under saline stress could be useful to obtain higher seed germination percentage in radish.

Also, PGPR can be used under pathogenic factor. Thus, different isolates of plant growth-promoting rhizobacteria (i.e., *B. pumilus* (INR-7), *B. subtilis* (GBO-3), *B. subtilis* (IN937b), *B. pumilus* (SE-34), *Brevibacillus brevis* (IPC-11), *B. pumilus* (T-4), and *B. amyloliquefaciens* (IN937a)) were used for seed treatment to suppress the seedling diseases caused by fungi. Among them, isolates GBO3, IPC-11, and INR-7 increased seed germination and seedling vigour to the greatest extent (Lokesh et al. 2007). Alike, Begum et al. (2003) reported that PGPR, *B. pumilus* (SE-34), *B. pasteurii* (T4), *B. subtilis* (IN937-b), and *B. subtilis* (GBO3) strains reduced the incidence of seed mycoflora, which indirectly enhanced the seed germination percentage and vigour index of the seedlings in okra. In another recent study, de Araujo (2008) reported that the inoculation of seeds with *B. subtilis* is a promising technological alternative for seed treatment owing to the fact that inoculation with *B. subtilis*, formulated with oyster meal, increased emergence in cotton and soybean.

2.4.3 Rooting of Cuttings

There are many physiological and environmental factors that influence root formation, with exogenous treatments on cuttings being particularly important (Couvillon 1998). Growers have attempted to stimulate rooting by applying growth regulators, various chemical substances, etc. However, the use of chemicals can produce environmental problems and increase proportion costs. Ecological problems have raised interest in environmental friendly sustainable agricultural practices (Salantur et al. 2005). Therefore, use of PGPR can overcome such problems associated with environment (Kaymak et al. 2008).

Recent studies showed that bacteria in several genera (*Agrobacterium*, *Bacillus*, *Streptomyces*, *Pseudomonas*, and *Alcaligenes*) induce root formation and growth in stem cuttings (Bassil et al. 1991; Hatta et al. 1996; Rinallo et al. 1999). More recently, PGPR such as *A. rubi* (A1, A16 and A18), *B. subtilis* (OSU142), *Bacillus* (BA16, RC03, RC23), *B. gladii* (BA7), *P. putida* (BA8), *B. megatorium* (M3 and RC01), *Paenibacillus polymyxa* (RC05), *Comamonas acidovorans* RC41, and *B. simplex* RC19 were effectively used for both hardwood and semi-hardwood cuttings to obtain higher rooting percentages in sour cherry (Ercisli et al. 2000; Esitken et al. 2003b), kiwifruit (Ercisli et al. 2003), grapevine (Köse et al. 2003), rose (Ercisli et al. 2004), pistachio (Orhan et al. 2006), tea (*Camellia sinensis* var. *Sinensis*) (Erturk et al. 2008), and mint (*Mentha piperita* L.) (Fig. 1) (Kaymak et al. 2008).

2.4.4 Nutrient Uptake

Living plants require 16 essential elements to survive. Three of 16 elements (carbon, hydrogen, and oxygen) are supplied primarily from air and water. The remaining 13 are normally absorbed by plant roots. Each of these essential elements has at least one specially defined role in plant growth (Swaider et al. 1992; Decateau 2000).

Fig. 1 Effect of inoculation with PGPR (*Agrobacterium rubi* A16, *Burkholderia gladii* BA7, *Peseudomonas putida* BA8, *Bacillus subtilis* OSU142, and *Bacillus megatorium* M3) on root formation of mint cuttings

PGPR have been promised as a component in approaching for maintaining adequate plant nutrition and reducing the negative environmental effects of fertilizers. PGPR might increase nutrient uptake from soils, thus reducing the need for fertilizers and preventing the accumulation of nitrates and phosphates in agricultural soils (Yang et al. 2009). It is known that phosphorous and nitrogen is the major and key nutrients limiting plant growth and important macronutrient required for plant growth (Kumar and Narula 1999; Sundara et al. 2002; Podile and Kishore 2006).

Additionally, some PGPR promote root development (Mantelin and Touraine 2004) by the production of phytohormones such as indole acetic acid (Kloepper et al. 2007). Given that root tips and root surfaces are sites of nutrient uptake, it is likely that one mechanism by which PGPR lead to increased nutrient uptake is via stimulation of root development (Yang et al. 2009). It has also been suggested that PGPR increase uptake of mineral ions via stimulation of the proton pump ATPase (Mantelin and Touraine 2004), although experimental evidence for this is lacking (Yang et al. 2009).

Several studies can be given about the relations with PGPR and enhancement of nutrient uptake. For example, Naveed et al. (2008) notified that PGPR application significantly enhanced N, P, and K uptakes. The *Pseudomonas fluorescens* biotype G (N-3) was found to be the best in increasing the grain yield of maize and nutrient uptake. In addition, the inoculation process with *Azospirillum* and *Bacillus* spp. showed positive response in enhancing higher accumulation of nitrogen, phosphorus, and potassium in the plant tissues, enhanced root dry weight and top growth of the oil palm seedlings under field nursery conditions (Amir et al. 2005).

In other recent study, Dursun et al. (2008) reported that *Burkholderia gladii* BA-7, *P putidita* BA-8, *B. subtilis* OSU-142 and MFD-5, *B. megaterium* M3, *A. rubi* A-1, A-16, and A-18 applications increased mineral contents particularly N, K, P, Zn, Fe, Mn, Na, Ca, and Mg in rocket leaves when compared with the control.

In a study aimed at assessment of effects of foliar application of bacteria *Bacillus* OSU-142, *Burkholderia* OSU-7, and *Pseudomonas* BA-8 on yield and growth of apricot, it was stated that application of bacteria resulted in an increase of N, P, K, Ca, and Mg contents of leaves (Esitken et al. 2005). In a similar study, Esitken et al. (2003a) suggested that N, P, K, Ca, and Mg contents of leaves were higher on OSU 142-treated trees than on the untreated control and OSU 142 has the potential to increase the yield of apricot trees.

Therefore, PGPR contributed significantly to the reducing nutrient build up in the soil. Several studies are underway that will further define the utility of PGPR in nutrient management strategies aimed at reducing fertilizer application rates and nutrient runoff from agricultural sources (Yang et al. 2009; Kumar et al. 2009).

3 Indirect Plant Growth Promotion

Induced systemic resistance (ISR), antibiosis, competition for nutrients, parasitism, production of metabolites suppressive to deleterious rhizobacteria are some of the mechanism that indirectly benefit plant growth.

3.1 Induced Systemic Resistance

More recently, biological control has been considered as an alternative strategy to manage soil-borne plant diseases. Available literature revealed positive effects of specific strains of rhizobacteria on growth of many plant species in soils in which more or less defined pathogens cause substantial losses. For this reason, several rhizobacteria have extensively been used as biological agents to control many soil-borne plant pathogens (Jeun et al. 2004; Dell'Amico et al. 2005; Rajkumar et al. 2005).

A strain, *P. fluorescens* WCS417, active against *Fusarium oxysporum* f. sp. *dianthi* was tested on carnation and results showed that bacteria, while remaining confined to the plant root system, were still protective when the pathogen was slash-inoculated into the stem (Van Peer et al. 1991). This protective effect had to be plant-mediated because in this case the rhizobacteria and the pathogenic fungus were never found to contact each other on the plant (Van Loon and Bakker 2006). Several strains of PGPR, which applied to roots of cucumber, and the leaves were subsequently challenged inoculation with the anthracnose fungus *Colletotrichum orbiculare* (Gang et al. 1991). The phenomenon was called ISR. (Van Loon et al. 1998; Vallad and Goodman 2004; Van Loon and Bakker 2006; Choudhary et al. 2007)

It is thought that the inducing rhizobacteria in the plant roots produce signal, which spreads systemically within the plant and increases the defensive capacity of the distant tissues from the subsequent infection by the pathogens. ISR thus extended the protective action of PGPR from their antagonistic activity against soil-borne pathogens in the rhizosphere to a defense-stimulating effect above the surface of the ground tissues against foliar pathogens (Van Loon and Bakker 2006).

ISR appears phenotypically similar to SAR, which is the phenomenon that once a plant has been infected by a pathogen and been able to effectively resist it, it has become more resistant to subsequent challenge inoculation by the same and other pathogens and, in some instances, even insects (Sticher et al. 1997; Van Loon et al. 1998; Van Loon and Bakker 2006). SAR occurs in distal plant parts following localized infection by a necrotizing pathogen. It is controlled by a signaling pathway that depends upon the accumulation of salicylic acid and the regulatory protein NPR1. In contrast, ISR is induced by selected strains of nonpathogenic PGPR. ISR functions independent from SA, but requires NPR1 and is regulated by jasmonic acid and ethylene (Walters and Heil 2007).

To reduce crop loss, pesticides are generally used. They are cost-effective and thus have become an integral part of modern agriculture. Environmental and human health-related concerns associated with use of hazardous chemicals have necessitated the search for eco-friendly alternatives. Such approaches must enhance and sustain agricultural productivity and at the same time be safe from environmental and health perspectives (Raj et al. 2003a).

Therefore, for economic reasons biological crop protectants can only seldom compete with highly effective chemicals. However, ISR is only one of the mechanisms that may be mobilized to counteract plant pathogens in an environmentally friendly and durable way. Integrating ISR-triggering PGPR into disease management programs in conjunction with other strategies will be a worthwhile approach to explore (Van Loon and Bakker 2006).

3.2 Suppression of Plant Diseases, Insects, and Nematodes by PGPR

Biocontrol is the process by which a pathogenic organism is maintained at low inoculum density or controlled or eradicated by beneficial organisms. Several microorganisms such as PGPR and insects present in the natural environment serve as potential biocontrol agents.

3.2.1 Bacterial Plant Diseases

The bacteria associated with plants exist as epiphytes, endophytes, and pathogens. Phytopathogens are comparatively few in both type and number, and all bacterial phytopathogens described to date fall within the domain Bacteria, formerly known as the *Eubacteria*. Bacterial phytopathogens that possesses a cell wall can be

subdivided into Gram-positive (*Clavibacter*, *Curtobacterium*, *Rathayibacter*, and *Streptomyces*) and Gram-negative (*Acidovorax*, *Agrobacterium*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Pantoea*, *Pseudomonas*, *Ralstonia*, and *Xanthomonas*) (Saddler 2002).

Bacterial soft rot of vegetables; blackleg of potato; fire blight of pome fruits; angular leaf spot or black arm, of cotton; bacterial blights of bean, lack rot of crucifers, southern bacterial wilt, bacterial wilt of cucurbits, ring rot of potato, bacterial canker of tomato, crown gall, hairy root, and cane gall, and common scab of potato are the more common bacterial diseases (Walker 1957; Waller et al. 2002).

Several cultural practises such as crop rotation, mixed cropping and intercropping, selection of cultivar, tillage, planting time, fertilization and irrigation, or highly effective chemical substances affect some diseases in different ways depending on the form of their application (Termorshuizen 2002). Recently, many microorganisms are increasingly used as inoculants for biocontrol (Romero et al. 2003; Chinnasamy 2005; Aliye et al. 2008; Xue et al. 2009). PGPR are nonpathogenic, environmental-friendly, cheaper to produce and easy to handle, and may create long-lasting effects (Chinnasamy 2005).

For instance, tomato is prone to a number of bacterial diseases, among which bacterial canker disease caused by *Clavibacter michiganensis* ssp. *michiganensis* is one of the most important diseases and nearly 100% crop loss can occur (Boudyach et al. 2001; Umesha 2006). Utkhede and Koch (2004) reported that treatments with *B. subtilis* (Quadra 136 and 137) and *Trichoderma harzianum* (R), *Rhodosporidium diobovatum* (S33), applied as a spray at 0.3, 0.6, 10 g^{-1}, have the ability to prevent the incidence of bacterial canker of tomato plants caused by *C. michiganensis* subsp. *michiganensis* under greenhouse conditions. Similarly, tomato seeds were treated with PGPR strains *B. subtilis* GBO3, *B. amyloliquefaciens* IN937a and *Brevibacillus brevis* IPC11 were recorded for maximum disease protection for bacterial canker under greenhouse conditions (Girish and Umesha 2005). Recent studies about the relations with bacterial diseases and PGPR are given in Table 2.

3.2.2 Fungal Plant Diseases

Fungal pathogens found on plants can be classified in different taxonomic groups. A few fungal pathogens such as rusts, powdery and downy mildews are obligate parasites. However, most of the plant pathogens are necrotrophs, killing plant tissues for their nutrition (Waller and Cannon 2002).

Exclusion or eradication of a disease from production areas, highly effective chemical substances or biological control of plant diseases have been suggested to protect the plants from fungal pathogens. Recently, PGPRs are increasingly and extensively used in biological control of fungal plant diseases (Altindag et al. 2006; Lourenco et al. 2006; Saravanakumar et al. 2007; Akgul and Mirik 2008; Sang et al. 2008; Dutta et al. 2008).

For example, apricot is the most important fruit crop grown in Anatolia, with approximately 600,000 tons of fruit produced annually, and Turkey dominates

Table 2 Examples of suppression of bacterial diseases by PGPR in different plant species

Phytopathogens	Species	PGPR	References
Pseudomonas syringae pv. *lachrymans*	Cucumber	*Pseudomonas putida* 89B-27 *Serratia marcescens* 90–166	Liu et al. (1995)
Pseudomonas syringae pv. *glycinea*	Soy bean	*Pseudomonas* sp. *Erwinia herbicola*	May et al. (1996)
Xanthomonas albilineans	Sugar cane	*Pentoena dispersa*	Zhang and Birch (1997)
Erwinia amylovora	Apple	*Erwinia herbicola* C9-1 *Pseudomonas fluorescens* A506	Pusey (1997)
		Single-strain treatments and three-way mixture of *Bacillus pumilus* INR7, *Curtobacterium flaccumfaciens* ME1 and *Bacillus subtilis* GB03	Raupach and Kloepper (1998, 2000)
Ralstonia solanacearum	Tomato	*Bacillus subtilis* B2G	Lemessa and Zeller (2007)
		Pseudomonas sp. (APF1) *Acinetobacter* sp. (Xa6) *Enterobacter* sp. (Xy3)	Xue et al. (2009)
Clavibacter michiganensis subsp. *michiganensis*		*Azospirillum brasilense* Sp7	Romero et al. (2003)
Xanthomonas campestris pv. *vesicatoria*		*Azospirillum* sp. (BNM-65)	
Ralstonia solanacearum	Eucalyptus	*Pseudomonas fluorescens* WCS417r *Pseudomonas putida* WCS358r	Ran et al. (2005)
	Potato	*Bacillus subtilis* PFMRI *Paenibacillus macerans* BS-DFS and PF9	Aliye et al. (2008)
Xanthomonas axonopodis pv. *malvacearum*	Cotton	*Bacillus cereus* MT5-5, MT5-6, L2-1 *Achromobacter xylosoxidans* L2-2, *Brevibacterium* sp. MT5-11	Ishida et al. (2008)

apricot production in the world (Ercisli 2009). Therewithal, brown rot caused by *Moniliana laxa* Ehr. is one of the most destructive diseases of apricot in Turkey. This pathogen is able to destroy the whole annual crop in the phase of blossom, although it can kill shoots up to 30 cm beyond the initial blossom infection, and management of brown rot in Turkey is in general carried out by fungicide application (Gulcan et al. 1999). Altindag et al. (2006) suggested that *Burkholdria gladii* OSU 7 has the potential to be used as biopesticide for effective management of brown rot disease on apricot.

Similarly, pepper (*Capsicum annum* L.) is one of the most important market vegetables grown worldwide, but the yield and quality of marketable peppers are frequently limited by Phytophthora blight. The incidence of this disease has

continued to increase production areas since the pathogen can infect roots, crowns, and even foliar parts of pepper plants through splashing rains or overhead irrigation waters (Ristaino and Johnston 1999; Hausbeck and Lamour 2004). Control of this disease has usually depended on chemical and cultural measures such as use of phenylamide fungicides or metalaxyl as well as crop rotation, soil amendments, use of protective mulches and water management (Matheron and Porchas 2000; Hausbeck and Lamour 2004). In a recent study, Sang et al. (2008) reported that *Pseudomonas corrugata* (CCR04 and CCR80), *Chryseobacterium indologenes* (ISE14), and *Flavobacterium* sp. (GSE09) showed consistently good control efficacy against *Phytophthora capsici*. Also, these strains could be applied by either drench or root-dip treatment as alternatives to agricultural chemicals to control Phytophthora blight of pepper. In another recent study, Akgul and Mirik (2008) also reported that *Bacillus megaterium* strains could be used for biocontrol of *Phytophthora capsici*.

The combination of *Pseudomonas* strains Pf1, TDK1, and PY15 was more effective in reducing sheath rot (*Sarocladium oryzae*) disease in rice plants compared with individual strains under glasshouse and field conditions (Saravanakumar et al. 2009).

Hernandez-Rodriguez et al. (2008) obtained that *Burkholderia* sp. MBf21, MBp1, MBf15, and *P. fluorescens* MPp4 stood out for their plant growth stimulation in maize and for the biological control exerted on *Fusarium verticillioides* M1. The strains *Burkholderia* sp. MBf21 and MBf15 showed the best results in disease suppression, which was achieved up to 80%.

The combined use of PGPR (*Bacillus cereus* strain BS 03 and a *Pseudomonas aeruginosa* strain RRLJ 04) and rhizobia (strain RH 2) were recommended for induction of systemic resistance against fusarial wilt (*Fusarium udum*) in pigeon pea (Dutta et al. 2008). Recent studies and more examples about the suppression of fungal diseases by PGPR are given in Table 3.

3.2.3 Viral Plant Diseases

Viruses are obligate parasites of submicroscopic size, with one dimension smaller than 200 nm. Virus particles, or virions, consist of segments of double or single-stranded RNA or DNA encased in protein structures, in some cases with lipid and additional substances (Waller 2002). So far at least 700 plant viruses has been discovered, many of which cause catastrophic diseases and have wide host ranges. They have been classified into three families and 32 groups (Martelli 1992; Waller 2002).

Some chemicals are used to produce virus-free plant material because they inhibit virus replication in agricultural crops. However, there are no therapeutic agents or viricides that can be applied to plants to control virus diseases. Consequently, control measures are based mainly on avoiding infection by using host plant resistance or disrupting the epidemic cycle of the disease. The use of

Table 3 Examples of suppression of fungal diseases by PGPR in different plant species

Phytopathogens	Species	PGPR	References
Rhizoctonia solani sclerotia	Cyclamen	*Serratia marcescens* B2	Someya et al. (2000)
Fusarium oxysporum f. sp. *cyclaminis*			
Fusarium oxysporium	Soybean	*Pseudomonas* PMZ2 *Bradyrhizobium japonicum*	Zaidi (2003)
Sclerospora graminicola	Pearl millet	*Bacillus pumilus* INR7 and SE34 *Bacillus subtilis* GB03	Raj et al. (2003b)
		Pseudomonas fluorescens UOM SAR 14	Raj et al. (2004)
Cronartium quercuum f.sp. *fusiforme*	Loblolly pine	*Bacillus pumilus* SE34 and T4	Enebak and Carey (2004)
Puccinia psidii	Eucalyptus	*Pseudomonas aeruginosa* FL2 *Pseudomonas* sp. MF4	Teixeira et al. (2005)
Didymella bryoniae	Muskmelon	Pseudomonas fluorescens	Sudisha et al. (2006)
Pythium ultimum, *Pythium debaryanum*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Phytophthora capsici*, *Botrytis cinerea*, *Botrytis allii*, *Cladosporium fulvum*, *Aspergillus* sp.	Sesame (in vitro)	*Paenibacillus polymyxa* E681	Ryu et al. (2006)
Exobasidium vexans	Tea	*Pseudomonas fluorescens* Pf1	Saravanakumar et al. (2007)
Fusarium spp. *Didymella bryoniae* *Myrothecium* spp.	Watermelon	*Bacillus subtilis* GBO-3 and *Brevibacillus brevis* IPC-11 *Bacillus pumilus* SE34 and T4	Lokesh et al. (2007)
Fusarium oxysporum f. sp. *lycopersici*	Tomato	*Paenibacillus lentimorbus* GBR158	Son et al. (2008)
Phytopthora capsici	Red pepper	*Bacillus subtilis* R33 and R13	Lee et al. (2008b)
Phytopthora capsici	Chili pepper	*Paenibacillus polymyxa* GBR-462	Kim et al. (2009)
Fusarium oxysporum L sp. *lycopersici*	Tomato	*Azospirillum brasilense* *Bacillus subtilis*	Abo-Elyousr and Mohamed (2009)
Rhizoctonia solani	Wheat	*Azotobacter* sp. WPR-51	Fatima et al. (2009)

genetically resistant cultivars provides effective control of many viral diseases. Mechanisms of resistance vary, some are explained to effects on vectors, whereas others may inhibit viral replication (Waller 2002).

Kirankumar et al. (2008) reported that *Pseudomonas* B-25 was highly efficient in promoting growth, fruit yield, and nutrient uptake of tomato in the presence of tobacco mosaic virus (TMV) pathogen, and the incidence of pathogenesis was markedly less after PGPR treatment. Similarly, biological control using PGPR

protected tomato plants against cucumber mosaic virus (CMV) under greenhouse and to a limited extent in the field conditions (Sikora and Murphy 2005). In another research, *P. fluorescens* strains were investigated for biocontrol efficacy against tomato spotted wilt virus (TSWV) in tomato. Virus concentration value clearly showed a reduction in viral antigen concentration in *P. fluorescens*-treated tomato plants corresponding to reduced disease ratings. All the *P. fluorescens*-treated tomato plants also showed enhanced growth and yield compared with control plants. Hence, it was suggested that PGPR could play a major role in reducing TSWV and increasing yield in tomato plants (Kandan et al. 2005). Banana bunchy top disease (BBTD) caused by Banana bunchy top virus (BBTV) is the most serious virus disease of banana plantations world wide. *P. fluorescens* Pf1 and CHA0 were significantly effective in reducing BBTV under field conditions, recording 33.33% infection with 60% reduction over control (Harish et al. 2008).

In a greenhouse experiment, *P. fluorescens* FB11 and *Rhizobium leguminosarum* FBG05 were tested alone and in combination as seed inoculants to induce systemic resistance in faba bean against bean yellow mosaic potyvirus (BYMV). The results demonstrated that BYMV challenged plants emerged from *Pseudomonas* inoculated seeds not only showed a pronounced and significant reduction in percent disease incidence but also a significant reduction in virus concentration in the challenged plants, compared with the nonbacterized seeds. *Rhizobium* alone also showed a significant reduction in both in percent disease incidence and in viral concentration value, but the reduction was less pronounced than that resulting from *Pseudomonas* inoculation (Elbadry et al. 2006).

In a recent study, the PGPR combinations (combinations included *B. subtilis* GB03 and IN937b, *B. pumilus* SE34, INR7 and T4, *B. amyloliquefaciens* IN937a) formulated with chitosan were referred to as biopreparations. The result indicated that treatment of tomato plants with biopreparations resulted in significant enhancement of plant growth and protection against infection by CMV (Murphy et al. 2003). Zehnder et al. (2000) reported that CMV symptom development was significantly reduced on PGPR-treated (*B. pumilus* SE34, *Kluyvera cryocrescens* IN114, *B. amyloliquefaciens* IN937a, and *B. subtilus* IN937b) plants compared with control, but the percentage of infected plants and tomato yields were not significantly different among treatments, suggested that PGPR-mediated induced resistance against CMV infection following mechanical inoculation into tomato can be maintained under field conditions.

Tomato plants treated with PGPR (*B. amyloliquefaciens* 937a, *B. subtills* 937b, and *B. pumilus* SE34), applied as an industrially formulated seed treatment, a spore preparation mixed with potting medium (referred to as powder), or a combined seed-powder treatment, were evaluated under field conditions for induced resistance to tomato mottle virus (ToMoV), resulted in reduced ToMoV incidence and disease severity. In some cases, a corresponding increase in fruit yield was observed. The use of PGPR could become a component of an integrated program for management of this virus in tomato (Murphy et al. 2000)

It was known that there are no highly effective chemical substances that can be applied to plants to control viral disease of agricultural or horticultural crops. For

exclusion or eradication of a viral disease from production areas, highly effective chemical substances cannot be suggested; however, biological control with PGPR may be suggested to protect these areas or plants from viral pathogens. Nevertheless, it is recommended that more work must be conducted because of the complexity and variability of virus diseases.

3.2.4 Nematodes

Plant–parasitic nematodes cause serious crop losses in production areas,e.g., yield loss of tomato due to root-knot nematodes (*Meloidogyne* spp.) ranges from 39.7 to 46.0% in India (Reddy 1985), and are among the most important agricultural pests (Koenning et al. 1999; Siddiqui and Akhtar 2008). The control of nematodes is difficult because nematodes mostly inhabit the soil and generally attack and settle around or inside the roots of the plants. During the last few decades, plant disease control has been based largely on the use of chemicals (Siddiqui et al. 2001). Although chemical nematicides are effective, easy to apply, and show rapid effects, they have begun to be withdrawn from the market in some developed countries owing to concerns about public health and environmental safety (Schneider et al. 2003; Nico et al. 2004). The search for novel, environmentally friendly alternatives with which to manage plant–parasitic nematode populations has, therefore, become increasingly important (Tian et al. 2007).

Biological control using microbial antagonists is one potential alternative to chemical nematicides (Burkett-Cadena et al. 2008). PGPR can also be used for the biological control of plant parasitic nematodes. Among the biological control agents that have been assessed are *B*. spp. and *Pseudomonas* spp. dominant populations in the rhizosphere that are able to antogonize nematodes (Tian et al. 2007).

Recently, rhizobacteria-mediated ISR in plants has been shown to be active against nematode pests. Plant growth-promoting rhizobacteria can bring about ISR by strengthening the physical and mechanical resistance of the cell wall of plants. They also change the physiological and biochemical ability of the host to promote the synthesis of defence chemicals against the challenge pathogen (van Loon et al. 1998; Ramamoorthy et al. 2001; Tian et al. 2007). Siddiqui and Shaukat (2004) concluded that fluorescent Pseudomonads ISR against root-knot nematode via a signal transduction pathway, which is independent of SA accumulation in roots.

In other words, PGPR may suppress pests and pathogens of plants and promote plant growth. For example, *P. aeruginosa* and *B. subtilis* exhibited nematicidal activity by killing the second stage larvae of *Meloidogyne javanica* to a varying degree. Especially, *B. subtilis* significantly suppressed root-knot infection and nematode population densities under greenhouse and field conditions and thereby enhanced plant growth and yield in mungbean (Siddiqui et al. 2001).

In a different example, *P. putida* promoted tomato growth in nematode-infected and nematode-free plants but growth promotion was higher in the infected ones. *P. putida* was better in reducing galling and nematode multiplication than arbuscular mycorrhizal fungus (Siddiqui and Akhtar 2008).

In another recent study, Li et al. (2005) reported that *Brevibacillus brevis* and *B. subtilis* exhibited strong nematicidal activity by killing the second stage larvae of *Meloidogyne javanica* to varying degrees in the greenhouse. The toxic principles of bacterium *B. subtilis* B7 that showed the highest juvenile mortality were partially characterized.

The influence of *P. fluorescens* as the treatment on the seed germination, migration, and penetration of *Meloidogyne incognita* in aubergine was evaluated under laboratory conditions. The results revealed that *P. fluorescens* promoted germination (87.5%) and was effective in reducing root penetration by *M. incognita* and the number of gall formation was also controlled by 70.3% (Inam-ul-Haq et al. 2003).

Rhizobacteria reported to show antagonistic effects against nematodes include members of different genera are given in Table 4.

3.2.5 Insects

Next to phytochemical insecticides, biocontrol agents of microbial origin play a role in pest management (Gandhi et al. 2006). Among the biocontrol agents, the strains of PGPR, *P. fluorescens* is the promising one (Commarea et al. 2002). They activate systemic resistance (Raupach and Kloepper 1998) by inducing plants' latent defense mechanisms and to control insect pests (Zehnder et al. 1997; Commarea et al. 2002) in addition to exerting beneficial effect on plant growth promotion (Gandhi et al. 2006).

Herman et al. (2008) notified that there are several examples of plants treated with PGPR, which showed a decrease in insect herbivory. Zehnder et al. (1997) used PGPR to reduce feeding by the spotted cucumber beetle, *Diabrotica undecimpunctata howardi* Barber. Boughton et al. (2006) reported that plants treated with defence elicitors caused the green peach aphid, *Myzus persicae*, to significantly decrease in their population growth when compared with that of the control plants. Similarly, Herman et al. (2008) notified that *B. subtilis* and *B. amyloliquefaciens* could be useful in *Myzus persicae* management program, for pepper plants grown in locations with consistently high aphid pressure. Additionally, white clover and *Medicago* plants grown in the presence of a *Pseudomonas*-like PGPR were better able to resist the effects of blue-green aphids (Kempster et al. 2002).

The talc-based formulation of two *P. fluorescens* PF1, FP7 and its mixture were tested against leaffolder in rice. The application of talc-formulation through seed, root, soil, and foliar spray significantly reduced leaffolder incidence both under greenhouse and field conditions. The mixture of two strains performed better than the individual strains. Additionally, *Pseudomonas* treated leaves altered the feeding behavior of leaffolder larvae and reduced larval and pupal weight, increased larval mortality and incidence of malformed adults under in vitro conditions. An increased population of natural enemies of leaffolder and predatory spider was noticed in *Pseudomonas* treated plots under field conditions, which yielded 12–21% more rice (Commarea et al. 2002). PGPR belonging to *Pseudomonas* spp. are being exploited

Table 4 Reported PGPR show antagonistic effects against nematodes

Nematodes	Species	PGPR	References
Meloidogyne incognita	Lettuce and tomato	*Pseudomonas* sp. W34 *Bacillus cereus* S18	Hoffmann-Hergarten et al. (1998)
Globodera pallida	Potato	*Agrobacterium radiobacter* G12A *Rhizobium etli* G12	Hackenberg et al. (1999) Reitz et al. (2000)
Meloidogyne incognita	Tomato and banana	*Bacillus cereus*, *B. subtilis*, *Pseudomonas fluorescens*, *Pseudomonas chlororaphis* *Burkholderia cepacia*	Jonathan et al. (2000)
	Bell pepper	*Burkholderia cepacia* Bc-2 *Burkholderia cepacia* Bc-F	Meyer et al. (2001)
Meloidogyne javanica	Tomato	*Pseudomonas aeruginosa* IE-6S(+) *Pseudomonas fluorescens* CHA0	Siddiqui and Shaukat (2002) Siddiqui and Shaukat (2003)
		Pseudomonas aeruginosa strain 7NSK2 *Pseudomonas fluorescens* CHA0	Siddiqui and Shaukat (2004)
Globodera rostochiensis	Potato	*Pseudomonas oryzihabitans*	Andreoglou et al. (2003)
Meloidogyne javanica	Lentil	*Pseudomonas putida*, *P. alcaligenes*, *Paenibacillus polymyxa*, *Bacillus pumilus*	Siddiqui et al. (2007)
Meloidogyne incognita	Tomato and soybean	*Pseudomonas fluorescens* CHA0	Siddiqui et al. (2005)
	Tomato	*Rhizobium etli* G12 *Bacillus amyloliquefaciens* FZB42	Reimann et al. (2008) Burkett-Cadena et al. (2008)
	Chickpea	*Pseudomonas alcaligenes* *Bacillus pumilus*	Akhtar and Siddiqui (2008)
Meloidogyne javanica	Chickpea	*Pseudomonas putida* 3604 *Pseudomonas alcaligenes* 493	Siddiqui and Akhtar (2009a)
Meloidogyne incognita	Tomato	*Bacillus subtilis*, *Paenibacillus polymyxa* *Burkholderia cepacia*	Siddiqui and Akhtar (2009b)
	Chickpea	*Pseudomonas putida* *Pseudomonas alcaligenes*	Akhtar and Siddiqui (2009)

commercially for plant protection to induce ISR against various pests and diseases. The performance of PGPR has been successful against certain pathogens, insect, and nematode pests under field conditions (Ramamoorthy et al. 2001). Murphy et al. (2000) studied the effects of PGPR treatment on whitefly nymphs number in field trials in Florida. They recorded significantly lower numbers of whitefly nymphs on PGPR-treated plants compared with the untreated tomato.

The metabolic pathways associated with insect-active secondary plant metabolites may be affected by induction of SAR or ISR, which could in turn effect changes in plant concentrations of insect feeding stimulants. Because induction of SAR and ISR involves different metabolic pathways, it is not unexpected that plants

treated with PGPR or other elicitors will vary in their suitability as insect host plants (Stout et al. 2002).

Consequently, it can be said that PGPR would be of great potential, especially to conserve natural enemies and to avoid potential problems encountered when some insecticides fail to control populations that have developed resistance (Wang et al. 2002).

4 Conclusions and Future Prospects

Since Kloepper and Schroth (1978) reported that microbial communities that exert benefit for plant growth have been called PGPR, there has been an increasing effort in advancing bacterial inoculants such as *Azotobacter*, *Azoarcus*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Gluconacetobacter*, *Klebsiella*, *Pseudomonas*, *Serratia*, *Rhizobium*, etc., for plant growth promotion in agriculture. Significant advances in the explanation of the mechanisms involved in plant growth promotion have been made, especially when using molecular biology approaches (Dobbelaere and Okon 2003). Mechanisms involved in plant growth promotion include biological nitrogen fixation, solubilization of insoluble phosphates, production of phytohormones, suppression of diseases, rooting of cuttings, increase germination and emergence of seeds under different conditions, promoted nutrient uptake of roots, total biomass of the plants, induce early flowering, increase in yield, etc.

Different PGPR have been examined under controlled and field conditions, and generally plant growth promotion such as yield increases in different crops, reduction of fertilizer and pesticides have been clearly demonstrated. The scientific basis of PGPR should continue to be investigated, tested, and explored for better and effective use of strains in the future, and free exchange of PGPR strains between researchers and countries (Podile and Kishore 2006) may help this. There is good possibility that the commercial mix of PGPR for various aims such as improved crop yield or suppression of pests and disease developed will be used extensively in the production of different crops in sustainable and environment friendly agriculture.

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Importance of Biofilm Formation in Plant Growth Promoting Rhizobacterial Action

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Abstract Among the diverse soil microflora, plant growth promoting rhizobacteria (PGPR) mark an important role in enhancing plant growth through a range of beneficial effects. This is often achieved by forming biofilms in the rhizosphere, which has advantages over planktonic mode of bacterial existence. However, the biofilm formation of PGPR has been overlooked in past research. This chapter

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focuses on new insights and concepts with reference to improved PGPR effects caused by the biofilm formation by PGPR and its impact on overall plant growth promotion, compared with the planktonic lifestyle of PGPR. Beneficial PGPR play a key role in agricultural approaches through quorum sensing in their biofilm mode. The in vitro production of biofilmed PGPR can be used to give increased crop yields through a range of plant growth mechanisms. They can be used as biofertilizers through improved N_2 fixation and micro- and macronutrient uptake. Further, higher levels of plant growth with PGPR have been observed due to their production of plant growth regulators and their abilities to act as biocontrol agents, which are carried out by the production of antibiotics and other antimicrobial compounds. The microbial inoculant industry would also benefit greatly by developing biofilmed PGPR with N_2 fixing microbes. Biofilmed PGPR can be manipulated to achieve results in novel agricultural endeavors and hence is as an area which needs a deeper probing into its potential.

1 Introduction

The soil represents a favorable habitat for diverse populations of microbes which have made inquisitive minds probe into their function and activities since time immemorial. The intrinsic roles they play in terrestrial ecosystems have a direct effect on plant growth and soil quality. This feature has led to considerable attention being paid to improve plant growth promotion using effective microorganisms in sustainable agriculture. By and large, this is attributed to the ability of microbes to "turnover" nutrients and to bind particles in soil which is essential for plant growth.

Among the plant associated soil microbial communities, root colonizing beneficial bacteria (rhizobacteria), known as plant growth promoting rhizobacteria (PGPR) (Lugtenberg and Kamilova 2009), are recognized as one of the predominant groups that wield a range of beneficial effects in enhancing plant growth. This is achieved by an array of activities including N_2 fixation, increasing the availability of phosphate and other nutrients in the soil, phytostimulation, suppression of plant diseases, synthesis of antibiotics and the production of phytohormones (Sivan and Chet 1992; Zehnder et al. 2001). Excellent reviews on the PGPR action on roots and mycorrhizosphere are found in Bending et al. (2006) and Spaepen et al. (2009). The success of PGPR in agriculture is attributed to their effective colonization of plant roots (Raaijmakers et al. 1995; Bolwerk et al. 2003) and subsequent growth to form microcolonies or biofilms, which is their common occurrence in a successful plant–microbe interaction (Saleh-Lakha and Glick 2006).

Biofilms are mass colonies of single or multispecies of microbial cells adherent to biotic or abiotic surfaces and/or in intimate contact with each other, encased in a self produced matrix of extracellular polymeric substances (EPS). Less complex biofilms with lower numbers of cells are variably described as microcolonies, aggregates, or cell clusters (Morris and Monier 2003; Ramey et al. 2004). The microcolony is the basic growth unit of a biofilm, and this mode of biofilms is

predominant in almost all natural environments (Lappin-Scott and Costerton 1995). The colonization of plant surfaces by plant-associated microbial populations shows similarities to the formation of biofilms on abiotic surfaces with certain genetic determinants common to both processes (Molina et al. 2003).

As outlined by Saleh-Lakha and Glick (2006), these bacterial assemblages have the capability to communicate chemically with one another through quorum sensing, functioning as a single unit. Thus, PGPR when they are in biofilm mode should perform well in inhibiting competing organisms, nutrient uptake, quick responses, and adaptation to changing environmental conditions. However, the natural existence of PGPR in the soil has not been adequately investigated, and the knowledge of biofilmed mode of PGPR and their actions is vastly unexplored. Some reports have highlighted that the plant-associated biofilms have a higher ability to protect themselves from external stress and microbial competition that are characteristic of the rhizosphere, and also to produce beneficial effects in plant growth promotion (Ramey et al. 2004; Seneviratne et al. 2008a, b, 2009). Additionally, it has been shown that naturally occurring or in vitro produced effective PGPR inocula have many potential uses evidently in agricultural and biotechnological settings (Seneviratne et al. 2008b).

Most bacteria appear to form biofilms and this multicellular mode of growth likely predominates in nature as a protective mechanism against hostile environmental conditions (e.g., *Pseudomonas aeruginosa*, Costerton et al. 1995; Costerton and Stewart 2000; Walker et al. 2004). Biofilms, in general, have unique developmental characteristics that are different to freely swimming planktonic cells or nonbiofilm-forming cells. Molecular and genetic studies have identified that biofilms differ considerably from individual microbes in planktonic mode of growth in vital characteristics such as gene expression (Davies et al. 1993; Vilain and Brözel 2006) and physiological functions (Dow et al. 2007). Further, Stoodley et al. (2002) reported that as a result of biofilm structure, physiological adaptation, and the adherent nature of microbial cells in biofilms, they show an elevated antimicrobial tolerance.

Thus, the role of biofilm architecture in plant–microbe interactions cannot be negligible and identification of plant growth improvements through developed biofilmed inocula would have a great scope in plant growth promotion. The impact of microbial biofilms in plant growth promotion has not received adequate attention and studies of beneficial biofilm communities are thus of special interest. This chapter focuses on new insights and concepts with reference to improved PGPR effects caused by the biofilm formation by PGPR and its impact on overall plant growth promotion, compared with the planktonic lifestyle of PGPR. In addition, their potentials in agricultural innovations are also discussed.

2 Occurrence of PGPR Biofilms in Plant–Microbe Interaction

It is well known that most microorganisms in the rhizosphere exist as biofilms rather than their planktonic mode (Watnick and Kolter 1999; Davey and O'Toole 2000). Biofilms associated with the plant roots have been found to be beneficial for

plant growth, yield, and crop quality. PGPR biofilm formation and plant growth promotion are governed by effective root colonization of the host plant (Saleh-Lakha and Glick 2006). However, to date biofilm-mediated PGPR actions have not been described adequately. Therefore, evidences found in literature for occurrence of PGPR biofilms in plant–microbe interactions and their possible mechanisms are discussed in this section.

Common plant-associated bacteria found on leaves, roots, and the soil such as *P. putida*, *P. fluorescens*, and related pseudomonads, together with a majority of other natural isolates, have been reported to form effective biofilms (Ude et al. 2006). Bloemberg et al. (2000) noted that the plant growth promoting *P. fluorescens* discontinuously colonized the root surface, developing as small biofilms along epidermal tissues. In contrast, dense and confluent biofilms on root surfaces were observed in studies analyzing pathogenic *Pseudomonas* spp. (Bais et al. 2004; Walker et al. 2004). Although the fundamental cause of these different observations is uncertain, it is evident that the root biofilms of *Pseudomonas* spp. can range from relatively small multicellular clusters to extensive biofilm networks.

Microbes in root-associated biofilms depend basically on root exudates for food and nutrition (Bais et al. 2006). Although the quantities of organic compounds exuding from plant roots are not large, seldom exceeding 0.4% of the C photosynthesized, they exert a very strong influence on the soil microorganisms and may be significant in affecting plant nutrient availability (Rovira 1969). By providing organic compounds as a nutrient source, these root exudates take a central role in being a major plant-derived factor and in triggering of root colonization (Lugtenberg et al. 1999) and biofilm associations (Walker et al. 2004). Some studies have also suggested that the biofilm formation at root sites is triggered by a plant-derived component similar to that seen in *Rhizobium*-legume and other bacterial interactions (de Ruijter et al. 1999), which has happened to be organic compounds of root exudates in this case. The role played by root exudates is further confirmed by Espinosa-Urgel et al. (2002) by observing that *P. putida* can respond rapidly to the presence of root exudates in soils, converging at root colonization sites and establishing stable biofilms.

Most species of bacteria use the quorum sensing to coordinate their gene expression according to the local density of their population. This signaling mechanism modulates and coordinates bacterial interactions with plants, including the control of tissue maceration, antibiotic production, toxin release, and horizontal gene transfer (HGT) (von Bodman et al. 2003). It is one of the main regulatory mechanisms in the formation of biofilms and it is seen that most beneficial phenotypes of PGPR are under its control (Loh et al. 2002). Quorum sensing of PGPR is mediated by an array of signal molecules which include (a) acylated homoserine lactones (AHLs) among proteobacteria; (b) gamma-butyrolactones in Streptomyces species; (c) *cis*-11-methyl-2-dodecanoic acid (also called DSF) in species of *Xanthomonas*, *Xylella*, and their relatives; and (d) oligopeptides among gram-positive microbes (Danhorn and Fuqua 2007). The AHLs-mediated cell-to-cell communication is mostly common among rhizospheric bacteria. The AHLs act as

population density sensors and facilitate the communication between different cells (Pierson et al. 1998). Although the AHLs-based quorum sensing is characterized by the proteins LuxI-type protein, AHL synthase, and LuxR-type protein, exceptions have been reported for *Vibrio harveyi* and *P. fluorescens* F113, as they replace the LuxI-type with LuxM AHL and HdtS AHL synthase, respectively (Case et al. 2008). The AHLs-mediated quorum sensing is widely detected in *Pseudomonas* spp. than any other root colonizing bacteria (Juhas et al. 2005). The root-associated biocontrol agent *P. fluorescens* 2P24 requires AHLs for biofilm formation and therefore control of take-all disease on wheat (Wei and Zhang 2006).

It is evident from above information that biofilm formation by PGPR is common in the rhizosphere and that quorum-sensing-based cell-to-cell communication could play a key role in the action of PGPR in green agricultural approaches. The importance of discovering effective forms of PGPR biofilms leads us to the next section, where we focus on their potential applications in futuristic agricultural systems.

3 PGPR Biofilms in Futuristic Agriculture

The current public concerns on the detrimental side effects in the use of agrochemicals have lead to search other avenues of gaining better crop productivity. Of these, an increasing interest has been shown in the use of biofertilizers comprising of beneficial microorganisms, which improves plant growth through the supply of plant nutrients in a manner sustaining environmental health and soil productivity (O'Connell 1992). However, an inconsistency in the field application of such microbial inocula has limited its widespread commercial application, most probably due to the incapability of such inocula to successfully compete with indigenous microflora in establishing themselves in the rhizosphere (Van Elsas et al. 1986; Bent and Chanway 1998).

This failure can be overcome by the introduction of bacterial inoculants in the form of biofilms, thus protecting the inoculants against adverse environmental conditions such as high salinity, tannin concentrations, low pH, heavy metals, predation by earthworms, the competition by native soil populations (Seneviratne et al. 2008b), and the resistance to protozoan grazing (Matz et al. 2004). In this respect, the use of well-characterized PGPR biofilms is remarkable than solitary PGPR since the biofilm formation is an added advantage for PGPR to colonize effectively on or in the plant root, where they can compete well with indigenous microflora along with improved plant growth promotion. This has been made evident by Timmusk et al. (2005) who reported that *Paenibacillus polymyxa* forms biofilms around the root tip and behaves as a root-invading bacterium attributing a possible mechanism in biocontrol and drought tolerance-enhancing activities. Apart from the root colonization, recent observations have been made that the bacterial colonization of biotic fungal surfaces leading to the formation of fungal–bacterial biofilms (FBB) renders the biofilms enhanced metabolic activities in comparison to monocultures,

leading to improved biofertilizing and biocontroling effects (Seneviratne et al. 2008a, b). Further, as speculated by Seneviratne and Jayasinghearachchi (2003), the establishment of such biofilms of rhizobia with common soil fungi provides a plausible strategy for the rhizobial survival.

This leads us to confirm that the in vitro production of such biofilmed inocula with PGPR can be utilized to satisfy the future demand of augmented crop production attributed to increased N_2 fixation, nutrient uptake, plant growth promoting agents, and biocontrol of diseases, through a range of mechanisms described below.

3.1 PGPR Biofilms as Biofertilizers

The plant growth promoting rhizobacterial species which flourish in the rhizosphere of plants have been seen to stimulate plant growth, yield, and crop quality by a plethora of mechanisms. This has led to a considerable number of PGPR being tested as biofertilizers, mainly because they provide inorganic nutrients to plants by mineralizing organic and insoluble inorganic forms of nitrogen, phosphorous, and sulfur that plants cannot utilize directly (Mendez-Castro and Alexander 1983) as well as providing essential micro and macro nutrients. This has been made evident by the possession of N_2-fixing properties in many PGPR species including *Bacillus* spp., *Azotobacter* spp., *Azospirillum* spp., *Beijerinckia* spp., *Pseudomonas* spp. (Dobereiner 1997; Reis et al. 1994; Vance 1997), and *Rhizobium* and *Bradyrhizobium* spp. (Dobereiner 1997; Vance 1997).

Such PGPR have been seen to valuably carry out their N_2-fixing ability in the biofilm mode as well, as shown by many studies. Jayasinghearachchi and Seneviratne (2004a) demonstrated that a fungal-rhizobial biofilm (FRB) (*Bradyrhizobium elkanii* SEMIA 5019 and *Penicillium* spp.) biologically fixed N_2 more effectively, as revealed by nitrogenase activity and N accumulation, in comparison to the rhizobial strain grown as a monoculture. A developed biofilmed inoculant of this FRB was also seen to significantly increase N_2 fixation (by ca. 30%) compared with a rhizobium-only (conventional) inoculant when applied to soybean (Jayasinghearachchi and Seneviratne 2004b). The ability to increase N availability by ca. twofold and a high nitrogenase activity, even under a very high soil NO_3^- concentration, were observed in the direct application of FRBs to soil, compared with the monocultures (Seneviratne and Jayasinghearachchi 2005). Yet another PGPR *Azospirillum brasilense*, a free-living N_2 fixer, was found to interact with roots of wheat and maize, forming dense biofilms and thereby promoting their host plant's growth (Assmus et al. 1995; Burdman et al. 2000).

Of the PGPR used to date, two genus most widely known are *Rhizobium* and *Bradyrhizobium* and their symbiotic N_2 fixation through inoculation to legume crops is well known (Dobereiner 1997; Vance 1997). Recent reports have indicated that these symbiotic bacteria may have the potential to be used as PGPR with nonlegumes. Seneviratne et al. (2009) have recently observed the heavy colonization of FBBs/FRBs on root hairs of rice (*Oryza sativa*), tea (*Camellia sinensis*),

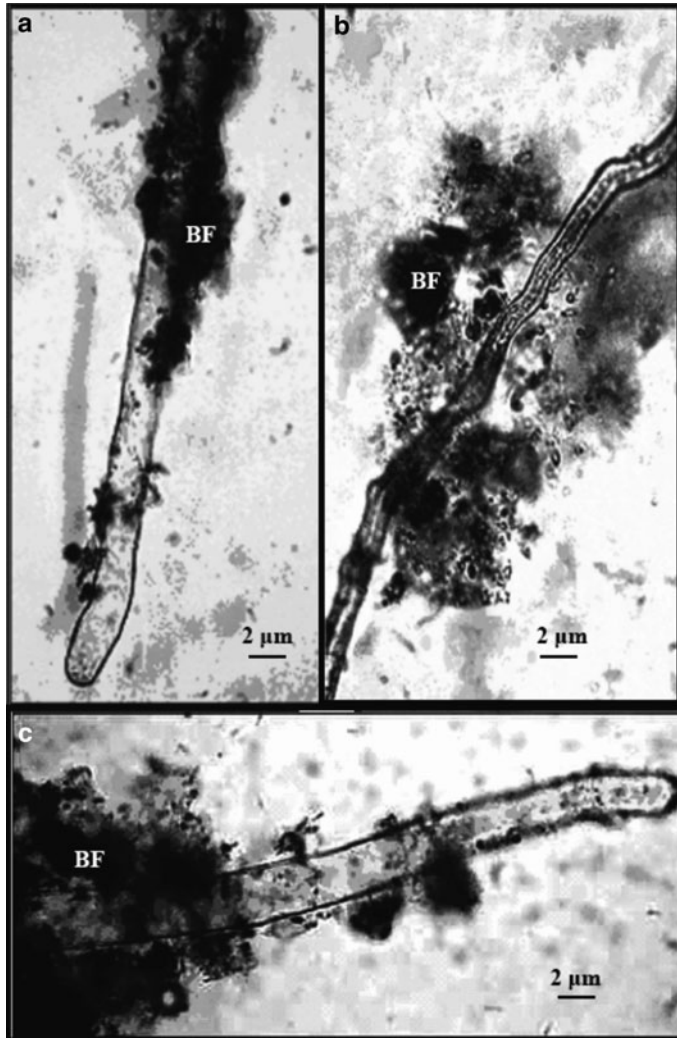

Fig. 1 Root hairs of rice (**a**), tea (**b**), and anthurium (**c**) colonized by microbial biofilms (BF), when fungal–bacterial biofilms (FBB) or fungal–rhizobial biofilms (FRB) were inoculated under axenic conditions. Darkness is due to cotton blue stain absorbed by the extra cellular polymeric substances (EPS) produced by the BF. Reprinted from Seneviratne et al. (2009)

Anthurium (*Anthurium andraeanum*), and wheat (*Triticum aestivum*) (Fig. 1). It has been suggested that such FRBs may act as "pseudonodules," fixing N_2 biologically on the roots of nonlegumes. Further, it was found that recommended chemical fertilizers may be reduced by about 50% by applying such in vitro produced biofilmed biofertilizers (BBs). When BBs were applied with chemical fertilizers to micropropagated Anthurium plantlets, leaf number and chlorophyll content increased by ca. 60% and 100%, respectively, compared with chemical fertilizers

alone application (KACN Seneviratne, unpublished). The BBs alone application increased root length of Anthurium by ca. 65%, compared with chemical fertilizers alone application.

Phosphorus (P) is a highly limited nutrient in some soils and hence phosphate-solubilizing bacteria play an important role in the P nutrition in plant growth. Seneviratne and Jayasinghearachchi (2005) have shown that the application of FRBs directly into soil increased P availabilities by 15-fold and that the biofilmed inocula can be effectively used in biosolubilisation of rock phosphate. This was amply demonstrated by an increased P solubilisation (up to ca. 230%) when biofilms developed from *Penicillium* spp., *Pleurotus ostreatus*, and *Xanthoparmelia mexicana*, a lichen fungus, were used compared with the fungus-only cultures (Jayasinghearachchi and Seneviratne 2006a; Seneviratne and Indrasena 2006).

Apart from the major nutrients required for plant growth, some studies have also shown that coinoculation of PGPR inocula enhanced the uptake of micronutrient such as Zn, Cu, and Fe (Bashan 1998). Coinoculation of *Pseudomonas* BA-8 + *Bacillus* OSU-142 increased Fe and Zn contents of leaves up to 50.5 and 35.5%, respectively, compared with the control (Esitken et al. 2005). Investigations of the modes of action by PGPR are increasing at a rapid pace to exploit them commercially as biofertilizers. The benefits of such combinations of mixed cultures or biofilms can be manipulated to overcome the challenges facing for more widespread utilization of PGPR as biofertilizers.

3.2 PGPR Biofilms as Plant Growth Promoting Agents

Numerous studies have demonstrated an improvement in plant growth and development in response to seed or root inoculation with various microbial inoculants capable of producing plant growth regulators (Zahir et al. 2004). Important plant growth promoting substances commonly produced by rhizosphere bacteria include auxins (indolyl-3-acetic acid), gibberellins, and cytokinins (Brown 1974).

Studies by Bandara et al. (2006) revealed that higher acidity and PGP hormone levels were produced by FBBs of beneficial rice endophytes than their mono- or mixed cultured forms with no biofilm formation. Their studies on a large collection of microbes also revealed the existence of a significant negative relationship between the production of indoleacetic-acid-like substances (IAAS) and pH in liquid culture media of FBBs, but not in mixed cultures with no biofilm formation. This high acidity reflects high IAAS production when biofilms are formed. Thus, the use of biofilmed inocula, rather than the conventional practice of plant inoculation with monocultures or mixed cultures of effective microbes, may help achieve the highest microbial effect. Another recent study on early growth of rice showed that the contribution of developed biofilmed inocula in enhanced release of organic acids and PGP substances led to ca. 25% increase in plant dry weight compared with conventional monocultured inocula (Seneviratne et al. 2009). In further studies, biofilmed inocula showed lower pH, higher IAAS, and rice plant dry

weights than the monocultured inocula (MLMAW Weerasekara, unpublished). The biofilmed inocula showed a fourfold increase in H^+ secretion to the culture medium, compared with the monocultured inocula. Negative relationships were observed between pH of both types of the inocula and plant dry weight (Fig. 2a) or soil NH_4^+ (Fig. 2b). This implies that the inoculated biofilmed inocula colonize the rhizosphere, producing high acidity and IAAS (Seneviratne et al. 2008a), and the high acidity in microsites causes to an increase of plant available NH_4^+ (Xu et al. 1997) in the soil solution near root hairs, which helps increase the plant growth. Therefore, in vitro production and application of more effective combinations of such beneficial biofilmed inocula would play an important role in the inoculant industry. However, this needs further research to fully understand the effects and potentials of the biofilmed inocula in the plant growth promotion. It is clear from the above studies that one of the most plausible mechanisms of plant growth promotion by PGPR is the production of plant growth regulators. Further, the effectiveness of using such PGPR in their biofilmed mode in the production of higher levels of plant growth promoting substances is also noticeable.

3.3 PGPR Biofilms as Biocontrolling Agents

Biocontrolling has been seen as a well-suited alternative or supplement in contrast to conventional methods of disease control of which microbial biocontrolling

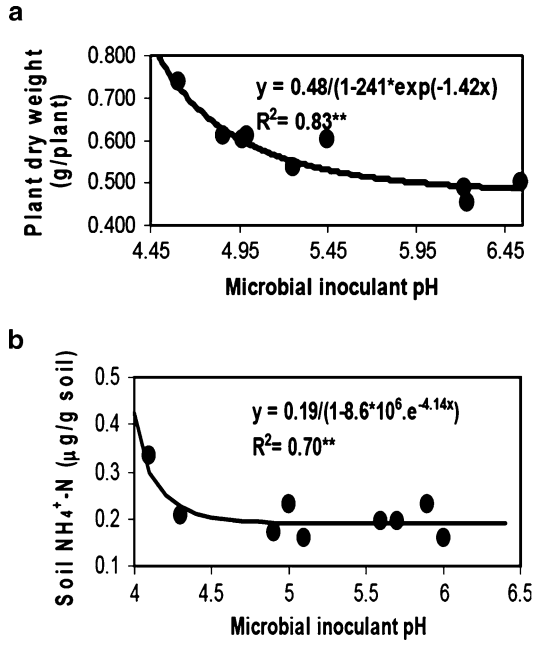

Fig. 2 Relationships between (**a**) microbial inoculant pH of both biofilmed and conventional inocula and rice plant dry weight, and (**b**) the microbial inoculant pH and soil NH_4^+, when inoculated in a soil pot experiment. The biofilmed inocula represent relatively low pHs

agents have emerged as favored options due to their complex mode of action and success in bringing out a reduced risk of resistance. For example, the extensive studies of root-associated pseudomonads have revealed that many of these promote the growth of host plants or are used as biocontrol agents (Lugtenberg et al. 2001). *P. fluorescens* has been reported to coat plant roots by forming a biofilm, which may protect roots against soil bacterial and fungal pathogens (O'Toole and Kolter 1998; Walker et al. 2004). The promising nature of PGPR strains as means of plant protection via disease suppression was amply demonstrated by Raupach and Kloepper (1998) in finding the occurrence of a consistent protection against pathogens when mixtures of PGPR were present, possibly in the biofilm mode.

An array of studies has confirmed that bacteria when they are in the biofilm mode perform well as biocontrol agents, mainly because the plant is made less sensitive to infection by the formation of biofilms by bacteria on the plant root (Bais et al. 2004; Rudrappa et al. 2008). Owing to the heterogeneous nature of biofilms, it is likely that the biofilm formation on the plant roots protects the plants against soil borne diseases through resistance mechanisms such as cell–cell communication via quorum-sensing (Danhorn and Fuqua 2007) and production of antibiotics against pathogens (Russo et al. 2006).

Biofilms bring about disease suppression through a variety of roles played by antibiotics. Such microbial communities have a significant resistance to antibiotics compared with planktonic bacteria of the same species (Stewart and Costerton 2001), while some biofilms have the ability to produce different antibiotics (Leifert et al. 1995; Raaijmakers et al. 2002; Yu et al. 2002; Risøen et al. 2004; Roberts and Stewart 2005).

In addition, biocontrolling agents of PGPR have been shown to successfully establish in plants, when they were applied as biofilmed inocula. Jayasinghearachchi and Seneviratne (2006b) confirmed this in vitro by using a *Pleurotus ostreatus* – *Pseudomonas fluorescens* biofilm which was seen to increase endophytic colonization of tomato by *P. fluorescens*, a biocontrolling agent, by over tenfold compared with inoculation of *P. fluorescens* alone. The PGPR *Paenibacillus polymyxa* provides protection from pathogens through the synthesis of several antibiotics, when it forms biofilms by predominantly colonizing the root tips of *Arabidopsis thaliana*, as revealed by fluorescence microscopy and electron scanning microscopy (Timmusk et al. 2005). *Bacillus subtilis*, another biocontrolling PGPR, protects plant roots from pathogenic bacteria by mechanisms which include biofilm formation and antibiotic and surfactin production (Bais et al. 2004; Cavaglieri et al. 2005). Surfactin possesses antimicrobial activity, and pathogens those reach inside the biofilms are killed by high surfactin concentrations (Bais et al. 2004).

Bacteria used to accomplish biocontrolling exert their action also through producing antimicrobial secondary metabolites, which target the competing microorganisms (Mazzola et al. 1992; Raaijmakers et al. 2002; Haas and Keel 2003). Some *Pseudomonas* strains secrete antimicrobial compounds such as exoproteases, antibiotics, HCN, or metabolites with antifungal activity known as phenazines (Molina et al. 2003). These compounds have the capacity to eliminate competititors from the rhizosphere with a plethora of studies demonstrating their prospect as

biocontrol agents (Thomashow 1996; Chin-A-Woeng et al. 2000; Kremer and Souissi 2001). Studies outlined above highlight the potential of using biofilmed PGPR with increased microbial action to carry out biocontrol feats in conventional agriculture and organic farming systems.

4 Conclusions

Although developing biofilms has been the axis around which many recent studies have evolved in diverse areas of biotechnology, the investigation of the involvement of PGPR in such biofilms is yet in its infancy. The capability of PGPR to colonize plant roots proficiently and carry out a range of benefits to the plant has made it one of the predominant soil microbial groups. Regulatory mechanisms, such as quorum sensing, exhibited by PGPR have made them stable partners in biofilms, placing them on a higher pedestal compared with their existence alone. PGPR biofilms have been shown to play a fundamental role in futuristic agricultural approaches such as biofertilizers, plant growth promoters, and biocontrolling agents. A heightened interest in recent times in inoculant technology has thrown much importance on the designing and developing of PGPR biofilmed inocula. The beneficial results they yield encourage the deeper delving into its applications and the innovative future perspectives. The importance of biofilm formation in PGPR action is thus an area which needs much more in depth exploration to bring out its true potential.

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Plant Growth Promoting Rhizobacteria: Constraints in Bioformulation, Commercialization, and Future Strategies

Naveen K. Arora, Ekta Khare, and Dinesh K. Maheshwari

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Abstract Bioformulations for plant growth promotion continue to inspire research and development in many fields. Increase in soil fertility, plant growth promotion, and suppression of phytopathogens are the targets of the bioformulation industry that leads to the development of ecofriendly environment. The synthetic chemicals used in the agriculture to increase yields, kill pathogens, pests, and weeds, have a big harmful impact on the ecosystem. But still the chemicals rule the agroindustry. The aim of the review is to assess the constraints associated with the effective

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development of bioinoculant industry particularly in developing countries. Another objective of the review is to evaluate what should be explored in the future to uplift the stature of the bioinoculants. Bioformulations offer an environmentally sustainable approach to increase crop production and health, contributing substantially in making the twenty-first century the age of biotechnology.

1 Introduction

The new challenge in the new millennium is to produce more and more food from shrinking per capita arable land, keeping the environment safe. As agricultural production intensified over the past few decades, producers became more and more dependent on agrochemicals. Chemical fertilizers and pesticides are presently accumulating in the environment harming the ecosystem, causing pollution, and spreading disease (Gerhardson 2002). Therefore, the urgent need of biological agents is accepted worldwide. Interest in biological control of plant pathogens has increased considerably over the past years, partly as a response to public concern about the use of hazardous chemical pesticides, but also because it may provide control of diseases that cannot or only partially be managed by other control strategies (Arora et al. 2008b, c).

For many decades, bacteria have been introduced into soil or on seeds, roots, bulbs, or other planting material to improve plant growth and health. The major objectives of bacterization include enhancement of symbiotic or associative nitrogen fixation, degradation of xenobiotic compounds, plant growth promotion, and biological control of plant pathogenic microorganisms (van Elsas and Heijnen 1990; Whipps 2001). To date, many bacterial genera are being used and tested in bacterization, including *Acinetobacter*, *Agrobacterium*, *Alcaligens*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Bradyrhizobium*, *Frankia*, *Pantoea*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Stenotrophomonas*, *Streptomyces*, and *Thiobacillus* (Whipps 2001; Lutenberg and Kamilova 2009). Some of the fungal taxa that have been successfully commercialized and are currently marketed as Environmental Protection Agency (EPA) registered biopesticides in United States belong to the genera, *Ampelomyces*, *Candida*, *Coniothyrium*, and *Trichoderma* (McSpadden Gardner 2002).

Although plant growth promoting rhizobacteria (PGPR) occur in soil, usually their number is not high enough to compete with other bacteria commonly established in the rhizosphere. Therefore for agronomic utility, inoculation of plants with target microorganisms at a much higher concentration than those normally found in soil is necessary to take advantage of their beneficial properties for plant yield enhancement (Subba Rao 1993). The erratic performances of bioinoculants under field conditions have raised concerns about the practical potential offered by microbial releases into soil (Arora et al. 2007b). Soil is an unpredictable environment and an intended result is sometimes difficult to obtain. The immediate response to soil inoculation with PGP bioformulations varies considerably depending on the bacteria (PGP agent), plant species, soil type, inoculum density, and

environment conditions. The inoculated bacteria sometimes do not survive in the soil when competing with the better adapted indigenous microflora (Bashan 1998). An effective PGP strain isolated from one region may not perform in the same way in other soil and climatic conditions (Johnsson et al. 1998). The aim of this review is to point out the status of bioformulations and constrains related with their application and to draw the focus on future research strategies for the development of better inoculants.

2 Plant Growth Promotory Bioformulations

Bioformulations are best defined as biologically active products containing one or more beneficial microbial strains in an easy to use and economical carrier materials. Usually, the term bioformulation refers to preparations of microorganism(s) that may be partial or complete substitute for chemical fertilization/ pesticides. Biological control of plant pests and pathogens continues to inspire research and development of formulations targeted at plant pathogens.

The first objective when considering inoculation with beneficial strain is to find the best bacteria available (Validov et al. 2007). Many potentially useful bacteria reported in the scientific literature never appear on the commercial market, perhaps because of inappropriate formulation. The carrier is the delivery vehicle of live microorganisms from the factory to the field; however, no universal carrier or formulation is presently available for the release of microorganisms into soil (Smith 1992). A good carrier should have the capacity to deliver the right number of viable cells in good physiological condition at the right time (Smith 1992; Arora et al. 2008a). Carriers can be divided into three basic categories: (1) soils (peat, coal, clays, and inorganic soil), (2) plant waste materials, (3) inert materials viz. vermiculite, ground rock phosphate, polyacrylamide gels, and alginate beads (Bashan 1998). Inoculants come in four basic dispersal forms (powders, slurries, granules, and liquids). The use of each type of inoculant depends upon market availability, choice of farmers, cost, and the need of a particular crop under specific environmental conditions.

3 Production and Marketing Constraints

Since bioformulation is a living product, utmost care is needed at all the steps beginning from the production till the end use to maintain the microbial load and vigor. Production technology of bioformulations requires proper care and aid of sophisticated equipments to ensure availability of quality products in the market. Kabi (1997) gave stress on the production of quality inoculants since these are very important not only in providing nutrient supply to the plants but also in rendering sustainability to farming systems. In developing countries, the insufficient knowledge, lack of adequate machinery, and improper distribution and importation laws

for live inoculants can lead to loss of their viability and effectiveness. The major constraints associated with effective bioformulation development are as follows:

3.1 High Cost of Production

Because of low price structure, hi-tech instrumentation required for producing bioformulations under completely sterile conditions is not getting acceptance. The potential hazards associated with bacterial contaminants should not be ignored as long as nonsterile carrier inoculants are widely used. However, one should note that the use of nonsterile carrier inoculants has caused no reported health hazards in decades of usage (Bashan 1998). The development of bacterial inoculants is claimed to be cheaper than that of agrochemicals, although the large-scale screening of strains with biological activity is still required (comparable to more than 1:20,000 screened molecules for a new chemical product) (Bashan 1998).

Deficiencies in handling procedure are a major cause of under performance in real life application. The high sensitivity to temperature and other external conditions of these "living" inputs calls for enormous caution at the stage of manufacture/culture, transportation/distribution, and application. This involves investment in packaging, storage, and use of suitable carrier materials (Arora et al. 2001).

Spurring the development of agricultural markets is the key factor for achieving targeted growth in bioformulation usage. In general, firms with larger production facilities are expected to invest more on networks to understand and access the market. A big obstacle is the registration procedure, which is often expensive and time consuming; especially, the cost of registration is the principal obstacle in the development of new products (Ehlers 2006).

3.2 Shelf Life

One of the main barriers faced by the producers of bioformulations and investors is inadequate demand and the inconsistent and seasonal nature of the existing demand, necessitating efficient storage. The storage of bioformulations requires special facilities and skills, which most producers, shopkeepers, and farmers do not possess. Shelf life is a culmination of several factors like production technology, carrier and packaging material used, mode and distance of transport, all these levels are desired to sustain the shelf life.

The most common solutions to this problem of survival time have been air-dried and lyophilized preparations (Kosanke et al. 1992). The lowered water content in the final product is responsible for long-term survival during storage. In this way, the bacteria in the formulation remain inactive, resistant to environmental stresses, insensitive to contamination, and are more compatible with fertilizer application (Bashan 1998). The dehydration phase is perhaps the most critical of the entire

formulation process especially for nonspore-forming bacteria (Shah-Smith and Burns 1997). Bacterial survival is affected by several variables: the culture medium used for bacterial cultivation, the physiological state of the bacteria when harvested from the medium, the use of protective materials, the type of drying technology used, and the rate of dehydration (Paul et al. 1993).

3.3 Inconsistent Performance: Fate of Inoculant Introduced in Soil

Inconsistent field performance is the major constrain associated with their marketing. These failures have raised concerns about the perspective of the great practical potential offered by microbial releases into soils. A key factor involved in the lack of success has been the rapid decline of the size of populations of active cells, to levels ineffective to achieve the objective, following introduction into soil. As soil is a heterogeneous system with a mixed biota under fluctuating local conditions, temporal and spatial aspects pertaining to the introduction should be critically evaluated for each release. This growth/survival-inhibitory effect of soil has been called soil microbiostasis (Ho and Ko 1985). It has been attributed to the scarcity of available nutrient sources to microbes in soil and the hostility of the soil environment to incoming microbes due to a myriad of adverse abiotic and biotic factors. The physiological characteristics of the inoculant organism determine to a great extent its fate and activity in soil. Hence, different species will show varying responses, in terms of survival and activity. The physiological traits that play a role in the capacity of inoculant bacteria to colonize soil and survive are often not well known. Therefore, a thorough selection procedure is required when searching for effective inoculants. Besides the intrinsic physiological characteristics of the organisms, abiotic and biotic soil factors play an important role. Abiotic soil factors (e.g., textural type, pH, temperature, and moisture) exert their (direct) effect on inoculant population dynamics by imposing stresses of various natures on the cells (Evans et al. 1993). They can also act indirectly by affecting the activity of the indigenous soil microflora. A correlation was found between the decline in populations of individual bacterial strains and the activity and increase in the abundance of protozoa in soil (Wright et al. 1995; Lutenberg and Kamilova 2009). Hence, protozoa play an important role as regulators of microbial inoculant population sizes in soil. Another biological factor, in line with the predation process, is the competition between inoculant and indigenous populations for available substrate and biological space.

Moreover, Elliott et al. (1980) showed that trophic interactions in soil, including nematode–protozoan–bacterium interactions, are influenced by the soil type as reflected in the pore space distribution. Colonization of soil particles and aggregates is assumed to be vital to ultimate inoculant survival in soil (Hattori and Hattori 1976). Under similar prevailing climatic conditions, the inoculant revealed higher

survival levels in fine-textured (clayey) than in coarser (sandy) soils. Vargas and Hattori (1986) clearly showed that in the presence of a cointroduced grazing protozoan species, the survival of inoculant bacteria localized in the interior parts of 1–2-mm soil aggregates was far better than that of cells present at external aggregate sites. This suggested that cells localized in the interior parts were physically protected from grazing by protozoa, presumably due to their localization in soil pores with small necks. The maintenance of sufficient activity of an inoculants population over a prolonged period after release often represents the main hurdle in the successful use of microbes as PGP agents. Furthermore, efficient introduction into soil during the growing season is a major technical constraint.

4 Research Areas for Development and Optimization of Bioformulations

Although the vast body of research on microbial inoculants deals with their ability to promote plant growth, there has been limited success in developing commercially viable products. For the development of successful bioformulation technology, progress must be made to meet numerous scientific challenges: (1) selection of improved strains having greater crop diversification, (2) survival during seed coating/pelleting, soil application and during storage at ambient temperatures is critical for the development of microbial inoculant products; therefore, it seems logical that these traits should form an integral part of any screening process for the development of new effective bioformulations, (3) more efficient plant growth promotory bacteria compete poorly with the rhizobacteria already in the soil. Ways to improve the competitive ability of inoculant should be explored, (4) study of environmental stresses that negatively affect nodulation, nitrogen fixation, and biocontrol ability such as soil pH, nutritional deficiencies, salinity, high temperature, and presence of toxic elements, (5) efficacy of microbial inoculants varies somewhat from site to site and year to year and this has to be considered and studied elaborately and, (6) understanding of interactions between the plant, beneficial rhizobacteria, and plant pathogens in the highly complex and dynamic rhizosphere environments is the ultimate need to overcome practical problems such as the inconsistency in field performance.

4.1 Microbial Diversity

Over the past 100 years, research has repeatedly demonstrated that phylogenetically diverse microorganisms can act as natural antagonists of various plant pathogens and promote plant growth (Cook 2000). The intensive screening of plant growth promoting microorganisms will allow the development of commercial

bioformulation(s). The rhizosphere is known to provide ecologically favorable niche for most of the beneficial soil organisms. The abundance of nitrogen fixing, phosphate solubilizing, and plant disease suppressing bacteria in the rhizosphere of crop plants assumes a natural significance from the agronomic point of view (Subba Rao 1999). A successful PGP agent must be an aggressive colonizer with better competence and storage conditions in its formulation and use. As plant pathogens survive and cause diseases at dry, nutrient-poor, and high soil-temperature conditions (e.g., *Rhizoctonia bataticola*), the biocontrol agent (BCA) must also be able to withstand more competitively in the same adverse environment. Growth at high temperature (45°C) and endospore-producing trait of *Paenibacillus* sp. (endophytic bacteria) makes it a more suitable bioinoculant and ensures its survival in soil when a host is not available (Senthilkumar et al. 2007). Endophytic bacteria probably have evolved an intimate relation with their host plants through coevolutionary process and may influence physiological process of plants. Moreover, their unique ability to survive in plants with no or little microbial competition makes them potential candidates for biological control (Bhowmik et al. 2002).

One important factor to be considered when screening new isolates is their activity in the range of environments in which they would be expected to be used; in particular different soil types (Ross et al. 2000). Saline conditions are known to suppress the growth of plants, causing a diminished yield. *Ochrobactrum* sp., the free-living α-proteobacteria, was reported to have the potential of plant growth promotion in saline soil conditions (Príncipe et al. 2007). Recent reports have described the isolation of *Ochrobactrum* from plant tissue of deep water rice (*Oryza sativa*) (Tripathi et al. 2006) as well as from soils and sediments.

PGPB that are effective in degradation of soil pollutants in laboratory conditions have not done well in presence of soil pollutants is another constraint for field application. Selection of pollutant-degrading rhizobacteria that live on, or are close to the root so that they can use root exudate as their major nutrient source is a promising strategy to solve this problem (Böeltner et al. 2008; Lutenberg and Kamilova 2009). Similar approach resulted in the isolation of novel *Sphingomonas* strains that are relatively efficient in the in situ removal of lindane (Böeltner et al. 2008). *Pseudomonas putida* PCL1444, effectively utilizes root exudate, degrades naphthalene around the root, protects seeds from being killed by naphthalene, and allows the plant to grow normally. Mutants unable to degrade naphthalene did not protect the plant (Kuiper et al. 2001). Validov et al. (2007) isolated two new BCAs, *Pseudomonas rhodesiae* and *Delftia tsuruhatensis*. *P. rhodesiae* was first isolated from natural mineral water and is taxonomically affiliated to the *Pseudomonas fluorescens* group (Anzai et al. 2000). The representatives of this species were known as degraders of aromatic compounds (Kahng et al. 2002) or as isonovalol producers (Fontanille and Larroche 2003), but had not been reported yet as control agents for plant disease. *Delftia* is a newly classified genus closely related to *Comamonas*. These bacteria were isolated for the first time from active sludge as degraders of terephthalate (Shigematsu et al. 2003). *Delftia terephthalate*, also been reported as a diazotrophic PGPR, is able to control blast and blight of rice caused by *Xanthomonas oryzae*, *Rhizoctonia solani*, and *Pyricularia oryzae* (Han et al. 2005).

During the past two decades research on marine bacteria has highlighted the tremendous potential of these microorganisms as a source of new bioactive secondary metabolites (Ahmed et al. 2000) and there is a growing awareness of the need for development of new antimicrobial agents for the treatment of human, animal, and plant diseases. Marine bacteria could represent a new scope of antibiotics, which are currently needed to combat emergent antibiotic-resistant pathogen. The strains of species isolated from different ecological niches also generally showed wide genetic diversity despite some strains having similarity in their biochemical characteristic. It has become essential to understand the bacterial community structure in relation to environmental factors and ecosystem functions to screen, select, and utilize the microbial diversity for development of bioformulations leading to environment safe for life.

4.2 Metagenomics

The majority of microorganisms on earth resist life in captivity, i.e., they cannot be grown in broth or on plates in the laboratory. An often-cited estimate is that as much as 99% or more of microbial life remains unculturable, and therefore, cannot be studied and understood in a way that microbial ecologists have become accustomed to over the past century. The metagenomic toolbox allows accessing, storing, and analyzing the DNA of nonculturable life-forms and thus can provide an otherwise hard-to-attain insight into the biology and evolution of environmental microorganisms, independent of their culturable status (Fig. 1).

Due to a historical bias to study those microorganisms that can be grown in the laboratory, there is limited knowledge on the abundance and activity of not-yet

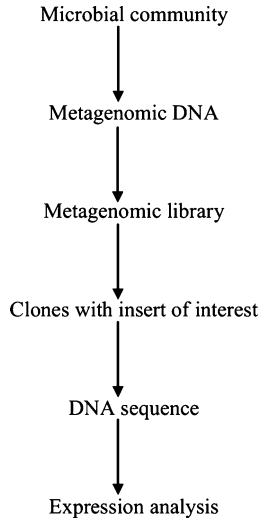

Fig. 1 Schematic representation of the classical metagenomic protocol

culturable PGPR. However, there are several examples of their existence and contribution to plant health, e.g., *Pasteuria penetrans*, a not-yet-culturable bacterium parasitic to plant-pathogenic nematodes (Fould et al. 2001), the nitrogen fixing activity by viable-but-not-culturable *Azoarcus* grass endophytes (Hurek et al. 2002), and the obligate biotrophism of arbuscular mycorrhizal (AM) fungi (Millner and Wright 2002). Bacteria belonging to the *Acidobacteria* and *Verrucomicrobia* are in many rhizospheres among the most abundant, difficult-to-culture representatives (Buckley and Schmidt 2003). However, it is not clear if and how their abundance is correlated to their contribution toward plant health. Several protocols have also been developed for the isolation of metagenomic bacterial DNA from inside plant material. Jiao et al. (2006) described an indirect method based on enzymatic hydrolysis of plant tissues to release associated microorganisms for subsequent DNA isolation and cloning. While optimized for leaves and seeds, this method seems readily adaptable for use with root material, and thus of great use to the metagenomic exploration of microorganisms in the rhizosphere.

There is a clear potential for metagenomics to contribute to the study of microbial communities of the rhizosphere, in particular PGPR. The other possible contributions of metagenomics in the study of PGPR include the discovery of novel PGP genes and gene products, and the characterization of not-yet culturable PGPRs. The tools of metagenomics offer many openings into a broadened view of the rhizosphere in general and of PGPR and their activities in particular. An analysis of the rhizosphere by comparative metagenomics holds the promise to reveal several important questions regarding the unculturable fraction of the rhizosphere community. For one, it could expose what actually constitutes this fraction from a comparison of metagenomic DNA isolated directly from rhizosphere to DNA isolated from all the colonies forming on solid media after plating from that same rhizosphere (i.e., the culturable fraction). The discovery of novel PGPR activities based on DNA sequence information from unculturables will add enormously to our understanding of the mechanistic variation that exists in PGPR phenotypes. It will also benefit our ability to improve existing PGPR, by adding to the pool of exploitable PGPR genes and utilization of this pool to develop PGPRs with enhanced performance (Timms-Wilson et al. 2004).

4.3 Plant–Microbe–Microbe Interactions

Rhizosphere is rich in microbial activity which takes part in biological and ecological processes important for plant health. To develop efficient and reliable bioformulation, understanding of the role of microbes in the panoply of processes and interactions which take place in the rhizosphere is essential. When analyzed within the context of biocontrol, the translocation processes of PGPR bacteria seem to warrant more attention. Motility on surfaces is an important mechanism for bacterial colonization of new environments. Furthermore, the ability to move in a directional manner may confer distinct advantage upon host-adapted prokaryotes.

There are few investigations reporting that motility is essential for the initial steps of development of microbial biofilms, which are often basic condition of beneficial effects of PGPR (Kinsinger et al. 2003). Avoidance of antimicrobial compounds produced either by the host or by competitors inhabiting the same niche also seems to be important for maintaining this contact.

Once a beneficial microbial strain has been able to colonize a host plant, it might be able to display a wide array of activities contributing to plant fitness. Expression of bacterial traits involved in biological control of plant pathogens is tightly regulated and N-acyl-homoserine lactones (AHL) signal molecules play an intriguing role in this respect. These AHL molecules have recently also been implicated in the sensing of bacteria by animals, more specifically *Caenorhabditis elegans* (Beale et al. 2006). Thus, these molecules play a role in communication within and between bacterial populations, in communication between bacteria and plants and vice versa (Teplitski et al. 2000; Schuhegger et al. 2006), and between bacteria and nematodes. In an environment containing all these organisms, like the rhizosphere, studying these interactions and predicting their outcome undoubtedly constitutes an exciting challenge.

The ever-increasing availability of plant and bacterial genome sequences and the development of "omic" technologies permit genome-wide approaches to unveil either microbial or plant functioning in the rhizosphere. Indeed, much has been done to investigate the global gene expression or transcriptomes of various plants when confronted with pathogens, symbiotic nitrogen-fixing bacteria, PGPR, or environmental conditions. However, the gene expression of microbes in the rhizosphere is much less studied largely due to the difficulty to obtain sufficient material under controlled conditions in this otherwise highly variable and irregular niche. The report by Matilla et al. (2007) constitutes the first on bacterial genomics in the rhizosphere. Secondary metabolites are often synthesized by multimodular, multidomain proteins called nonribosomal peptide synthetases (NRPS), and polyketide synthases (PKS). Both NRPS and PKS systems are molecular assembly lines for successive linking of multipleamino/hydroxyl acids or acyl-CoA precursors, respectively, into complex polymers which are often further modified into unique structures. A novel "genomisotopic" approach uses a combination of genomic sequence analysis and isotope-guided fractionation to identify unknown compounds synthesized by NRPS gene clusters (Gross et al. 2007). A phage display method was developed for high-throughput mining of gene clusters encoding PKS and NRPS systems, which can be applied to genomes of unknown sequence and metagenomes (Yin et al. 2007), providing opportunities for exploiting the potentially rich source of natural products from unculturable microbes. The ever-increasing pace of microbial genome sequencing is revealing a plethora of new NRPS/PKS gene clusters, mostly of unknown function. A major challenge for the next decade is to back this up with characterization of the chemical structures and biological activities of these secondary metabolites, so that we can chart Nature's unique repertoire of natural products and exploit them for the directed synthesis of novel molecules of agricultural utility (Arora et al. 2007a). Future developments in functional genomics (including proteomics and metabolomics) will be useful to

identify the genes expressed in the rhizosphere, while the use of promoters to drive gene expression specifically at the root–soil interfaces will allow the engineering of microorganisms for beneficial purposes.

4.4 Formulation Design

Formulation is the crucial issue for inoculants containing an effective bacterial strain and can determine the success or failure of a biological agent. Since natural soil commonly represents a hostile environment to inoculant cells (Ho and Ko 1985), the use of inoculant formulations involving carrier materials for the delivery of microbial cells to soil or the rhizosphere is an attractive option. Carrier materials are generally intended to provide a (temporarily) protective niche to microbial inoculants in soil, either physically, via the provision of a protective surface or pore space (creating protective microhabitats), or nutritionally, via the provision of a specific substrate (Trevors et al. 1992). Peat and soil rich in organic matter are generally used in the preparation of legume inoculants and constitute a suitable carrier for the purpose. Peat and lignite, though good carriers, are not easily available and are expensive. The low cost and easily availability of carrier material are the major requirements for bioformulations in developing countries (Saha et al. 2001).

The microbial inoculant is not merely a suitable carrier containing the bacteria. Other materials might be involved in the final formulations. Evidence suggests that the addition of nutrients to seed pellets may be a useful strategy for improving inoculant survival (Moënne-Loccoz et al. 1999). Furthermore, carbon sources and minerals have been shown to have an important role in antifungal metabolite production by *Pseudomonas* BCAs, suggesting that nutrient amendments to formulations may also be a useful strategy for improving biocontrol efficacy (Duffy and Défago 1999). Soil amendment with chitin showed increase of the chitinolytic microbial populations and significantly reduced the incidence of fungal diseases in celery (Bell et al. 1998). Chitin supplementation supports the survival of *Bacillus cereus* and *B. circulans* in the groundnut phylloplane and resulted in better control of early and late leaf spot disease (Kishore et al. 2005). These improved disease control results are associated with an increase in the population of the introduced BCAs in presence of chitin.

Drying is a part of many procedures for development of formulation of microbial inoculants. The drying procedures are not very suitable for incorporation in a formulation protocol. However, Amiet-Charpentier et al. (1998) reported that it is possible to formulate nonsporulating bacteria using both freeze- and spray-drying. It was demonstrated that a methacrylic copolymer carrier, an ethyl-cellulose, and a modified starch product all increase survival of rhizosphere bacteria during spray-drying (Palmfeldt et al. 2003). Remarkably low percentage of endospore formers was observed that survived after drying (Validov et al. 2007). Designing of formulation that allow inoculant survival during drying procedure and support high

colony forming units of PGP agents on short storage in the grower's warehouse (which in developing countries usually lack refrigeration) was an important necessity for commercialization of the technology.

One factor which can have a detrimental effect on dried microorganisms over the long term is humidity in the environment; increasing moisture content of the dried sample compromises viability. Storage under vacuum or in an inert atmosphere can prevent this (Johnsson et al. 1998), but is costly and unwieldy. Manzanera et al. (2004) have shown how osmotic preconditioning of bacteria, followed by drying in the presence of glass-forming protectant molecules, such as trehalose or hydroxyectoine, results in a high level of desiccation tolerance, where viability is maintained throughout extended storage periods at above-ambient temperatures and its potential application as a seed coating. This has been termed anhydrobiotic engineering (Fages 1992), in reference to anhydrobiotic organisms which naturally exhibit extreme desiccation tolerance (Validov et al. 2009). Similar observations of García de Castro et al. (2000) demonstrate the potential of this novel biotechnology for stabilizing nonsporulating organisms. Storage of culture collections and libraries could be simplified using a plastic encapsulation procedure, for example, since there is no requirement for freezing or storage under vacuum or in an inert atmosphere.

5 Integrated Management

In the era of integrated use and management of various agro-inputs for maximization of crop yields, a comprehensive knowledge about the compatibility of various components to each other is very much required. Recommendations on combined use of such inputs, like treatment of seeds both with fungicides and biofertilizers, must accompany appropriate information on their compatibility to each other. Inhibitory effects have been observed on some nitrogen fixing microorganisms by insecticides (Sarkar and Balasubramanayam 1978) and seed dressing chemicals (Chitriv 1986). Knowledge of multiple microbial interactions is also of extreme value for development of bioformulations. The majority of interactions considered so far concern a single pathogen and a single BCA in the rhizosphere. However, one way of improving biocontrol in the rhizosphere may be to add combinations of BCAs, particularly those exhibit different or complementary mode of action or abilities to colonize root microsides. Application of a combination of *Paenibacillus* sp. and a *Streptomyces* sp. suppressed *Fusarium* wilt of cucumber than when either was used alone (Singh et al. 1999). The combination of *Pseudomonas aeruginosa* and *Pochonia chlamydospora* caused greater suppression of fungal phytopathogens and promoted plant growth compared with their individual application (Siddiqui and Shaukat 2002). Combinations of fungi and bacteria have also been shown to provide enhanced biocontrol (Duffy et al. 1996).

However, it is important when considering the use of combinations of strains that no member of the mixture is inhibitory to another or interferes excessively with

the existing, normal, and nonpathogenic microbiota associated with the roots. Various reports indicate that coinoculation of beneficial organisms generally increased plant growth and/or decreased plant disease relative to single inoculation with a sole beneficial organism (Whipps 2004; Raimam et al. 2007). Most of the effects of the individual microorganisms in coinoculation are additive, although a synergistic effect has been reported in some cases (Ravnskov et al. 2006; Kohler et al. 2007). However, neutral or negative effects have been reported (Akköprü and Demir 2005) indicating that the outcome of coinoculation of these microorganisms on plant health and productivity should be determined on a case-by-case basis. There is evidence to suggest that *Pseudomonas* BCAs can affect the growth and subsequent nodule occupancy of certain *Sinorhizobium meliloti* strains in gnotobiotic systems (Neimann et al. 1997). Within commercial scale field trials, however, a *Pseudomonas* BCA did not affect nodulation in the foliage of a red clover rotation crop (Moënne-Loccoz et al. 1998), again demonstrating the necessity of conducting impact analysis experiments within agronomically relevant parameters. Only when the symbiosis is well understood are we likely to be able to exploit it to provide optimum growth enhancement of the host and control of phytopathogens (Arora et al. 2008a).

6 Conclusion

Because of current public concerns about the side effects of agrochemicals, there is an increasing interest in improving the understanding of cooperative activities among rhizosphere microbial populations and how these might be applied to agriculture. Certain cooperative microbial activities can be exploited as a low-input biotechnology and form basis for a strategy to help sustainable, environmentally friendly practice fundamental to the stability and productivity of both agricultural systems and natural ecosystems (Kennedy and Smith 1995). Recent survey of both conventional and organic growers indicates an interest in using biological products (Rzewnicki 2000), suggesting that the market potential of bioformulations will increase in coming years. It is estimated that the total global market for synthetic pesticides which was valued at US$ 26.7 billion in 2005 will decline to US$ 25.3 billion in 2010. On the other hand, the global market for biopesticides will increase from US$ 672 million in 2005 to over US$ 1 billion in 2010 (Fig. 2). While Europe, at an average annual growth rate (AAGR) of 15%, is projected to lead the growth in biopesticide use, Asia will be no far behind with an average AAGR of 12%. The global market is divided into 43.5% of sales in North American Free trade Agreement countries (including Mexico), 20.7% in Europe, 12.2% in Asia, 11.2% in Oceania (including Australia), 8.3% in Latin America (excluding Mexico), and 3.9% in Africa (Bolckmans 2008). Furthermore, a detailed report about nitrogen-fixing bacteria as biofertilizers, for which the market is also growing, was published by Bhattacharjee et al. (2008).

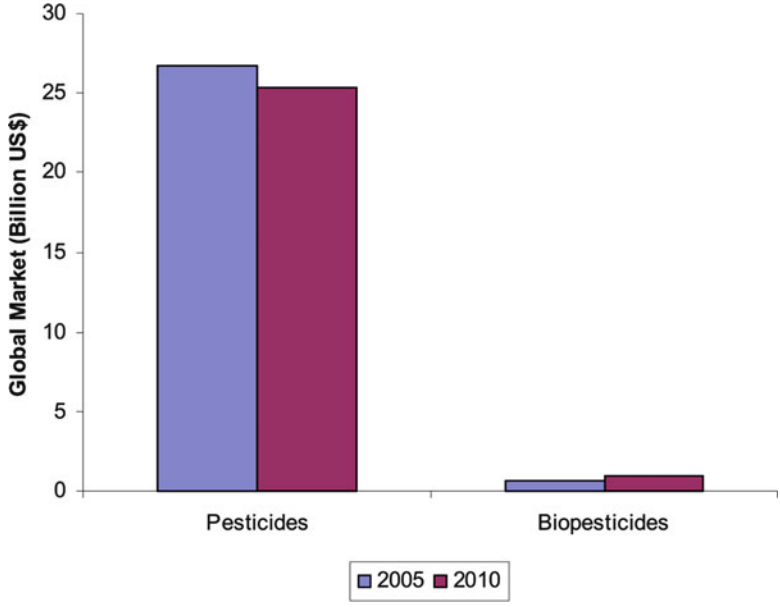

Fig. 2 Change in global market of synthetic pesticides and biopesticides in 5 years

The effects of soil on the physiology and ecology of introduced microorganisms are still poorly understood at the microscale (pore) level. Future research in this area should aim for a better understanding of the in situ physiology of inoculant cells, as well as for possible ways to manipulate it. Molecular techniques are being used in microbial ecology to understand the soil ecosystem, for the production of microbial inoculates, and for monitoring these inoculates after field release. These inoculants may or may not be genetically modified strains. Thus, future research in rhizosphere biology will rely on the development of molecular and biotechnological approaches to increase our knowledge of rhizosphere biology and to achieve an integrated management of soil microbial populations. Future investigation in the field of development of microbial formulation for plant growth promotion will include: (1) advances in visualization technology; (2) analysis of the molecular basis of root colonization; (3) signaling in the rhizosphere; (4) functional genomics; (5) mechanisms involved in beneficial cooperative microbial activities; (6) engineering of microorganisms for beneficial purposes; and (7) biotechnological developments for integrated management. A variety of research questions remain to be fully answered about the nature of bioformulations and the means to most effectively manage it under production conditions. As our understanding of the complex environment of the rhizosphere, of the mechanisms of action of PGPR, and of the practical aspects of inoculant formulation and delivery increases, we can expect to see new PGPR products becoming available.

On the applied side, and given the history of failures or variabilities of previous microbial releases, it is interesting to test the concept of application of mixtures of

ecologically diverse strains with similar functions instead of limited function of single strains. Such consortia might consist either of mixtures of completely natural strains or of different strains into which similar functions had been engineered. By this way, beneficial functions might be expressed more continually in a soil or rhizosphere system, even under ecologically different and/or variable conditions.

One of the major challenges for the inoculant industry is to develop an improved formulation that provides high shelf-life, high number of viable cells, protection against soil environment, convenience to use, and cost effective (Smith 1992). More studies on the practical aspects of mass-production and formulation need to be undertaken to make new bioformulations that are stable, effective, safer, and more cost-effective. There is an urgent need to develop a definite correlation between agriculturists, microbiologists, biotechnologists, industrialists, and farmers (Fig. 3).

Generally, inoculants are being used for legume crops and to a certain extent for cereal crops. Fresh alternatives should be explored for the use of bioinoculants for other high value crops such as vegetables, fruits, and flowers. This will not only

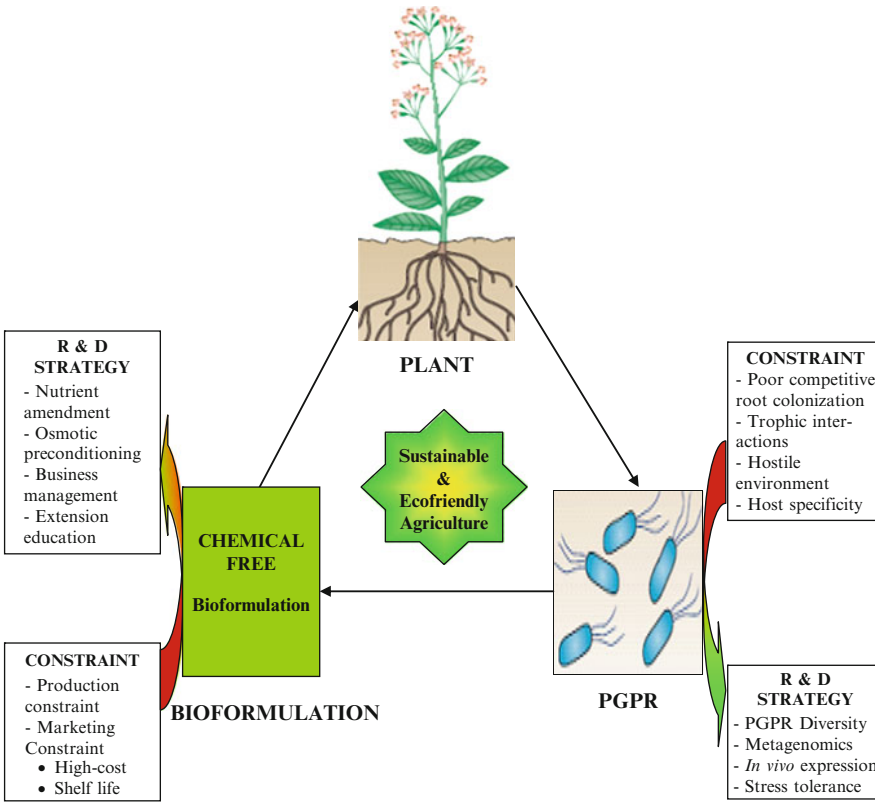

Fig. 3 Research and development strategies for commercialization of bioformulation technology

increase the field of the inoculants but also create confidence among the farmers for their use. As a recent approach, new environment friendly, genetically modified, microbial inoculates are being produced commercially and used to protect plants from disease and to promote plant growth. Numerous studies on technological evolution emphasized the developmental role of a firm and the strength of its sales network, creating market and drawing market feedback, for its success. In addition, future marketing of bioinoculant products and release of these transgenics into the environment as eco-friendly alternations to agrochemicals will depend on the generation of biosafety data required for the registration of PGP agents. Clearly, the future success of the bioformulation industry will depend on innovative business management, product marketing, quality product, extension education, and research.

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Antifungal Compounds of Plant Growth Promoting Rhizobacteria and Its Action Mode

C.S. Quan, X. Wang, and S.D. Fan

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Abstract Plant growth promoting rhizobacteria (PGPR) are bacteria that colonize plant roots and then promote plant growth and/or reduce disease or insect damage via exudation of some active metabolites. Antagonistic PGPR have attracted much attention in their role in reducing plant diseases, especially strains of the genus *Bacillus*, *Pseudomonas*, and *Burkholdeira*, and there is now an increasing number of PGPR being commercialized for crops. In this chapter, we present three major antagonistic PGPR (*Bacillus* spp., *Pseudomonas* spp., and *Burkholdeira* spp.) and their antifungal metabolites including the chemical structure first, and then introducing the mode of action and biosynthesis pathway of these antifungals.

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1 Introduction

Plant fungal diseases reduce yield and productivity of several economical crops all over the world. Resistant plant cultivars, cultural practices, and chemical applications are often used to control plant disease. However, resistant cultivars for every disease are not available and cultural practices are not always economically or technologically feasible. Moreover, available chemical fungicides are often expensive and also have bad effects on human beings. Environmentally friendly control of plant disease is a pressing need for agriculture in new century (Emmert and Handelsman 1999). Biological control using antibiotics and antifungal rhizobacteria to suppress plant diseases offers a powerful alternative to the use of synthetic chemicals.

There have been many studies regarding the use of antagonistic microbes as an alternative to synthetic chemical pesticides in biocontrol systems, because the latter has given rise to human and ecological risk. Many bacteria and fungi have been reported as antagonistic microbes against phytopathogenic fungi (Bonsall et al. 1999; Lee et al. 2001). Most of the interactions between antagonistic and phytopathogenic microbes have been summarized as deriving from the inhibition of the pathogen by antimicrobial materials (Raaijmakers et al. 2002), competition for nutrients (Mondal and Hyakumachi 2000), the inaction of pathogen germinating factors (Whipps 1997), and degradation of the pathogenicity factor (Steijl et al. 1999). The usage of antagonistic microorganisms with antifungal effects as biocontrol agents to inhibit or reduce the rate of propagation of deleterious fungi during storage is considered a safer and more environmentally friendly alternative. Biological control of plant pathogens is strongly based on the production of antifungal factors such as antibiotics, hydrolytic enzymes, and siderophores by the bacterial antagonists (Becker and Cook 1988; Howell and Stipanovic 1980; Keel et al. 1990; Thomashow and Weller 1988; Vincent et al. 1991).

Plant growth promoting rhizobacteria (PGPR) are free-living soil bacteria that can either directly or indirectly facilitate rooting and growth of plants. PGPR indirectly enhance plant growth via suppression of phytopathogens by a variety of mechanisms. These include the ability to produce siderophores that chelate iron, making it unavailable to pathogens; the ability to synthesize antifungal metabolites such as antibiotics, fungal cell wall-lysing enzymes, or production of volatiles such as hydrogen cyanide, which suppress the growth of fungal pathogens; the ability to successfully compete with pathogens for nutrients or specific niches on the root; and the ability to induce systemic resistance (ISR). Taxonomically, PGPR represent a variety of bacterial species from different genera such as *Pseudomonas*, *Bacillus*, *Burkholderia*, *Enterobacter*, and *Azospirillum* (Lodewyckx et al. 2002). Among PGPR bacteria, *Bacillus*, *Pseudomonas*, and *Burkholderia* have been intensively investigated as biological control agents with regard to the production of antimicrobial metabolites.

The purpose of this chapter is to provide an up-to-date overview of the current knowledge of the structural diversity and activity of antifungal compounds

produced by plant-associated PGPR, in particular the antagonistic *Pseudomonas* spp., *Bacillus* spp., and *Burkholderia* spp. A detailed description of structures, mechanism of action mode, and genes involved in the biosynthesis of antifungals is presented.

2 Antagonistic PGPR and Its Antifungal Metabolites

PGPR suppress various group of plant pathogens, thus protect the plants against different diseases. This protective effect is mainly due to production of antifungal metabolites produced by various species of *Bacilli, Pseudomonads*, and *Burkholderia* in particular.

2.1 Bacillus and Its Antifungal Metabolites

Spore-forming bacteria, typically *Bacillus* species, are one of the major types of soil bacteria. *Bacillus* species offer several advantages for protection against root pathogens because of their ability to form endospores and the broad-spectrum activity of their antibiotics. *Bacillus* species produce 167 biological compounds active against bacteria, fungi, protozoa, and viruses (Bottone and Peluso 2003).

The first successful application and commercial production of PGPR is a *B. subtilis* strain A13. *B. subtilis* A13 was isolated more than 25 years ago in Australia based on in vitro inhibitory activity to all of nine pathogens tested and was subsequently shown to promote plant growth. Since 1990, *Bacillus* spp. have been developed as fungal disease control agents. Strains of *B. megaterium*, *B. cereus*, and *B. subtilis* have been used for the biocontrol purpose (Idris et al. 2008; Kildea et al. 2008), in the form of the commercial product namely, Serenade, EcoGuard, Kodiak, Yield Shield, and BioYield.

Bacteria of the genus *Bacillus* are capable of producing antibiotics, as well as a variety of fungal cell-wall-degrading enzymes, such as chitinase, cellulases, amylases, glucanses, etc. Most of the antibiotics are peptides effective against Gram-positive, Gram-negative bacterial species, and filamentous fungi, and also with a high stability attributable to their structure. Several antifungal peptides synthesized by *Bacillus* species are active against filamentous fungi and yeasts. According to structural features of peptides, it can be divided into cyclic lipopeptide (CLP), phosphono-oligopeptide, and dipeptide. Many *Bacillus* strains produce small circular peptides (such as Iturin, Fengycin, Bacillopeptins, and Surfactin) with a long fatty acids moiety. They are composed of seven (surfactins and iturins) or ten α-amino acids (fengycins) linked to one unique β-amino (iturins) or β-hydroxy (surfactins and fengycins) fatty acid. The length of this fatty acid chain may vary from C-13 to C-16 for surfactins, from C-14 to C-18 in the case of fengycins (Ongena and Jacques 2007). Iturin and fengycins display a strong antifungal

activity and are inhibitory for the growth of a wide range of phytopathogens (Hsieh et al. 2008; Vanittanakoma and Loeffler 1986). Bacilysocin produced by *B. subtilis* has a phospholipid structure, and it may be derived from phosphatidyl glycerol through acyl ester hydrolysis. Phosphatidyl glycerol is the major component of phospholipids in *B. subtilis*, which constitutes about 75% of the total phospholipids (Tamehiro et al. 2002). Bacilysin is a nonribosomally synthesized dipeptide antibiotic that is active against a wide range of bacteria and some fungi (Rajavel et al. 2009).

Production of polyketide-like compounds with antimicrobial activity by wild-type isolates of *Bacillus* spp. has been described previously (Hofemeister et al. 2004). The polyene antibiotics, difficidin and oxydifficidin, are highly unsaturated 22-member macrolides with a rare phosphate group (Wilson et al. 1987). Another antibiotic, bacillaene, is a linear molecule with two amide bonds: the first links an α-hydroxy carboxylic acid to a β-amino carboxylic acid containing a conjugated hexaene, and the second links the hexaene-containing carboxylic acid to an (ω-1) amino carboxylic acid containing a conjugated triene.

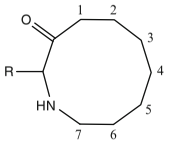
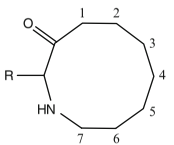
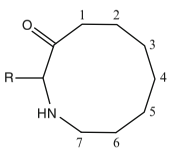
Numerous cell-wall-degrading enzymes, especially chitinase, have been isolated from *Bacillus* species. Many strains of *Bacillus* can produce a high level of chitinolytic enzymes (Xiao et al. 2009; Huang et al. 2005). Moreover, many researches have shown that chitinase is involved in antifungal activity and can enhance the insecticidal activity of *Bacillius* sp. (Table 1).

2.2 *Pseudomonas and Its Antifungal Metabolites*

Pseudomonas species are ubiquitous inhabitants of soil, water, and plant surfaces that belong to the Gamma-subclass of Proteobacteria. Many pseudomonades live in a commensal relationship with plants, utilizing nutrients exuded from plant surfaces and surviving environmental stress by occupying protected sites provided by the plant's architecture. Bacteria of *Pseudomonas* genus are the most popular PGPR and some species were also used in practice for biocontrol of *Gaeumannomyces graminis* var *tritici*, *Rhizoctonia solani*, *Erwinia carotovora* var. *carotovora*, *Pythium ultimum*, and *Fusarium oxysporum*. The mechanism suggested for achieving such inhibition includes: production of antibiotics, iron chelating compounds, hydrolytic enzymes, and biosurfactants; competition for favorable nutritional sites; and ISR and even due to their action as mycorrhization-helper bacteria (MHB).

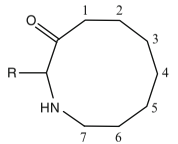
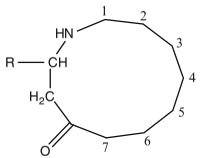
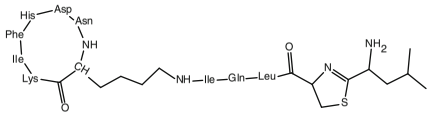
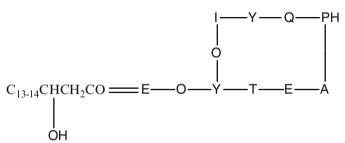
Pseudomonads have an exceptional capacity to produce a wide variety of metabolites, including antibiotics (Pyrrolnitrin, Pyoluteorin (Plt), Phenazine compounds) and chitinase that are toxic to plant pathogens. Antibiotic production by *Pseudomonas* spp. enhances the fitness of the producing strain and suppresses pathogens that would otherwise disserve plant health. Certain antibiotic-producing *Pseudomonas* spp. have received great attention in the world as biological control agents to enhance agricultural productivity.

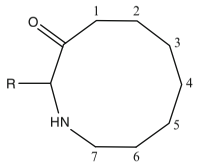
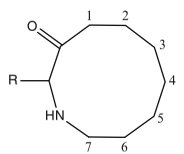
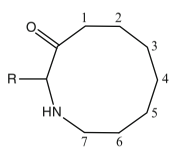
Table 1 Antifungal metabolites produced by *Bacillus* spp.

Antifungals	Type	Source	Structure	Pathogen	Reference
Bacillomycin D, F, L, Lc	Cyclic lipopetide	*B. subtilis* *B. amyloliquefaciens*	 1: L-Asn; 2: D-Tyr; 3: D-Asn; 4: L-Pro; 5: L-Glu; 6: D-Ser; 7: L-Thr R: n-C_{14}, i-C_{15}, ai-C_{15} bacillomycin D	*Fusarium graminearum*, *Alternaria alternata*, *Rhizoctonia solani*, *Cryphonectria parasitica*, *Phytophthora capsici*	Eshita and Roberto (1995) Zhao et al. (2010) Ongena and Jacques (2007)
			 1: L-Asn; 2: D-Tyr; 3: D-Asn; 4: L-Gln; 5: L-Pro; 6: D-Asn; 7: L-Thr R: i-C_{16}, i-C_{17}, ai-C_{17} bacillomycin F		
			 1: L-Asp; 2: D-Tyr; 3: D-Asn; 4: L-Ser; 5: L-Gln; 6: D-Ser; 7: L-Thr R: n-C_{14}, i-C_{15}, ai-C_{15} bacillomycin L		

(*continued*)

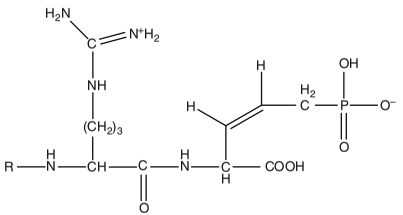
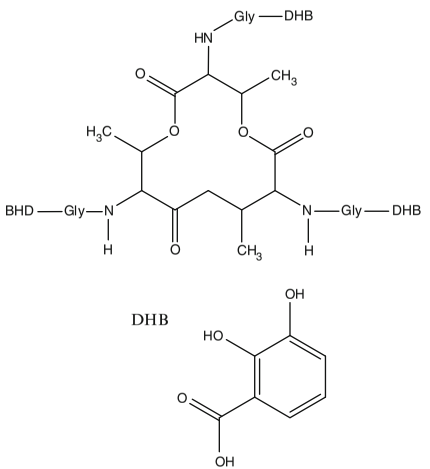
Table 1 (continued)

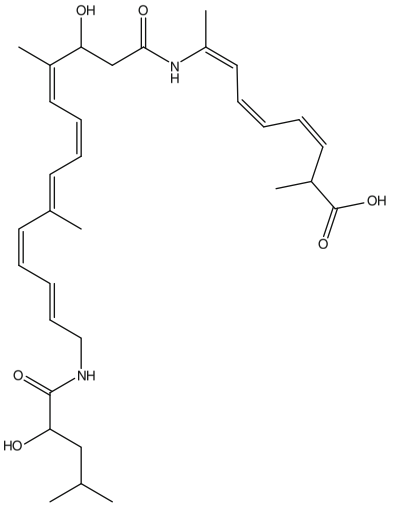
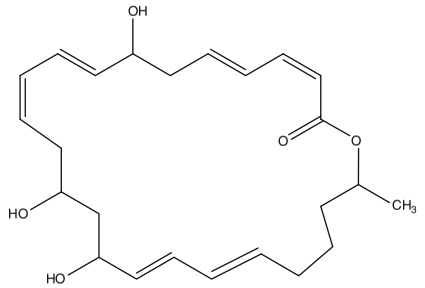
Antifungals	Type	Source	Structure	Pathogen	Reference
			 1: L-Asn; 2: D-Tyr; 3: D-Asn; 4: L-Ser; 5: L-Glu; 6: D-Ser; 7: L-Thr R: n-C_{14}, i-C_{15}, ai-C_{15}, i-C_{16} bacillomycin C		
Bacillopeptins A, B, C	Cyclic lipopetide	*B. subtilis*	 1: L-Thr; 2: D-Ser; 3: L-Glu; 4: L-Ser; 5: D-Asn2; 6: D-Tyr; 7: L-Asn1 R A: $CH_3(CH_2)_9CH_2$ B: $CH_3CH(CH_3)(CH_2)_8CH_2$ C: $CH_3CH(CH_3)(CH_2)_9CH_2$	With broad spectrum antibacterial activity	Kajimura et al. (1995)
Bacitracin	Cyclic lipopetide	*B. subtilis* *B. licheniformis*		*Streptococcus aureus*, *S. faecalis*	Azevedo et al. (1993) Haavik and Froyshov (1975)
Fengycin	Cyclic lipopettide	*B. thuringiensis* *B. subtilis* *B. amyloliquefaciens*		*C. gloeosporioides*, *F. oxysporum*, *F. solani*, *R. solani*, *B. cinerea*, *P. capsici*, *Sclerotium cepivorum*, *Colletotrichum*	Kim et al. (2004) Wang et al. (2007) Vanittanakoma and Loeffler (1986)

				coccodes, *Trichoderma harzianum*	
Iturin(A–E)	Cyclic lipopetide	*B. subtilis* *B. amyloliquefaciens* *B. circulan*	 1: L-Asn; 2: D-Tyr; 3: D-Asn; 4: L-Gln; 5: L-Pro; 6: D-Asn; 7: L-Ser R: n-C_{14}, i-C_{15}, ai-C_{15} Iturin A	*Mycobacterium smegmatis*, *Agrobacterium tumefaciens*, *Penicillium chrysogenum*, *P. notatum*, *R. solani*, *B. cinerea*	Peypoux et al. (1978) Besson et al. (1984a, b) Hsieh et al. (2008)
Mycosubtilin	Cyclic lipopetide	*B. subtilis*	 1: L-Asn; 2: D-Tyr; 3: D-Asn; 4: L-Gln; 5: L-Pro; 6: D-Ser; 7: L-Asn R: n-C_{16}, i-C_{15}, ai-C_{17}	*Candida* spp., *Saccharomyces cerevisiae*, *Pythium aphanidermatum*	Duitman et al. (1999)
Mycobacillin	Cyclic peptide	*B. subtilis*	 1: L-Asn; 2: D-Tyr; 3: D-Asn; 4: L-Gln; 5: L-Pro; 6: L-Ser; 7: D-Asn; R: n-C_{14}, i-C_{15}, ai-C_{15}	*C. albicans*, *Aspergillus niger*	Majumder et al. (1988)

(*continued*)

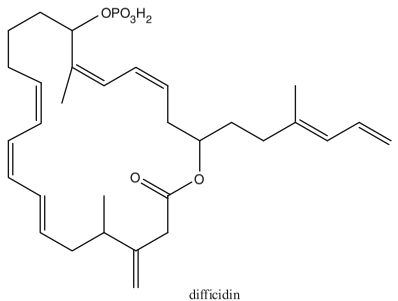
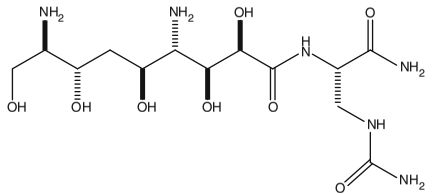
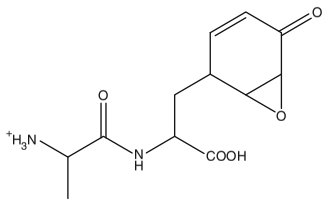
Table 1 (continued)

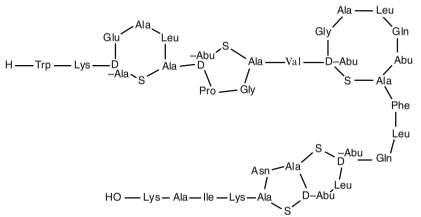
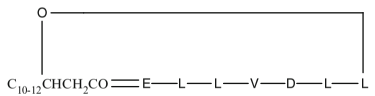
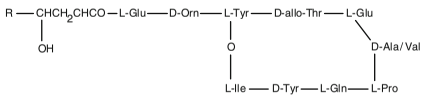
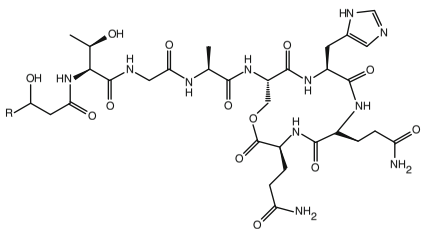
Antifungals	Type	Source	Structure	Pathogen	Reference
Rhizocticins	Phosphono-oligopeptides	*B. subtilis*		With broad spectrum antifungal activity	Rapp et al. (1988)
Alboleutin	Peptide	*B. subtilis* AF-8	Unknown	Unknown	Omura et al. (1980)
Bacillibactin	Iron-siderophore	*B. amyloliquefaciens*		With broad spectrum antibacterial activity	Chen et al. (2009)

Bacillaene	Polyketides	*B. subtilis* *B. amyloliquefaciens*		*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Serratia marcescens*, *B. thuringiensis*, *S. aureus*	Chen et al. (2009)
Macrolactin	Polyketides	Marine *Bacillus* strains		*S. aureus*, murine melanoma cancer cells, *Herpes simplex* viruses	Schneider et al. (2007)

(*continued*)

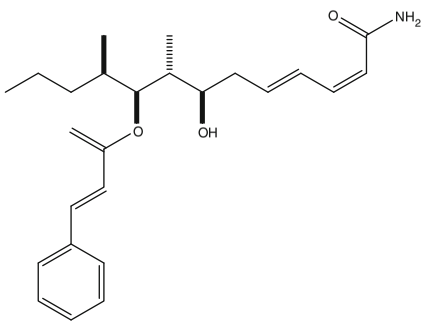
Table 1 (continued)

Antifungals	Type	Source	Structure	Pathogen	Reference
Difficidin (oxydifficidin)	Polyketides	*B. subtilis* *B. amyloliquefaciens*	 difficidin	With broad spectrum antibacterial activity	Wilson et al. (1987)
Zwittermicin A	Aminopolyol	*B. thuringiensis* *B. cereus*		*Phytophthora medicaginis*, *P. aphanidermatum*	Nair et al. (2004) Silo-Suh et al. (1998)
Bacilysin	Dipeptide	*B. subtilis* *B. amyloliquefaciens*		*E. coli* *Absidia* sp.	Phister et al. (2004) Ozcengiz and Alaeddinoglu (1991)

Subtilin	Peptide	*B. subtilis*		*Micrococcus luteus*, *Lactococcus lactis*	Chan et al. (1993)
Surfactin	Biosurfactant	*B. subtilis* *B. polyfermenticus* *B. amyloliquefaciens*		With broad spectrum antibacterial activity	Nakano et al. (1988) Kim et al. (2009)
Plipastatin	Lipopeptide	*B. subtilis* *B. cereus*		*A. mali*, *B. cinerea*, *Pyricularia oryzae*	Tsuge et al. (2007)
Kurstakins	Lipopeptide	*B. thuringiensis*		*Stachybotrys charatum*	Hathout et al. (2000)
Fungicin M-4	Peptide	*B. licheniformis*	Unknown	*Microsporum canis*, *Mucor mucedo*, *M. plumbeus*, *Sporothrix schenckii*	Lebbadi et al. (1994)

(*continued*)

Table 1 (continued)

Antifungals	Type	Source	Structure	Pathogen	Reference
Bacisubin	Protein	*B. subtilis*		*Magnaporthe grisease*, *Sclerotinia sclerotiorum*, *R. solani*, *A. oleracea*, *A. brassicae*, *B. cinerea*	Liu et al. (2007)
YM-47522		*Bacillus* sp. YL-03709B		*Rhodotorula species*, *Pichia angusta*, *C. albicans*, *C. tropicalis*	Shibazaki et al. (1996), Sugawara et al. (1996)
Chitinase		*B. licheniformis* *B. subtilis* *B. cereus*		*Gibberella saubinetii*, *A. niger*	Xiao et al. (2009) Yang et al. (2009) Chang et al. (2007)
Cellulases, Amylases, Glucanases	Fungal cell-wall-degrading enzymes	*B. subtilis* *Bacillus* sp.		With broad spectrum antifungal activity	Fukumori et al. (1986) Roncero 1983
Others	Protein or peptide	*Bacillus* sp.		*Geotrichum candidum*, *Bipolaris maydis*, *A. brassicae*, *A. niger*, *Cercospora personata*	Maldonado et al. (2009) Zhang et al. (2008)

Pyrrolnitrin (Prn, 3-chloro-4-(29-nitro-39-chlorophenyl)-pyrrole) is a secondary metabolite derived from tryptophan and has strong antifungal activity. Most of *Pseudomonas* and *Burkholderia* strains produce this antibiotic. Production of Prn has been correlated with the ability of some bacteria to control fungal plant pathogens and diseases, including the damping-off pathogen, *R. solani*. Prn and its production by *Pseudomonas* species was first described by Arima et al. (1964). This highly active metabolite has been used as a clinical antifungal agent for the treatment of skin mycoses, and a phenylpyrrole derivative of Prn has been developed as an agricultural fungicide (Tawara et al. 1989).

Pyoluteorin (Plt) is an antibiotic that inhibits *P. ultimum* and suppresses plant diseases. Plt is composed of a resorcinol ring, derived through polyketide biosynthesis, which is linked to a bichlorinated pyrrole moiety whose biosynthesis remains uncharacterized. The production of the antifungal metabolite 2,4-diacetylphloroglucinol (2,4-DAPG) by many fluorescent *Pseudomonas* spp. has been seen to play a major role in the biocontrol of a range of plant pathogens, including *P. ultimum*, *G. graminis* var. *tritici*, and *Thielaviopsis bassicola* (Keel et al. 1990; Vincent et al. 1991; Fenton et al. 1992; Levy et al. 1992).

Kim (2003) reported that *P. aeruginosa* excrete two different types of siderophores (pyoverdine and pyochelin) at low iron concentration. When free iron concentration in environment reached to 10^{-17} mol/L, binding ability of pyochelin with iron ions is 1.5×10^{-7} mol/L, whereas binding ability of pyoverdine is higher than 10^{-20} mol/L (Chen et al. 2008).

Phenazines (Phz) are N-containing heterocyclic pigments. Currently, over 50 naturally occurring Phz compounds have been described and mixtures of as many as ten different Phz derivatives can occur simultaneously in one organism. Growth conditions determine the number and type of Phz synthesized by an individual bacterial strain. For example, *P. fluorescens* 2–79 produces mainly phenazine 1-carboxylic acid (PCA), whereas *P. aureofaciens* 30–38 not only produces PCA but also lesser amounts of 2-OH-phenazines (Dwivedi and Johri 2003).

Antifungal proteins, such as chitinase, are key components of defense and offense mechanisms of many groups of fungi and bacteria. These microbial lytic enzymes are being exploited widely for crop disease management. These enzymes hydrolyze the chitin present in the fungal cell wall, leading to lysis. Production of these lytic enzymes is considered to be the major antagonistic activity of fluorescent pseudomonads belonging to PGPR.

Plant-associated *Pseudomonas* spp. also produces diversified CLPs with potential antimicrobial, cytotoxic, and surfactant properties. Based on the length and composition of the fatty acid tail as well as the number, type, and configuration of the amino acids in the peptide moiety, CLPs of *Pseudomonas* spp. were classified into four major groups (i.e., the viscosin, amphisin, tolaasin, and syringomycin groups). In brief, CLPs of the viscosin group are composed with nine amino acids linked at the N terminus to, in most cases, 3-hydroxy decanoic acid (3-HDA). CLPs in the amphisin group consist of 11 amino acids in the peptide part coupled to 3-HDA. For several members of this group, including amphisin and tensin, the crystal structure has been resolved (Henriksen et al. 2000; Sorensen et al. 2001). For

both tensin and amphisin, the structures are mainly helical, with the cyclic peptide wrapping around a water molecule. Compared with the viscosin and amphisin groups, CLPs in the tolaasin group are much more diverse due to multiple variations in both the composition and length of the peptide chain (19–25 amino acids) and the lipid tail (3-HDA or 3-hydroxyoctanoic acid). In terms of shear numbers of amino acids in the peptide moiety, CLPs in the syringomycin group are structurally similar to the CLPs in the viscosin group. However, macrolides, polyenes, quinone-type antibiotics, and hydroxyphenol have not been found so far among the secondary metabolites produced by the *Pseudomonas* (Table 2).

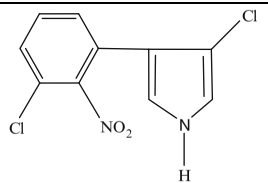
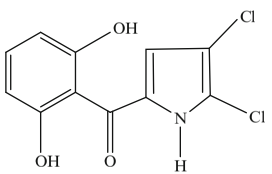
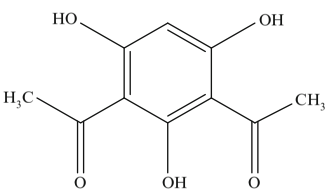
2.3 *Burkholderia* and Its Antifungal Metabolites

The genus *Burkholderia* comprises more than 40 different species, which inhabit a wide array of ecological niches. Among others, they occur in soil and water, in the plant rhizosphere and endophytically in roots and shoots, but also in and on fungal mycelia. *Burkholderia* species are also well known for their biological and metabolic properties, which can be exploited for biological control of fungal diseases but also for bioremediation and plant-growth promotion. *Burkholderia* spp. can antagonize and repress many soil-borne plant pathogens. Particularly, *B. cepacia* complex (Bcc) is known to be a ubiquitous inhabitant in soil, which has been used as an effective biocontrol agent for *Pythium*-induced damping-off, *Aphanomyces*-induced root rot of pea, and *R. solani*-induced root rot of poinsettia. Bcc is a group of genetically distinct but phenotypically similar bacteria that are divided into at least nine species. The effectiveness of Bcc isolates as biocontrol and PGP agents is based on a wide array of beneficial properties including the production of indole-acetic acid (IAA), the ability to fix atmospheric nitrogen, and the production of a wide array of compounds with antimicrobial activity, including cepacin, cepaciamide, cepacidines, altericidins, pyrrolnitrin, quinolones, phenazine, siderophores, and a lipopeptide. In the early 1990s, these useful properties led to the registration of four Bcc strains for use as biopesticides by the EPA (the U.S. Environmental Protection Agency), three of which were later classified as *B. ambifaria* and one as *B. cenocepacia*.

Cepaciamides A and B are fungitoxic 3-amino-2-piperidinone-containing lipids effective against *Botrytis cinerea* and *Penicillium expansum*, which cause the storage rot of beet roots, and are considered to be a promising biocontrol agent (Toshima et al. 1999). A peptide antibiotic complex, altericidins (altericidin a, B, and C), was isolated from the culture broth of *P. cepacia* KB-1 (Kirinuki et al. 1984). The alteridins inhibit the growth of a wide range of fungi and yeasts, but show no effect on the growth of bacteria species.

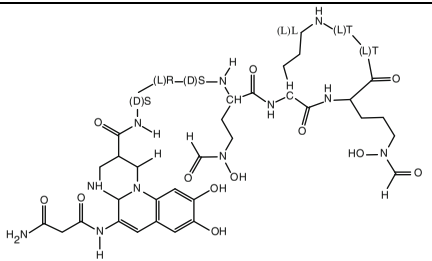
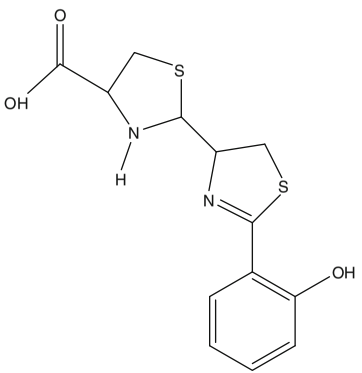
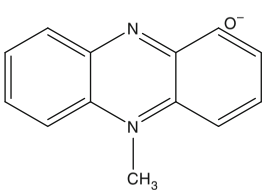
Cepacidine A is a novel glycopeptides with a potent antifungal antibiotic produced by *P. cepacia* AF 2001 (Lee et al. 1994). Cepacidine A exhibited a broad antifungal spectrum against various animal and plant pathogenic fungi. In particular, cepacidine A was highly active against dermatophytes, namely

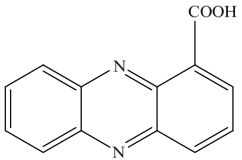
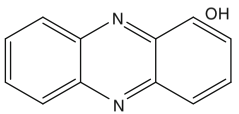
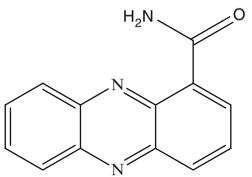
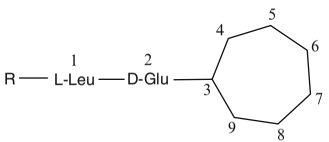
Table 2 Antifungal metabolites produced by *Pseudomonas* spp.

Antifungals	Type	Source	Structure	Pathogen	Reference
Pyrrolnitrin	N-containing heterocycles	*P. fluorescens* *P. cepacia*		*Fusarium* sp., *Pythium ultimum*, *R. solani*, *Alternaria* spp., *Pyricularia oryzae*, *Botrytis cinerea*, *Penicillium expansum*, *Verticillium dahiae*	Loper and Gross (2007) Hammer et al. (1997) Ligon et al. (2000)
Pyoluteorin	N-containing heterocycles	*P. fluorescens*		*P. ultimum*, *R. solani*	Howell and Stipanovic (1980) Maurhofer et al. (1995)
2,4-diacetylphloro-glucinol	Polyketide	*P. fluorescens*		*Gaeumannomyces graminis*, *P. ultimum*	Bangera and Thomashow (1999) Keel et al. (1996)

(*continued*)

Table 2 (continued)

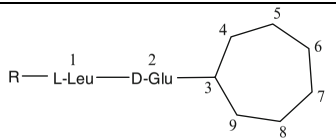
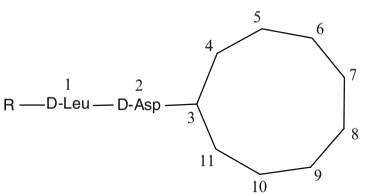
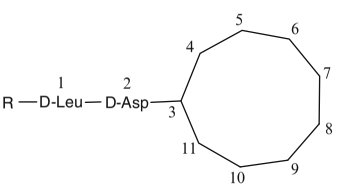
Antifungals	Type	Source	Structure	Pathogen	Reference
Pyoverdine	Siderophore	*P. fluorescens* *P. aeruginosa* *P. putida*		*F. graminearum*	Lamont and Martin (2003)
Pyochelin	Siderophore	*P. fluorescens*		*Candida* sp., *Aspergillus* sp.	Cox et al. (1981)
Pyocyanin	Phenazine compounds	*P. aeruginosa*		*C. albicans*, *A. fumigatus*	Budzikiewicz (1993)

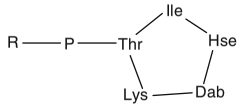
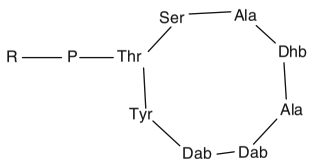
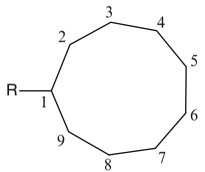
Phenazine-1-carboxylic acid	Phenazine compounds	*P. aeruginosa* *P. chlororaphis* *P. fluorescens*		*Caenorhabditis elegans*, *Gaeumannomyces graminis*	Denning et al. (2003) Liu et al. (2008)
1-hydroxyphenazine	Phenazine compounds	*P. aeruginosa*		*C. albicans*, *A. fumigatus*	Loper and Gross (2007)
Phenazine-1-carboxamide	Phenazine compounds	*P. aeruginosa* *P. Chlororaphis*		*F. oxysporum*	Loper and Gross (2007) Woeng et al. (1998)
Hydrogen cyanide Chitinase Viscosin	Cyclic lipopeptide	*P. fluorescens* *P. pseudomonuds* *P. fluorescens*		*P. ultimum* *F. oxysporum* *F. culmorum*	Ramette et al. (2003) Ajit et al. (2006) Braun et al. (2001)

R: C_{10}HO acid; 3: D-aThr; 4: D-Val; 5: L-Leu; 6: D-Ser; 7: L-Leu; 8: D-Ser; 9: L-Ile

(*continued*)

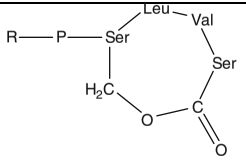
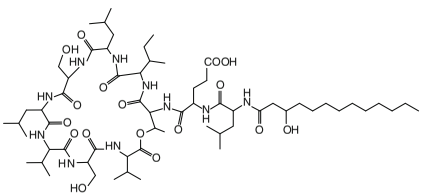
Table 2 (continued)

Antifungals	Type	Source	Structure	Pathogen	Reference
Massetolide (A, D)	Cyclic lipopeptide	*P. fluorescens*	 R: C_{10}HO acid; 3: D-aThr; 4: D-aIle; 5: L-Leu; 6: D-Ser; 7: L-Leu; 8: D-Ser; 9: L-Ile(A)/ Leu(D)	*Mycobacterium tuberculosis*, *M. aviumintracellulare*	De Souza et al. (2003)
Amphisin	Cyclic lipopeptide	*Pseudomonas* sp.	 R: C_{10}HO acid; 3: D-aThr; 4: D-Leu; 5: D-Leu: 6: D-Ser; 7: L-Leu; 8: D-Gln; 9: L-Leu; 10: L-Ile; 11: L-Asp	*P. ultimum*, *R. solani*	Koch et al. (2002)
Arthrofactin	Cyclic lipopeptide	*Pseudomonas* sp.	 R: C_{10}HO acid; 3: D-Thr; 4: D-Leu; 5: D-Leu; 6: D-Ser; 7 : L-Leu; 8: D-Ser; 9: L-Ile; 10: L-Ile; 11: L-Asp	With broad spectrum antifungal activity	Roongsawang et al. (2003)

Tolaasin	Lipopeptide	*Pseudomonas* sp. *P. tolaasii*	 R: C_8HO acid P: Dhb-Pro-Ser-Leu-Val-Ser-Leu-Val-Val-Gln-$(Leu)_5$-Val-Dhb	With broad spectrum antifungal activity	Rainey et al. (1993) Godfrey et al. (2001)
Syringopeptin	Lipopeptide	*P. syringae* pv. *syringae*	 R: $CH_3(CH_2)_8CHOHCH_2CO$ P: Dhb-Pro-Val-Val-Ala-Ala-Val-Val-Dhb-Ala-Val-Ala-Ala-Dhb	With broad spectrum antifungal activity	Feil et al. (2005) Scholz-Schroeder et al. (2003) Kang and Gross (2005)
Syringomycin	Cyclic Lipopeptide	*P. syringae* pv. *syringae*	 R: 3-hydroxy fatty acyl; 1: L-Ser; 2: D-Ser; 3: D-Dab; 4: L-Dab; 5: L-Arg; 6: L-Phe; 7: Z-Dhb; 8: L-Asp(3-OH); 9: L-Thr(4-Cl)	With broad spectrum antifungal activity	Kang and Gross (2005) Vaillancourt et al. (2005)

(*continued*)

Table 2 (continued)

Antifungals	Type	Source	Structure	Pathogen	Reference
Putisolvin	Cyclic Lipopeptide	*P. putida*	 R: $CH_3(CH_2)_4CO$ P: Leu-Glu-Leu-Ile-Gln-Ser-Val-Ile-Ser	*Candida* sp.	Kuiper et al. (2004)
Orfamide A	Cyclic Lipopeptide	*P. fluorescens*		Unknown	Loper and Gross (2007)

Microsporum canis, *Trichophyton* spp., and *Epidermophyton* spp., and true yeast at concentrations lower than 0.049 μg/ml. The activities of cepacidine A were greater than those of amphotericin B in almost all strains. However, no antibacterial activity against *B. subtilis*, *E. coli*, *Staphylococcus aureus*, and *P. aeruginosa* was detected (MIC $>$ 100 μg/ml).

Two acetylenic antibiotics, cepacins A and B, have been isolated from the fermentation broth of *P. cepacia* SC 11,783. Cepacin A has good activity against *Staphylococci* (MIC 0.2 mg/ml) but weak activity against *Streptococci* (MIC 50 mg/ml) and the majority of Gram-negative organisms. Cepacin B has excellent activity against *Staphylococci* (MIC less than 0.05 mg/ml) and some Gram-negative organisms (Parker et al. 1984). Similarly, Glidobactins are acylated tripeptide derivatives that contain a 12-membered ring structure consisting of the two unique nonproteinogenic amino acids, *erythro*-4-hydroxy-l-lysine and 4(*S*)-amino-2(*E*)-pentenoic acid (Schellenberg et al. 2007). The antibiotic 2-Hydroxymethyl-chroman-4-one isolated from *Burkholderia* sp. MSSP exhibited good activities against phytopathogens such as *P. ultimum*, *Phytophthora capsici*, and *S. sclerotiorum*. 2-Hydroxymethyl-chroman-4-one was used to mediate for synthesis of benzopyranones (Kang et al. 2004).

Most antibiotics isolated from *Burkholderia* culture filtrates, namely, Prn, Phz, Plt, and indole derivatives, belong to the class of N-containing heterocycles and have been shown to originate from intermediates or end products of the aromatic amino acid biosynthetic pathways. Prn is a chlorinated phenylpyrrole antibiotic that was first isolated from *B. pyrrocinia* (Arima et al. 1964) and later from other microorganisms, including *P. fluorescens*, *P. chlororaphis*, *P.aureofaciens*, *B.cepacia*, *Enterobacter agglomerans*, *Myxococcus fulvus*, and *Serratia species*. Prn has activity against several bacteria and soil-borne fungi, in particular *R. solani*. Prn is also effective against postharvest diseases caused by *B. cinerea* on apple, pear, and on cut flowers and has been used to treat humans infected by opportunistic fungi. Plt is a phenolic polyketide that was first isolated from *P. aeruginosa* and later from *P. aeruginosa* strain S10B2 and *P. fluorescens* strains Pf-5 and CHA0 (Takeda 1958). Plt has bactericidal, herbicidal, and fungicidal activities, in particular against *Pythium* spp. Application of pure Plt to cotton seeds resulted in significant suppression of *P. ultimum*-induced damping-off (Howell and Stipanovic 1980).

B. contaminans strain MS14 isolated from disease-suppressive soil produced a cyclic glycopeptide antibiotic, Occidiofungin. This compound inhibited the growth of a broad range of fungal pathogens, and the high-resolution mass spectrometry data revealed the existence of two structural variants of this antifungal peptide (Lu et al. 2009).

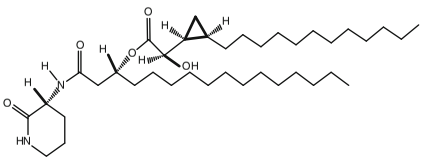
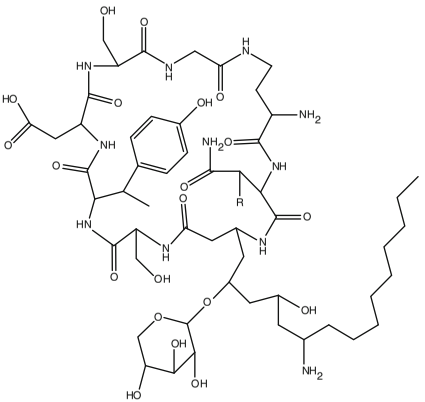
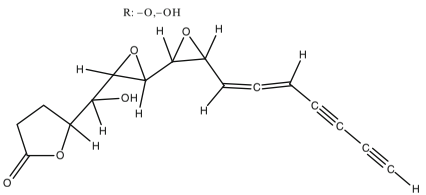
Many 4-quinolone compounds have antifungal activity. 4-Hydroxy-2-alkylquinolines (HAQs) have been long known as a class of antimicrobials produced by the opportunistic bacterial pathogen such as *P. aeruginosa* and *B. cepacia*. Many HAQs also act as iron chelators and even immune modulants. HAQs especially 3,4-dihydroxy-2-heptylquinoline (*Pseudomonas* quinolone signal) and its precursor, 4-hydroxy-2-heptylquinoline, have recently attracted much attention because of their role as intercellular signaling molecules in bacteria (Vial et al. 2008).

Members of the Bcc produce up to four different siderophores (ornibactin, pyochelin, cepabactin, and cepaciachelin). The siderophores produced by the Bcc contain most of the bidentate ferric iron-chelating groups commonly present in bacterial siderophores and includes catechols (present in cepaciachelin), linear hydroxamate and ahydroxycarboxylate groups (both present in ornibactin), a cyclic hydroxamate (hydroxypyridonate) moiety (as in cepabactin), and 2-hydroxyphenyl-thiazoline/-oxazoline and thiazolidine-carboxylate moieties. Pyochelin, 2-(2-o-hydroxyphenyl-2-thiazolin-4-yl)-3-methylthiazolidine-4-carboxylic acid, is derived from the condensation of salicylic acid with two molecules of cysteine, each of which undergoes cyclisation to thiazoline and thiazolidine ring derivatives following their incorporation into the molecule. Natural pyochelin is present as two spontaneously interconvertible stereoisomers, pyochelins I and II, due to isomerisation at the C-2″ position of the thiazolidine ring. Ornibactin, L-Orn1(N5-OH, N5-acyl)-D-threo-Asp(b-OH)-L-Ser-L-Orn4 (N5-OH, N5-formyl)-1,4-diaminobutane, is a linear tetrapeptide derivative that chelates iron by providing three bidentate metal chelation groups. These groups (two hydroxamates and an ahydroxycarboxylate) are generated by modification of the sidechains of three of the amino acids in the peptide (the N- and C-terminal ornithines, and the D-aspartate), with the serine residue serving only as a spacer. Cepabactin, 1-hydroxy-5-methoxy-6-methyl-2(1H)-pyridinone, is a cyclic hydroxamate (i.e., a hydroxypyridonate) and for that reason can also be considered as a heterocyclic catecholate. It was first identified as a metal-binding antibiotic that is secreted into the culture medium by *P. alcaligenes strain* NCIB 11492 and was termed G1549. Cepaciachelin, 1-N-[2-N′,6-N″-di(2,3-dihydroxybenzoyl)-L-lysyl]-1,4-diaminobutane, is a catecholate siderophore first isolated from the culture supernatant of *B. ambifaria* strain PHP7 (LMG 11351), a rhizosphere isolate, grown under iron-limiting conditions. It is comprised of a single molecule of lysine derivatised with 2,3-dihydroxybenzoic acid (DHBA) on the a and e amino groups, and with diaminobutane (putrescine) on the carboxyl group (Table 3).

3 Antifungal Mechanisms

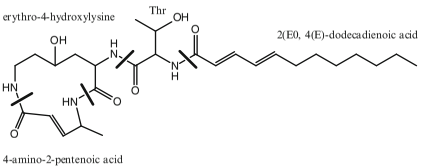
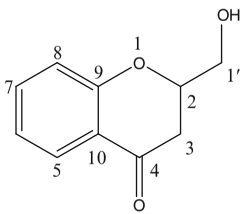
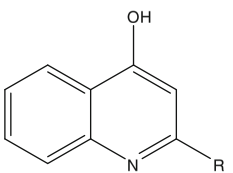
Antagonistic PGPR, including *Bacillus* and *Pseudomonas*, are often considered microbial factories for the production of a vast array of biologically active CLPs (*Bacillus*: surfactin, iturin, and fengycin families; *Pseudomonas*: viscosin, amphisin, tollasin, and syringomycin group) potentially inhibitory for phytopathogen growth. One of the main modes of action of CLPs produced by antagonistic PGPR involves the formation of ion channels in the host plasma membrane leading to cytolysis. Pore formation results in the alkalization of the intercellular fluid and in the release of multiple cellular compounds. These antibiotics gave important modifications in the membrane permeability which permitted nucleotides proteins, polysaccharides, and lipids to escape from cells. At high concentrations (well above the critical micelle concentration), CLPs can directly solubilize plasma membranes

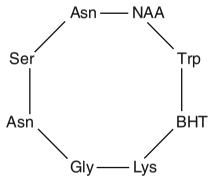
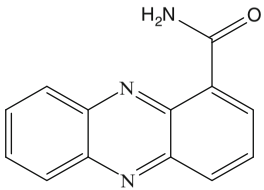
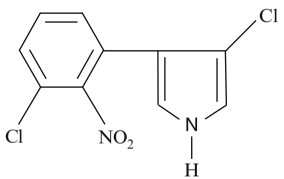
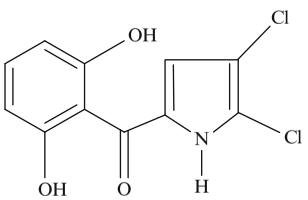
Table 3 Antifungal metabolites produced by *Burkholderia* spp.

Antifungals	Source	Structure	Pathogen	Reference
Altericidins (peptide)	*B. cepacia* KB-1	Unclear	A wide range of fungi and yeasts	Kirinuki et al. (1984)
Cepaciamides A, B	*B. cepacia* D-202		*B. cinerea*, *P. expansum*	Toshima et al. (1999)
Cepacidines/xylocandins (glycopeptide)	*B. cepacia* AF 2001		*Microsporum canis*, *Trichophyton* spp., *Epidermophyton* spp.	Lee et al. (1994)
Cepacin	*B. cepacia*		With broad spectrum antibacterial activity	Parker et al. (1984)

(*continued*)

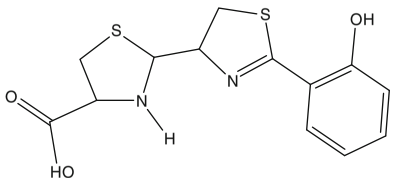
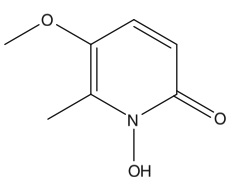
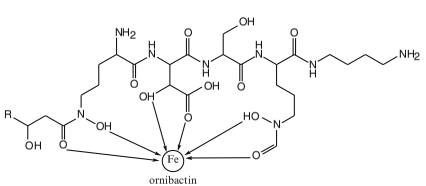
Table 3 (continued)

Antifungals	Source	Structure	Pathogen	Reference
Glidobactins	*B. cepacia*		*Thielaviopsis basicola*	Shoji et al. (1990)
2-hydroxymethyl-chroman-4-one	*Burkholderia* sp.		*P. ultimum*, *Phytophthora capsici*, *Sclerotinia sclerotiorum*	Kang et al. (2004)
4-hydroxy-2-alkylquinoline	*B. pseudomallei*, *B. thailandensis*, *B. ambifaria*		Antibacterial activity	Vial et al. (2008)
Lipopeptide-AFC-BC11	*B. cepacia* BC11	Unclear	*R. solani*, *P. ultimum*, *Colletotrichum* sp., *B. cinerea*, *Helminthosporium maydis*, *Fusarium* sp., *Rhizopus stolonifer*, *Rhodotorula glutinis*, *Sclerotium rolfsii*, *Scopulariopsis brevicaulis*	Kang et al. (1998)

Occidiofungin A (glycopeptide)	*B. contaminans*		A broad range of fungal pathogens	Lu et al. (2009)
Phenazine	*B. cepacia*		A broad range of fungal pathogens	Cartwright et al. (1995)
Pyrrolnitrin	*B. cepacia* NB-1 *B. cepacia* 5.5B *B.cepacia* *B. cepacia* K87 *B. pyrrocinia*		*R. solani*, *B. cinerea*, *C. gloeosporioides*, *Pyricularia oryzae*	Hwang et al. (2002)
Pyoluteorin	*Burkholderia* spp.		*Pythium* spp.	De Souza et al. (2003)

(*continued*)

Table 3 (continued)

Antifungals	Source	Structure	Pathogen	Reference
Quinolinones (pseudanes)	*P. cepacia* PC II			Moon et al. (1996)
Pyochelin (Siderophore)	*B. cepacia* complex	 pyochelin	*Pythium* sp.	Thomas (2007)
Cepabactin (Siderophore)	*B. cepacia* complex	 cepabactin	Antifungal activity	Thomas (2007)
Ornibactins (Siderophore)	*B. cepacia* complex	 ornibactin	Antifungal activity	Thomas (2007)

(Raaijmakers et al. 2006). The antifungal activity has been studied for many different CLPs and for a wide variety of plant and human-pathogenic fungi and yeasts, including *R. solani*, *Phoma lingam*, *Podosphaera fusca*, *P. aphanidermatum*, *Alternaria brassicae*, *S. sclerotiorum*, *G. candidum*, *B. cinerea*, *Ophiostoma ulmi*, *Aspergillus* spp., *Fusarium* spp., *Penicillium digitatum*, *Cryptococcus neoformans*, *C. albicans*, and *C. glabrans*. In vitro studies showed that CLPs adversely affected mycelia of *R. solani* and *P. ultimum*, causing reduced growth and intracellular activity, hyphal swellings, increased branching, and rosette formation (Hansen et al. 2000; Thrane et al. 1999, 2000). The site of action on yeast cells was demonstrated to be cytoplasmic membrane. CLPs of iturin group can lyses spheroplasts of *S. cerevisiae* (Besson et al. 1984a, b). Moreover, a rapid leakage of potassium ions found in the presence of this antibiotics is directly associated to the killing effects. These results are consistent with a disruption of the structural integrity of the cytoplasmic membrane correlated to the loss of viability of the yeast cells.

Bacisubin secreted from *B. subtilis* strain B-916 is an antifungal protein and is strongly inhibited mycelial growth in *R. solani*, *M. grisease*, *S. sclerotiorum*, *A. oleracea*, *A. brassicae*, and *B. cinerea*, especially some species of *Alternaria* and *Botrytis*. The IC_{50} values of antifungal activity of bacisubin toward *A. brassicae*, *A. oleracea*, *R. solani*, and *B. cinerea* were as low as 0.055, 0.087, 4.01, and 2.74 mM, respectively. Bacisubin inhibited the growth of *R. solani* and induced increase in mycelial apex offshoot, distortion, tumescence, and rupture (Liu et al. 2007).

Apart from peptides, polyketides are the other dominant family of secondary metabolites having antimicrobial, immunosuppressive, antitumor, or other physiologically relevant bioactivities. Although polyketides are widespread secondary metabolites from bacteria, only a few (difficidin/oxydifficidin, bacillaene, and macrolactin) have been isolated and characterized from *Bacillus*. Difficidin has been shown to inhibit protein biosynthesis (Zweerink and Edison 1987), but the exact molecular target remains unknown. Patel et al. (1995) described that Bacillaene is a highly unstable inhibitor of prokaryotic protein synthesis with a partially characterized open-chain polyenic structure with the empirical formula C35H48O7. However, this formula was suspected to be incorrect, because the NRPS modules indicated the presence of two nitrogen atoms. Macrolactin is the third polyketide with macrolide-like structure; it is originally detected in an unclassified deep-sea marine bacterium and has been previously reported from several other *Bacillus* strains. The macrolactin carbon skeleton contains three separate diene structure elements in a 24-membered lactone ring. Until now, at least 17 macrolactins have been described and one of them, 7-O-malonyl-macrolactin A, has been recently reported as efficient against Gram-positive bacterial pathogens. A broad-spectrum antibiotic, 2,4-di-acetylphloroglucinol (2,4-DAPG), is a polyketide compound produced by many fluorescent pseudomonads, exhibits antifungal, antibacterial, antihelmenthic, and phytotoxic activities. Previous study has demonstrated that root-associated fluorescent *Pseudomonas* spp. with the capacity to produce 2,4-DAPG are the key components in biological control. It is synthesized by condensation of three molecules of acetyl CoA with one molecule of malonyl CoA to produce the

precursor monoacetylphloroglucinol (MAPG), which is subsequently transacetylated to generate DAPG. The exact mechanism of DAPG action is still unclear, although it is known that disease suppression by this antifungal molecule is a result of interaction of specific root-associated microorganisms and the pathogen. This antibiotic also appears to cause ISR in plants. Plt is an aromatic polyketide antibiotic consisting of a resorcinol ring, which is derived through polyketide biosynthesis. This in turn is linked to a bichlorinated pyrrole moiety, whose biosynthesis remains unknown. Plt is produced by several *Pseudomonas* sp., including strains that suppress plant diseases caused by phytopathogenic fungi. Plt mainly inhibits the oomycetous fungi, such as *P. ultimum*.

Phzs are N-containing heterocyclic pigments synthesized by *Brevibacterium*, *Burkholderia*, *Pseudomonas*, and *Streptomyces*, and these compounds have been identified as virulence factors in a number of in vivo model systems. The Phz secreted by *Pseudomonas* are PCA, pyocyanin, 1-hydroxyphenazine (1-HP), and phenazine-1-carboxamide. Almost all Phz exhibits broad spectrum activity against bacteria and fungi. In addition to inhibiting fungal pathogenesis, Phz play an important role in microbial competition in rhizosphere, including survival and competence. The broad-spectrum activity exhibited by Phz compounds against fungi and other bacteria is not well understood. However, it is considered that Phz can accept electrons, yielding a relatively stable anion radical that readily undergoes redox cycle. It includes biosynthesis of Mn-containing superoxide dismutase (MnSOD) which causes enhanced production of O_2^- (superoxide radical). There is a distinct possibility that the antibiotic action of pyocyanin is actually a result of toxicity of O_2^- and H_2O_2 produced in increased amounts in its presence.

Prn was thought to inhibit bacterial growth by complexing with phospholipids of cell membranes. Although Prn inhibited the respiration of intact cells, the oxidative phosphorylation of mitochondria isolated from *C. utilis* was not inhibited. Tripathi and Gottlieb (1970) concluded that inhibition of electron transfer in yeast was the site of action of Prn. Previous study clearly shows that Prn inhibits respiration of fungal mitochondria and mammalian respiratory system (Wong and Airallb 1970). Complete inhibition of electron transport requires its higher concentration.

Most organisms require iron as an essential element in a variety of metabolic and informational cellular pathways. More than 100 enzymes acting in primary and secondary metabolism possess iron-containing cofactors such as iron-sulfur clusters or heme groups. The reversible Fe(II)/Fe(III) redox pair is best suited to catalyze a broad spectrum of redox reactions and to mediate electron chain transfer. Furthermore, several transcriptional and posttranscriptional regulators interact with iron to sense its intracellular level or the current status of oxidative stress to efficiently control the expression of a broad array of genes involved mainly in iron acquisition or reactive oxygen species protection. However, in most microbial habitats, Fe(II) is oxidized to Fe(III) either spontaneously by reacting with molecular oxygen or enzymatically during assimilation and circulation in host organism. In the environment, Fe(III) forms ferric oxide hydrate complexes ($Fe_2O_3 \cdot nH_2O$) in the presence of oxygen and water at neutral to basic pH. These complexes are very stable, leading to a free Fe(III) concentration of 10^{-9}–10^{-18} M. Many microorganisms

produce siderophores that bind iron and enhance microbial growth by solubilizing ferric iron and by accelerating iron transport.

Siderophores are themselves growth inhibitors of various phytopathogenic fungi, such as *P. parasitica*, *P. ultimum*, *F. oxysporum* veri *dianthi*, and *S. sclerotiorum.* Siderophores, whose chemical structures depend upon their producer microorganism, may provide iron (III) to some vegetable cells. These metabolites, due to their antagonistic capability against pathogenic microorganisms, could act as growth factors in plants.

4 Understanding Biosynthesis of Antifungal Metabolites at the Molecular Level

CLPs from *Pseudomonas* species are produced by nonribosormal peptide synthesis (NRPSs) via a thiotemplate process. NRPSs possess a modular structure and each module is a building block resulting in the stepwise incorporation and modification of one amino acid unit. Their substrates are not restricted to the usual proteinogenic amino acids but also can incorporate multiple nonproteinogenic D-amino acids, carboxy acids, or fatty acids. For CLP-producing *Pseudomonas* spp., a number of partial and complete sequences of NRPSs have been obtained over the past decade. Two of the best-characterized biosynthetic templates are the synthetase clusters for arthrofactin and syringomycin.

Bacteria of the *Bacillus* genus produce a wide variety of antibacterial and antifungal antibiotics. Some of these compounds, like subtilin, subtilosin A, TasA, and sublancin, are of ribosomal origin, but others, such as bacilysin, chlorotetain, mycobacillin, rhizocticins, bacillaene, difficidin, and lipopeptides belonging to the surfactin, iturin, and fengycin families, are formed by nonribosomal peptide synthetases and/or polyketide synthases (PKS)(Leclére et al. 2005). The model organism *B. subtilis* 168 and the plant root-colonizing *B. amyloliquefaciens* FZB42 produce a wide variety of antibacterial and antifungal antibiotics, and their gene clusters involved in antibiotics biosynthesis have been identified. In *B. amyloliquefaciens* FZB42 (Chen et al. 2007), the nine gene clusters (*srf*, *bmy*, *fen*, *nrs*, *dhb*, *bac*, *mln*, *bae*, *dfn*) direct the synthesis of bioactive peptides and polyketides by modularly organized megaenzymes defined as nonribosomal peptide synthetases (NRPSs) and PKS. Four gene clusters (*bmy*D, *pks*2, *pks*3, and *nrs*) are not found in *B. subtilis* 168. Except for the gene cluster encoding bacilysin synthesis, the functional activities of the remaining gene clusters depend on Sfp, an enzyme that transfers 4′-phosphopantetheine from coenzyme A to the carrier proteins of nascent peptide or polyketide chains.

Prn and Plt are broad-spectrum antibiotics produced by several strains of *Pseudomonas* and *Burkholderia* species. The *prn* operon has been completely sequenced; *prnABCD* spans 5.8 kb DNA which encodes Prn biosynthetic pathway in which four ORFs (Hammer et al. 1997), *prnA*, *prnB*, *prnC*, and *prnD*, are

involved. All four ORFs are located on a single transcriptional unit. The four genes encode proteins which are identical in size. Among these, *prnA* gene product catalyses the chlorination of L-trp to 7 chloro-L-trp. The *prnC* gene product chlorinates it at the 3-position to form an amino pyrrolnitrin. The *prnD* gene product catalyses the oxidation of aminopyrrolnitrin to a nitro group to form pyrrolnitrin. The organization of *prn* genes in the operon is identical to the order in which the reactions are catalyzed in the biosynthetic pathway. Phz nucleus is formed by the symmetrical condensation of two molecules of chorismic acid, and the amide nitrogen of glutamine serves as the immediate source of N in the heterocyclic nucleus. PCA is the first phenazine formed, which gets converted to PCA and acts as the key intermediate in the synthesis of other phenazine in *Pseudomonas* and *Burkholderia* species. Phz compounds reported previously are pyocyanin, 1-hydroxyphenazine, and phenazine-1-carboxamide. Seven genes, *phzABCDEFG*, are involved in the synthesis of PCA (Mavrodl et al. 2001). These are localized within a 6.8 kb fragment in *P. fluorescens* 2-79. The phenazine biosynthetic loci in *P. fluorescens* 2-79, *P. aeruginosa* PAO1, and *P. chlororaphis* PCL 1394 are highly conserved. Each *phz* locus contains a set of seven gene core operons, regulated in a cell density-dependent manner by homologues of LuxI and LuxR.

The pathways of biosynthesis of siderophores from members of *Pseudomonas* and *Burkholderia* have been investigated clearly, and genes involved in these siderophores have been identified as shown in Table 4.

5 Concluding Remarks and Further Perspectives

PGPR can promote plant growth directly or indirectly. Some PGPR can directly cause plant growth promotion by producing and secreting plant growth regulators such as auxins, gibberellins, and cytokinins. Other PGPR affect plant growth by indirect mechanisms that involve suppression of bacterial, fungal, and nematode pathogens. To date, PGPR represent a variety of bacterial species from more than 20 genera, but the mechanism is yet unclear. At present, most research focus on the PGPR as biocontrol agent; however, in fact biological control bacteria can not only produce inhibitory metabolites but also produce some metabolites with growth promoting activity. Although the chemical structure and biosynthesis genes of many antifungal compounds are known, its regulatory mechanisms and the relationship between growth-promoting activity and antifungal activity do not understand. Furthermore, particular bacteria can produce a variety of antibiotics simultaneously, and some of them are considered as signal molecules that can regulate plant growth or pathogenicity of pathogenic fungal (bacteria). The solution of these problems will contribute to the extensive application of PGPR in agriculture and development of agricultural biotechnology.

Table 4 Genes involved in the biosynthesis of anbiotics of *Bacillu*, *Pseudomonas*, and *Burkholderia*

Antibiotics	Species/strain	Gene/Protein information	GenBank accession no.	References
Viscosin	*P. fluorescens* PfA7B	Nonribosomal peptide synthetases	No sequence available	Braun et al. (2001)
Massetolide A	*P. fluorescens* R1SS101	Nonribosomal peptide synthetases	AY303770; AY303771	De Souza et al. (2003)
Amphisin	*Pseudomonas* sp. DSS73	*amsY*, peptide synthetase	AJ416154	Koch et al. (2002)
Arthrofactin	*Pseudomonas* sp. MIS38	*arfA B C*; nonribosomal peptide synthetases	AB107223	Roongsawang et al. (2003)
Tolaasin	*P. tolaasii*	TL1, TL2, TL3; high molecular weight protein	No sequence available	Rainey et al. (1993)
Tolaasin	*Pseudomonas* NZ17	Homology to syringomycin synthetase	No sequence available	Godfrey et al. (2001)
Syringopeptin	*P. syringae* pv. *syringae* B728a; *P. syringae* pv. *Syringae* B301D	Syringopeptin synthetase genes; *sypABC*; syringopeptin synthetase; *pseABC*; tripartite	CP000075; AF286216	Feil et al. (2005); Scholz-Schroeder et al. (2001, 2003); Kang and Gross 2005
Syringomycin	*P. syringae* pv. *syringae* B728a; *P. syringae* pv. *Syringae* B301D	Syrinogmycin synthetase genes; *syrE*, *syrB1*, *syrC*, *syrB2*, *syrD*, putative ABCtransporter protein pseABC	CP000075, AF047828, U25130, M97223	Feil et al. (2005); Guenzi et al. (1998); Scholz-Schroeder et al. (2001); Zhang et al. (1995); Vaillancourt et al. (2005);
Putisolvin	*P. putida* PCL1445	*psoA*, putisolvin synthetase	DQ151887	Kuiper et al. (2004); Dubern et al. (2005)
Orphan	*P. fluorescens* Pf-5	*ofaA*, *ofaB ofaC*	YP259252.2, YP259253.1, YP_259254.1	Loper and Gross (2007)
2,4-Diaacetyl phloroglucinol	*P. fluorescens* Q2-87 *P. fluorescens* F113	*phlABCD*, *phlF*	U41818, AF129856	Dwivedi and Johri (2003); Delany et al. (2000)
Pyoluteorin	*P. fluorescens* Pf-5	*pltABCDEFG*	PFL2784-PFL2800	Paulsen et al. (2005)
Pyrrolntin	*P. fluorescens*	*prnABCD*	PFL3599-PFL3627	Paulsen et al. (2005)
Phenazines	*P. fluorescens* 2-79 *P. aeruginosa PAO1* *P. chlororaphis PCL 1394*	*phzABCDEFG*		Dwivedi and Johri (2003)

(*continued*)

Table 4 (continued)

Antibiotics	Species/strain	Gene/Protein information	GenBank accession no.	References
Pyochelin	*P. fluorescens* Pf-5	*pchABCDEF*	PFL3473-PFL3504	Paulsen et al. (2005)
Pyoverdine	*P. aeruginosa PAO1*	*pvdADEFIJ*	PA 2386-2388, PA2396-2402, PA	Lamont and Martin (2003)
Hydrogen cyanide	*P. fluorescens* Pf-5 *P. fluorescens* CHA0	*hcnABC*	PFL2577-PFL2579	Paulsen et al. (2005)
Lipopeptide-AFC-BC11	*B. cepacia* BC11	*afcABCD*	AF076477	Kang et al. (1998)
Pyrrolntin	*B. cepacia* LT4-12-W,	*prnABCD*	AF161183	Hammer et al. (1997)
Glidobactin	*Burkholderia* sp.	*Glb ABCDEFGH*	ZP_00417086, NP_929149, NP_929145, ZP_01144206 YP_111276, YP_111275, YP_111274, YP_111273,	Schellenberg et al. (2007)
Ornibactin	*B. cenocepacia*	*OrbGKL*, *pvdAF*	BCAL1690, BCAL 1698, BCAL 1699, BCAL 1701, BCAL 1702	Thomas (2007)
4-hydroxy-2-alkylquinoline	*B. pseudomallei*, *B. thailandensis*, *B. ambifaria*	*pqsABCDE*	BPSS00481-BPSS00485 BTH II1935-BTH II1931	Vial et al. (2008)
Subtilin	*Bacillus subtilis* ATCC 6633	*spa BCD*	M83944	Chung et al. (1992)
Surfactin	*B. amyloliquefaciens* FZB42 *B. subtilis 168*	*srfABCD*, *aat*, *334*, *ycx*, *CycxD*, *sfd*, *yczE; srfAA*, *AB*, *AD*, *ycxABCD*		Chen et al. (2007)
Bacillomycin D	*B. amyloliquefaciens* FZB42	*bmyBCAD*		Chen et al. (2007)
Fengycin	*B. amyloliquefaciens* FZB42 *B. subtilis 168*	*fenABCDE; PpsABCDE*		Chen et al. (2007)
Bacillibactin	*B. amyloliquefaciens* FZB42 *B. subtilis 168*	*dhbABCDEF*		Chen et al. (2007)

Bacilysin	*B. amyloliquefaciens* FZB42 *B. subtilis 168*	*BacABCDE*, *ywfG; ywfCDEFG*		Chen et al. (2007)
Macrolactin	*B. amyloliquefaciens* FZB42	*mlnABCDEFGHI*		Chen et al. (2007)
Bacillaene	*B. amyloliquefaciens* FZB42 *B. subtilis 168*	*baeBSDE*, *acpK*, *baeGHIJLMNRS; pksBCDE*, *acpK*, *pksGHIJLMNRS*		Chen et al. (2007)
Difficidin	*B. amyloliquefaciens* FZB42	*dfnAYXBCDEFGHIJKLM*		Chen et al. (2007)
Iturin A	*Bacillus subtilis* RB14	*ituABCD*	AB050629	
Zwittermicin A	*Bacillus cereus* UW85	*zmaDERG*, *zmaHI*, *zmaJL*, *zmaM*, *zmaNP*, *zmaS*, *zmaT*, *zmaU*, *zmaV*	FJ430564	Kevany et al. (2009)

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Role of Plant Growth Promoting Rhizobacteria in Biocontrol of Plant Diseases and Sustainable Agriculture

Mohd. Sayeed Akhtar and Zaki A. Siddiqui

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Abstract The management of plant diseases in the sustainable agriculture has become a challenge for plant pathologist. Increasing knowledge and growing concern of pesticide applications on environment have aroused interest in alternative methods of plant protection. Plant growth promoting rhizobacteria (PGPR) are the important group of microorganisms, which play a major role in the biocontrol of plant pathogens. PGPR can profoundly improve seed germination, root development, and water uptake by plants. These rhizobacteria stimulate plant growth directly by producing growth hormones and improving nutrient uptake or indirectly

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by changing microbial balance in favor of beneficial microorganisms in the rhizosphere and can suppress a broad spectrum of bacterial, fungal, nematode, and even some viral diseases. Although significant control of plant pathogens has been demonstrated by PGPR in laboratory and greenhouse studies, results in the field trials have been inconsistent. Recent progress in our understanding of their diversity, colonizing ability, and mechanisms of action, formulation, and their application may facilitate their development as reliable biocontrol agents against plant pathogens. Use of PGPR has become a common practice in many regions of the world, and greater application of PGPR is possible for sustainable agriculture in near future.

1 Introduction

Management of plant diseases has become a challenge for the plant pathologist for sustainable agriculture. Increasing knowledge and health hazards associated with the applications of pesticides have aroused interest in alternative methods of plant protection. One of the best methods that may be used by plant pathologists is Biocontrol. Out of different organisms used for biocontrol, rhizosphere microorganisms may provide a front line defense against pathogen attack and are ideal for use as biocontrol agents (Weller 1988; Siddiqui 2006). Biocontrol involves harnessing of disease-suppressive microorganisms to improve plant health (Handelsman and Stabb 1996). Disease suppression by biocontrol agents is the manifestation of interactions among the plant, the pathogen, the biocontrol agent, the microbial community on and around the plant, and the physical environment. Among the wide range of beneficial microorganisms, plant growth promoting rhizobacteria (PGPR) play a vital role in the management of plant diseases (Kloepper and Schroth 1978; Glick 1995; Siddiqui 2006). PGPR are free-living bacteria that may impart beneficial effects on plants. PGPR inhabit the rhizosphere, the volume of soil under the immediate influence of the plant root system, and favors the establishment of a large amount of active microbial population. Plants release metabolically active cells from their roots and deposit as much as 20% of the carbon allocated to roots in the rhizosphere, suggesting a highly evolved relationship between the plant and rhizosphere microorganisms (Handelsman and Stabb 1996), and the dynamic nature of the rhizosphere creates interactions that lead to biocontrol of diseases (Rovira 1965, 1969, 1991; Hawes 1991; Waisel et al. 1991).

Biocontrol of plant diseases is particularly complex because diseases mostly occur in the dynamic environment at the interface of the plant root as well as in the aerial parts of plants. PGPR enhance seedling emergence, colonize roots, and stimulate overall plant growth. PGPR also improve seed germination, root development, mineral nutrition and water uptake/utilization. They can also suppress diseases of plants. Numerous recent reviews present comprehensively the variety of microbial biocontrol agents (Weller 1988; Handelsman and Stabb 1996; Siddiqui and Mahmood 1995a, 1996, 1999; Whipps 2001; Weller et al. 2002; Bakker et al.

2003; Compant et al. 2005; Siddiqui 2006). This chapter focuses on the potentiality of PGPR, mechanisms involved in the biocontrol of plant diseases to understand the behavior and interaction of mycorhizospheric organisms with PGPR. This understanding will facilitate the application of PGPR for the biocontrol of plant diseases under field conditions.

2 Mechanisms of Disease Suppression

There are different ways by which PGPR can affect the plant growth directly: by fixing atmospheric nitrogen, synthesizing several plant hormones and enzymes, and solubilizing minerals that can modulate plant hormone levels.

A particular plant growth promoting bacterium may possess one or more of these mechanisms (Compant et al. 2005; Siddiqui 2006). The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one more phytopathogenic organism by producing siderophores that limit the available iron to the pathogen, producing antibiotics that kill the pathogen, antibiosis, and inducing systemic resistance in plant (Fig. 1). PGPR also cause cell wall structural modifications and biochemical/physiological changes leading to the synthesis of proteins and chemicals involved in plant defense mechanisms. PGPR has been successfully used for the biocontrol of nematode, fungal, bacterial, and viral diseases of plants in different parts of the world (Tables 1–4). Some of the biocontrol mechanisms that have been dealt and will be discussed as follows:

- Interactions of PGPR with pathogens
- Interactions of PGPR with plants
- Interactions of PGPR in the rhizosphere

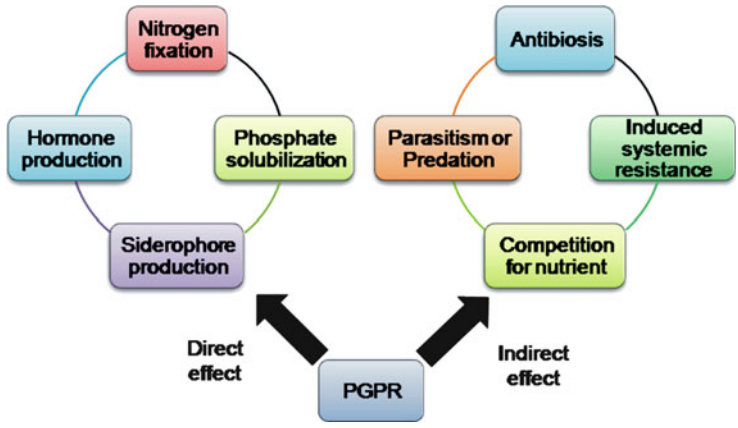

Fig. 1 Some direct or indirect effects of plant growth promoting rhizobacteria on the plant growth

3 Interactions of PGPR with Pathogens

During interaction process of PGPR with phytopathogens, the former produce certain antibiotics, cell wall degrading enzymes, siderophore, etc., and release of such metabolites decides the fate of the pathogen.

3.1 Antibiotic Production

One of the most effective mechanisms to prevent proliferation of phytopathogens is the synthesis of antibiotics by PGPR. There are numerous reports of the production of antifungal metabolites by bacteria in vitro that may also have activity in vivo. A variety of metabolites such as amphisin, butyrolactones, 2,4-diacetylphloroglucinol (DAPG), cyclic lipopeptide, HCN, kanosamine, oligomycin A, oomycin A, phenazine-1-carboxylic acid (PCA), pyoluterin (Plt), pyrrolnitrin (Pln), tensin, tropolone, viscosinamide, xanthobaccin, and zwittermycin A are produced by PGPR (Defago 1993; Milner et al. 1996; Keel and Defago 1997; Whipps 1997; Kang et al. 1998; Kim et al. 1999; Nakayama et al. 1999; Thrane et al. 1999; Nielsen et al. 2002; Raaijmakers et al. 2002; de Souza et al. 2003; Compant et al. 2005). To demonstrate a role for antibiotics in biocontrol, mutants lacking production of antibiotics or antibiotics over-producing mutants have been used (Bonsall et al. 1997; Chin-A-Woeng et al. 1998; Nowak-Thompson et al. 1999). Alternatively, the use of reporter genes or probes to demonstrate the production of antibiotics in the rhizosphere is becoming more common (Kraus and Loper 1995; Raaijmakers et al. 1997; Chin-A-Woeng et al. 1998). Indeed, isolation and characterization of genes or gene clusters responsible for antibiotic production have now been achieved (Kraus and Loper 1995; Bangera and Thomashow 1996; Hammer et al. 1997; Kang et al. 1998; Nowak-Thompson et al. 1999). Significantly, both Phl and PCA have been isolated from the rhizosphere of wheat following introduction of biocontrol strains of *Pseudomonas* (Thomashow et al. 1990; Bonsall et al. 1997; Raaijmakers et al. 1999), confirming that such antibiotics are produced in vivo. Further, Ph1 production in the rhizosphere of wheat was strongly related to the density of the bacterial population present and the ability to colonize roots (Raaijmakers et al. 1999). PCA from *Pseudomonas aureofaciens* has even been used as a direct field treatment for the control of *Sclerotinia homeocarpa* on creeping bent grass (Powell et al. 2000).

The first antibiotics clearly implicated in biocontrol by fluorescent pseudomonads were the phenazine derivatives (Handelsman and Stabb 1996). *P. fluorescens* strain 2-79 and *P. aureofaciens* strain 30-84 contribute to disease suppression of take-all of wheat (Weller and Cook 1983; Brisbane and Rovira 1988). *P. fluorescens* strain CHA0 produces hydrogen cyanide, 2,4-diacetylphloroglucinol, and pyoluteorin, which directly interferes with the growth of various pathogens and contributes to the disease suppression (Voisard et al. 1989; Keel et al. 1992; Maurhofer et al. 1994b; Duffy and Defago 1999). Furthermore, a quantitative relationship between antibiotic production and disease suppression is suggested

by the enhanced production of 2, 4-diacetylphloroglucinol and pyoluteorin accomplished by adding extra copies of a 22-kb fragment of DNA that improves suppression of *Pythium* on cucumber (Maurhofer et al. 1992). Antibiotic DAPG has been shown to act as the inducing agent in CHA0-mediated induced systemic resistance (ISR) in tomato against root-knot nematode *M. javanica* (Siddiqui and Shaukat 2003) and suggest that more antibiotics may be capable of eliciting ISR in plants. The role of individual antibiotic compound in suppression of root pathogens has been clearly established using mutation analysis and molecular genetic tools, and purified antibiotics compounds viz. DAPG overproducing mutant of *P. fluorescens* offered a better protection against the take all of wheat and bacterial wilt of tomato (Hongyou et al. 2005).

3.2 Enzyme Production

Biocontrol of *Phytophthora cinnamomi* causing root rot of *Banksia grandis* was obtained using a cellulase-producing isolate of *Micromonospora carbonacea* (El-Tarabily et al. 1996) and *Phytophthora fragariae* var. *rubi* causing raspberry root rot that was suppressed by the application of actinomycete isolates selected for the production of β-1,3-, β -1,4-, and β -1,6-glucanases (Valois et al. 1996). Chitinolytic enzymes produced by both *Bacillus cereus* and *Pantoea* (*Enterobacter*) *agglomerans* also appear to be involved in the biocontrol of *Rhizoctonia solani* (Chernin et al. 1995, 1997; Pleban et al. 1997). Tn*5* mutants of *E. agglomerans* deficient in chitinolytic activity were unable to protect cotton, and the expression of the *chiA* gene for endochitinase in *E. coli* allowed the transformed strain to inhibit *R. solani* on cotton seedlings. Similar techniques involving *Tn*5 insertion mutants and subsequent complementation demonstrated that biocontrol of *Pythium ultimum* in the rhizosphere of sugar beet by *Stenotrophomonas maltophila* was due to the production of extracellular protease (Dunne et al. 1997). The incidence of plant disease caused by the phytopathogenic fungi *R. solani*, *Sclerotium rolfsii*, and *P. ultimum* was reduced by using a β-1, 3-glucanse producing strain of *Pseudomonas cepacia*, which was able to degrade the fungal mycelia. Many of the bacterial enzymes that can lyse fungal cells, including chitinases and β-1, 3-glucanse, are encoded by a single gene.

3.3 Siderophores Production

Iron is an important micronutrient used by bacteria and it is essential for their metabolism. In the soil, it is unavailable for direct assimilation by microorganisms because ferric iron (FeIII), which predominates in nature, is only sparingly soluble and too low in concentration to support microbial growth (Rachid and Ahmed 2005). To survive, soil microorganisms synthesize and secrete low-molecular-

Table 1 Effects of PGPR on plant parasitic nematodes

Nematode	PGPR	Effect	References
Meloidogyne incognita			
M. incognita	*Bacillus thuringiensis*	Prevented *M. incognita* from forming galls on tomato	Ignoffo and Dropkin (1977)
M. incognita	244 isolates	Only 125 bacterial isolates imparted positive effect on tomato and cucumber, rarely on both and negative effect on nematodes.	Zavaleta-Mejia and VanGundy (1982)
M. incognita	*Serratia marcescens*	Bacterium produced a volatile metabolite and was nematoxic.	Zavaleta-Mejia (1985)
M. incognita	354 isolates	*Pseudomonas fluorescens* (strains JOB204, JOB 209) and *Bacillus* (JOB203) were most effective and clover plants treated with these bacteria had fewer galls and large roots.	Becker et al. (1988)
M. incognita	*Bacillus licheniformis*, *Pseudomonas mendocina*	*B. licheniformis* caused greater reduction in nematode multiplication than *P. mendocina* on tomato.	Siddiqui and Husain (1991)
M. incognita	*Bacillus licheniformis*, *Alcaligenes faecalis*	*B. licheniformis* caused a greater reduction in nematode multiplication than *A. faecalis* on chickpea.	Siddiqui and Mahmood (1992)
M. incognita race3	*B. subtilis*	*B. subtilis* reduced nematode multiplication and improved growth of chickpea.	Siddiqui and Mahmood (1993)
M. incognita	*B. subtilis*	Reduced nematode population on tomato.	Gautam et al. (1995)
M. incognita	*B. subtilis*	Seed treatment with bacteria reduced nematode multiplication on chickpea.	Siddiqui and Mahmood (1995b)
M. incognita	Endophytic bacterial stains	Reduced galling of cotton roots by root-knot nematode.	Hallmann et al. (1997)
M. incognita	*P. fluorescens* PRS9, *B. polymyxa*	Reduced the galling and nematode population on tomato.	Khan and Akram (2000)
M. incognita	*B. subtilis*, *Azotobacter chroococcum* *Azospirillium lipoferum*	Reduced the no. of galls per root system, egg-mass production and nematode population on mung bean.	Khan and Kounsar (2000)
M. incognita	*P. aeruginosa*	Reduced galling on tomato.	Siddiqui and Haque (2001)
M. incognita	*P. fluorescens* (strains GRP3 and PRS9)	GRP3 strain was better in reducing galling and nematode multiplication than PRS9.	Siddiqui et al. (2001)
M. incognita	*Pseudomonas fluorescens*	Seed treatment significantly reduced the galling on okra.	Devi and Dutta (2002)
M. incognita	*P. fluorescens*, *A. brasilense*, *Azotobacter chroococcum*, Microphos	Best management of *M. incognita* was obtained when Microphos culture (mixture of *P. straita*, *Paenibacillus. polymyxa* and *Aspergillus niger*) was used with *A. chroococcum* and *A. brasilense* on brinjal.	Siddiqui et al. (2002)

M. incognita	*A. chroochoccum*, *Azospirillum* sp.	*Azotobacter* was better in reducing galling than *Azospirillum* sp. in okra.	Sharma and Mishra (2003)
M. incognita	*P. fluorescens*, *A. chroococcum*	Greater biocontrol of *M. incognita* was observed when *P. fluorescens* was used with the straw of *Zea mays*.	Siddiqui and Mahmood (2003)
M. incognita	*P. fluorescens*, *A. chroococcum*, *Azospirillum brasilense*	*P. fluorescens* was better at improving tomato growth and reducing galling and nematode multiplication than *A. chroococcum* or *A. brasilense*.	Siddiqui (2004)
M. incognita	*Pseudomonas straita*	Reduced reproduction of *M. incognita* on pea.	Siddiqui and Singh (2005a)
M. incognita, *H. cajani*	*Bacillus* and fluorescent pseudomonads isolates	Four isolates of *Pseudomonas* and 2 of *Bacllus* (Pa70, Pf18, Pa116, Pa324, B18, and B160) were considered potentially useful for the biocontrol of nematodes.	Siddiqui et al. (2005)
M. incognita	*Bacillus* consortium (Strains INR7, T4 and IN 937b)	*Bacillus* spp. had no significant differences over untreated control in term growth of micro- propagated papaya but the inoculation of *Bacillus* with AM fungi reduced the development of *M. incognita* in plants.	Jaizme-Vega et al. (2006)
M. incognita	20 isolates of fluorescent pseudomonads	Out of 20, four isolates of fluorescent pseudomonads (Pf604, Pf605, Pf611 and Pa616) have inhibitory effect against the hatching and penetration of nematodes.	Siddiqui and Shakeel (2006)
M. incognita	*P. putida*, *Paenibacillus polymyxa*	Inoculation of plants with *P. putida* most effectively reduces galling and nematode multiplication than *P. polymyxa* on chickpea.	Akhtar and Siddiqui (2007)
M. incognita	*P. aeruginosa*	*P. aeruginosa* caused a significant reduction in galling and nematode multiplication on chickpea	Siddiqui and Akhtar (2007)
M. incognita, *H. cajani*	*Bacillus* isolates	Isolate B 615 and B 603 were found more promising for the control of nematodes.	Siddiqui and Shakeel (2007)
M. incognita	*Bacillus* and *Pseudomonas* isolates	Out of 18 isolates, B 28 was best in improving tomato growth of *M. incognita* inoculated plants.	Siddiqui et al. (2007b)
M. incognita, *H. cajani*	*Bacillus* and *Pseudomonas* isolates	Combined use of Pa324 with B18 provided better biocontrol of nematodes than use of either of them on pigeon pea.	Siddiqui et al. (2008)
M. incognita	*P. straita*, *Rhizobium* sp	Inoculation of *Rhizobium* caused a greater increase in chickpea growth than caused by *P. straita*.	Akhtar and Siddiqui (2008a)
M. incognita	*Pseudomonas alcaligenes*, *B. pumilus*	*P. alcaligenes* caused a greater increase in shoot dry in plants inoculated with nematodes than did *B. pumilus* on chickpea.	Akhtar and Siddiqui (2008b)
M. incognita	*Paenibacillus polymyxa* GBR-1	Culture filtrate of *Paenibacillus polymyxa* GBR-1 under in vitro significantly reduced egg hatch and caused substantial mortality of *M. incognita* juveniles.	Khan et al. (2008)

(*continued*)

Table 1 (continued)

Nematode	PGPR	Effect	References
M. incognita	*Pseudomonas putida*	Use of composted manure with *P. putida* was more beneficial for tomato growth than the use of urea with bacterium.	Siddiqui and Akhtar (2008a)
M. incognita	*P. putida*	Use of *P. putida* caused 39% reduction in galling and nematode multiplication on tomato.	Siddiqui and Akhtar (2008b)
M. incognita	*P. putida*	Combined use of neem leaf litter with *P. putida* plus *G. intraradices* was best in improving growth of nematode infected tomato.	Siddiqui and Akhtar (2008c)
M. incognita	*P. putida*, *P. alcaligenes*, *Pseudomonas* isolate Pa 28	*P. putida* caused greatest reduction in galling and nematode multiplication followed by *P. alcaligenes* and Pa 28.	Akhtar and Siddiqui (2009)
M. incognita	*A. chroococcum*, *B. subtilis*, *P. putida*	Highest increase in the growth of nematode inoculated plants was observed when *P. putida* was used with cattle manure on tomato.	Siddiqui and Futai (2009)
M. incognita	*B. subtilis*, *P. polymyxa*, *Burkholderia cepacia*	The greatest increase in growth of nematode inoculated plants and reduction in nematode galling was observed when *P. polymyxa* was used with *P. lilacinus* on tomato.	Siddiqui and Akhtar (2009a)
M. incognita	*B. cepacia*, *B. subtilis*	Application of *B. cepacia* caused 36% increase in shoot dry mass of nematode inoculated plants followed by *B. subtilis* (32%) on tomato.	Siddiqui and Akhtar (2009b)
M. incognita	10 isolates of *Pseudomonas* and *Bacillus*	Fluorescent Pseudomonads isolates (Pf1, Pa2, Pa3, Pa4, and Pf5) caused greater inhibitory effect on the hatching and penetration of *M. incognita* than *Bacillus* isolates (B1, B2, B3,B4 and B5) on pea.	Siddiqui et al. (2009)
M. javanica			
M. javanica	*B. cereus*	Inhibited penetration of nematodes on tomato roots.	Oka et al. (1993)
M. javanica	*P. fluorescens*	Reduced nematode multiplication and morphometrics of *M. javanica* females on tomato in different soil.	Siddiqui and Mahmood (1998)
M. javanica	*B. subtilis*	Greatest growth of tomato and high reduction in nematode multiplication occurred when ammonium sulphate was used with *B. subtilis* and *G. mosseae*.	Siddiqui and Mahmood (2000)
M. javanica	*P. aeruginosa*	Reduced the galling and nematode population on tomato.	Siddiqui et al. (2000)
M. javanica	*P. fluorescens*, *Azotobacter chroococcum*, *Azospirillum brasilense*	Use of *P. fluorescens* with *Glomus mosseae* was better at improving chickpea growth and reducing galling and nematode multiplication than other treatments.	Siddiqui and Mahmood (2001)

M. javanica	*P. aeruginosa*, *P. fluorescens*	Bare root dip or soil drench treatment reduced nematode penetration into tomato roots.	Siddiqui and Shaukat (2002)
M. javanica	*P. fluorescens* CHA0	Use of *P. fluorescens* with ammonium molybdate reduced the nematode penetration in mung bean.	Hamid et al. (2003)
M. javanica	*Brevibacillus brevis* or *B. subtilis*	Use of B7 strain as seed dressing was found to be most effective in reducing nematode population on mung bean.	Li et al. (2005)
M. javanica	*P. putida*, *P. alcaligenes*, *P. polymyxa*, *B. pumilus*	Use of all PGPR strains reduced the galling and nematode reproduction in lentil but *P. putida* was found best in reducing galling and nematode reproduction.	Siddiqui et al. (2007a)
M. javanica	*P. fluorescens* EPS291 and EPS817	Both the isolates significantly increased the plant growth and reduced nematode reproduction in micropropagated banana.	Rodriguez-Romero et al. (2008)
M. javanica	*P. putida* *P. alcaligenes*	Individually all the PGPR strains significantly reduced the disease severity in chickpea.	Siddiqui and Akhtar (2009c)
M. exigua			
M. exigua	*Paenibacillus macerans*	Culture filtrate under in vitro condition showed potential against the root-knot nematode *M. exigua* juveniles.	Oliveira et al. (2009)
Globodera pallida			
Globodera pallida	Number of bacterial isolates	Seed treatment reduced nematode penetration in potato roots.	Racke and Sikora (1985)
Globodera pallida	*Agrobacterium radiobacter*	Reduced nematode infection by 40% when sprayed on seed pieces of potato.	Sikora et al. (1989)
Globodera pallida	*B. sphaericus*, *A. radiobacter*	Rhizobacteria systemically induced resistance against potato cyst nematode.	Hasky-Günther et al. (1998)
Heterodera cajani			
H. cajani	*B. subtilis*	Bacteria reduced nematode multiplication on pigeon pea	Siddiqui and Mahmood (1995c)
M. incognita, *H. cajani*, *H. zeae*, *H. avenae*	*B. subtilis*, *B. cereus*, *B. pumilus*, *Pseudomonas* sp.	Most effective isolates against all tested species were *B. subtilis* and *B. pumilus*. The non-cellular extract exhibited high larvicidal properties.	Gokte and Swarup (1988)
H. cajani	*P. fluorescens*	Reduced multiplication of *H. cajani* on pigeon pea.	Siddiqui et al. (1998)

(*continued*)

Table 1 (continued)

Nematode	PGPR	Effect	References
Heterodera schachtii			
H. schachtii	290 isolates	Eight isolates were antagonistic to *H. schachtii*, 3 isolates were identified as *P. fluorescens*.	Oostendorp and Sikora (1989a)
H. schachtii	8 isolates	Nematode penetration was reduced by 6 of 8 isolates tested.	Oostendorp and Sikora (1989b)
Other nematodes species			
Rotylenchulus reniformis, Meloidogyne spp.	*B. subtilis*	Reduced nematode reproduction and galling on cotton, tomato, peanut, and sugar beet.	Sikora (1988)
Criconemella xenoplax	*Pseudomonas aureofaciens*	One strain inhibited nematode multiplication in greenhouse test.	Westcott and Kluepfel (1992)
Criconemella xenoplax	*Pseudomonas aureofaciens*	Bacteria suppressed population of ring nematode.	Kluepfel et al. (1993)
Panagrellus sp.	*P. fluorescens*	Bacteria cultivated on plate count broth reduced nematodes up to 57.4%.	Weidenborner and Kunz (1993)
C. elegans, R. reniformis, P. penetrans	*Bacillus thuringiensis*	Isolate 371 of bacterium reduced nematode populations on tomato and strawberry.	Zuckerman et al. (1993)
R. reniformis	*Pseudomonas solanacearum*	Slight inhibition of nematode activity on aubergine roots.	Kermarrec et al. (1994)
R.. similis, Meloidogyne spp.	*P. putida, P. fluorescens*	Inhibited invasion of *R. similis* and *Meloidogyne* spp. in banana, maize, and tomato.	Aalten et al. (1998)
Heterodera cruciferae	Fluorescent pseudomonads	Growth and hatching of nematode eggs were inhibited	Aksoy and Mennan (2004)

Table 2 Effects of PGPR on fungal diseases of plants

Fungus	PGPR	Effect	References
Gaeumannomyces sp.			
Gaeumannomyces graminis var. *tritici*	*P. fluorescens*	Strains of *P. fluorescens* may be involved in the suppression of *G. graminis var. tritici*	Cook and Rovira (1976)
G. graminis	*Pseudomonas* spp. (fluorescent strains)	27% yield increase due to biocontrol of bacteria in winter wheat under field conditions.	De Freitas and Germida (1990)
G. graminis var. *tritici*	*P. aureofaciens Q2-87*	Inhibition of fungus was demonstrated both in vitro and in vivo.	Harrison et al. (1993)
G. graminis, *Rhizoctonia solani*	*Bacillus subtilis*, *B. cereus* isolates, *P. corrugata*	*Bacillus* isolate A47 and *B. subtilis* B908 reduced the take-all disease in sodic acid soil while *B. subtils* B931 was more effective in reducing Rhizoctonia root-rot in calcareous sandy loam soil of wheat.	Maarten et al. (1998)
Pythium spp.			
Pythium sp.	*P. fluorescens*	In *Pythium* contaminated sites, significant increases were observed in plant height, number of heads and grain yield of winter wheat.	Weller and Cook (1986)
P. ultimum P17	Fluorescent pseudomonads	Significantly suppressed root-rot disease on tulip.	Emma (1990)
P. aphanidermatum	*P. corrugate*, *P. aureofaciens*	Induced systemic resistance in cucumber roots.	Chen et al. (1999)
Pythium sp.	*P. putida*, *B. subtilis*, *E. aerogenes*, *E.agglomerans*, *B. cereus*	Most strains increased root length of cucumber in *Pythium*-infected plants *in vitro*.	Uthede et al. (1999)
P. aphanidermatum, *F. o.* f. sp. *cucurbitacearum*	*B. subtilis*, *P. putida*	Growth and yield of lettuce and cucumber were increased and disease severity reduced.	Amer and Utkhede (2000)
P. aphanidermatum, *Aspergillus* sp. *F. oxysporum* f. sp. *ciceri*, *Rhizoctonia solani*	*Pseudomonas* isolates	Two strains MRS23 and CRP55P have shown antifungal activity.	Goel et al. (2002)
P. aphanidermatum	*P. fluorescens*, *P. putida*	*P. fluorescens* isolate Pf1 was effective in reducing the damping-off incidence in tomato and hot pepper.	Ramamoorthy et al. (2002a)

(*continued*)

Table 2 (continued)

Fungus	PGPR	Effect	References
P. aphanidermatum OP4	Fluorescent *Pseudomonas* (CH31, CH1)	Suppressed the root-rot disease on cucumber.	Moulin et al. (1996)
Fusarium spp.			
Fusarium sp.	*P. fluorescens*	Observed induced resistance and phytoalexin accumulation in carnation.	Van Peer et al. (1991)
F. udum	*B. subtilis*	Increased shoot dry weight and reduced wilt of pigeon pea.	Siddiqui and Mahmood (1995c)
F. oxysporum f. sp. *raphani* *A. brassicicola*, *F. oxysporum*	*P. fluorescens*	Protected radish plants through induction of systemic resistance against these pathogens.	Hoffland et al. (1996)
F. culmorum	*P. chlororaphis 2E3, O6*	Strong inhibition of the fungus on spring wheat in the field.	Kropp et al. (1996)
F. udum	*B. subtilis*	Seed treatment with *B. subtilis* significantly reduced the incidence of wilt of pigeon pea.	Podile and Laxmi (1998)
F. udum	*P. fluorescens*	Wilt incidence was reduced in pigeon pea.	Siddiqui et al. (1998)
F. oxysporum f. sp. *lycopersici*	*P. fluorescens* PRS9, *B. polymyxa*	Reduced the wilting index and rhizosphere population of fungus on tomato.	Khan and Akram (2000)
F. oxysporum *M. phaseolina* *F. solani* *R. solani*	*P. aeruginosa*	Significantly suppressed growth of root infecting fungi on tomato.	Siddiqui et al. (2000)
F. oxysporum f. sp. *lycopersici*	*B. subtilis*, *P. fluorescens*, *Aspergillus awamori*, *A. niger*, *P. digitatum*	Use of all the PSM increased the yield and also reduced the rhizospheric population of wilt fungus by 23–49% on tomato.	Khan and Khan (2001)
F. oxysporum	Fluorescent Pseudomonads isolates	All the five isolates have shown the antifungal activity against the pathogen on pea.	Kumar et al. (2001)
F. udum, *Aspergillus niger*	*B. subtilis* AF1	AF1 supplemented with chitin or chitin material showed better control of crown rot and wilt diseases of ground nut and pigeon pea.	Manjula and Podile (2001)

F. moniliformae, *F. graminearum*, *Macrophomina phaseolina*	*Pseodomonas* sp. EM85 *Bacillus* sp. (MR-11(2), MRF)	All these isolates had the ability to suppress the diseases caused by *F. moniliforme*, *F. graminearum* and *M. phaseolina* on maize.	Pal et al. (2001)
F. solani f. sp. *phaseoli*, *R. solani*, *F. oxysporum*	*B. subtilis* GBO3, MBI600	Seed treatment with these isolates caused increase in biomass and decrease in disease severity in glasshouse on bean.	Estevez de Jansen et al. (2002)
F. oxysporum f. sp. *ciceri*, *Aspergillus* sp., *P. aphanidermatum*, *R. solani*	*Pseudomonas* isolates	Two strains MRS23 and CRP55P have shown antifungal activity.	Goel et al. (2002)
F. oxysporum, *R. solani*	*P. fluorescens*	Out of 40 strains, 18 strains showed strong antifungal activity.	Kumar et al. (2002)
F. oxysporum f. sp. *lycopersici*	*P. fluorescens* Pf1	Pf1 protected tomato plants from wilt disease.	Ramamoorthy et al. (2002b)
F. udum, *F. oxysporum* f. sp. *ciceris*	*P. aeruginosa PNA 1*	*P. aeruginosa* protected pigeon pea and chickpea from *Fusarium* wilt.	Anjaiah et al. (2003)
F. chlamydosporium	*P. fluorescens*	Reduced the severity of disease on *Coleus*.	Boby and Bagyaraj (2003)
F. oxysporum f. sp. *melonis*	*P. putida*	Control on muskmelon achieved by seed treatment of *P. putida* strain 30 was 63% and 46–50% for strain 180.	Bora et al. (2004)
F. udum	*Bacillus* and fluorescent pseudomonads isolates	Four isolates, namely Pa116, P324, B18 and B160, have shown antifungal activity.	Siddiqui et al. (2005)
F. oxysporum f. sp. *ciceri*	*Paenibacillus lentimorbus* NRRL B-30488	Seed treatment with B-30488 caused greater mortality in non-bacterized seedlings compared to bacterized seedlings of chickpea.	Dasgupta et al. (2006)
F. udum	Fluorescent *Pseudomonas*	Four isolates, namely Pf604, Pf605, Pf611 and Pa616 have shown antifungal activity but isolate Pf605 reduced the wilt disease index of pigeon pea under pot condition.	Siddiqui and Shakeel (2006)
F. oxysporum f. sp. *lycopersici*	Fluorescent Pseudomonads	Significantly reduced the disease severity on tomato but the results were more pronounced when applied in combination with *T. harzianum*.	Yigit and Dikilitas (2007)
F. oxysporum f. sp. *radicis-lycopersici*	*Bacillus subtilis* EU07	Inoculation of *B. subtilis* (EU07) reduced the disease incidence up to 75% on tomato.	Baysal et al. (2009)

(*continued*)

Table 2 (continued)

Fungus	PGPR	Effect	References
Rhizoctonia spp.			
R. solani	*P. cepacia* R55, R85 *P. putida* R104	Increase of 62–78% of dry weight of winter wheat grown in *R. solani* infected soil.	De Freitas and Germida (1991)
R. solani	*B. subtilis* RB14	Antibiotics production by *B. subtilis* RB14 suppressed the damping off disease of tomato in vitro and in pot conditions.	Asaka and Shoda (1996)
R. solani	*Bacillus megaterium* (B153-2-2)	Seed treatment significantly reduced damage caused by *R. solani* on soybean in different soil.	Zheng and Sinclair (2000)
R. solani	*Pseudomonas fluorescens*	Mixture of 3 strains reduced disease and promoted growth of rice.	Nandakumar et al. (2001)
R. solani	*Bacillus subtilis*, *Burkholderia cepacia*	Combination of *B. subtilis* RB14-C with *B. cepacia* BY can lead to greater damping-off suppression on tomato than by these strains separately.	Szczech and Shoda (2004)
R. solani	*P. fluorescens* A6RI	Increased the growth of pathogen inoculated plants.	Berta et al. (2005)
	Fluorescent pseudomonads	Out of 103 isolates, only 52 isolates showed antifungal activity against the *R. solani*, in vitro condition.	Ahmadzadeh and Tehrani (2009)
Macrophomina phaseolina			
M. phaseolina	*B. licheniformis*, *A. faecalis*	Reduced root-rot disease of chickpea.	Siddiqui and Mahmood (1992)
M. phaseolina	*B. subtilis*	*B. subtilis* was superior to *P. lilacinus* for the management of *M. phaseolina* on chickpea.	Siddiqui and Mahmood (1993)
M. phaseolina	*B. subtilis*	*B. subtilis* resulted in greater shoot dry weight of chickpea than with any fungal filtrate.	Siddiqui and Mahmood (1995b)
M. phaseolina	*P. fluorescens* 4-92	*P. fluorescens* increased disease resistance by 33% in chickpea.	Srivastava et al. (2001)
M. phaseolina	Fluorescent *Pseudomonas* GRC_2	Seed bacterization with *Pseudomonas* isolates GRC_2 strain reduced the charcoal rot disease of peanut in *M. phaseolina* infested soil.	Gupta et al. (2002)
M. phaseolina	*P. fluorescens*	Seed treatment with *P. fluorescens* and neem cake reduced the root rot indices on green gram.	Begum and Kumar (2005)
M. phaseolina			

	Pseudomonas alcaligenes *Bacillus pumilus*	*P. alcaligenes* caused greater reduction against the root-rot disease than *B. pumilus* on chickpea.	Akhtar and Siddiqui (2008b)
Colletotrichum spp.			
Colletotrichum orbiculare	*P. putida*, *S. marcescens*, *Flavomonas oryzihabitans*, *B. pumilus*	PGPR mediated ISR was operative under field conditions against naturally occurring anthracnose of cucumber.	Wei et al. (1996)
Colletotrichum orbiculare	*B. pumilis*, *B. subtilis*, *Curtobacterium flaccumfaciens*	Mixture of these PGPR strains as seed treatment caused disease reduction on cucumber.	Raupach and Kloepper (1998)
Colletotrichum orbiculare	*S. marcescens* 90-166	Seed treatment suppressed anthracnose of cucumber.	Press et al. (2001)
Colletotrichum capsici	*Pseudomonas fluorescens*	Increased accumulation of enzymes involved in phenyl propanoid pathway and PR-proteins in hot pepper.	Ramamoorthy and Samiyappan (2001)
Colletotrichum lindemuthianum	*Pseudomonas aeruginosa*, *Pseudomonas fluorescens*	*P. aeruginosa* induced resistance only in resistant interactions while *P. fluorescens* induced resistance in susceptible and moderately resistant interactions on bean.	Bigirimana and Hofte (2002)
Colletotrichum falcatum	*P. fluorescens*	Induced systemic resistance against red rot of sugarcane.	Viswanathan and Samiyappan (2002)
Colletotrichum gloeosporioides	*P. fluorescens* FP7	Suppressed the anthracnose pathogen on mango leading to improved yield attributes.	Vivekananthan et al. (2004)
Other fungi			
Sclerotium rolfsii, *Fusarium* sp.	*P. putida* *P. fluorescens* *P. alcaligenes*	Reduced the incidence of disease caused by *S. rolfsii* in bean, and fusarium wilt of cotton and tomato.	Gamliel and Katan (1993)
Verticillium dahliae	*Pseudomonas PsJN*	Reduced disease incidence in tomato.	Sharma and Nowak (1998)

(*continued*)

Table 2 (continued)

Fungus	PGPR	Effect	References
Cronartium quercuum f. sp. *fusiforme*	*B. pumilus* SE34 *S. marcescens* 90-166	Two bacterial isolates out of 8, significantly reduced number of galls and induced systemic resistance against fusiform rust on Loblolly pine.	Enebak and Carey (2000)
Botrytis cineria	*Pseudomonas* PsJn	PsJn inhibits growth of *B. cineria* by disrupting cellular membrane and cell death.	Barka et al. (2002)
Curvularia lunata	*Bacillus* species BC121	Showed high antagonistic activity against *C. lunata*.	Basha and Ulaganathan (2002)
Cnaphalocrocis medinalis	*P. fluorescens* strains Pf1, FP7	Mixture of two strains performed better than the individual strains in reducing sheath blight of rice.	Radja Commare et al. (2002)
Phytophthora infestans	*P. fluorescens* 89B61 *B. pumilus* SE34	Elicited systemic protection against late blight of tomato and reduced disease severity.	Yan et al. (2002)
Sclerospora graminicola	*Bacillus pumilus*	Out of 7 PGPR strains, maximum vigor index resulted from treatment with strain INR7 followed by IN937b.	Niranjan Raj et al. (2003)
S. graminicola	*Pseudomonas fluorescens*	The isolates offered protection ranging from 20 to 75% against downy mildew to pearl millet.	Niranjan-Raj et al. (2004)
Alternaria triticina	*P. fluorescens* *A. chroococcum*	*P. fluorescens* caused greater reduction in *A. triticina* infected leaf area than *A. chroococcum*.	Siddiqui and Singh (2005b)
A. triticina	*Bacillus* and Fluorescent Pseudomonads	Out of 6 isolates, B28 was found best in improving plant growth and also caused reduction in percent leaf infected area of wheat.	Siddiqui (2007)
Exobasidium vexans	*Pseudomonas* and *Bacillus*	Seed treatment with PGPR strains reduced the disease severity on tea under field condition.	Saravanakumar et al. (2007)

Table 3 Effects of PGPR on bacterial diseases of plants

Pathogenic bacteria	PGPR	Effect	References
Xanthomonas compestris pv. *citri*	*P. fluorescens*	Control of citrus canker by siderophore production.	Unnamalai and Gnanamanickam (1984)
Erwinia carotovora	*P. putida* W4P63	Increased yield of Rosset Burbank potato and suppressed soft rot potential of tubers.	Xu and Gross (1986)
E. amylovora	*P. fluorescens* A506	Reduction in the population size of *E. amylovora* in pear flowers with *P. fluorescens* was due to competition.	Wilson and Lindow (1993)
P. syringae pv. *tomato*	*P. fluorescens* WCS417	*P. fluorescens* protected radish through induction of systemic resistance against a virulent bacterial leaf pathogen.	Hoffland et al. (1996)
P. solanacearum	*P. fluorescens* M29 and M40	Isolate M40 reduced tomato wilt significantly.	Kim and Misaghi (1996)
P. syringae pv. *lachrymans*	*P. putida*, *S. marcescens*, *Flavomonas oryzihabitans*, *B. pumilus*	PGPR strains caused significant protection against pathogen on cucumber.	Wei et al. (1996)
E. amylovora	*P. fluorescens* A506	Strain A506 and antibiotics acted additively in the control of frost and fire blight disease.	Lindow et al. (1996)
P. syringae pv. *lachrymans*, *Erwinia tracheiphila*	*B. pumilis*, *B. subtilis*, *Curtobacterium flaccumfaciens*	Seed treatment of strains mixture caused reduction in angular spot and wilt of cucumber.	Raupach and Kloepper (1998)
Ralstonia solanacearum	Fluorescent pseudomonads	All three strains suppressed wilt of tomato and increased yield.	Jagadeesh et al. (2001)
Xanthomonas oryzae pv. *oryzae*	*P. fluorescens*	Showed resistance to the rice bacterial blight pathogen.	Vidhyasekaran et al. (2001)
P. syringae pv. *tomato*	*Azospirillium brasilense*	Prevented bacterial speck disease development and improved tomato growth.	Bashan and Bashan (2002)
R. solanacearum	*Serratia* J2, *Pseudomonas*, *Bacillus* BB11	All the three strains suppress wilt of tomato and increase yield.	Guo et al. (2004)

(*continued*)

Table 3 (continued)

Pathogenic bacteria	PGPR	Effect	References
Xanthomonas compestris pv. *compestris*	*B. cereus, B. lentimorbus, B. pumilus*	Incidence and severity of black rot of cabbage were reduced when antagonists were applied.	Massomo et al. (2004)
R. solanacearum	*Bacillis* and *Pseudomonas* isolates	Out of 120, six isolates (PFMRI, BS-DFS, PF9, PF20, BC, and BS-wly) having antagonistic activity against the bacterial wilt on potato in vitro condition.	Aliye et al. (2008)

Table 4 Effects of PGPR on viral diseases of plants

PGPR	Viruses	Effects	References
Tobacco mosaic virus	*B. uniflagellatus*	Cultures and extracts from cultures reduced numbers of lesions from TMV.	Mann (1969)
Tobacco necrosis virus	*P. fluorescens* CHA0	Reduction in TNV leaf necrosis in *P. fluorescens* treated tobacco plants.	Maurhofer et al. (1994a)
Cucumber mosaic virus	*P. fluorescens, Serratia marcescens*	Treatment of cucumber or tomato plants with PGPR induced systemic resistance against CMV.	Raupach et al. (1996)
Tomato mottle virus	*B. amyloliquefaciens, B. subtilis, B. pumilus*	Disease severity ratings were significantly less in all PGPR powder based treatments.	Murphy and Zehnder (2000)
Cucumber mosaic cucumo virus (CCMV)	*B. amyloliquefaciens, B. subtilis, B. pumilus*	PGPR mediated ISR occurred against CCMV following mechanical inoculation on tomato.	Zehnder et al. (2000)
Pepper mild mottle virus (PMMoV)	*Bacillus amyloliquefaciens*	*Bacillus* induced systemic resistance against PMMoV in tobacco via salicyclic acid and jasmonic acid dependent pathways.	Ahn et al. (2002)

weight iron-binding compounds (400–1,000 daltons) known as siderophores. Siderophores bind FeIII with a very high affinity (Whipps 2001). The bacterium that originally synthesized the siderophores takes up the iron siderophore complex by using a receptor that is specific to the complex and is located in the outer cell membrane of the bacterium. Once inside the cell, the iron is released and is then available to support the microbial growth. PGPR can prevent the proliferation of fungal and other pathogens by producing siderophores that bind most of the FeIII in the area around the plant root. The resulting lack of iron prevents pathogens from proliferating in this immediate vicinity (Loper and Henkels 1999; Siddiqui 2006). The siderophores synthesis in bacteria is generally regulated by iron sensitive fur proteins, global regulators (GasS and GasA), the sigma factors (RpoS, PvdS, and Fpv1), quorum sensitive autoinducers (*N*-acyl homoserine lactone), and many site-specific recombinase (Cornelis and Matthijis 2002; Ravel and Cornelis 2003; Compant et al. 2005). A myriad of environmental factors can also modulate the siderophore synthesis, pH, iron level and forms of iron ions, presence of trace elements, and an adequate supply of C, N, and P (Duffy and Defago 1999). Microbial siderophores vary widely in overall structure but most contain hydroxamate and catechol groups, which are involved in chelating the ferric ion (Neilands 1995).

Suppression of soil borne plant pathogens by siderophore producing pseudomonads was observed (Bakker et al. 1987; Becker and Cook 1988; Loper 1988), and the wild type siderophore producing strain was more effective in suppressing disease compared with non-siderophore-producing mutants. Siderophore production is an

important feature for the suppression of plant pathogens and promotion of plant growth. In another study, siderophore producing mutant *P. putida* was most effective than the wild type in suppression of Fusarium wilt of tomato (Vandenburgh and Gonzalez 1984), while a siderophore-deficient mutant of *P. aeruginosa* lost its biocontrol activity (Buysens et al. 1994). Fluorescent siderophores production was observed as a mechanism of biocontrol of bacterial wilt disease in the fluorescent pseudomonads RBL 101 and RSI 125 (Jagadeesh et al. 2001), while Akhtar and Siddiqui (2009) reported that siderophore producing Pseudomonads strains significantly reduced the root-rot disease in chickpea. Press et al. (2001) reported the catechol siderophore biosynthesis gene in *Serratia marcescens* 90–166 associated with induced resistance in cucumber against anthracnose, while *P. fluorescens* inhibited the growth of *Fusarium culmorum* in vitro (Kurek and Jaroszuk-Scisel 2003).

The capacity to utilize siderophores is important for the growth of bacteria in the rhizosphere (Jurkevitch et al. 1992) and on the plant surface (Loper and Buyer 1991). Specific siderophore producing *Pseudomonas* strains rapidly colonized roots of several crops and resulted in increased yield (Schroth and Hancock 1982). Enhanced plant growth caused by pseudomonad strains was often accompanied by the reduction in pathogen populations on the roots. There is convincing evidence to support a direct role of siderophore mediated iron competition in the biocontrol activity exhibited by such isolates (Leong 1986; Loper and Buyer 1991). The antagonism depends on the amount of iron available in the medium; siderophores produced by a biocontrol agent and sensitivity of target pathogens (Kloepper et al. 1980; Weger et al. 1988). Production of ALS 84 and siderophores contributed to the biocontrol of crown gall by *Agrobacterium rhizogenes* K84 especially under conditions of iron limitation (Penyalver et al. 2001).

Iron nutrition of the plant influences the rhizosphere microbial community structure (Yang and Crowley 2000), and the role of the pyoverdine siderophores produced by many *Pseudomonas* species has been clearly demonstrated in the control of *Pythium* and *Fusarium* species (Loper and Buyer 1991; Duijff et al. 1993). Pseudomonads also produce two other siderophores, pyochelin and its precursor salicylic acid. Pyochelin is thought to contribute to the protection of tomato plants from *Pythium* by *P. aeruginosa* 7NSK2 (Buysens et al. 1996). Different environmental factors can also influence the quantity of siderophores produced (Duffy and Defago 1999).

4 Interactions of PGPR with Plants

Inoculation with PGPR imparts resistance in various plant species against a variety of pathogens including bacteria, viruses, and fungi. And apart from inducing certain morphological changes in the plant itself, it also generates accumulation of phenolics and increases the levels of certain enzymes.

4.1 Induced Resistance

Use of selected PGPR strains was shown to trigger a plant-mediated resistance in above ground plant parts (Van Peer et al. 1991; Wei et al. 1991). This type of resistance is often referred as ISR and has been demonstrated in many plant species including bean, carnation, cucumber, radish, tobacco, tomato, and *Arabidopsis thaliana* (van Loon et al. 1998). Rhizobacteria-mediated ISR resembles phenotypically with classic pathogen induced resistance, in which noninfected parts of a previously pathogen infected plant become more resistant to further infection. This form of resistance is referred as systemic acquired resistance (SAR) (Ross 1961). The difference between ISR and SAR is that ISR is induced by nonpathogenic rhizobacteria, while SAR is induced systemically after inoculation with necrotizing pathogens. Moreover, ISR is independent of salicyclic acid but involves jasmonic acid and ethylene signaling, while SAR requires salicyclic acid as a signaling molecule in plants. ISR is accompanied by the expression of sets of genes distinct from the PR genes whereas SAR is accompanied by the induction of pathogenesis related proteins. Both ISR and SAR are effective against a broad spectrum of plant pathogens (Kuc 1982; van Loon et al. 1998).

The effectiveness of ISR and SAR to a range of viral, bacterial, fungal, and oomycete pathogens was tested on *Arabidopsis*. *Arabidopsis thaliana* L. has many features favoring its use as a model in studies of PGPR (O-Callaghan et al. 2000). In this model system, the nonpathogenic rhizobacterial strain *P. fluorescens* WCS417r was used as the inducing agent (Pieterse et al. 1996) to trigger ISR in several plant species (Van Peer et al. 1991; Leeman et al. 1995; Duijff et al. 1998; Bigirimana and Hofte 2002). Colonization of *Arabidopsis* roots by *P. fluorescens* WCS417r protected the plants against different plant pathogens (Pieterse et al. 1996; Van Wees et al. 1997; Ton et al. 2002). Protection against different pathogens was expressed both in reduction in disease symptoms and inhibition of pathogen growth. Since rhizobacteria were spatially separated from pathogens, the mode of disease suppression in the plants is through ISR. The ability to develop ISR appears to depend on the host/rhizobacterium combination (Pieterse et al. 2002) and suggests that specific recognition between the plant and the ISR-inducing rhizobacterium is required for the induction of ISR. Several bacterial components as potential inducers of ISR are involved including outer membrane lipopolysaccharides and iron regulated siderophores (Leeman et al. 1995; van Loon et al. 1998).

Changes that have been observed in plant roots exhibiting ISR include the following: (1) strengthening of epidermal and cortical cell walls and deposition of newly formed barriers beyond infection sites including callose, lignin, and phenolics (Benhamou et al. 1996a, b, c, 2000; Duijff et al. 1997; Jetiyanan et al. 1997; M'Piga et al. 1997); (2) increased levels of enzymes such as chitinase, peroxidase, polyphenol oxidase, and phenylalanine ammonia lyase (M'Piga et al. 1997; Chen et al. 2000); (3) enhanced phytoalexin production (Van Peer et al. 1991; Ongena et al. 1999); and (4) enhanced expression of stress-related genes (Timmusk and Wagner 1999). However, not all of these biochemical changes are found in all

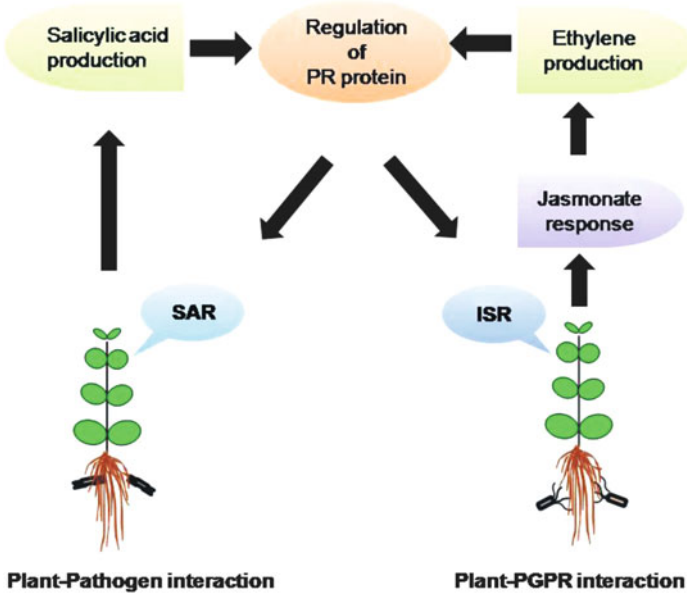

Fig. 2 Signaling pathway in plants responsible for the disease resistance in plants

bacterial–plant combinations (Steijl et al. 1999). Protection from diseases by biocontrol and its consistency in the field are generally not sufficient to compete with conventional methods of disease control. Combined use of antagonistic microorganisms with different mechanisms of action may improve efficacy and consistency of biocontrol agents (De Boer et al. 1999). Moreover, combination of ISR and SAR that results in an enhanced level of protection against specific bacterial pathogens (Van Wees et al. 2000) offers great potential to integrate both forms of induced resistance in agricultural practices. Induced resistance appears to be more useful for the management of viral diseases of plants where other management strategies are not generally successful (Fig. 2).

4.2 Root Colonization

Rhizosphere colonization is important not only as the first step in pathogenesis of soil borne microorganisms but also is crucial in the application of microorganisms for beneficial purposes (Lugtenberg et al. 2001). PGPR generally improves plant growth by colonizing the root system and pre-empting the establishment of, or suppressing deleterious rhizosphere microorganisms (Schroth and Hancock 1982). PGPR must be able to compete with the indigenous microorganisms and efficiently colonize the rhizosphere of the plants to be protected. Colonization is widely believed to be essential for biocontrol (Weller 1983; Parke 1991), and a biocontrol agent should grow and colonize the root surface. The ineffectiveness of PGPR in

the field has often been attributed to their inability to colonize plant roots (Benizri et al. 2001; Bloemberg and Lugtenberg 2001). Colonization or even initial population size of the biocontrol agent has been significantly correlated with disease suppression (Parke 1990; Bull et al. 1991).

Cell surface characteristics influence the attachment of bacteria to roots, which may be necessary for colonization (Vesper 1987; Anderson et al. 1988). Certain mutants that affect accumulation of secondary metabolites also influence colonization of roots in the field (Mazzola et al. 1992; Carroll et al. 1995). Analysis of mutants indicates that prototrophy for amino acids and vitamin B1, rapid growth rate, utilization of organic acids and lipopolysaccharide properties contribute to colonization (Lugtenberg et al. 1996). A variety of bacterial traits and specific genes contribute to colonization but only few have been identified (Benizri et al. 2001; Lugtenberg et al. 2001). These include motility, chemotaxis to seed and use specific components of root exudates, production of pili or fimbriae, production of specific cell surface components, ability of protein secretion, and quorum sensing (Lugtenberg et al. 2001). Competition of introduced bacteria with indigenous microorganisms already present in the soil and rhizosphere of the developing plant is another important aspect for root colonization.

4.3 Genetic Variations in the Host

Plants vary in their ability to support and respond to beneficial microorganisms (Handelsman and Stabb 1996). The ability to support certain biocontrol agents varies among plant species and among cultivars. Some plants appear to attract and support biocontrol agents, which are antagonistic to pathogens (Neal et al. 1973; Azad et al. 1985). Legumes vary in their response to *P. polymyxa* (Chanway et al. 1988), and *Bacillus* isolates from wheat roots enhanced growth of wheat in a cultivar-specific manner (Chanway et al. 1988). Plant species vary in their ability to induce genes for pyoluteorin biosynthesis in *P. fluorescencs* (Kraus and Loper 1995) probably because of variation in composition of root exudates among species. Moreover, different cultivars vary in terms of survival or disease incidence in the presence of a pathogen and biocontrol agent (Liu et al. 1995; King and Parke 1996). Strains of *P. fluorescens* that overproduce pyoluteorin and 2,4-diacetyl-phloroglucinol provide superior disease suppression compared with the parent strain in some host–pathogen combinations and not others, and effect correlate with host, and not pathogen, besides sensitivity to antibiotics (Maurhofer et al. 1995).

5 Interactions of PGPR in the Rhizosphere

Soil being a sink of microorganisms, therefore, influences the ability of introduced PGPR strain to interact with microbial community comprising beneficial and deleterious rhizospheric microorganisms.

5.1 Interactions with the Microbial Community

Many biocontrol agents suppress disease effectively in the laboratory but fail to do so in the field. These biocontrol agents may be affected by indigenous soil microbial communities and may also influence the community into which they are introduced. Certain fluorescent pseudomonads displace resident microflora in some cases reducing populations of deleterious microorganisms (Yuen and Schroth 1986). Manipulation of introduced PGPR populations may lead to enhanced suppression of other soil borne plant pathogens. Limited induced soil suppressiveness can also be achieved through stimulation in microbial community structure and function by several cultural practices (Kloepper et al. 1999). This may include the application of organic manures and plant straw (Siddiqui and Mahmood 2003; Siddiqui 2004; Siddiqui and Akhtar 2008c), inclusion of antagonistic plants in cropping systems and other integrated pest management approaches.

5.2 Interactions of PGPR Strains

In general, a single biocontrol agent is used for biocontrol of plant disease against a single pathogen (Wilson and Backman 1999). On the one hand, this may sometimes account for the inconsistent performance by the biocontrol agent, because a single agent is not active in all soil environments or against all pathogens that attack the host plant. On the other hand, mixtures of biocontrol agents with different plant colonization patterns may be useful for the biocontrol of different plant pathogens via different mechanisms of disease suppression. Moreover, mixtures of biocontrol agents with taxonomically different organisms that require different optimum temperature, pH, and moisture conditions may colonize roots more aggressively, improve plant growth and efficacy of biocontrol. Naturally occurring biocontrol results from mixtures of biocontrol agents rather than from high populations of a single organism. The greater suppression and enhanced consistency against multiple cucumber pathogens was observed using strain mixtures of PGPR (Raupach and Kloepper 1998).

Incompatibility of the coinoculants may sometimes arise and thus inhibit each other as well as the target pathogens (Leeman et al. 1996). Thus, an important prerequisite for successful development of strain mixtures appears to be the compatibility of the coinoculated microorganisms (Baker 1990; De Boer et al. 1997). A biocontrol product composed of a mixture of strains is more costly than a product composed of single strain due to increased costs of production and registration of such product. However, greater emphasis on the development of mixtures of biocontrol agents is needed, because they may better adapt to the environmental changes that occur throughout the growing season and protect against a broader range of pathogens. Mixtures of microorganisms may increase the genetic diversity of biocontrol systems that persist longer in the rhizosphere

and utilize a wider array of biocontrol mechanisms (Pierson and Weller 1994). Multiple organisms may enhance the level and consistency of biocontrol by a more stable rhizosphere community and effectiveness over a wide range of environmental conditions. In particular, combination of fungi and bacteria may provide protection at different times, under different conditions, and occupy different or complementary niches.

6 A Practical Control System Using PGPR

Selection of effective strains of bacteria is of prime importance for the biocontrol of plant pathogens. Isolation of bacteria from pathogen suppressive soils may increase the chances of finding effective strains (Cook and Baker 1983). The suppressive soil becomes apparent where the severity or incidence of disease is lower than the expected when compared with that in the surrounding soil (Cook and Baker 1983). To obtain effective strains, the isolation of bacteria should be conducted from the same environment in which they will be used (Weller et al. 1985). The ability to colonize roots and resistance against antibiotics are other parameters necessary to screen the effective strains (Siddiqui et al. 2005). Screening of biocontrol agents by a seedling bioassay chamber is required to determine the compatibility of an antagonist with the microflora of a field soil (Randhawa and Schaad 1985). Selection of field-effective strains can also be facilitated by a greenhouse assay. The important considerations in the development of the assays in the greenhouse are the inoculum potential of the pathogen (Weller et al. 1985), environmental conditions, and dose of the bacterium (Xu and Gross 1986). Many factors such as temperature, soil moisture, and soil texture influence the survival and establishment of bacteria. Formulation and application methods are often of paramount importance in effecting biocontrol (Papavizas and Lumsden 1980).

PGPR have great potential in the biocontrol of plant pathogens but the use of these rhizobacteria by farmers in the field is still lacking. The most obvious reasons for the limited use are the limited numbers of PGPR formulations available and inconsistent performance of these formulations. Mixtures of different strains are required to overcome inconsistency in the biocontrol performance. These mixtures of rhizobacteria may be used as seed treatment, which may be useful in reducing the quantity of bacterial inoculum required. This will facilitate systemic spread of the bacterial inoculum along the surface of the developing root system, and their antagonistic activity on the root surface during the early root infection by the pathogens. Rhizobacteria suspensions or formulations can also be mixed with organic manures in large vessels. They can be stored at 30–35°C for 5–10 days, mixing each day with water to keep them moist (Siddiqui and Mahmood 1999). Within 10 days, bacteria will attain high populations and this organic manure can be used at planting or after planting for the biocontrol of plant pathogens and better plant growth in the field.

7 Conclusion

Revelations about the mechanisms of PGPR action open new door to design strategies for improving the efficacy of biocontrol agents (Wang et al. 2000; Morrissey et al. 2004). Numerous studies have indicated that PGPR have great potential in the biocontrol of plant pathogens but most of the studies have been conducted in sterilized soil and in pots. There is an urgent need to conduct studies under field conditions. Colonization of root by PGPR is also important to increase their potential as biocontrol agents. Studies on the physical and chemical factors of soil, which affect root colonization, are needed. Moreover, use of mixture of effective strains of PGPR is advisable compared with use of single strain. The application of organic amendments with effective strains of PGPR is recommended because organic materials encourage the growth of organisms that compete with or destroy pathogens (Siddiqui and Mahmood 1999; Siddiqui and Akhtar 2008a, c). PGPR may also be used with fungal biocontrol agents and with arbuscular mycorrhizal fungi for greater beneficial effects. The absence of commercial interest in the biocontrol of plant pathogens by PGPR is also a major obstacle to progress. It is hoped that the future will see greater use of PGPR for the biocontrol of plant pathogens.

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Potential of Bacilli for Biocontrol and Its Exploitation in Sustainable Agriculture

Olga Susana Correa and Marcelo Abel Soria

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Abstract Plant diseases are caused mainly by fungi, bacteria, viruses, and nematodes, and their control is necessary to feed an increasing population. Control of plant diseases often rely on chemical pesticides, which have contributed to improvements in crop productivity and quality over the past years. However, the intensive use of agrochemical pesticides results in soil and groundwater pollution. Consequently, there are worldwide efforts to develop other alternatives to chemical pesticides for controlling plant diseases. Among them, the use of microorganisms and their products, referred as biological control, are regarded as promissory alternatives to reduce the use of chemical products. Different *Bacillus* species excrete peptides and lipopeptides to the culture medium, such as fungicine, iturin, bacillomicine and others, that have antifungal antibacterial and surfactant activity. In addition, these species produce spores that are resistant to heat and desiccation, which allows the preparation of more stable and durable formulations. A variety of biological control products based on *Bacillus* species are available for agronomical use; but in order to translate these developments into a broader and more effective use, a greater understanding of the complex interactions among plants, microorganisms, and the environment is required. This chapter describes some mechanisms of

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biocontrol exhibited by species of *Bacillus*, the current status of research and application of biological control using *Bacillus* species, constraints to microbial biocontrol implementation, and briefly outlines the future directions that might lead to the development of more diverse and effective biological controls for plant diseases.

1 Introduction

Agricultural production in the twenty-first century faces the challenge of increasing food production without negatively affecting the environment. The effective control of diseases is an essential component in every crop production system. To this end, resistant plant cultivars, cultural practices, and chemical applications are routinely used to provide disease control. Among these, the use of resistant cultivars and the careful management of cultural practices have the least aggressive effect on the environment. However, not every disease has a corresponding resistant or tolerant plant cultivar and through natural selection, the pathogens frequently overcome the resistance present in current cultivars in a few years (Cook 1993; Howarth 1991; Rusell 1995). Besides, cultural practices are not always economically or technologically feasible. Since World War II, numerous synthetic pesticides have been developed and successfully used for the control of crop pests and diseases. On the other hand, chemical pesticides also lose their effectiveness because of the development of genetic resistance in pathogen populations or they are banned or its use restricted by new regulations. In addition, available chemical pesticides are often expensive and also have adverse effects on human beings and the environment (Gupta 2004; Bortoli et al. 2009). So, in order to keep the pace of increase food demands, we need to search new solutions to control plant disease problems by alternative methods that result in effective control with minimum impact on humans, animals, and the environment.

Despite the synthetic pesticides dominating the phytosanitary market worldwide, their irrational selection and misuse have determined a decline in their use since 2000, thus increasing the need for new strategies of phytopathogen control. Therefore, biological control appears to constitute an appropriate alternative for controlling diseases in an environmentally friendly manner. Biological control can be defined as the use of one organism to reduce the population density of another organism and thus can include the control of animals, weeds, and diseases (Bale et al. 2008). The use of beneficial microorganisms for controlling plant diseases represents an environmentally friendly alternative to chemical pesticides, and can be used where conventional pesticides should be avoided because of residue concerns or in organic farming. Moreover, biological control can be applied together with chemicals in order to reduce the doses of chemicals and pathogen resistance, and as part of an integrated pest management (IPM) schema. The final goal is to minimize the use of synthetic pesticides.

Another undesirable effect of an excessive use of agrochemicals for management of plant diseases is their detrimental impact on the microbial biodiversity of the agroecosystems. Many of the chemical pesticides kill not only the target species of pathogen but also other non-harmful or beneficial organisms (Hanazato 2001). Two examples of beneficial microorganisms affected by chemical product meant to control pathogens are the nitrogen-fixing symbiotic bacteria and the fungi that form mycorrhizal associations with plants. In addition, during the past few years, there is a growing and widespread concern about the use of non-sustainable technologies for food production (Allen et al. 2008; Saifi and Drake 2008).

The use of bacteria as biocontrol agents has been extensively studied (Expert and Digat 1995; Asaka and Shoda 1996; Podile and Prakash 1996; Kim et al. 1997; Mao et al. 1997; Singh et al. 1998; de Vrije et al. 2001). Most of the bacterial biopesticides belong to the genera *Agrobacterium*, *Bacillus*, and *Pseudomonas* (Adesemoye et al. 2008). Currently, the contribution of biocontrol to plant health management is small but it is expected that it will increase in the next years (Ongera and Jacques 2008).

The process of developing biological control begins with in vitro and in vivo screenings that continues with the study of mechanisms of control such as competition, antibiosis, and induced systemic resistance. The next stage, the production of large amounts of efficient biomass at a low cost, requires studies of microbial physiology and the use of biotechnological processes. Adequate formulations and application methods have to be designed to ensure that the microbial biomass will attain a high level of biocontrol activity (Schisler et al. 2004). The legal registration procedures is usually the hardest part, it is a time-consuming and expensive process that must prove the effectiveness of the product and also that it does not entail any significant adverse effect on human health and the environment.

2 Mechanism Involved in Microbial Biological Control

The main mechanisms by which microbial biocontrol agents (MBCAs) can control other microorganisms are direct competition for space and nutrients, antibiosis or toxin production, predation or parasitism, and induced host resistance (Compant et al. 2005). Most MBCAs exhibit only one of these mechanisms, whereas some can use more than one. The molecular bases of biological control activity are diverse. Several biochemical pathways and gene regulatory networks are involved in the different processes that lead to pathogen control.

There are variations in the range of antibiotics between species and even within species, at the level of strains (Nagórska et al. 2007). This variability enhances the effectiveness of the use of *Bacillus* as a biocontrol tool, since the greater the spectrum of antifungals released the more difficult it becomes to the pathogen to adapt by natural selection. It also has another practical implication, because the life span of a product in the market can be expected to extend for several years before the target organism can develop genetic resistance. The same reasoning applies to

biosurfactant production: there are differences across strains in types and relative amounts of compounds produced, all of which can have differential activities against a diverse set of targets. Some strains have good surfactant and poor antibiotic biosynthetic activities and vice versa; since it has been postulated that both types of compounds act synergistically, it makes sense to combine both types of strains in the same product.

The genus *Bacillus* has several bacterial species that produce lipopeptides with biological actives for inhibiting plant pathogens (Ongera and Jacques 2008). These molecules have antagonistic activity against bacteria, fungi, and oomycetes. In Fig. 1 a clear antagonisms effect in a dual Petri dish culture is showed. *Bacillus amyloliquefaciens* strain BNM122 has showed high antagonistic activity both in vitro and in vivo against several fungi that cause plant diseases (Souto et al. 2004). The antagonistic fungal activity exhibited by strain BNM122 was related to the coproduction of iturin, which has antifungal activity and surfactin, which has surfactant properties (Souto et al. 2004).

Most of the biological activity of these compounds is related to their effect on the lipids of the cell membrane, where they can promote, depending on concentration, irreversible pore formation in the double layer of phospholipids (Fig. 2).

These antifungal peptides inhibit the growth of a large number of fungi, including *Aspergillus*, *Penicillium*, and *Fusarium* species (Munimbazi and Bullerman

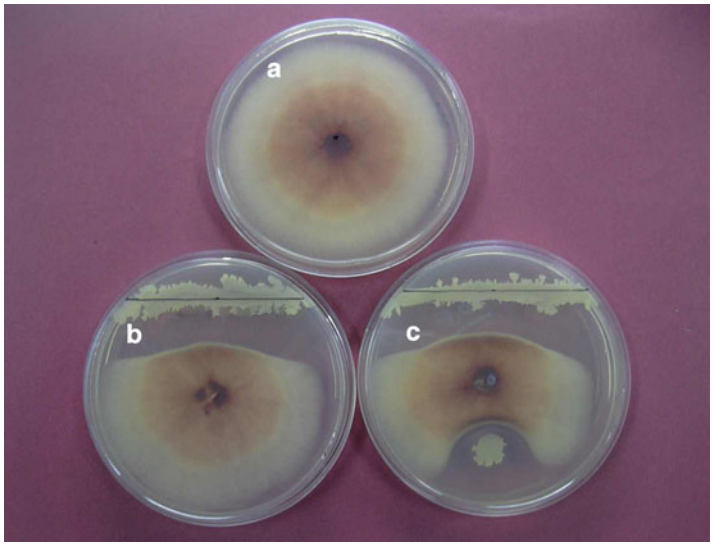

Fig. 1 In vitro antagonism of *Bacillus amyloliquefaciens* strain BNM122 against *Fusarium oxysporum* in dual culture on Petri dishes with potato dextrose agar medium. An inoculum of *F. oxysporum* was placed in the *middle* of the plates. (**a**) Growth of *F. oxysporum* with no bacterial inhibition; (**b**) Fungal growth inhibition by strain BNM122 streaked on one edge of the plate; (**c**) Fungal growth inhibition by strain BNM122 streaked (at the *top*) and spotted (at the *bottom*) on plate edges. Clear zones of fungal growth inhibition are observed toward the growth of BNM122

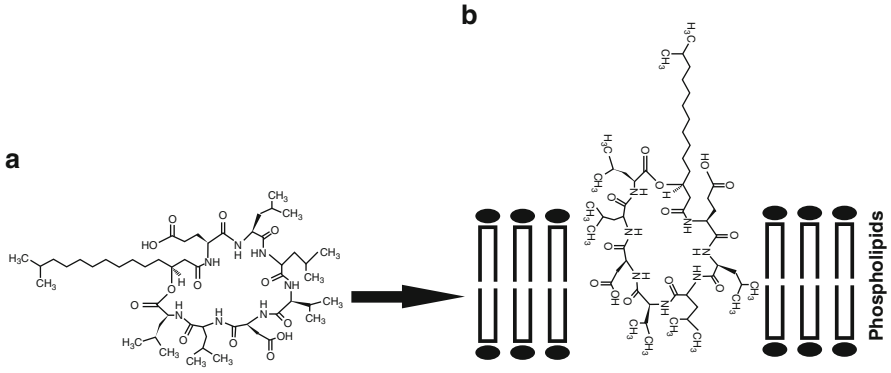

Fig. 2 Chemical structure of surfactin (**a**) and its action mode on fungal plasma membrane (**b**)

1998), as well as some yeasts (Thimon et al. 1995). In addition to their antagonistic activity against pathogens, *Bacillus* lipopeptides also have others more specific and important functions. Several of these compounds are involved in plant tissue colonization and in inducing plant resistance to phytopathogens (Ongera and Jacques 2008), whereas others like surfactin and mycosubtilin are important for bacterial surface motility and as wetting agents, reducing surface tension (Leclère 2006).

In the past few years, *Bacillus* species have also been proposed as biological control of plant parasitic nematodes belonging to the genera *Meloidogyne*, *Heterodea*, and *Rotylenchulus* (Tian et al. 2007). Nematodes cause great crop losses and are one of the most important agricultural pests. They are difficult to control because they inhabit the soil and attack the underground parts of the plants. Despite there are several chemical nematicides that are effective and easy to apply, they are being withdrawn from the market because of concerns regarding public and environmental safety. Hydrolytic enzymes such as proteases that degrade nematode cuticle are the main mechanisms involved in nematode biocontrol by *Bacillus* species (Lian et al. 2007). In Table 1, some of these useful substances and the *Bacillus* species that produce them are consigned.

3 Sustainability of Plant Disease Control Using Bacilli

Biological control products based on *Bacillus* species have huge potential in systems of IPM in order to reduce environmental contamination and to obtain safe and healthy foods. Cultural control, crop rotation, chemical pesticides, resistant host, and biocontrol agents are all part of IMP. Probably, one of the best known examples of integrated management is the application of fungicides and biocontrol agents for seed treatments. In the USA, almost all cotton planted is protected with Kodiak, a *B. subtilis* GB03 product, and fungicides. Biocontrol agents and fungicides in

Table 1 Bacterial species, the lipopeptide synthesized and main mechanism of action

Bacteria	Lipopeptide	Action as	References
B. subtilis	Bacillomycin	Antifungal	Peypoux et al. (1981)
B. subtilis and *B. amyloliquefaciens*	Fengycin	Antifungal/surfactant	Koumoutsi et al. (2004)
B. subtilis and *B. amyloliquefaciens*	Iturin	Antifungal	Delcambe (1965)
B. subtilis	Mycosubtilin	Antifungal/surfactant	Peypoux and Michel (1976)
B. cereus	Kanosamine, Zwittermycin A	Antifungal	Emmert and Handelsman (1999)
B. licheniformis, *B. coagulans*, *B. pumilis*	Lichenysin	Antifungal/surfactant	Huszcza and Burczyk (2006)

combination provide effective control of plant pathogens, which in many cases are not controlled by the available fungicides alone (Brannen and Kenney 1997). However, the potential use of lower fungicide doses when combined with a *Bacillus*-based biocontrol product has not been well explored yet, although some studies did report increases in disease control and reduction in chemical pesticide doses when bacilli-based products and chemicals are combined. In this sense, Cook et al. (2002) reported significant increases in winter wheat yield when *Bacillus* sp. strain L-324-92 was used in association with difeconazole plus mefoxam.

The integration of biological control *Bacillus*-based products with disease-resistant host plants should also be considered as part of IPM (Jacobsen et al. 2004). Published studies have demonstrated that the protective effect of *B. mycoides* Bm J against *Cercospora* leaf spot and that the control of *Fusarium* wilt with *B. subtilis* GB03 were more effective when the more resistant plant cultivar was used (Jacobsen et al. 2002; Hervas et al. 1998). These results highlight the importance of integrating several tools to gain stability in disease management programs. Also, mixtures of organisms with different modes of action may enhance the spectrum of activity, but unfortunately, there is limited knowledge and understanding of the interactions of such mixtures (Fravel 2005).

One of the main topics of discussion about the practical applications of biocontrol agents is how effective they are in real field applications. Since biological products can be very sensitive to environmental conditions, such as temperature, humidity, and sunlight exposure among other factors, they tend to be less stable than their traditional chemical counterparts. Ojiambo and Scherm (2006) conducted a statistical meta-analysis of 53 reports published between 2000 and 2005 that accounted for 149 combinations of target organism, biocontrol agent, plant host, and cultural treatments. These authors found that after normalization the range of observed results was quite wide, going from cases in which the biocontrol agent potentiated the pathogen to highly effective biocontrol. However, overall, the effect of biocontrol agents was positive and statistically significant. At a finer level of aggregation, they discovered that there were no differences in the effects observed between field or greenhouse conditions; soilborne or aerial diseases or when

Table 2 *Bacillus* species and strains; pathogens and diseases controlled, and host plants

Antagonistic species	Pathogen disease	Host plant	References
B. subtilis ZJY-116	*Fusarium* head blight	Wheat and barley	Zhang et al. (2005)
B. subtilis 6051	*Pseudomonas syringae* pv. tomato	*Arabidopsis*	Bais et al. (2004)
B. subtilis M4	Damping-off	Tomato, bean	Ongera et al. (2005)
B. subtilis RC8	*Fusarium verticillioides*	Maize	Cavaglieri et al. (2004)
B. subtilis AF1	Wilt in pigeon pea	Pigeon pea	Manjura and Podile (2001)
B. amyloliquefaciens MET0908	Anthracnose	Watermelon	Kim and Chung (2004)
B. amyloliquefaciens RC-2	Mulberry anthracnose	Mulberry	Hiradate et al. (2002)
B. amyloliquefaciens B94	*Rhizoctonia solani*–Damping-off	Soybean	Yu et al. (2002)
B. amyloliquefaciens BNM122	*Rhizoctonia solani*–Damping-off	Soybean	Souto et al. (2004)
B. cereus UW85	*Phytophthora megasperma*	Alfalfa	Handelsman et al. (1990)
B. mycoides BacJ	Cercospora leaf spot	Sugar beet	Bargabus et al. (2002)

considering the disease intensity. Interestingly, they did find a significant difference regarding host lifestyle: biocontrol agents were more effective controlling pathogens attacking annual plants compared to perennials. Some examples of bacilli reported as biological control agents of different plant diseases are shown in Table 2. Also, *Bacillus*-based products seemed less effective to other biocontrol species. However, a more detailed analysis showed that many experiments used commercial products that included strains of *Bacillus* species, probably due to its wide availability, even for conditions when they would not be recommended, such as treatment of aerial pathogens. A further test removing entries with potential misuse was performed, and it showed that *Bacillus*-based products were as effective as other biocontrol organisms. Another interesting conclusion from this meta-analysis study was that there were no differences between bacterial or fungal biocontrol agents, and that they could effectively control both bacterial and fungal targets. However, there was a significant difference between r- and K-strategists agents. Irrespective of whether they were fungal or bacterial, r-type organisms achieved greater controlling effects. Microorganisms with an r-strategy can divide very fast under favorable conditions and reach a high population size, a requisite for disease suppression.

Although *Bacillus*-based products are more effective controlling soil-borne pathogens compared to aerial targets, there is an interest among researchers in finding and overcoming the factors hindering the development of foliar formulations. The discoveries in this field can mutually benefit with those related to the use of microorganisms as biopesticides. The work of Ojiambo and Scherm (2006) found that one or two spray applications can be enough for controlling an aerial

microbial target, a convenient rate of application from an economical and management point of view. However, Gan-Mor and Matthews (2003) pointed out that the configuration and handling of field equipment requires special attention in the case of biopesticide, because the optimal settings and adequate procedures are different from those recommended for chemical products. These observations suggest that some failures in controlling aerial pathogens could be caused by improper handling and not only by strictly biological factors. Clearly, the successful deployment of biological control agents in the field needs specialized training of farm personnel.

4 Causes That Restrict the Adoption of Biological Control

Despite the advantages of biological control for a sustainable agriculture, few products are commercially available. There are many reasons for this; one is the difficulty to obtain a formulation with a good shelf live, others are the lack of knowledge about their modes of action, and the differences among regulatory policies in different countries that complicate the inscription process. Sporulating Gram positive bacteria, like those belonging to the genus *Bacillus*, offer a solution to the formulation problem. Their spores have high resistance to dryness which constitutes and advantage for the production of certain classes of commercial products. The spores can be formulated as dry powders and can be stored for a long period of time without loss of concentration and effectiveness (Emmert and Handelsman 1999).

Another limitation to the extensive use of biocontrol agents is that most of the research reports focus only on the control of the target pathogen without further investigations on their impact on the agroecosystem and the environment in general. Some exceptions are the studies made with *B. cereus* UW85, a biocontrol agent of *Phytophthora* damping-off and root rot of soybean in the USA (Osburn et al. 1995). For this organism, researchers have studied the basis for disease biocontrol, the interaction with the plant, with the pathogen, and also the impact of strain UW85 on soil microbial communities (Handelsman et al. 1990; Silo-Suh et al. 1994; He et al. 1994; Gilbert et al. 1993; Halverson et al. 1993; Milner et al. 1995). In the same research line, Souto et al. (2004) and Correa et al. (2009) have studied the mechanisms of action of *B. amyloliquefaciens* strain BNM122 and their impact on the microbial community of soybean rhizosphere. Using culture-dependent and -independent methods, the authors demonstrate that this bacterium have a lower impact on soil microbial communities and non-target microorganisms than that exhibited when a chemical fungicide was applied (Fig. 3).

Soybean plants, whose seeds had been inoculated with *B. amyloliquefaciens* strain BNM 122 or treated with fungicides did not show differences in plant growth (mg pl^{-1}) and nodulation (nodules per plant) but a significant reduction was observed in the mycorrhizal symbiosis (Fig. 3). The important reduction observed in this beneficial non-target fungal symbiosis can be attributed to the wide spectrum

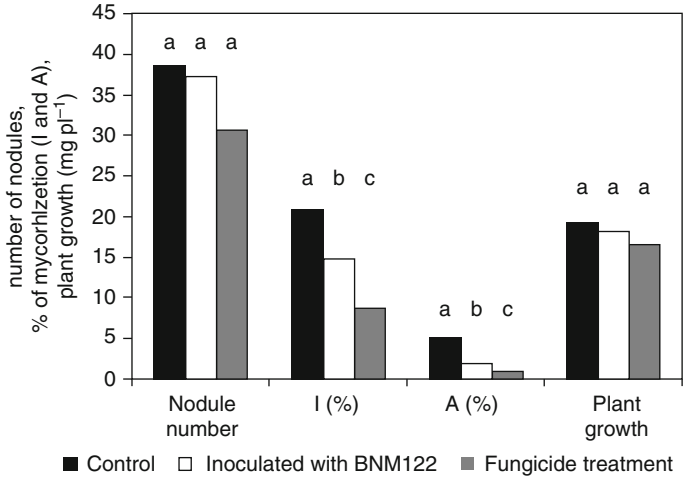

Fig. 3 Effect of seed inoculation with strain BNM122 on soybean nodule number, percentage of intensity and abundance (*I%* and *A%*) of mycorrhization, and plant growth (mg pl^{-1}). Plants were grown in pots, in greenhouse, under natural condition of light and temperature. Before sowing, all seeds were inoculated with the nodulating nitrogen-fixation bacteria *Bradyrhizobium japonicum* Treatments were non-inoculated seeds (control), inoculated with the biocontrol strain BNM122, and seeds treated with a mixture of chemical fungicides (carbendazim and thiram). Different *letters* indicate significant differences ($p < 0.05$) among treatments. Adapted from Correa et al. (2009)

of action displayed by both treatments. However, it is worth pointing out the lesser negative effect on root mycorrhization exhibited by the *Bacillus* biocontrol agent.

5 Conclusions and Future Considerations

Although scientists have been working for more than 50 years on biological control and IPM systems, the commercial importance of the business grows slowly and biological control products represent less than 2% of the plant protection market worldwide (Kiewnick 2007). If our objective is to deploy environmentally sounder alternatives to plant disease control, research in biological control should be well supported and funded. We have antecedents of how well some bacterial biocontrol agents have performed in the past. The success of *B. thuringiensis* and *B. sphaericus*, two larvicides, that have been successfully used to replace DDT and malathion. Both have proved to be extremely effective and to pose lower human health and environmental risk and to be useful for resistance management (Grisolia et al. 2009). All in all, it is important to bear in mind that these products do not have the efficacy of chemical counterparts; although in many occasions biological control is a valuable complement of chemical protection. For these reasons, the use of biological control pesticides is expected to increase in the coming years,

especially in developing countries, together with the application of integrated disease management schemas. Despite the great interest in the exploitation of MBCAs, there are still barriers that impede their adoption in agriculture. Among them, the main obstacles are the long time for product registration and the slow adoption of new developments in a market more accustomed to chemical alternatives (Marrone 2007).

In the near future, the greater sustainability and lesser environmental risks associated with MBCAs will be factors balancing their lower performance and will become drivers in the search of better and diversified products, to enhance competitiveness and greater market penetration (Bailey et al. 2010). This will require government support such as tax benefits and incentives. Also of great importance is to increase the level of biocontrol-related education, to ensure the availability of detailed extension information and the training of distributors and farmers.

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Plant Growth Promoting Rhizobacteria as Biocontrol Agents Against Soil-Borne Plant Diseases

Nico Labuschagne, T. Pretorius, and A.H. Idris

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Abstract Soil-borne diseases are responsible for major crop losses worldwide. Alternatives to the use of synthetic chemicals for disease control are increasingly being sought due to among other reasons, the detrimental effects of these compounds on the environment. In this chapter, biological control of soil-borne plant diseases by means of plant growth promoting rhizobacteria (PGPR) is reviewed with emphasis on cereals. The concepts and definitions of PGPR, biocontrol agents, biopesticides, biofertilizers, and soil inoculants are discussed and overlap between these categories are illustrated. Advantages and disadvantages of the use of PGPR

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as biocontrol agents are mentioned. Biocontrol of soil-borne diseases of crops is discussed and illustrated by means of specific examples of effective application of growth promoting rhizobacteria for control of soil-borne pathogens on cereals such as wheat and sorghum. The modes of action of PGPR with biocontrol activity is discussed with reference to the production of antibiotics, siderophores, and cell wall degrading enzymes as well as induction of systemic resistance, root colonization efficacy, and rhizosphere competence.

1 Introduction

Research in the area of plant growth-promoting rhizobacteria (PGPR) has opened up a fascinating world of remarkable diversity not only in terms of the rhizobacteria but also in terms of the multifaceted beneficial plant–microbe interactions and effects involved. These interactions and effects encompass both enhancement of plant growth directly and indirectly through biological control of plant pathogens.

From the volume of scientific publications appearing on the topic of biocontrol by means of PGPR, it is evident that this is an active and growing field of science. Some of the reasons for the sustained interest in PGPR and also biocontrol by means of PGPR include the following:

(a) Huge crop losses sustained due to diseases including soilborne diseases
(b) The increase in production costs, especially fertilizer costs
(c) The global trend toward the use of more environmentally friendly production methods

Huge amounts of money are being spent on application of synthetic pesticides to control soilborne diseases worldwide. The application of rhizobacteria that colonize the roots of crop plants and suppress soilborne diseases is becoming an alternative choice to the use of chemical fungicides because of increased environmental and health concerns as mentioned earlier (Raupach and Kloepper 1998; Walsh et al. 2001; Kobayashi et al. 2002). The use of PGPR as soil inoculants for control of soilborne diseases, therefore, constitutes a viable biological alternative.

Rhizobacteria with biocontrol efficacy often provide long-term protection from soilborne pathogens at the root surface because they are often rhizosphere competent, that is, they have the capacity to rapidly colonize the rhizosphere and spread down the root from a single seed treatment or drench application into the soil (Rangarajan et al. 2003; Whipps 2007).

The literature on PGPR is voluminous and in the last 10 years there have been more than 26 reviews, including some chapters in books, on the topic of biocontrol by means of rhizobacteria. However, many of these reviews did not only deal with PGPR as biocontrol agents but also discussed micro-organisms other than PGPR (Avis et al. 2008; Compant et al. 2005; Fravel 2005; Lucy et al. 2004; Pielach et al.

2008; Preston 2004; Raaijmakers et al. 2008; Whipps 2001; Zahir et al. 2004). The review by Lucy et al. (2004) gives an extensive summary of examples of free-living PGPR tested on various crop types.

The current chapter is focused on biological control of soilborne diseases and mechanism of biocontrol agents (PGPR) with special emphasis on soilborne diseases of cereals.

1.1 Concepts and Definitions

At the outset, it is necessary to clarify some key concepts and definitions. In the literature on PGPR and biocontrol agents (BCA), different definitions and classifications are being used. PGPR as a group of free-living bacteria occupying the rhizophere and rhizoplane of plants finds itself in between a number of different groupings. Some authors consider PGPR and BCAs to be separate groups while others consider BCAs to be a subgroup of PGPR. Bashan and Holguin (1998), for example, proposed the division of PGPR into two classifications namely "biocontrol-plant growth promoting bacteria" and "plant growth promoting bacteria." Clearly there is overlap between these groupings. For example, a PGPR can have plant growth enhancing activity as its primary effect and as its secondary effect, it reduces the disease by enabling the plant to outgrow and thereby "escape" the disease. However, there are many specific examples of PGPR with direct biocontrol activity as will be discussed later in this chapter.

In addition to PGPR and BCAs, there are also the classifications of "biofertilizers," "biopesticides," "biofungicides," and "soil inoculants." Depending on the definition one ascribes to, some PGPR can be classified as biofertilizers (purely enhancing plant growth), biocontrol agents (suppressing or controlling plant disease), and biopesticides (controlling plant pests). "Soil inoculants" are mostly used as a general term for biological products (microbials), which are applied to the soil.

We define PGPR as a group of free-living rhizosphere occupying bacteria that enhances plant growth and can also be classified as biocontrol agents, biofertilizers, or biopesticides, depending on their activities/abilities. We concur with the definition of Menn and Hall (1999) for biopesticides as microbials or products derived from microbes, plants, and other biological entities, applied for control of plant pests. Furthermore, we concur with the definition for biofertilizers proposed by Vessey (2003) as a substance that contains living microorganisms, which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply and availability of primary nutrients to the host plant. In the current review, we define biocontrol agents more specifically as microbials capable of suppressing or controlling plant diseases. The relationships and overlap between some of these groupings are illustrated in the proposed model in Fig.1.

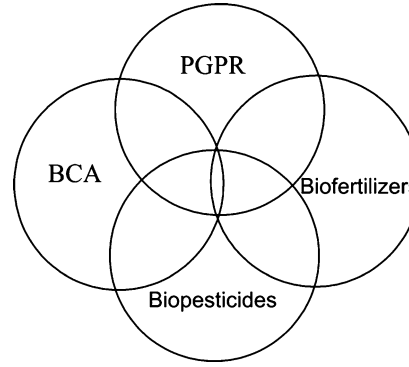

Fig. 1 Proposed model illustrating the relationships/ overlaps between PGPR, BCAs, biopesticides, and biofertilizers

1.2 Advantages and Disadvantages

PGPR as biocontrol agents have certain advantages over conventional chemical control compounds. Firstly, PGPR are beneficial, naturally occurring micro-organisms, which are environmentally friendly and nontoxic. Secondly, from an ecological perspective, their application is sustainable (long term). Another advantage of PGPR is the fact that they possess a diverse range of modes of action including antibiosis, production of siderophores, cell wall degrading enzymes, bio-surfactants and volatiles, and also induces systemic resistance in plants. The fact that some PGPR by definition directly enhances the growth of plants is an additional advantage.

There are also, however, certain disadvantages to the use of PGPR based BCAs compared with conventional chemical control compounds. Firstly, being live micro-organisms, they are more sensitive to environmental conditions such as temperatures, soil conditions desiccation, etc. Shelf life of commercial PGPR or BCAs in general is shorter than that of the chemical pesticides or fungicides. Secondly, and probably the most important disadvantage, is the fact that up to now, efficacy of PGPR and BCA in general has been inconsistent under field conditions. Many scientific publications report effective biocontrol under environmentally controlled conditions in vitro or in greenhouses, but much fewer data exist regarding efficacy under field conditions. However, this does not detract from the fact that PGPR as BCAs is constantly becoming more effective as researchers are gaining more knowledge on the factors and mechanisms involved in biological control of plant diseases by means of PGPR and the factors that play a role in biocontrol of plant diseases.

Another area for application of PGPR as BCAs is that of formulation and application of the commercial product. Formulating a live micro-organism into a commercial product in such a way that it remains viable and that it can be applied by growers on a large scale is evidently more difficult than formulating a chemical pesticide.

We concur with the view of various other authors that biological products, be they BCAs or biopesticides, should not be seen as replacements for chemical

pesticides on agricultural crops, but rather as important components of an integrated disease control program.

2 Biocontrol of Soilborne Diseases by Means of PGPR with Emphasis on Cereal Crops

The importance of soilborne diseases is indirectly illustrated by the fact that soil fumigation often results in increases in production of between 7 and 100%, for example, in wheat (Cook 1992), although other factors apart from disease control are also involved in this phenomenon. Soilborne diseases affect all crops and encompass the whole spectrum of plant pathogens including fungi, bacteria, and nematodes. Several groups of soilborne fungi attack most of the economically important crops causing infection resulting in huge yield losses (Gohel et al. 2006).

Cereals are as much affected by soilborne diseases as any other crop. Crown rot of wheat and barley in the Pacific Northwest in the US, for example, caused by a complex consisting of mainly *Fusarium* spp, can cause yield losses of up to 35% in commercial fields (Smiley et al. 2005). The economic as well as socioeconomic importance of cereals, such as wheat (*Triticum aestivum* L), rice (*Oryza sativa*), and maize (*Zea mays* L), which the most important crops worldwide, makes control of cereal root diseases a priority.

There are many examples of effective control of soilborne diseases by means of PGPR (Whipps 2001; Lucy et al. 2004), and many bacterial strains have been shown to have potential for development as biocontrol agents on cereals (Table 1). The biocontrol potential of *Bacillus* spp. as important agents to combat root and soilborne pathogens has been reported in many crops including chickpea (Landa et al. 1997). Several *Bacillus s*pp. isolated from the rhizosphere of chickpea had shown antagonistic activity against fusarium wilt caused by *Fusarium oxysporum.* Similarly, several strains of *Bacillus* spp. isolated from the rhizosphere of sorghum in Ethiopia and wild grass spp. in South Africa have shown effective biocontrol of the root and crown rot pathogens *F. oxysporum* and *Pythium ultimum*, respectively, in sorghum under greenhouse conditions (Figs. 2 and 3) (Idris et al. 2007, 2008). Effective control of crown and root rot of wheat, caused by *F. oxysporum*, has been achieved with a strain of *Paenibacillus alvei* in South Africa (Labuschagne and Idris, unpublished data) (Fig. 4). Apart from disease control, this strain has also been demonstrated to induce about 40% increase in wheat shoot mass in the absence of pathogens. On the basis of this and other data, *P. alvei* strain has been included together with another PGPR strain in a commercial product, which is being marketed as a soil inoculant in South Africa under the trade name BacUp®.

Several commercial biocontrol products are currently available on cereals and a variety of other crops (Coping 2001; McSpadden and Fravel 2002) and new products are constantly entering in the market. Although there are several PGPR products available as soil inoculants on cereals, most of these are marketed as biofertilizers and not as biocontrol agents (Ryder et al. 1999).

Table 1 Examples of biocontrol of fungal plant pathogens on cereal crops by means of rhizobacterial application

Biocontrol agent	Plant pathogen	Crop	Mode of action of biocontrol agent	Reference
Pseudomonas fluorescens	*Microconidium nivale*/ *Fusarium nivale*	Wheat	Growth promotion, siderophore production, in vitro antibiosis	Amein et al. (2008)
Acinetobacter, Pseudomonas, Staphylococcus, Bacillus, Enterobacter, Pantoae, Alcallgenes	*Fusarium oxysporum, F. culmorum, F. solani, Pythium ultimum, Alternaria alternate, Botrytis cinerea, Phytophtora cryptogea*	Wheat	Antagonism and growth promotion	Egamberdieva et al. (2008)
Pseudomonas fluorescens	*Fusarium culmorum*	Rye	Fe(III) chelating compounds (including siderophores)	Kurek and Jaroszuk-Scisel (2003)
Bacillus sp. L324-92	*Gaeumannomyces graminis* var *tritici*, Rhizoctonia root rot, R. *solani* AG8, Pythium root rot, *Pythium irregulare P. ultimum.*	Wheat	Not specified	Kim et al. (1997)
Bacillus subtilis and *B. cereus*	Take all (*G. graminis* var *tritici*) Rhizoctonia root rot (R. *solani* AG8)	Wheat	Growth promotion	Ryder et al. (1999)
Bacillus spp, *Pseudomonas fluorescens*	*G. graminis*, *R. solani*, *R. oryzae*, *P. ultimum*,	Wheat	Not specified	Cook et al. (2002)
Bacillus subtilis CE1	*Fusarium verticilloides*	Maize	Not specified	Cavaglieri et al. (2005)
Pseudomonas chlororaphis	*Macrophomina phaseolina* (charcoal rot of sorghum)	Sorghum	Extracellular antibiotics, production of volatiles, siderophores, effective root colonization	Das et al. (2008)
	Fusarium oxysporum	Sorghum		Idris et al. (2007)

Bacillus stearothermophilus, *B. cereus*, *B. licheniformis*, *B. circulans*, *Chromobacterium violaceum*			Antibiotic production, chitinolytic enzymes, efficient root colonization	
Bacillus cereus, *Brevibacterium laterosporus*, *Pseudomonas fluorescens*, *Serratia marcescens*	*Pythium ultimum*	Sorghum	Antibiotic production, siderophores, induction of systemic resistance	Idris et al. (2008)
Pseudomonas fluorescens MKB 100 and MKB 249, *P. frederiksbergensis* 202, *Pseudomonas* spp. MKB 158	*Fusarium culmorum*	Wheat and barley	Induced resistance, antibiotic production, pathogenesis related proteins (induced resistance) in wheat	Khan et al. (2006)

[a]Additional examples of biocontrol agents on other crops can be found in the reviews by Whipps (2001) and Lucy et al. (2004), the latter review including a comprehensive table of isolates reported to be effective against soilborne pathogens of several crops

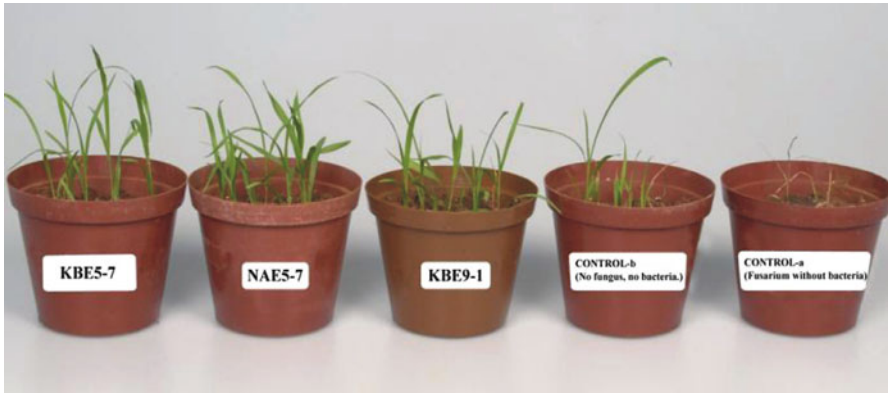

Fig. 2 Greenhouse experiment illustrating the efficacy of three *Bacillus* strains for biocontrol of root and crown rot of sorghum caused by *F. oxysporum*. All plants treated with *F. oxysporum* alone died (Control a, *far right*) whereas 100% of the plants inoculated with both the pathogen and Bacillus isolates KBE5-7, NAE5-7, and KBE9-1 survived, showing no symptoms of infection. (Adopted from Idris et al. 2007)

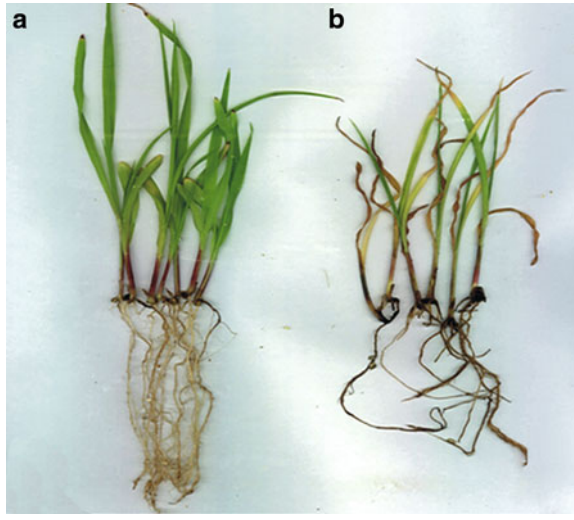

Fig. 3 Example of suppression of *P. ultimum* root rot in 4-week-old sorghum seedlings by bacterial strains isolated from the rhizosphere of wild grasses in South Africa. (**a**) Plants inoculated with *P. ultimum* and treated with rhizobacterial isolates. (**b**) Control plants that were treated only with *P. ultimum* developed visible root rot and necrotic leaves. (Adopted from Idris et al. 2007)

However, most putative biocontrol agents fail in the field. Factors that can affect biocontrol efficacy in the field include effects of the environment, ecological factors, and difficulties in production, formulation, and delivery of BCAs. Suggested solutions to overcome these constraints include combination of BCAs with

Fig. 4 Effective control of *F. oxysporum* crown and root rot of wheat with a strain of *Paenibacillus alvei* in the greenhouse (Labuschagne N and Idris A H, unpublished data). Plants on the *left*: inoculated with *F. oxysporum* and treated with *Paenibacillus alvei*. Plants in the *middle*: pathogen free (uninoculated) and untreated. Plants on the *right*: inoculated with *F. oxysporum* only

chemical pesticides/fungicides, modification of agronomic practices, application of BCA mixtures, and genetic manipulation. The efficacy of biocontrol is also affected by the screening and sourcing protocol used in the development of BCAs (Spadaro and Gullino 2005; Fravel 2005). It has been shown that root colonization is an important aspect, which has a determinative impact on biocontrol efficacy (Van Bruggen et al. 2008).

The outcome (i.e., success) of a biocontrol agent treatment depends on the following:

1. The method of inoculation/application
2. The physiological state of the BCA
3. The concentration and dosage of the BCA
4. The presence or absence of nutrients
5. The presence or absence of adjuvants such as adhering or protective agents (Knudsen et al. 1997)
6. The media used for BCA production
7. The volume of treatment (Levenfors et al. 2008)
8. The plant type and cultivar. Both plant and cultivar specificity has been observed for some BCAs (Khan et al. 2006)

Other indirect factors include the effect of fungi on BCA colonization as reported for wheat roots (Mazzola and Cook 1991) and host plant–BCA interaction (Lugtenberg et al. 2002). Consideration should also be given to the effect of the

BCA application on the microbial ecology and occurrence of phenomena such as disease replacement where a particular root disease is controlled but another takes its' place (Kim et al. 1997).

3 Modes of Action of PGPR as Biocontrol Agents

For successful and sustainable biocontrol under field conditions, it is imperative that the mode of action of the BCA strains being used is known. The mode of action involved will be a determining factor in the type of disease control strategy to be implemented.

3.1 Production of Antifungal Metabolites

PGPR including those associated with cereal crops produce various types of antifungal metabolites capable of reducing or suppressing infection by pathogenic fungi in several crops (Ongena et al. 1999; Bloemberg and Lugtenberg 2001; Raaijmakers et al. 2002).

3.1.1 Antibiotics

Antibiosis is an attractive and a highly effective mode of action of rhizobacteria in the suppression of soilborne infections in a number of crops (Handelsman and Stab 1996). Most biocontrol strains of PGPR produce one or several groups of antibiotics, which inhibit fungal pathogens (Haas and Defago 2005). Antibiotics produced by these biocontrol PGPR reduce or suppress soilborne infections of cereal crops including wheat, rice, maize, chickpea, and barley (Raaijmakers et al. 2002). Some of these antibiotics cause membrane damage to pathogens such as *Pythium* spp. and inhibit zoospores formation (de Souza et al. 2003). Others such as the phenazines inhibit electron transport in disease causing organisms and also act by damaging lipids and other macromolecules (Haas and Defago 2005).

Genetic analysis of many biocontrol strains of *Pseudomonas* indicated that there is a positive correlation between disease suppression and antibiotic production (Vincent et al. 1991). It was demonstrated that with increasing populations of *Pseudomonas* spp., which produce the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG), there was a rapid decline in take-all disease in wheat caused by the fungus *Gaeumanomyces graminis* var. *tritici* (Raaijmakers and Weller 1998; de Souza et al. 2003). The production of phenazine-1-carboxylic acid (PCA), another group of antibiotics by *Pseudomonas fluorescens* and *Pseudomonas aureofaciens* strains, has also been described elsewhere. Bacterization of wheat seeds with *P. fluorescens*

strains 2–79 producing the antibiotic PCA resulted in significant suppression of take-all in about 60% of field trials (Weller 2007).

There is a growing list of reports of *Bacillus* spp. as biocontrol agents in various crops. Kim et al. (1997), for instance, isolated and discovered a potential biocontrol strain, *Bacillus* sp. L324-92, with a broad spectrum inhibitory activity against take-all, root rot caused by *Rhizoctonia. solani*, *Pythium irregulare*, and *Pythium ultimum*. In other experiment (El-Meleigi et al. 2007), treatment of spring wheat seeds with antibiotic producing strains of *Bacillus* spp. has been reported as a powerful tool to control root rot causing fungal pathogens in dry land fields. According to this work, application of *Paenibacillus polymyxa* to wheat seeds suppressed infection by root rot pathogens *Fusarium graminearum* and *Cochliobolus sativum*.

The potential uses of antibiotic producing PGPR as biocontrol agents have been reported in many other cereals including maize, sorghum, rice, and chickpea. In maize for instance, *Fusarium verticilloides*, causing root rot and yield loss, has been significantly suppressed by the application of *Bacillus amyloliquifaciens* as seed treatment (Pereira et al. 2009). Von der Weid et al. (2005) recently described *Paenibacillus brasilensis* PB177, a new strain isolated from the rhizosphere of maize in Brazil that produces antimicrobial substances suggesting that it could be a potential biocontrol agent in the rhizosphere of maize.

In another biocontrol experiments, Idris et al. (2007, 2008) demonstrated the bio-control of *F. oxysporum* and *Pythium ultimum* on sorghum with *Bacillus* spp. (mentioned under point 2 earlier in this chapter). It was demonstrated that the bacterial strains produce antimicrobial metabolites, possibly antibiotics, which suppressed the growth of the fungal pathogens in vitro (Fig. 5).

3.1.2 Siderophores

Biocontrol PGPRs also exert their antagonistic activity against plant pathogens by means of secretion of siderophores. These low molecular weight compounds

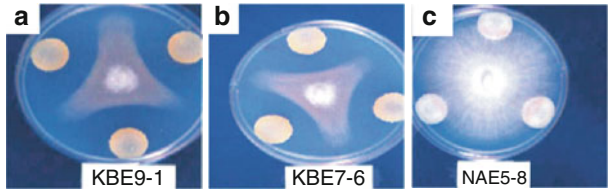

Fig. 5 Dual culture assay for screening of biocontrol agents based on the production of antifungal metabolites in agar plates. *Bacillus* strains KBE9-1, KBE7-6, and NAE5-8 were inoculated in three equidistant positions at the margin of Potato Dextrose Agar (PDA) plates with *F. oxysporum* agar block placed in the centre. The growth of the fungal mycelium was significantly inhibited by strains KBE9-1 (**a**) and KBE7-6 (**b**) showing prominent inhibition zones on the plates. Strain NAE5-8 does not produce any inhibition zones, indicating the absence of antifungal metabolites (**c**). (Adopted from Hassen 2007, PhD thesis)

(400–1, 500 Da) preferentially chelate iron (Fe^{+++}) and transport it into the cell across the cell membrane (Neilands 1995; Wandersman and Delepelaire 2004). The siderophores bind most of the Fe^{+3} in the rhizosphere and effectively prevent the proliferation of fungal pathogens by depriving them of available iron (Kloepper et al. 1980; O' Sulivan and O' Gara 1992). Suppression of the pathogens arises because iron deficiency causes growth inhibition, decrease in nucleic acid synthesis, inhibition of sporulation, and causes changes in cell morphology (Mathiyazhagan et al. 2004).

Among the biocontrol rhizobacteria, the fluorescent *Pseudomonas* spp. are efficient competitors for iron (Fe^{+3}) in the rhizosphere of various crops producing two major types of siderophores: the fluorescent pigmented pyoverdins (pseudobactins) (Lemanceau et al. 1993) and the nonfluorescent siderophore called pyochelins (Leeman et al. 1996). Siderophores produced by certain strains of the *P. fluorescens-putida* group are responsible for enhanced plant growth and biocontrol and are most often associated with fungal suppression in the rhizosphere of several crops (Battu and Reddy 2009). According to these workers, siderophore mediated the suppression of rice fungal pathogens *R. solani* and *Pyricularia oryze* in an in-vitro assay on Kings-B medium. Earlier, Becker and Cook (1988) reported the role of siderophores produced by *Pseudomonas* strain B324 in the suppression of *Pythium* root rot of wheat. Mutants deficient in pyoverdins production are less effective than parental strains in suppression of fungal pathogens (Loper and Henkels 1999). It is thus believed that siderophore production is another important mechanism by which some strains of bacteria protect plants against root pathogens.

3.1.3 Cell Wall Degrading Enzymes

One of the major mechanisms used by biocontrol agents to control soilborne pathogens involves the production of cell wall degrading enzymes (Chet et al. 1990; Kobayashi et al. 2002). Cell wall degrading enzymes such as β-1, 3-glucanase, chitinase, cellulase, and protease secreted by biocontrol strains of PGPR exert a direct inhibitory effect on the hyphal growth of fungal pathogens. Chitinase and β-1,3-glucanase degrade chitin, an insoluble linear polymer of β-1,4-N-acetylglucoseamine, which is the major component of the fungal cell wall.

The β-1, 3-glucanase synthesized by strains of *Paenibacillus* and *Streptomyces* spp. lyse fungal cell walls of pathogenic *F. oxysporum*. In a similar manner, *Bacillus cepacia* synthesizes β-1,3-glucanase, which destroys the cell walls of the soilborne pathogens *R. solani, P. ultimum*, and *S. rolfsi* (Compant et al. 2005). Potential biocontrol agents with chitinolytic activities include *B. licheniformis*, *B. cereus*, *B. circulans*, and *B. thuringiensis* (Sadfi et al. 2001). Among the Gram-negative bacteria, *Serratia marcescens*, *Enterobacter agglomerans*, *Pseudomonas aeruginosa*, and *P. fluorescens* have been found to have chitinolytic activities (Nelson and Sorenson 1999).

Cell wall degrading enzymes of rhizobacteria affect the structural integrity of the walls of the target pathogen (Budi et al. 2000). Someya et al. (2000) studied the chitinolytic and antifungal activities of a potent biocontrol strain of *S. marcescens* B2 against the soilborne pathogens *R. solani* and *F. oxysporum*. The mycelia of the fungal pathogens coinoculated with this strain showed various abnormalities such as partial swelling in the hyphae and at the tip, hyphal curling or bursting of the hyphal tip. Examples of protection from phytopathogenic infection as a result of the activity of cell wall degrading enzymes include control of *Sclerotium rolfsii* and *F. oxysporum* on beans (Felse and Panda 1999).

3.2 Induction of Systemic Resistance

Induced systemic resistance (ISR) is the state of defensive capacity developed by the plant when stimulated by diverse agents including rhizobacteria (van Loon et al. 1998). Once resistance is induced in plants, it will result in nonspecific protection against pathogenic fungi, bacteria, and viruses (Silva et al. 2004). The mode of action of disease suppression by nonpathogenic rhizosphere bacteria should be distinguished from pathogen induced systemic acquired resistance (SAR) (Bakker et al. 2003). Colonization of the plant root system by rhizobacteria can indirectly lead to reduced pathogen attack through induction of systemic resistance (Kloepper and Beauchamp 1992). PGPR elicit ISR in plants by increasing the physical and mechanical strength of the cell wall as well as changing the physiological and biochemical reactions of the host. This results in the synthesis of defense chemicals such as chitinase, peroxidase, and pathogenesis-related proteins (Ramamoorthy et al. 2001; Nandakumar et al. 2001; Silva et al. 2004).

In rice, *P. fluorescens* strains showed inhibitory effect on the mycelial growth of *R. solani* by inducing resistance in the plant (Radjacommare et al. 2004). The bacteria induced resistance against the sheath blight fungus by activating chitinase genes in rice (Nandakumar et al. 2001). Another biocontrol PGPR, *S. marcescens* strain B2, which inhibits several soil borne pathogens including *F. oxysporum* under greenhouse conditions, could not inhibit the same pathogens in a dual culture assay indicating that this is due to the induction of systemic resistance (Someya et al. 2002).

In beans, *P. aeroginosa* ISR against infection by *Collehotricum lindemuthianum* (Bigirimana and Hofte 2002). Benhamou et al. (1996) investigated ISR in *Pisum sativum* and found that pea roots inoculated with *P. fluorescens* strain 63–28 produced more chitinase at the site of penetration by *F. oxysporum* f. sp. *pisi*. Several strains of *Bacillus* spp. also have the capacity to induce systemic resistance in various crops against a wide range of pathogens. *Bacillus subtilis* AF1 isolated from soils suppressive to pigeon pea (*Cajanus cajan*) wilt caused by *Fusarium* sp. caused lysis of *Aspergillus niger* by stimulating the production of phenylalanine ammonia lyase and peroxidase by the plant thereby eliciting induction of systemic resistance (Kloepper et al. 2004).

Similar to other modes of action, rhizobacterial-mediated ISR can be an important additional means of environmentally friendly plant disease control (van Loon et al. 1998).

3.3 Root Colonization and Rhizosphere Competence

Root colonization is an important prerequisite for bacteria to be considered as true PGPRs, and it is commonly believed that a biocontrol agent should colonize the rhizosphere and the surface of the plant it protects (Silva et al. 2003; Handelsman and Stab 1996; Benizri et al. 2001). Therefore, any given PGPR is often ineffective as a biocontrol agent against root disease if it does not colonize the roots efficiently (Montealegre et al. 2003).

Pseudomonas and *Bacillus* spp. are the most important root colonizing PGPR in various crops. Several members of this group have widespread distribution in the soil, are efficient colonizers of the rhizosphere, and produce various types of metabolites inhibitory to a wide range of pathogens in plants (Rangarajan et al. 2003). Many other root colonizing strains of PGPR have also been found to have antifungal properties toward a number of pathogens in soil.

However, for many of the potential biocontrol strains including *Pseudomonas* and *Bacillus* spp., biological control of soilborne diseases is often inconsistent. One of the major factors associated with this inconsistency is insufficient root colonization by introduced bacteria (Bloemberg and Lugtenberg 2001). Correlation of poor biocontrol performance of a biocontrol agent with inefficient root colonization has been confirmed by means of mutants of *Pseudomonas* strains, which had lost their biocontrol activity. In this regard, it is essential to understand the bacterial traits that contribute to root colonization.

It is now possible to detect and enumerate microorganisms in situ on plant surfaces using molecular techniques. In the study of root colonization of bacteria in situ, one of the approaches was the use of marker genes such as the *gfp* gene encoding the green fluorescent protein (GFP). GFP transformed bacteria can be monitored and visualized using confocal laser scanning microscopy (CLSM) (Bloemberg and Lugtenberg 2001). Apart from visualizing root colonization, the GFP technique can also be used to study the colonization patterns of different biocontrol agents.

4 Latest Advances and Future Prospects of PGPR as Biocontrol Agents in Plants

With the advancement and innovations of current biotechnological research over the past ten years, there is now vastly improved knowledge on the beneficial effects of both biocontrol and growth enhancing PGPR. Several strategies have so far been

exploited to increase the efficacy of biocontrol strains to develop them for widespread use in agriculture. Because of their metabolic versatility, excellent root colonization capability, and their capacity to produce a wide range of antifungal metabolites, intense biotechnological research is being done on the soil borne fluorescent Pseudomonads (Walsh et al. 2001). For example, the antifungal metabolite 2,4-diacetylphloroglucinol (2,4 DAPG) is an important metabolite produced by these biocontrol strains. In this regard, the development of sensitive in situ detection methods of 2,4-DAPG helped to understand the relationship between effective BCA pseudomonads and suppressive soils in the suppression of take-all disease caused by *Gaeumanomyces graminis* var. *tritici* (Raaijmakers et al. 1999; Walsh et al. 2001).

Improving the biocontrol efficacy of potential rhizobacteria by means of genetic modifications involves, for instance, the construction of strains that produce increased levels of antimicrobial and growth enhancing metabolites (Walsh et al. 2001). By transforming *P. fluorescens* CHAO with the gene coding for 1-aminocyclopropane-1-carboxylic acid deaminase, for instance, the plant growth promotion and biocontrol capacity of this strain have been increased (Wang et al. 2000). Novel perspectives are emerging regarding biocontrol and optimizing the application of biocontrol strains for future use.

The identification of *P. fluorescens* genes associated with root colonization and that are specifically expressed in the rhizosphere (*rhi* genes) by means of in-vivo expression technology (IVET) is another important innovation (Bloemberg and Lugtenberg 2001). Many such root colonizing genes and traits from *P. fluorescens* have been identified and used to improve root colonization patterns of wild type *Pseudomonas* strains (Lugtenberg and Dekkers 1999) In some biocontrol PGPR, efficient root colonization is linked to a site-specific recombinase gene, and transfer of this gene from a rhizosphere competent *P. fluorescens* strain to a noncompetent strain improved its root colonization ability (Compant et al. 2005).

5 Conclusion

Two important principles pointed out by Baker and Cook (1974) should be borne in mind. First, there is no one system by which biological control works, each relationship is unique. Second, analysis of the microorganisms involved, as well as their relationships and interactions on biochemical/molecular level, becomes a means of perfecting the result obtained and is not a necessary precursor to attempting biological control.

In conclusion, as there are numerous examples of effective biocontrol candidates, the future challenge is not to prove that biocontrol is possible, but to improve efficacy and durability of biocontrol in the field. This will only be achieved through a better understanding of the biocontrol mechanisms, plant–microbe interactions and processes as well as microbial ecology in the soil and rhizosphere. The necessary molecular tools for studying these processes and interactions are already

available. If this is achieved, the efficacy of biocontrol could conceivably be improved through application of this knowledge to develop improved screening protocols, formulation, and application procedures as well as new innovative integrated disease management practices.

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Sustainable Approaches for Biological Control of Fusarium Wilt in Pigeon Pea (*Cajanus cajan* L. Millspaugh)

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Abstract *Cajanus cajan* (Pigeon pea) is an important crop of Indian subcontinent and African countries, cultivated in the tropics and subtropics. *Fusarium* wilt is one of the major yield and growth-limiting factors of pigeon pea. Along with nematodes such as *Meloidogyne incognita* and *Heterodera cajani*, *F. udum* result in highly destructive wilt disease complex, which is a major constraint for the successful cultivation of pigeon pea. *F. udum* from the same or different geographical origin have shown that the fungus is highly variable in cultural characteristics and pathogenicity. Although development and use of resistant cultivars is effec tive, economical, and environmentally sound strategy for disease control, still variable

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responses with cultivation conditions had been a matter of concern. For an eco-friendly and sustainable management of fusarium wilt, biological control with the application of PGPR offers a potential nonchemical means for disease management. Several strains of *Pseudomonas* and *Bacillus* have been widely reported as effective biocontrol agents for pigeon pea wilt, though combination of several organisms have been proved more effective in field conditions.

1 Introduction

C. cajan L. Millspaugh, a multipurpose species, is extensively used as food grain and green manure crop for soil fertility amelioration in cropping systems (Tobita et al. 1994; Adu-Gyamfi et al. 1996). It is an important pulse crop in India and is a major source of protein for most of the vegetarian population worldwide (Nene et al. 1996). In India, cultivation area of pigeon pea increased from 2.2 million hectare (1.7 metric tons) in 1950–1951 to 3.8 M ha (2.9 M tons) in 1996–1997, while the productivity dropped from 780 to 753 kg/ha in the same period. In Asia, between 1972 and 2003, pigeon pea recorded 57% increase in area (2.44–3.81 m ha) and 61% increase in production (1.72–2.77 m tons). Globally, pigeon pea area has been recorded an increase of 43% since 1970. It is currently grown on 4.3 m ha (Anonymous 2007). In India, it had a low growth rate of 0.8% in production between 1949–1950 and 2004 because of various biotic and abiotic stresses (Singh et al. 2005). Kenya stands next to India in annual pigeon pea production. Kenya dedicates 200,000 ha of cultivated land annually to pigeon pea cultivation (Odeny et al. 2009). With more than 150,000 ha under cultivation, mostly located in the dry regions of the Eastern part of the country, Kenya is the main producer of pigeon pea in East-Africa and the second highest producer in the world, after India (Johansen et al. 1993).

The wilt disease complex is a major constraint for the successful cultivation of pigeon pea in India, and therefore there is an urgent need to workout a suitable biocontrol of wilt disease complex of pigeon pea (Hasan 1984; Siddiqui and Mahmood 1996, 1999). However, it suffers major economic loss, associated with poor yield mainly due to wilt caused by fusarial infection. *Fusarium* wilt of pigeon pea causes a loss of several million US$ (Reddy et al. 1990), an estimated yield loss of US$36 million in India and $5 million in eastern Africa (Kannaiyan et al. 1984). Previous studies (Songa et al. 1991; Khonga and Hillocks 1996) highlighted *Fusarium* wilt as one of the most important and wide spread diseases in Kenya with wilt incidence estimated at 60% (Kannaiyan et al. 1984). *Fusarium udum* (Singh 1983) along with *Heterodera cajani* (Husain et al. 1989) were reported to induce wilting and cause destruction to the pigeon pea crop in certain states of northern India (Perveen et al. 1999). Since then, the two decades have witnessed some very effective work for its control, though a definite practical measure is yet to be adapted.

2 The Universal Pathogen: *Fusarium* spp.

Hundreds of species of *Fusarium* are known, which play multiplicity of role in the environment. *Fusarium* species are important pathogens invading seeds, seedlings, and older plants of almost all kinds of vegetables, flowers, and cereals, as well as many fruit and forest tree. Most of the interest in this fungus arises because of its ability to cause diseases of economically important plant hosts, but it is near ubiquity in soils worldwide and its ecological activities indicate a much more diverse role in nature (Alves-Santos et al. 1999).

Fusarium sp. come in contact with host surface and recognizes the host. Sometimes, macroconidia release an extra cellular material from their tip, which is involved in adhesion (Schuerger and Mitchell 1993). Plant recognizes pathogen when physical contact occurs between them. Some of the cell wall components act as elicitors in their recognition by host (Ren and West 1992). *Fusarium* spp. penetrate the cell wall of host by producing several hydrolytic enzymes. Production of cutinase is supposed to be of major importance (Lin and Kolattukudy 1980). Fusaria are known to produce extracellular polygalacturonase and/or pectate lyase, the pectin enzymes, to breach pectinaceous barrier (Peres-Artes and Tena 1989). These deadly pathogens are known to cause wilting in host plant by affecting xylem tissues.

2.1 Fusarium Wilt of Pigeon Pea

Wilt disease of pigeon pea was first reported in 1906 by E.J. Butler from the state of Bihar, India. He was unable to distinguish the pigeon pea wilt pathogen from *F. vasinfectum* that attack cotton and sesamum (Butler 1906). He reported that wilt disease of pigeon pea is responsible for 15.25% mortality of plants, and the wilting may rise to more than 50% in epidemic year. In 1940, Padwick studied cultural characteristics of *F. udum* and found that it differed from *F. vasinfectum* because it produced abundant spores in sporodochia, and these spores are strongly hooked at the apex and so he proposed the name *F. udum* Butler var. *cajani* for the wilt pathogen of pigeon pea. On the basis of the specific shape and prominent hook at the apex of macroconidia, Booth (1977) proposed the name *F. udum* Butler, which is now widely accepted.

The *F. udum* grows systematically in taproot, lateral root, collar, stem branches, leaflets, petioles, rachis, pedicel, and pod hull. It is mainly soil borne but in the tolerant cultivars also carried in seeds. The fungus can survive for 2–3 years in soil (Kannaiyan et al. 1984). The pioneering studies on ecology of *F. udum* revealed that the fungi in root regions of healthy and diseased *C. cajan* differed qualitatively and quantitatively, as *F. udum* was always recorded on the rhizoplane of wilted plants and about 90% of the total fungal population of the rhizosphere of wilted plants was *F. udum* (Upadhyay and Rai 1982). In an early report, Sarojini (1951) isolated several *F. udum* strains from Coimbatore (India) sick soil and compared the virulence with respect to micro nutrient requirements.

Fig. 1 (**a**) Wilted infected plant of pigeon pea against a green, healthy plant; (**b**) *Fusarium* infested field of pigeon pea with infected plants; (**c**, **d**) Pigeon pea plant infected with *F. udum*, with brown streak of on stem

Limited studies on variability in the wilt fungus *F. udum* have indicated that the fungus exhibits physiologic specialization (Shit and Sen Gupta 1978; Reddy and Raju 1993). *F. udum* shows great deal of variation in cultural and morphological characteristics (Booth 1977; Rai and Upadhyay 1982; Kiprop 2002). The high variation in cultural and morphological characteristics of these pathogens is supposed to be because of environmental conditions, age of isolates, subculturing, method of storage, and culturing conditions. Wide variation in virulence to different genotypes of pigeon pea among *F. udum* isolates has been suggested, mainly because of environmental conditions and inoculation techniques (Shit and Sen Gupta 1978; Kiprop 2002).

Kiprop et al. (2002) isolated 79 single-spore isolates of *F. udum*, the causal agent of wilt disease of pigeonpea, from Kenya, India, and Malawi and characterized according to their cultural characteristics, pathogenicity, and vegetative compatibility group (VCG). They observed that isolates exhibited high variation in pathogenicity on a wilt-susceptible pigeonpea variety, and in mycelial growth and sporulation on potato dextrose agar medium. Further, the 79 isolates were categorized into two virulence groups, two groups of radial mycelial growth, and four groups of sporulation. Further, 38 *F. udum* isolates from pigeon pea were tested for variability in VCG and amplified fragment length polymorphism (AFLP). All the isolates were placed in single VCG with two subgroups, and one AFLP with more than ten AFLP groups (Kiprop et al. 2005). The disease symptoms of fusarium infestation of pigeon pea are given in Fig.1.

2.2 *Resistant Varieties of Pigeon Pea: An Effective Strategy for Wilt Control (?)*

A lot of research has been conducted on *Fusarium* wilt since the 1930s, especially in India, yet the genetics of resistance to this disease remains to be understood

(Saxena 2008). Some of the reports available (Shaw 1936; Joshi 1957; Jain and Reddy 1995; Pandey et al. 1996; Singh et al. 1998) are conflicting and inconclusive regarding the genetics of this destructive disease (Odeny et al. 2009). Pal (1934) reported that resistance to wilt in pigeon pea was controlled by multiple factors while Shaw (1936) observed two complementary genes. Later studies by Pathak (1970) confirmed the presence of two complementary genes while Pawar and Mayee (1986) reported the control of this trait by a single dominant gene. Ten pigeon pea lines were developed for use in African countries, which were resistant or tolerant to *F. udum* early maturing, short in height, and high yielding (Kimani et al. 1994).

It was found that germplasm from Asia and Africa possess different genetic mechanisms for resistance to *Fusarium* wilt (Odeny et al. 2009), rendering it difficult to raise a resistant variety with consistent performance in field conditions. Singh et al. (2004) checked the combined effect of root knot nematode, *Meloidogyne javanica*, and wilt pathogen, *F. udum*, in ten wilt resistant/tolerant accessions of pigeon pea. They found that presence of *M. javanica* with *F. udum* increased wilting from 8 to 33% in KPL 44, 15 to 60% in AWR 74/15, 25 to 50% in ICP 8859 and ICPL 89049, and 15 to 50% in ICP 12745, and hence proposed serious concerns over these cultivars. However, in other five accessions, wilting was not increased much in presence of nematodes. The lowest root knot index was observed in KPL 43 (1.50) and GPS 33 (1.75). Further, reaction to fusarium wilt as well as agronomic performance of elite pigeon pea germplasm was evaluated in three different countries during the 2001/2002 cropping season using wilt-sick plots (Gwata et al. 2006). The genotype ICEAP 00040 consistently showed a high (<20.0%) level of resistance to the disease in all three countries. ICEAP 00068, a short duration but susceptible to fusarium wilt (Gwata et al. 2007) cultivar was used to develop elite germplasm by breeding with three long-duration genotypes that were either resistant (ICEAP 00040; ICEAP 00020) or moderately resistant (ICP 13076) to fusarium wilt.

RAPD has been used to tag wilt resistance in pigeon pea (Kotresh et al. 2006; Dhanasekar et al. 2010). ICP 8863 (ICRISAT 1993) and ICP 9145 (ICRISAT 1994) are popular wilt resistant varieties. During 1978–1983, 61 pigeon pea lines and cultivars were screened for *F. udum* wilt at 15 wilt-endemic locations in India, and lines ICP 4769, 8863, 9168, 10958, 11299, and cultivars C 11 (ICP 7118) and BDN 1 (ICP 7182) were found to be resistant in all the years of testing at most of the locations, suggesting stability and broad-based resistance (Nene et al. 1985). Isozymes variability among different pigeon pea cultivars for resistance against wilt caused by *F. udum* and to assess the genetic variability among the resistant and susceptible cultivars was reported (Prasad et al. 2003).

Marley and Hillocks (2007) suggested that mechanisms of resistance to fusarium wilt (*F. udum*) were mainly because of phytoalexin synthesis. Wilt-susceptible (Malawi local) and wilt-resistant (ICP 9145) plants were stem-inoculated with a spore suspension containing 2×10^6 conidia/ml of the pathogen. Four fungitoxic isoflavonoid phytoalexins – hydroxygenistein, genistein, cajanin, and cajanol – were isolated from plants, 15 days after inoculation. Cajanol was identified as the

main antifungal compound. Still it is evident that environmental and cultivation practices affect the wilt susceptibility. Therefore, it may be advised to use biocontrol agents and other PGPR (described below) to enhance yield and minimize loss with wilt resistant varieties.

3 Plant Growth Promoting Rhizobacteria

The term "plant growth promoting rhizobacteria" (PGPR) was first defined by Kloepper and Schroth (1978), to include soil bacteria that colonize the roots of plants following inoculation onto seed and enhance plant growth. The definition was revised as beneficial free-living soil bacteria that enhance plant growth, referred to as PGPR (Kloepper et al. 1989) or yield increasing bacteria (YIB) (Tang 1994). The bacteria useful to plants were proposed to be characterized into two general types: bacteria forming a symbiotic relationship with the plant, and another the free-living ones found in the soil but are often found near, on, or even within the plant tissues (Kloepper et al. 1988; Frommel et al. 1991). The premier examples of plant growth enhancing agents occur in many genera including *Actinoplanes*, *Agrobacterium*, *Alcaligens*, *Amorphosporangium*, *Arthrobacter*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Cellulomonas*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Gluconacetobacter*, *Micromonospora*, *Pseudomonas*, *Rhizobia*, *Serratia*, *Streptomyces*, *Xanthomonas* as stated by large number of microbiologists (Kloepper et al. 1989; Tang 1994; Weller and Thomashao 1994; Glick 1995; Glick et al. 1995, 1998, 1999; Lucy et al. 2004). Recently, a new PGPR *Delftia tsuruhatensis* HR4, having both nitrogen fixing and biocontrol activity, was reported (Han et al. 2005). Further, Burelle et al. (2006) reported beneficial effect of PGPR and their application methods on bacterial survival, rhizosphere colonization, growth, yield, and selected indigenous rhizosphere microorganisms, without adversely affecting the beneficial indigenous microbial population.

PGPR had also been classified according to their beneficial effects (1) biofertilizers that fix nitrogen, subsequently used by the plant, thereby improving plant growth when the amount of nitrogen in the soil is limiting as observed by number of workers (Bartsev et al. 2004); (2) phytostimulators that can directly promote the growth of plants, usually by the production of hormones; and (3) biocontrol agents that are able to protect plants from infection by deleterious microorganisms (Bloemberg and Lugtenberg 2001). PGPR provide an effective and eco-friendly alternative to the agrochemicals, as they have been established to improve the yield and growth of crop plants (Vyas 2003; Vessey 2003).

Microbial inoculants as a source of biofertilizers have become a hope for most of the countries in relation with economical and environmental points of view (Ramamoorthy et al. 1994; Burelle et al. 2002; Singhal et al. 2003; Compant et al. 2005; Tilak et al. 2006a, b; Gahukar 2006).

3.1 *Biocontrol Agents: A Sustainable Eco-Friendly Strategy for Pathogen Control*

Interest in biocontrol has increased recently fuelled by public concerns over the use of chemicals in the environment in general, and the need to find alternatives to use of chemicals for disease control in particular (Whipps and Davies 2000; Whipps and Lumsden 2001). Seed treatment with fungicides does not protect the crop for long periods. Soil drenching with fungicides are not economical and they may establish imbalances in the microbial community unfavorable for activities of beneficial organisms (Jeyarajan et al. 1991). In addition, continuous use of the same fungicides for the same pathogen results in the development of resistant strains of the pathogen, besides polluting the environment (Pandey and Maheshwari 2007a). It is now widely recognized that biological control of plant pathogens using antagonistic fungi and bacteria is a distinct possibility for the future and can be successfully utilized especially within the framework of integrated disease management system (Muthamilan and Jeyarajan 1996).

The use of microorganisms for biological control instead of chemicals is a boon as it does not cause any harm to plant and free it from plant pathogens (Cook et al. 1995) and also exhibit their effect in stable form for long duration (Waage and Greathead 1988).

Studies on biological control of fusarium wilts have a long history (Alabouvette et al. 1998) as various disease suppressive mechanisms of biocontrol agents has been suggested, including siderophore-mediated competition for iron (Bakker et al. 1988; Raaijmakers et al. 1995), competition for substrate (Couteaudier and Alabouvette 1990), induction of systemic resistance (Van Peer et al. 1991; Van Loon 1997), and production of antibiotics (Chin-A-Woeng et al. 1998). Role of rhizobia in biocontrol of *Fusarium* has been suitably compiled (Deshwal et al. 2003).

There had been several mechanisms suggested for biocontrol mechanism including siderophore. The bacterial siderophores are known to sequester the limited supply of iron available in the rhizosphere making it unavailable to pathogenic fungi, thereby restricting their growth (O'Sullivan and O'Gara 1992). Recently, Bae et al. (2007) reported siderophore production from *Burkholderia gladioli* but based on their findings, they discarded its role in biocontrol activity.

Certain volatiles of bacterial origin including hydrogen cyanide (HCN), which is produced by many fluorescent pseudomonads in the exponential growth phase in media containing $FeCl_3$ or inorganic phosphate may also influence plant root pathogen (Voisard et al. 1989) and suppresses the diseases (Glick 1995). In one historical experiment, the effect of added cyanide was tested directly in the field, where "sick" soil was treated with $Ca(CN)_2$, which is a cheap water-soluble cyanide that is used by the mining industry and is known as "cyanogas." This treatment killed fungi en masse, significantly reduced "grey speck" disease of oats, and induced oat grain yield with no side effects on the fauna (Timonin 1947). Chanway et al. (1988) suggested that HCN produced by rhizobacteria form stable complexes with several divalent metal ions, and also cytochrome oxidase of many organisms is

strongly inhibited by cyanide. Pandey et al. (2006) isolated a *Bacillus* sp., which produced HCN in vitro and reduced the radial growth of *F. udum*. Similarly, Siddiqui et al. (2008) isolated a *Pseudomonas* strain Pa324, a strong antagonist of *F. udum*, which had ability to produce HCN and siderophore in excessive amount.

Rhizobia are major biocontrol agents in natural and agricultural ecosystems. Tu (1979) suggested that rhizobia achieve this bioprotection by parasitizing the hyphal tips of the fungal pathogens and decreasing contact with the host plant cells. Different rhizobial strains were reported to successfully protect field-grown leguminous (soybean, mungbean) and nonleguminous (sunflower, okra) plants from infection by the root-borne pathogens including *Fusarium* species, irrespective to the mode of application including – seed dressing or soil drench (Ehteshamul-Haque and Ghaffar 1993). There had been substantial reports where rhizobia had been used for control of fusarium infections. Antoun et al. (1978) found 49 strains of *Sinorhizobium meliloti* that inhibited growth of *F. oxysporum* by up to 50%. Chakrabarty and Chakrabarty (1988) reported that presence of *R. meliloti* increased the production of phytoalexin 4-hydroxy-2, 3, 9-trimethoxypterocarpan, which inhibited *F. solani* f. sp. *pisi* affecting pea. Nautiyal (1997) screened the biological control activity of 256 rhizobial strains and noticed that *Rhizobium* NBRI9513 completely inhibited growth of *F. oxysporum*, *R. bactaticola*, and *Pythium* sp. in vitro condition. However, reports on rhizobial control of *F. udum* are still limited (Pandey and Maheshwari 2007a; Siddiqui and Shakeel 2009).

3.2 Biological Control of Fusarium *Wilt*

Biological control of fusarial wilt has attracted attention throughout the world. Currently, the idea of controlling soil-borne plant pathogens, including *Fusaria*, with chemical pesticides or fungicides is being challenged by the approach that biological control can have an important role in sustainable agriculture.

3.2.1 Use of *Bacillus* or *Pseudomonas*

Production of chitinases is an important attribute of biocontrol bacteria. Most of the fungi contain chitin (a homopolymer of β-1, 4 linked *N*-acetylglucosamine) in the cell wall, which ranges from 22 to 40% (Muzzarelli 1977). Therefore, formulations based on chitinases producing organisms offer potential biocontrol agents (Boller 1985). In a very early report, Mitchell and Alexander (1961) demonstrated biological control of *Fusarium* sp. and *Pythium sp.* by bacteria that degrades the cell wall of these plant pathogens. Biological control of *Fusarium* wilt of pigeon pea had been reported with chitinolytic activity of *Alcaligenes xylosoxydans* (Vaidya et al. 2001, 2003a). Further, they employed random mutagenesis through physical (UV, gamma radiation) and chemical agents (ethyl methane sulphonate

[EMS]) to obtain improved mutants for chitinase producing biocontrol strain of *A. xylosoxydans* (Vaidya et al. 2003b).

Bacillus is one of the most commonly found soil bacteria, which has been reported as excellent biocontrol agent by a number of workers (Dal-Soo et al. 1997; Bacon et al. 2001; Basha and Ulaganathan 2002; Chaurasia et al. 2005). *Bacillus* species as a group has been suggested to offer several advantages over other bacteria on protection against root pathogens because of their ability to form endospores and the broad spectrum activity of their antibiotics (Cavaglieri et al. 2005). *Bacillus brevis* inhibited the growth of pigeon pea pathogen – *F. oxysporum* f. sp. *udum* because of production of unknown antibiotic substance (Bapat and Shah 2000). Similarly, in vitro interaction of *F. udum* and a biocontrol strain of *Bacillus subtilis* AF 1 showed that the fungus forms chlamydospore-like structures and increases vacuolation, when both cultures are simultaneously inoculated into potato dextrose broth. Though, in their experiments, extracellular proteins of *B. subtilis* AF 1 reduced the growth of *F. udum* in proportion to the concentration of the protein precipitate, still formation of chlamydospore-like structures and vacuolated portions in mycelium of *F. udum* in the presence of AF 1 led the authors to conclude that *F. udum* has a mechanism to tolerate mycolytic activity (Harish et al. 1998). Recently, Siddiqui and Shakeel (2007) reported that two *Bacillus* strains (B615 and B603) had biocontrol potential against *F. udum*, in addition to inhibitory effect on the hatching and penetration of *H. cajani* and *Meloidogyne incognita* along with colonization of pigeon pea roots. In fact, the latter two nematodes cause serious damage in wilt disease complex of pigeon pea. Although restricted to pot trials, this work provides substantial evidence for PGPR to be used as broad spectrum control strategy of wilt disease in pigeon pea.

Anjaiah et al. (2003) found that *Pseudomonas aeruginosa* PNA1, an isolate from chickpea rhizosphere in India, protected pigeonpea from fusarium wilt disease. They also measured root colonization of pigeon pea using a *lacZ*-marked strain of PNA1, and observed tenfold lower root colonization of susceptible genotypes than that of moderately tolerant genotypes, indicating that this plant–bacteria interaction could be important for disease suppression in this plant. Further, strain PNA1 produced two phenazine antibiotics, phenazine-1-carboxylic acid and oxychlororaphin, in vitro, and its Tn*5* mutants (FM29 and FM13), which were deficient in phenazine production, caused a reduction or loss of wilt disease suppression in vivo, which suggest that phenazine production by PNA1 contributes to the biocontrol of fusarium wilt diseases in pigeon pea. The root nodulating bacterial isolate *Burkholderia* sp. MSSP (Pandey et al. 2007a) produces antibiotic 2-hydroxymethyl-chroman-4-one, because of which it shows antifungal properties against *F. udum* and many other phytopathogens (Kang et al. 2004).

In a similar kind of work, several pseudomonads were checked for biocontrol potential and strain Pf736 was found to cause greater increase in plant growth and higher reduction in nematode multiplication and wilting index followed by other Pa737, Pf718, and Pf719 pseudomonad strains (Siddiqui and Shakeel 2009). The use of these isolates along with *Rhizobium* (pigeon pea strain) further increased plant growth and reduced nematode multiplication and wilting index.

3.2.2 Use of Combination of Microorganisms

Combination of several organisms has been checked by many workers. *Bacillus subtilis*, *Bradyrhizobium japonicum*, and *Glomus fasciculatum* were used alone and in combination for the management of a wilt disease complex of pigeon pea caused by *H. cajani* and *F. udum* (Siddiqui and Mahmood 1995). Application of all the three management agents alone or in combination to plants inoculated with the pathogens increased shoot dry weight, number of nodules, phosphorus content, and reduced nematode multiplication and wilting index. Interestingly, in their experiments, another phenomenon in pigeon pea rhizosphere biology was identified as combined application of *G. fasciculatum* and *B. japonicum* increased root infection by *G. fasciculatum* whereas combined use with *B. subtilis* reduced mycorrhizal colonization.

In a related study, Siddiqui et al. (2008) experimented with six potential isolates of *Bacillus* and *Pseudomonas* under pot and field conditions for the biocontrol of wilt disease complex of pigeon pea. Under field condition, isolate Pa324 was best in reducing wilt disease complex followed by B18. Combined use of Pa324 with B18 provided better biocontrol of wilt disease complex than the use of either of them. Application of these isolates (Pa324 and B18) with *Rhizobium* sp. caused about 30% increase in yield under field condition and provided substantial protection against wilt disease complex of pigeon pea.

Jayalakshmi et al. (2003) also observed that the seed treatment with *Trichoderma viride* followed by *T. harzianum* was found to be effective in reducing the wilt disease incidence in pigeon pea by controlling *F. udum* effectively, when compared with individual treatments. Singh et al. (2002) checked *Aspergillus flavus*, *Aspergillus niger*, *Bacillus licheniformis* (strain-2042), *Gliocladium virens*, *Penicillium citrinum*, and *Trichoderma harzianum* for biological control of *F. udum*. They claimed that these were the most potent organisms in inhibiting the radial colony growth of the test pathogen. They observed maximum reduction of the wilt disease was with application of *G. virens* (50%) both in pots and in the fields, followed by *A. niger* (38%), *P. citrinum* (33%), and *T. harzianum* (28%), although the mechanism of biocontrol was not described.

Prasad et al. (2002) studied the efficacy of *T. harzianum* on various levels of *F. udum*. They applied *T. harzianum* as seed treatment (10 and 20 g/kg seed) and as a soil amendment (10 and 20 g/9 m^2) in field plots infested with the pathogen at three inocula levels (log 3.04, log 4.98, and log 5.34 colony-forming units (cfu)/g of soil). They observed that *Trichoderma* population increased to more than 10^8 cfu/g soil by 60 days in treated plots, whereas for seed treatments, fungal population reached a maximum of $10^{4.62}$ cfu/g soil within 45 days, and thereafter started to decline. However, even at the highest pathogen density (log 5.34), soil amendment with *T. harzianum* at 10 g gave about 30% disease reduction.

A novel mycolytic strain *Pantoea dispersa* was evaluated against *F. udum*, as a biocontrol agent in comparison with chemical fungicide Bavistin and antifungal biocontrol agent *Trichoderma* Monitor WP in both pot and field experiments (Maisuria et al. 2008). In the pot experiment, *P. dispersa* the treated pigeon pea

(T-15-15) seeds showed higher percentage of seed germination and decreased wilt incidence when compared with chemical fungicide; Bavistin and antifungal biocontrol agent *Trichoderma* Monitor WP treatments. Moreover, the root, shoot lengths, and growth were also found to be higher. The results of field study during three cropping seasons (2004/2007) suggested that the seed dressing by *P. dispersa* reduced wilt incidence (47%) during field trials, which was greater than Bavistin (41%) and *Trichoderma* Monitor WP (36%) treatments. Similarly, *T. harzianum* and *A. niger* were evaluated as biocontrol agents against *F. udum* in combination to two fungicides, Foltaf 80W (Captafol 80%) and Blue Copper-50, for the treatment of pigeon-pea wilt (Bhatnagar 1995). It was observed that the disease was more effectively controlled when biocontrol agents were applied with chemical fungicides, in comparison to the fungicides that were used alone.

More recently, Kumar et al. (2010) reported wilt disease management of *C. cajan* (L.) var. Manak by root nodulating *Sinorhizobium fredii* KCC5 and rhizospheric *Pseudomonas fluorescens* LPK2 amended with chemical fertilizers. Combinations of *S. fredii* KCC5 and *P. fuorescens* LPK2 with low dose of chemical fertilizers provided better disease management of wilt in *C. cajan*. The microbial combinations involving *S. fredii* KCC5 and *P. fuorescens* LPK2 reduced wilt disease, proved the most effective in reducing disease incidence due to *F. udum*.

3.2.3 Use of Bioformulations

Pandey and Maheshwari (2007a) formulated an effective bioformulation utilizing *Burkholderia* sp. MSSP, a known PGPR using green fluorescent protein (*gfp*) to monitor its population in carriers, including sugarcane – bagasse, sawdust, cocoa peat, rice husk, wheat bran, charcoal, rock phosphate; and paneer – whey. They concluded that whey and wheat bran proved to be efficient carrier materials for the bioformulation. Interestingly, viability of MSSP was also assessed in wheat bran and whey-based consortium, having three other bacterial strains, namely *Sinorhizobium meliloti* PP3, *Rhizobium leguminosarum* Pcc, and *Bacillus* sp. B1. Presence of other plant growth promoting bacteria did not have any detrimental effect on viability of MSSP. In fact, MSSP and PP3 strains were known to enhance seedling growth in mixed-species, coinoculated consortium (Pandey and Maheshwari 2007b), while *Bacillus* sp. B1 had biocontrol activity against *F. udum* (Pandey et al. 2006). Efficiency of wheat bran based multispecies consortium was studied on growth of pigeonpea in field conditions. Considerable increase in plant biomass, nodule number and weight, and number of pods was recorded when compared with individual trials, as well as control.

Similarly, seed treatment of groundnut and pigeonpea with peat based formulation of *B. subtilis* supplemented with 0.5% chitin or with 0.5% of sterilized *Aspergillus* mycelium controlled wilt of pigeon pea. It also increased growth promotion even in the presence of inoculum pressure (Manjula and Podile 2001). For formulation details, Nakkeeran et al. (2005) may be referred.

Treatment of pigeon pea seeds with talc based formulation of *P. fluorescens* (Pf1) effectively controlled fusarial wilt of pigeon pea under greenhouse and field conditions (Vidhyasekaran et al. 1997). More specifically, Seed treatment of pigeon pea with talc-based formulation of fluorescent pseudomonads at the rate of 4 g/kg of seed followed by soil application at the rate of 2.5 kg/ha at 0, 30, and 60 days after sowing controlled pigeonpea wilt incidence under field conditions.

Biocontrol agents *T. harzianum* and *P. fluorescens*, isolated from rhizosphere soil samples collected from various pigeon pea-growing fields, were immobilized in wheat bran, rice bran, paddy straw, and neem cake (Niranjana et al. 2009). It was found that boiled rice bran increased the growth of both biocontrol agents. Talc and sodium alginate formulations of mass-multiplied biocontrol agents were prepared and evaluated for their effects against fusarium wilt under greenhouse conditions. The fresh cultures of both biocontrol agents were found to increase seedling emergence and reduce fusarium wilt disease incidence when compared with the control and the formulations.

3.2.4 Others

A *Bacillus cereus* strain BS 03 and a *P. aeruginosa* strain RRLJ 04 were studied for their effect on induction of systemic resistance against *F. udum* wilt in pigeon pea, both individually and in combination with a rhizobial strain RH 2 (Dutta et al. 2008). They observed increased level of defense-related enzymes, viz., l-phenylalanine ammonia lyase (PAL), peroxidase (POX), and polyphenol oxidase (PPO), in coinoculated plants. Production of β-1, 3-glucanase and polymethyl galacturonase by the pathogen in culture medium was also sharply reduced in the presence of both the PGPR strains.

On the basis of results of an interesting set of experiments, Prasad et al. (2002) reported that "preinoculation" or "simultaneous inoculation" of pigeon pea seedlings with soilborne fungi nonpathogenic to pigeon pea, viz., *Fusarium oxysporum* f. sp.*niveum; F. oxysporum* f. sp.*ciceris; F. solani* f. sp. *pisi*; and *Cephalosporium sacchari*, before challenge inoculation with the pathogen *F. udum*, was effective in controlling wilt of pigeon pea to a great extent. Inoculation with the nonpathogens before the challenge inoculation was more effective than simultaneous inoculation and gave up to 81.6% protection.

In an unrelated work, fungitoxic effects of different plant extracts on *F. udum* was examined (Singh and Rai 2000). At 10% concentration of leaf extract from *Adenocallyma alliaceum*, the radial growth of *F. udum* was completely arrested. A leaf extract of *Citrus medica*, a root extract of *Asparagus adscendens*, rhizome extracts of *Curcuma longa* and *Zingiber officinale*, and a bulb extract of *Allium sativum* inhibited up to 100% growth at higher concentrations. The population of *F. udum* was found to be markedly reduced following treatments with plant powders. On the basis of these results, it may be proposed to evaluate that these plant materials as carrier for the formulation of PGPR amended bioinoculant for pigeon pea.

4 Conclusion

Pertaining to the economic importance of crop and extent of loss, the *Fusarium* wilt of pigeon pea has received considerable attention from scientists working in diverse field. Raising elite disease-resistant varieties or study of variability in virulence of *F. udum*, every attempt is toward understanding the disease complex for its management. Biological control had been proved very effective in lab as well as field conditions. Along with *Bacillus*, *Pseudomonas*, or *Trichoderma*, several other biocontrol agents (either monoculture or in combination) had been reported to be very effective by workers. Definite measure is required to popularize these technologies to completely replace the traditional use of fungicides.

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Plant Growth Promoter Rhizobacteria in Plants Inhabiting Harsh Tropical Environments and Its Role in Agricultural Improvements

Suikinai Nobre Santos, Vanessa Nessner Kavamura, João Luiz da Silva, Itamar Soares de Melo, and Fernando Dini Andreote

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Abstract The importance of the interactions between plants and bacteria is well known for plant development and success of agriculture. A number of succeeded examples are reported in the literature for the improvement of plant yields and protection against pathogens and pests. However, some specific niches where these interactions are essential are still unexplored, like the environments where the agriculture is not practiced due to the harsh conditions found; mangroves and

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the Brazilian semiarid caatinga. Digging into the bacterial diversity associates to plant growth promotion in such spots can help on the description of new species and features related to the plant growth-promoting rhizobacteria character under harsh tropical conditions. This chapter gives an overview of examples of such niches, where the bacterial community must be adapted to survive and support the plant development. Possible bacterial characteristics related to this ability will be discussed, as the production of biofilms and exopolysaccharides. Furthermore, the application of these biotechnological products will be evaluated and discussed allowing the reader to have a snapshot on this yet nonexplored biodiversity.

1 Introduction

Plant growth-promoting rhizobacteria, known as PGPR, are those with the ability in stimulating the plant development, acting in the nutritional and water supplementation, hormonal production, and plant protection against pathogens and pests. A number of studies have focused on the role of PGPR in a variety of crops cultivated all over the world. Although the plant species and cultivation techniques are variable in these studies, and regardless its importance, new approaches and new plant niches should be explored to enhance our knowledge about such interaction between plants and bacteria. Hence, to access the diversity of bacteria which are able to promote the plant growth in new environments can contribute in many fields; (1) the discovery of new microbial species and genotypes, which are capable of surviving in stressful environmental conditions; (2) the development of new technologies for the improvement of agricultural practices in soils where the availability of water and nutrients are low, the salinity is high and extreme temperatures are usual.

In this chapter, we explore some important features found in bacteria inhabiting harsh tropical conditions, where the practice of agriculture is not usual. The explored environments are the mangroves, where the salinity and the exposition to the sea effects are intense and the endogenous Brazilian caatinga, where the high temperatures and low water availability compose a harsh soil where plants have to develop. The bioprospection of PGPR in these niches might contribute in a better description of interactions going on under these conditions. It can also name new microbial species as the first coming candidates for the usage in program of plant protection by bacterial inoculation, leading to the safety and viable agricultural practices in lands now considered out of order for this activity.

2 Soil Structure and Microbial Community

Soils can be defined as the mineral layer used by plants to play their roles in the ecosystems (Paul and Clark 1996), subdivided into layers called horizons (Brock et al. 1994). However, if we consider the symbiotic relations, the soil can be defined

as the superficial portion of the Earth which present the essential conditions for animal, plant, and microbial life. Although very heterogeneous and variable according to the depth, physical–chemical properties and location in distinct geographic regions, an average soil is formed by approximately 25% of air, 25% of water, and 50% of solids, divided in 45% minerals divided into sand, silt, and clay, and 5% of organic matter (4.5% inert organic matter and 0.5% of live organisms) (Stotzky 1972; Siqueira et al. 1994).

The microbial fraction in the soil (5%) is constituted by a diversity determined by the combination of the environmental conditions, which interact with the phenotypes of the microbes, resulting in higher populations to adapted genotypes and low populations to less adapted microbes. It also results in the way of life, as active microbial communities, or dormant cells, which can last for a period of inactivity of low dense populations. Concerning the niche occupied by microbes in soils, these species can live in association to clay particles, organic matter, in spaces between soil particles as well as in association to plants, colonizing the roots surfaces. But it is hard to assume that the life in soil is always easy and abundant in water and nutrients. The soil is a very stressful environment, where the competitiveness is constant or the niche occupation and nutrients uptaking. Such a balance and the importance of distinct features make the soil very densely inhabited and constituted by a wide diversity of microbial species (Dommergues et al. 1978; Siqueira et al. 1994).

Moreover, soils located in specific environments present even harsher conditions for the development of life, but do not limit its occupation by adapted species of microbes and plants. Such soils are deficient in nutrients and organic matter, present high acidity and salinity, extreme low or high capacity of cationic exchange, limiting its recovering and further usage as common soils. Some examples of these soils are those found in the caatinga and mangroves, which have extreme conditions of humidity – that is, low in caatinga and high in mangroves and limited amount of nutrients, due to the low content of organic matter in caatinga and the anoxic conditions in mangroves, limiting the organic matter processing (Stotzky 1972).

Considering the name of microbes that lives in soils, a number of studies are available; however, none of them have final conclusions about the main species inhabiting this niche. Consistent results have named bacterial groups found in soil, like *Proteobacteria*, *Actinobacteria*, and *Acidobacteria* as major soil inhabitants. Controversially, it is well known that the number of organisms in soil is immensurable, with amounts of 10^8 to 10^{10} microbial cells per gram of soil. Hence, a remark should be made for the density and diversity of bacteria in soils, possibly related to its essentiality to the maintenance of the functionality of this ecosystem, cycling nutrients, and harnessing the compounds degradation.

The quantity of bacterial cells in soils is dependent on environmental variables, like soil depth, pH, humidity, and temperature. Kuske et al. (2002) studying an arid soil, observed that the superficial layer from 0 to 10 cm, the bacterial counting was significantly higher than 20–30 cm. The amount of DNA extracted has also decreased with increase in depth. The culturability also decreases with depth increment (Sait et al. 2002).

3 The Plant-Associated Bacteria

In addition to microorganisms inhabiting soils, there is a group of microorganisms associated to plants that are extremely important to plant metabolism. They are found in synergism with plant roots and are called rhizosphere microorganisms. A wide diversity of bacteria can interact with plants, composing bacterial communities with important roles in plant development and health status (Hallmann et al. 1997). For a review of the bacterial communities associated with plants and how to assess them, please read Andreote et al. (2009). These interactions can vary according to the host plant in a process similar to those widely known for pathogenic microorganisms (Liu et al. 1995). Bacterial populations are distributed in the rhizosphere, epiphytic, and endophytic communities.

The rhizosphere (Fig. 1) was first defined by Hiltner in the beginning of the twentieth century as the volume of soil influenced by root plants (Hiltner 1904; Melo 2002), its extent varies with soil type and plant species (Campbell and Greaves 1990).

In rhizosphere, the quantities and types of substrate are different from those in the bulk soil and lead to colonization by different populations of bacteria, fungi, protozoa, and nematodes. Other physiochemical factors, which can be different in this region, are acidity, moisture and nutrients status, electrical conductivity, and

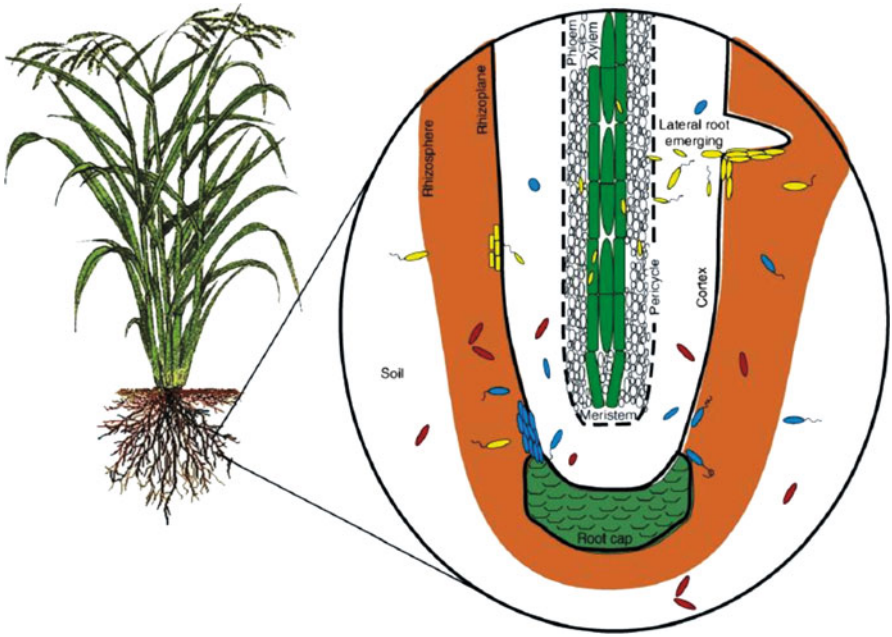

Fig. 1 Scheme of the rhizosphere system in detail and its role on the supplying of endophytes for plant colonization. Adapted and authorized
Source: Hardoim et al. (2008)

redox potential. The total rhizosphere environment is determined by an interacting trinity of the soil, the plant and the organisms associated with the roots (Campbell and Greaves 1990). A more recent definition state the rhizosphere as the soil compartment influenced by the root, including the root itself (Hartmann et al. 2008). In this way, roots associated bacteria became into the context.

Epiphytic and endophytic bacteria are characterized by the colonization of surface and inner tissues of plants, respectively. There is an ongoing discussion toward a better definition of these microorganisms; a commonly used definition of endophytes is those whose isolates form on surface-disinfected plant tissues (Hallmann et al. 1997). In addition to these definitions is the separation of endophytes according to their essentiality in niche occupations. In that case, the endophytic community is divided into "passenger" endophytes, i.e., bacteria that eventually invade internal plant tissues by stochastic events and "true" endophytes, those with adaptive traits enabling them to strictly live in association with the plant (Hardoim et al. 2008). Due to the novelty of this separation, and the problems involved in the methodological separation of these endophytic groups, we will consider in this review that the endophytic community is those bacteria that colonize inner tissues of healthy plants.

The cells in the rhizosphere, plant-surface or endophyte communities are variable. A superficial analysis of these communities could lead to the conclusion that there is a strict specificity for niche colonization. However, a more realistic scene is represented by the gradient of population distribution along plants. If a didactic approach is applied to explain bacterial communities associated with plants, it would divide these bacteria into distinct communities, with separation between epiphytic and endophytic communities in accordance with plant organs, such as roots, stems, and leaves. However, in nature the gradient of distribution will prevail over separation. It is important to note that bacteria in the rhizosphere are often similar to those in the endophytic community and on leaf surfaces. Concerning the role in plant growth promotion, both, the rhizosphere or endophytic bacteria can act supplying plants with their need and stimulating the plant development. It is remarkable that similar bacteria can be present in the rhizosphere and also colonizing inner tissues of the host plant (Hardoim et al. 2008) (Fig. 1). Chi et al. (2005) demonstrated that similar bacteria were distributed over the rice plant, from roots to leaves. However, the abundance of bacterial types along the different niches can differ, mainly due to differences in these niches in nutrient supply, atmospheric conditions, and competitiveness with other components of these communities (Rao et al. 2006). The behavior of these populations and how they colonize plants is determined by environmental conditions, like formation of biofilms that help bacteria fix to cell walls, avoiding the migration driven by sieve transportation. Similarly, in the parenchymatic region, being single-celled can enable better contact with cells and so better nutritional supply for the bacterium.

Among these bacteria, an important group is named rhizobacteria, which occupy diverse niches in the roots-plants system. Such bacteria have the ability to promote the plant growth by a diversity of mechanisms like the production of antibiotics, the nutritional supplying of the plants and by the induction of systemic resistance.

Major groups for the induction of systemic resistance are the genera *Bacillus* and *Pseudomonas*, while other bacteria like *Agrobacterium*, *Serratia*, *Enterobacter*, and *Rhizobium* can interfere in the recognition process between nematode and plants (Seldin et al. 1984; Melo 2002; Von der Weid et al. 2005; Tian et al. 2007).

In summary, the close-to-plant environment is the main niche for bacteria occupation, leading to the importance of these interactions for plant healthy. One can also consider that if these interactions are important ion common conditions of plants cultivation, it might be even more important or essential, when plants are developing under harsh environmental conditions.

4 Plant Growth-Promoting Rhizobacteria

The objective of this section is to give an overview about the PGPR, leaving the major responsibility of this task for other chapter, where experts are writing about it. PGPR were first defined by Kloepper and Schroth (1978) as being bacteria that colonize the roots of plants and help them in their growth and development (Zahir et al. 2003). This is achieved by several mechanisms such as nitrogen fixation, plant-growth hormone production, protection against diseases, and pathogens (Table 1). In this way, PGPR can be used as inoculants in assays of biofertilization, phytostimulation, and biocontrol (Bloemberg and Lugtenberg 2001) with application in agriculture, forests and environmental restoration (Lucy et al. 2004). Again, the strict division of tasks is merely didactic, considering that more than one mechanism can be present in one bacterial species.

The isolates from a sample can be examined for a wide array of traits associated with growth promotion. Cattelan et al. (1999) studied this by analyzing the siderophore, indoleacetic acid, chitinase, β-1,3-glucanase, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and cyanide production as well as phosphate solubilization of soil and rhizosphere isolates from soybean – *Glycine max*. After this screening, they have chosen 23 isolates positive for these traits and also tested their ability associated with biocontrol, bradyrhizobial inhibition, and rhizosphere competence. Ahmad et al. (2008) also screened some bacteria in vitro for the production of indoleacetic acid, ammonia, hydrogen cyanide, siderophore, phosphate solubilization, and antifungal activity.

The use of PGPR to inoculate plants can be convenient for reforestation purposes as shown by Requena et al. (1997). They have tested the ability of two arbuscular mycorrhizal fungi (AMF), one native and one exotic; two native *Rhizobium* bacteria and two PGPR, one exotic and one native in the combination of microbial inoculants. The native microorganisms were isolated from the rhizosphere of *Anthyllis cytisoides* and the other ones were obtained from existing collections. The native microorganisms were more effective inoculants than the exotic ones when biomass accumulation, nutrient uptake, and nitrogen fixation were evaluated. This report highlights the importance of previously knowledge of the microorganisms

Table 1 Examples of plant growth promotion features found in distinct bacterial species

Application	Plant growth-promoting rhizobacteria	References
Undescribed plant growth – promotion feature	Not-identified rhizobacteria: *PGB4*, *PGG2*, *Pseudomonas* sp., *Variovorax* sp., *Agrobacterium* sp., *Phyllobacterium* sp., *Bacillus firmus*, *B. mycoides*, *B. stearothermophilus*, *B. subtilis*, *B. subtilis/amyloliquefaciens*, *B. circulans*, *Brevibacillus brevis*, *Paenibacillus lautus and Stenotrophomona maltophilia*, *Pseudomonas alcaligenes PsA15*, *P. denitrificans PsD6*, *Bacillus polymyxa BcP26 and Mycobacterium phlei MbP1*, *Unindentified PGPR strains*, *Bacillus edaphicus*, *Pseudomonas putida*	Asghar et al. (2002), Ashrafuzzaman et al. (2009), Bertrand et al. (2001), Díaz et al. (2009), Egamberdiyeva (2009), Höflich (2004), Khalid et al. (2004), Sheng (2005), Trivedi and Pandey (2007)
Protection against drought stress	*Pseudomonas corrugata*, *Bacillus thuringiensis*	Kumar et al. (2007), Marulanda et al. (2006)
Indole acetic acid production	PGB4, PGG2, unindentified PGPR	Ashrafuzzaman et al. (2009), Khalid et al. (2004)
Phosphate solubilization	PGB4, PGG2, *Pseudomonas putida*	Ashrafuzzaman et al. (2009), Trivedi and Pandey (2007)
Nodulation	*Bacillus endophyticus*, *B. pumilus*, *B. subtilis*, *Paenibacillus lautus*, *P. macerans*, *P. polymyxa*, *Bacillus* sp.	Figueiredo et al. (2008), Camacho et al. (2001)
Nutrient uptake	Strain YAS34, *Azospirillum* sp., and *Azotobacter* sp.,*Bacillus endophyticus*, *B. pumilus*, *B. subtilis*, *Paenibacillus lautus*, *P. macerans*, *P. polymyxa*, two PGPR (A2 and E)	Alami et al. (2000), Biari et al. (2008), Figueiredo et al. (2008), Requena et al. (1997)
Revegetation	Two PGPR (A2 and E)	Requena et al. (1997)
Promotion of soil aggregation	Strain YAS34, *Pseudomonas mendocina*	Alami et al. (2000), Kohler et al. (2006)

inhabiting the environments, as well as their physiological and genetic adaptation, so they can be useful for further applied researches.

The indigenous rhizosphere bacteria are able to break a great variety of contaminants, but not all of them are necessarily known as plant growth-promoting rhizobacteria (PGPR) (Lucy et al. 2004). However, some PGPR can help in the productivity of some culture in soils with low nutrient content or even in contaminated soils. It occurs mainly when the microbial communities are involved in the control and absorption of metals and nutrients by surrounding plants (Stout and Nüsslein 2005). This role can be extended to the use of the term biofertilizer to PGPR, once this term is related to increase in nitrogen fixation, nutrient availability,

and root growth (Vessey 2003). The nitrogen fixation can be increased as showed by Zhang et al. (1996). They have coinoculated nine growth-promoting rhizobacteria with *Bradyrhizobium* to test their ability to reduce the negative effects at suboptimal root zone temperatures in *G. max* in the nodulation and nitrogen fixation. They have observed that in certain temperatures some strains increased the number of nodules as well as the amount of fixed nitrogen when coinoculated with *Bradyrhizobium japonicum*. The most stimulating ones were *Serratia proteamaculans* in 15°C and 17.5°C; *Aeromonas hydrophila* in 17.5°C; and *Serratia liquefaciens* in 25°C.

5 Bacterial Community in Harsh Tropical Ecosystems

Microorganisms in general and bacterial community in particular adapt to grow under abiotic stresses. Such bacteria have been isolated from extreme environments of low and high temperature, sodic, and acidic habitats. These bacteria have the ability to recognize physio-chemical environment based on genetic features.

5.1 The Adaptation of Microbes to Harsh Environments

The microorganisms are able to grow using different carbon and nitrogen sources and inhabit a wide variety of ecological niches, such as extreme semiarid environments, mangroves, and desert areas. The key for the microbial adaptability may be related to their capacity of expressing only the genes of enzymes and biochemical traits which are required to a maximum growth rate in the particular environment, like the soils where they are found. This is possible due to their ability to recognize chemical and physical composition of the environment. This ability is codified by a cluster of genes that are only expressed when it is necessary. Therefore, the well-succeeded growth of a microbial population reflects its adaptation grade to the physical and chemical composition of a particular environment (Dick 1992).

The biodiversity of microorganisms allows their survival in several habitats (Parkinson and Coleman 1991). Among microorganisms, the bacteria represent the group with the greatest physiological diversity, which provides major adaptability. That is why, among certain limits, it is possible to select organisms that tolerate several stressful factors such as high temperature and soil acidity like in semiarid and mangrove regions, extremely low temperatures, minimum levels of carbon source, and high solar incidence like in polar region (Parkinson and Coleman 1991). The existence of a microorganism in determined time and place results from its evolution, from the existence of favorable abiotic factors and from its diverse biological relationships with competitors, antagonists, and predators. With the soil environment modification, the adaptation capacity of a community varies in function of its genetic constitution and it is known as “biological buffer of the soil.”

However, little is known about the impacts that the environmental changes may have over the soil microorganisms.

The microorganisms in environments with special characteristics, such as high salinity as present in mangroves and low water and organic nutrients rate as in semiarid climate, may present physiological and genetic mechanisms responsible for the survival capacity, even in environments considered extremes. These characteristics allow the discovery of compounds with bioprospection potential and industrial applicability. In the presence of this scenery, the rhizosphere of plants from extreme environments can be considered as a promissory source for bioprospection of new microorganisms and products that result from microbial metabolism, aiming a biotechnological application (Melo 2002). According to Idris et al. (2007b) and Kishore and Pande (2007), several members of the *Bacillus* genera, such as *B. cereus*, *B. subtilis*, and *B. licheniformis* as well as *Pseudomonas* spp., isolated from rhizospheric soil of semiarid regions, synthesize natural metabolites that act as biological control agents and growth promoters. The biofilm formation is a mechanism that can also accomplish its role in the protection of microbes to harsh environments, as will be discussed in the next section.

5.1.1 Biofilm Formation: The Role of Exopolysaccharides

Biofilm is defined by the community of microorganisms when attached to a surface (O'Toole et al. 2000) or associated with interfaces (Davey and O'Toole 2000). According to O'Toole et al. (2000), biofilms can comprise one single microbial species or several and can also be formed on a wide variety of biotic and abiotic surfaces, depending on a response to a specific environmental condition. The matrix where those microorganisms attach is made of a chain of polysaccharides that can be produced in the interior of the microorganism and then be eliminated to the exterior as exopolysaccharides. They are produced by a large variety of microorganisms (Sutherland 1998) and are accumulated in the surface of cells (Coronado et al. 1996) (Fig. 2).

The use of exopolysaccharides is being associated to a mechanism of adaptation of microorganisms to a wide variety of environmental conditions such as salinity, shifts in temperature, and water stress. They make possible the degradation of some substances, help the colonization, virulence, and survival of some phytopathogens in the host (Roper et al. 2007) and can also protect against stressful environmental conditions (Coronado et al. 1996). Iwabuchi et al. (2000) discovered that *Rhodococcus rodochrous* was able to produce an exopolysaccharide containing D-glucose, D-galactose, D-mannose, D-glucuronic acid, and lipids that contributed to the bacterial tolerance to the aromatic faction of crude oil. They have added some exopolysaccharide in sea water with high nutrient content and some aromatic fraction of crude oil, resulting in a growth promotion ability of indigenous bacteria and increase in the degradation of crude oil by bacteria (Iwabuchi et al. 2002).

Chang et al. (2007) suggested that a strain of *Pseudomonas putida* must produce an exopolysaccharide called alginate that influences the development and the

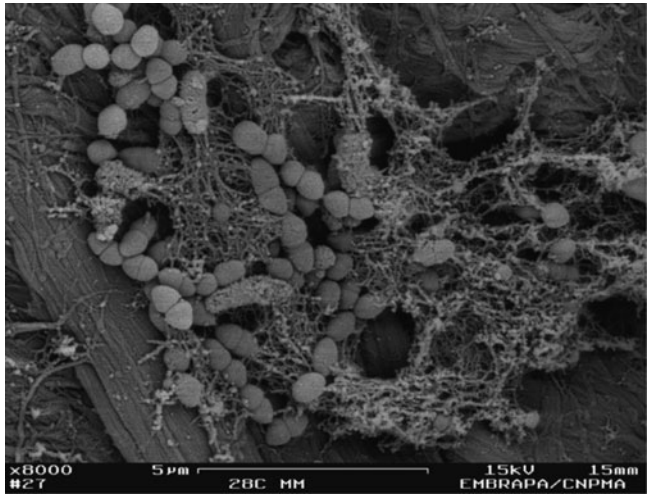

Fig. 2 Scanning electron microscopy showing some bacteria capable of producing exopolysaccharides. Note that cells are embedded in a net formed by a polymeric matrix
Source: EMBRAPA – Meio Ambiente 2009

physical–chemical properties of exopolysaccharide in response to a limited water supply. These responses should facilitate the maintenance of a hydrated environment, protecting the microorganisms against desiccation. Mutant bacteria without the gene responsible for alginate production displayed sensitivity to heat, paraquat, and hydrogen peroxide (Keith and Bender 1999). In the case of the terrestrial cyanobacterium *Nostoc commune*, exopolysaccharide production showed to be crucial to the stress tolerance during desiccation, freezing, and thawing (Tamaru et al. 2005).

Alami et al. (2000) studied the effects of a rhizobacterium capable of producing exopolysaccharide in the growth promotion of sunflower (*Helianthus annus*) under water stress. The inoculation of the strain modified the soil structure in the root system, acting against the negative effect of the lack of water in the growth. There is a factor called AlgU (AlgT) that controls the production of exopolysaccharide that is important for the adaptation of *Pseudomonas fluorescens* in dry environmental conditions (Schnider-Keel et al. 2001).

With this in mind, bacteria inoculated in the root system of plants can help in their survival in environments where water is a limiting factor. Marulanda et al. (2006) innoculated *Glomus intraradices and Bacillus thuringiensis* in *Retama sphaerocarpa*, observing an increase in the root growth of 201%, as well as higher water absorption. *Medicago sativa* plants were submitted to drought and an analysis of the involvement of the carbon metabolism and oxidative stress in the reduction of nitrogenase activity was performed. In a very severe drought, the activity of the enzyme was inhibited in 82% (Naya et al. 2007).

5.2 The Brazilian Caatinga

In Brazil, the semiarid region encompasses several minor-environments with singular conditions of climate, soil, and vegetation heterogeneity. This zone is located almost exclusively in the Northeast of the country, and is one of the five Brazilian geopolitical regions with nine states (Fig. 3). This large dry lands, stretching between 3 and 17°S and 35–45°W, covers approximately 8% of the Brazilian territory and occupies an area of about 900,000 km^2 (Giulietti 2006). The climate of this region is one of the most complex systems in the world (Giulietti et al. 2002), not only due to the size of this huge land and its diverse physiography, but also due to the conjunction of two major weather system, provided by the northeast and southeast trade winds, which create an enormous diversity and instability in rainfall patterns. These physical and climatic conditions provide the great diversity of vegetation types that characterize the semiarid region. The precipitation within the region varies from being extremely wet, with an annual rainfall of up to around 2,000 mm along the coast, to only 300–500 mm in the semiarid zone, where the rainfall is usually restricted to a few months during the year. It is indeed this factor of water availability, which is the controlling influence over the vegetation (Fig. 4) and fauna, as well as, to a great extent, human exploitation of natural resources, throughout the region.

The set of contrasting physical and climatic factors has combined to provide the diversity of vegetation types that characterize the semiarid region as a mosaic, reflecting the microlocal conditions particular to each region. Such change of rainfall regime can be modulated by the altitude, where the presence of hills or mountains provides a gradual range of condition of raining and temperature, modulating the landscapes compositions. From the coast to about 100–200 km inland, the vegetation is dominated by Atlantic Forest, with its lush, evergreen canopy leaves. Further inland, as rain is scarcer, the rain forest gives space to a forest in which the canopies semideciduous or leaves-free in dry season species are more abundant. More into the continent, the extreme dry conditions make the deciduous forest dominant, free of leaves and bleached due to the intensity of the sun. From these forests, the last two delimit the caatinga, excluding only the rain forest, which is more related to the coastline area. The structure of these forests can vary considerably from forest composed of often spiny trees, 6–10 m tall, deciduous or semideciduos, and often with a ground-layer of small deciduous shrubs and annual herbs, with predominance of Leguminosae, cacti, bromeliads, and Euphorbiaceae (Giulietti et al. 2006).

The caatinga biome covers about 735,000 km^2, and it is one of the most degraded vegetation in the semiarid region, with less than 1% of its area protected in permanent reservoirs. Recently, the Brazilian government put efforts on initiatives to better preserve its biodiversity. Areas of extreme biological interest were selected overlapping information of different groups of organisms and endogenous regions were proposed for the biome preservation (Giulietti et al. 2006). In addition, the scientific community has raised the question about the biotechnological

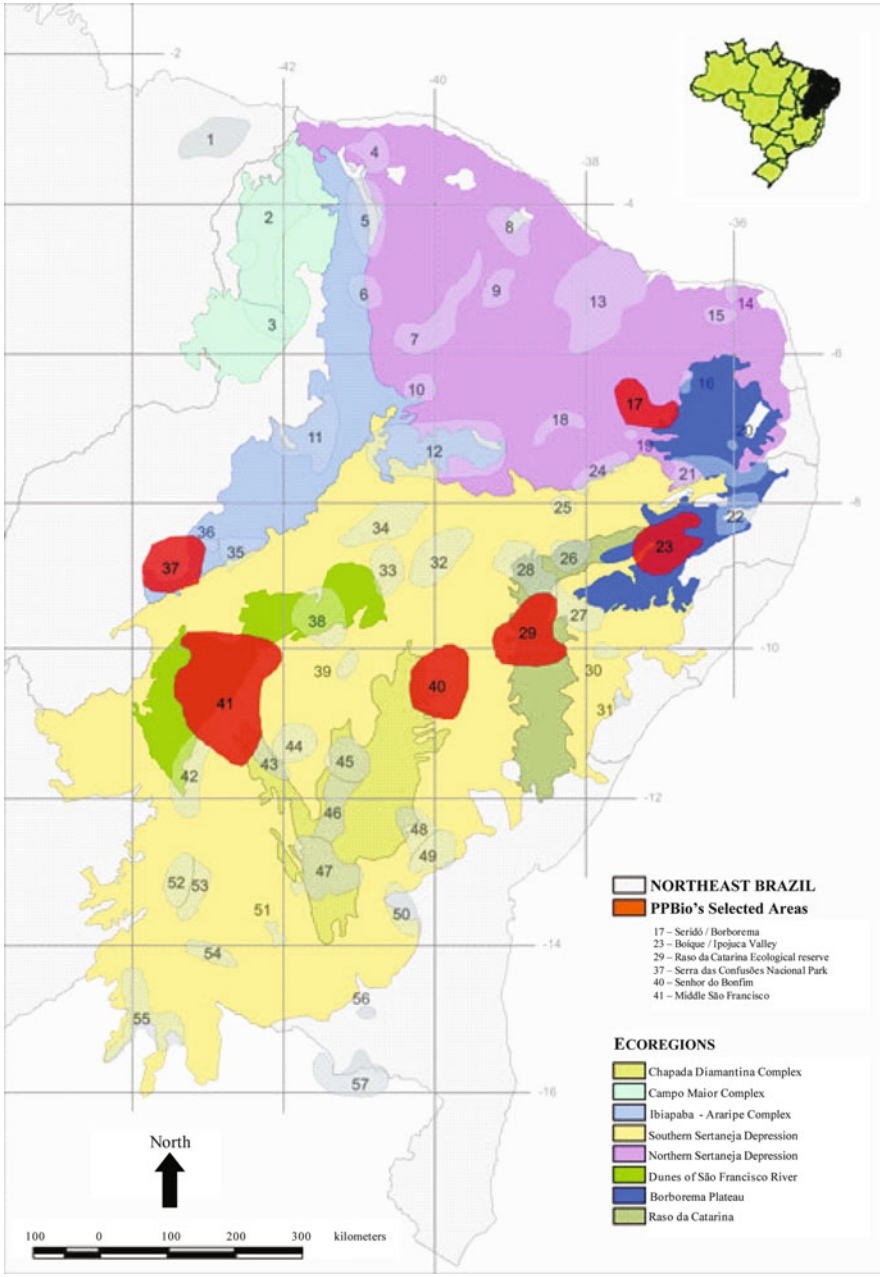

Fig. 3 Location of the semiarid climate in Brazil. Map of the Brazilian Northeast showing the ecoregions of the Caatinga Biome and the 57 priority areas for conservation (modified from Giulietti et al. 2006); with a remark to six (*red*) areas of Extreme Biological Importance

Fig. 4 Pictures from the Caatinga area in Northeast of Brazil. Typical vegetation and sight scene are shown, with a remark for plants from the Cactaceae family

potential found in species endogenous to the caatinga biome, where a remark is made for the microbial community, believed to be highly important in the biome functioning and maintenance.

A great example of such potential is represented by the bacteria associated to caatinga-plants. It is reasonable to consider that the plant life is not easy in such environmental conditions, and microbes associated to plants might have important roles in plant development. The first coming hypothesis for biotechnological possibilities is that, microbes associated to plants at caatinga might help the plant cultivation in dry agricultural lands, where water regime is not similar every year. Also, such bacteria can help the initial plant development and during the fruiting process, when the missing of water result in expressive decreasing of production. Other possibilities are still open to explored by enthusiastic scientists on the field of plant growth promotion rhizobacteria.

5.3 The Mangrove: Ecosystem and the Interaction of Plant–Bacteria in this Niche

Mangrove is a typical tropical ecosystem comprised of a coastal biome, located at the transition between the land and the sea (Sjõling et al. 2005; Zhou et al. 2006). This biome covers around 60–75% of the world's tropical and subtropical coastlines (Holguin et al. 2001) (Figs. 5 and 6), and it is characterized by the periodic tidal flooding which makes environmental factors such as salinity and nutrient availability highly variable (Crump et al. 2004; Holguin et al. 2006; Alongi 1989).

This constant change in environmental conditions makes the microbial diversity highly responsive, in order to adapt to these shifts, controlling and maintaining the

Fig. 5 The mangrove ecosystem shown in different distances. The remark should be made to the soil under the water film, responsible for the harsh conditions of anaerobiosis combined with the high salinity

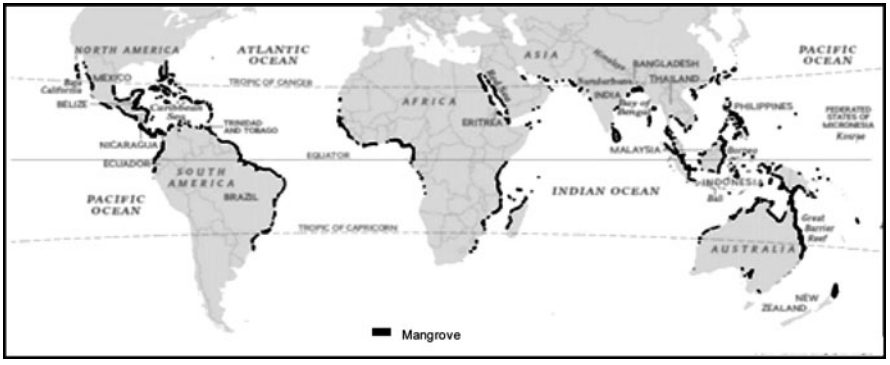

Fig. 6 The abundance and occurrence of mangrove coastline areas in the world represented by lines drawn in the color black

functioning of the mangrove (Holguin et al. 2006). However, a phylogenetic and functional description of microbial diversity in the mangrove ecosystem has not been addressed to the same extent as that of other environments (Zhou et al. 2006). A more thorough description of the bacterial diversity and distribution in a

mangrove would improve our understanding of bacterial functionality and microbial interactions found in that ecosystem (Kathiresan et al. 2006 and Selvam 2006).

In the mangrove ecosystem, rhizobacteria are present and very important in the plant protection against the salinity. These bacteria are also able to fix nitrogen, supplying the plants with this essential nutrient (Holguin et al. 2001). The main bacterial groups and their role found as rhizobacteria in mangrove plants are affiliated to the genus *Vibrio*, *Listonella*, *Phyllobacterium*, the free-living nitrogen fixers; *Bacillus*, *Paenibacillus*, *Xanthobacter*, and *Vibrio* responsible for phosphate solubilization; and *Desulfovibrio*, *Desulfotomaculum*, *Desulfosarcina*, and *Desulfococcus* known as sulfate reducers.

The importance in the knowledge of plant-associated bacteria in mangroves resides on its biotechnological potential. The microbes in such conditions might help plants to survive in the mangrove, where the salinity and the depletion of oxygen exert a particular environment. A possible application to such microbes is the protection of plants against the salinity of soils, commonly found in newly available areas for agricultural practice.

5.4 Plant Growth-Promoting Rhizobacteria in Harsh Environments

Some PGPR are able to protect plants against abiotic stress such as drought and salinity. It was demonstrated the protection of soybean plants in saline soil by the inoculation with two PGPR *Bacillus subtilis* and *Bacillus megaterium* (Han and Lee 2005).

In semiarid ecosystems like those presented in Northeast of Brazil and in some Mediterranean regions, where the temperature is very high in the summer, the rainfalls are regularly scarce and the evaporation rate is considerably high. In cases where plants are submitted constantly to drought, it is interesting to apply rhizobacteria that could improve plant fitness reducing the growth inhibition ameliorating the drought. This was showed by Jaleel et al. (2007) who inoculated *P. fluorescens* in *Catharanthus roseus* and submitted to drought. There was an increase in fresh and dry weights in inoculated plants. Then, plants and microorganisms in such niches must be very well adapted to these conditions. The mechanisms by which PGPR tolerate some abiotic stress remain unknown. However, Yang et al. (2009) have proposed that PGPR are able to elicit the induced systemic tolerance (IST) of plants by physical and chemical changes that result in increased tolerance to abiotic stress. In soils with high salinity, the application of PGPR can alleviate salt stress on plant growth by the production of phytohormones and growth regulators (Egamberdiyeva 2009). The growth promotion can be attributed to the production of indole-3-acetic acid (IAA) in the case of *Azospirillum brasiliense*, which is triggered by some nutrient stresses as well as environmental fluctuations (Malhotra and Srivastava 2009). It has been demonstrated that

the synthesis of auxins depend on tryptophan and it is also related to plant growth promotion, as showed by Idris et al. (2007a) for *Bacillus amyloliquefaciens*. Ethylene, a plant growth regulator, has also been established as a stress hormone. Under stress conditions the production of ethylene increases, affecting the root growth and the growth of the plant as a whole. The enzyme ACC deaminase is responsible for the regulation of ethylene biosynthesis. So, several studies have been focusing on the introduction of ACC deaminase genes into plants to regulate ethylene level in plants for optimum growth under stressed conditions (Saleem et al. 2007). In the case of mangrove ecosystems, although rich in organic matter, this niche is defective in nutrients, especially nitrogen and phosphorus (Vazquez et al. 2000). The supply of nutrients can be raised by the action of PGPR. This was shown by Biari et al. (2008) when they inoculated some PGPR in maize – *Zea mays* – seeds. They observed that the uptake of nutrients such as N, P, K, Fe, Zn, Mn, and Cu was significantly influenced by this inocula as well as the increment in plant height and shoot and seed dry weight.

6 Agricultural Improvements: The Role of Plant Growth-Promoting Rhizobacteria

The soil vegetation protects it against erosion and helps in the maintenance of the equilibrium between the factors related to its formation and degradation. The disruption of this relation provokes physical, chemical, and biological alterations that can cause a decay in the productivity and a degradation in the ecosystem. Tropical regions are considered to have the greater agricultural potential due to the abundance in light, water, and heat, essential to plant development. However, these areas are being more subjected to soil degradation, because of the high precipitation rates, weak soil structure, low organic matter content, and inadequate handling. The relation between the components of agriculture production and their environmental consequences depends on the production system and the type of exploration (Knoepp et al. 2000).

Due to the special features of PGPR presented in this chapter, Ashrafuzzaman et al. (2009) state that the use of these microorganisms is a very attractive option to replace the use of chemical fertilizers, pesticides, and other substances in agriculture. In this way, several researches have been conducted to improve growth and yield of many agricultural products by increasing nitrogen, phosphorus, and potassium availability, stimulating root nodulation and phytohormone production. PGPR has already been used as inoculum for rice, maize, wheat, soybean, and others. Phosphate is important for plant growth and in soils where this nutrient in unavailable, PGPR can be used for phosphate solubilization. In this way, Trivedi and Pandey (2007) immobilized cells of *P. putida* on sodium alginate beads to test the efficiency of wheat growth promotion by the solubilization of insoluble phosphate. The use of genetic manipulation by recombinant DNA methodology can also

be useful for some agricultural purposes. Rodríguez et al. (2000) first reported the achievement of improved phosphate solubilizing ability from rhizobacterial strains by genetic construction using a gene involved in mineral phosphate solubilization. Potassium is also an important nutrient required for plant growth and development (Ashley et al. 2006). Sheng (2005) studied the ability of a potassium-releasing bacterial strain *Bacillus edaphicus* to promote cotton and rape growth in K-deficient soil. The strain was able to mobilize potassium as well as promote root and shoot growth. It is known that nitrogen is one of the most limiting nutrient for growth of plants (Stacey et al. 1992). The associative interaction of rhizobia to leguminous plants has evolved to help the nitrogen fixation, thus providing nitrogen to plant to develop its function. However, this association depends on the nodulation of the leguminous plants, allowing the rhizobia to penetrate plant tissues. In this way, PGPR can also help in this process. Inoculation of the common bean – *Phaseolus vulgaris* – with PGPR stimulated nodulation resulting in higher levels of nitrogen accumulation (Figueiredo et al. 2008).

When there is a concern about crop productivity, PGPR can be used for this purpose, by increasing growth rate and production of biomass. Ashrafuzzaman et al. (2009) tested the efficiency of ten isolates for rice growth enhancement, resulting in a significant increase in plant height, root length, production of root and shoot dry matter, and seed germination. One strain was also able to produce indoleacetic acid and solubilize phosphorus. The potential of growth promotion by rhizobacteria may be due to the expression of more than one trait, as it was observed by Dey et al. (2004). Inoculation of *Pseudomonas* spp. in *Arachis hypogea* displayed a suppression of phytopathogens, solubilization of tri-calcium phosphate, production of siderophore, and nodulation promotion, and all of these characteristics might have contributed to the enhancement of growth, yield, and nutrient uptake of peanut. Khalid et al. (2004) screened some PGPR for the potential of in vitro auxin production, applying four isolates in wheat seedlings, which resulted in increase in root and shoot elongation, root and shoot dry weight. *Pseudomonas corrugata* showed to be an appropriate inoculum for maize under rainfed conditions (Kumar et al. 2007).

When looking for an optimal crop production it is interesting to make a coinoculation of more than one PGPR as well as in combination with other rhizobia and fungi, i.e., Arbuscular Mycorrhizal Fungi (AMF), so they can act synergistically in the increase of plant growth (Gamalero et al. 2004). To examine this, Camacho et al. (2001) coinoculated a strain of *Bacillus* sp. with *Rhizobium tropici*, observing an increased bean nodulation.

7 Conclusion

This chapter gives an overview of typical harsh conditions found in specific environments exclusively found in tropical areas of the world. Exploiting these niches for PGPR functions may help on the development of biotechnological

processes to save plants cultivated in common land against environmental harsh conditions. Also, an elusion could be made for the use of new land areas and for the maintenance of the agricultural yield under the climate changing process.

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Cold-Tolerant Agriculturally Important Microorganisms

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Abstract Cold-tolerant microorganisms are endowed with the ability to grow at 0°C, though their growth optima lie in the mesophilic range. To overcome the stress induced by low temperatures they have evolved a variety of adaptive responses at

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the cellular and molecular levels. Multiple cell membrane modifications ensure that solute transport is not impaired at low temperatures. Other mechanisms include the synthesis of cold-shock proteins (Csps), cold acclimation proteins (Caps), cryoprotectants, ice nucleation factors, cold-adapted enzymes, and RNA degradosomes. The agricultural importance of such microbes stems from the fact that the world over temperate agro-ecosystems are characterized by low temperatures and short growing seasons that subject both plant and microbial life to cold temperature induced stress. Hence, there is a need to identify potential microbes that retain their functional traits under low temperature conditions. Such microbes can be profitably used as inoculants in agricultural production systems in the temperate regions of the world. This chapter deals with the cold tolerance/resistance mechanisms operating in microorganisms and the utility of cold-tolerant microbes in improving soil quality and productivity of agricultural crops.

1 Introduction

Among the various environmental stresses that microbes encounter due to their ubiquitous distribution on earth, cold temperature induced stress assumes paramount importance. This is due to the fact that most life processes are temperature-dependent and life almost comes to a standstill under suboptimal temperatures. Cold temperatures affect the cell interiors and a myriad of cellular processes, rendering microbial cells inactive or often irreversibly damaged. Since more than 80% of the earth's biosphere is exposed to temperatures below 5°C, throughout the year (Herbraud and Potier 1999), microorganisms capable of coping with low temperature stress have naturally evolved in several environments. Considering their ubiquity and dominance, cold-adapted microorganisms are widely regarded as the most successful colonizers of our planet (Russell 1990). During the past two decades, considerable research attention has been devoted to cold-adapted microorganisms driven by the realization that such microbes and their enzymes have a great potential for exploitation in biotechnology (Kottmier and Sullivan 1990). The agricultural importance of cold-tolerant microorganisms arises due to the fact that the cropping cycle in several parts of the world is subject to transient cold periods, which are deleterious to microbial processes such as symbiotic and asymbiotic nitrogen fixation, plant growth promotion, and disease suppression.

Life under low temperatures was identified by Forster (1887), who reported that microorganisms isolated from fish could grow at 0°C. Since then, a number of organisms particularly bacteria, yeasts, unicellular algae, and fungi have been reported to successfully colonize low temperature environments and contribute to nutrient cycling processes and primary biomass production. Following nearly two decades of debate over the term psychrophiles (named from the Greek word for "cold-loving"), the definition given by Morita in 1975 became widely accepted. He based his definitions of cold-adapted bacteria on their cardinal growth temperatures, viz. lower limit, optimum, and upper limit. Psychrophiles grow at or below

zero (0°C) and have an optimum growth temperature at 15°C and an upper limit of 20°C. In contrast, psychrotolerant microbes (also called psychrotrophs) can also grow close to 0°C, and also grow at mesophilic temperatures with a growth optima usually above 30°C, hence they could be considered as cold-tolerant mesophiles (Morita 1975). Such organisms are much more widely distributed than psychrophiles and can be isolated from soils and waters in temperate regions, as well as from refrigerated food products. Though psychrotolerant organisms do grow at 0°C, they have highly extended lag periods, before the appearance of visible colonies on growth media under in vitro conditions.

2 Ecological Diversity of Cold-Tolerant Microorganisms

Generally, it is widely perceived that more extreme the environmental conditions of a niche, the lower the diversity of organisms. But most cold inhospitable environments are dominated by a variety of microorganisms, thereby making them the most versatile of all life forms. The lowest temperature limit for life seems to be around −20°C, which is the value reported for bacteria living in permafrost soil and in sea ice. Microbial activity at such temperatures is restricted to small amounts of unfrozen water inside the permafrost soil or the ice and brine channels. These contain high concentrations of salts, exopolymeric substances, and/or particulate matter, and fluid flow is maintained by concentration and temperature gradients (D'Amico et al. 2006). Cold-tolerant microorganisms are also widely encountered in refrigerated environments and have become a major cause of concern in the food processing and storage industry. In nature, the alpine soil environments are characterized by dramatic seasonal shift in physical and biochemical properties, due to intermittent snow cover and fluctuating sub freezing temperatures in winter and intense desiccating sunshine punctuated by infrequent rains during summer (Greenland and Losleben 2001). It is not uncommon to come across a wide variety of cold-tolerant microorganisms in the alpine and subalpine landscapes. Bacteria, archaea, and eukaryotes like yeast occur in cold environments. While bacteria dominate and are present in greater diversity than archaea in polar environments, archaea are wide spread in cold, deep ocean waters (Karner et al. 2001; Deming 2002). Morphological types encountered in cold environments include spore-formers, nonspore formers, and filamentous bacteria. Together they cover a wide range of metabolic types ranging from aerobes to anaerobes and include both heterotrophs and autotrophs.

3 Cold Temperature Effects on Microbial Cells

Temperature can influence the response of a microorganism either directly or indirectly. Direct effects include decreased growth rate, enzyme activities, alteration of cell composition, and differential nutritional requirements. Indirect

effects are usually observed on the solubility of solute molecules, diffusion of nutrients, osmotic effects on membranes, and cell density (Herbert 1986). As temperature falls, the lag phase that precedes growth extends, leading to a decrease in the growth rate and the final cell number. During the lag phase that precedes growth in mesophiles, many physiological changes occur, including a decrease in the saturation of fatty acids and inhibition of DNA, RNA, and protein synthesis (Panoff et al. 1998).The effect of cold temperatures is largely felt on the solute transport system. The lipid bilayer which is the basic structure of the microbial membranes must have proper fluidity to maintain the cell permeability and movement of essential solutes. The functional state of this bilayer is a liquid–crystalline phase, but a decline in temperature induces a gel phase transition and a drastic loss of the membrane properties. A major difference between mesophiles and psychrotrophs is the ability to transport sugars into the cell at temperatures near 0°C (Wilkins 1973). The effect of the rapid cold shock on the membrane correlated with high rates of cell inactivation (90 and 70%) in *Escherichia coli* and *Bacillus subtilis*, respectively. Thus, membrane alternation seems to be the principal cause of cold-shock injury in *E. coli* and *B. subtilis* (Hoang et al. 2007).

In some bacteria, production of pigments and other enzymatic activities are enhanced at low temperatures, e.g., lipase and proteinase production by *Pseudomonas* and certain other genera occurs preferentially at low temperatures (Witter et al. 1966; Olson and Nottingham 1980).The prior temperature history of the cell has been found to be an important factor for the survival and growth of organisms because of its effects on the extent of lag phase before onset of growth (Dufrenne et al. 1997). A decrease in the poly-β-hydroxybutyrate (PHB) content of non-cold acclimated *Rhizobium* DDSS69 cultures was observed by Sardesai and Babu (2001a). Cold stress induces a shift in the carbon source utilization and enhances the susceptibility of bacteria to antibiotics (Ponder et al. 2005). *Vibrio cholerae* is known to enter the viable but nonculturable state in response to cold shock (Escalana et al. 2006). Since the agro-ecosystem is characterized by transient cold stress followed by warmer temperature regimes, we will focus mainly on the cold tolerance mechanisms of psychrotolerant microbes and their role in agriculture.

4 Cold Tolerance Mechanisms in Microorganisms

Unsaturation of fatty acids, reduction in the average fatty acid chain length, maintenance of membrane fluidity, synthesis of several cryoprotectant compounds, cold acclimation proteins (Caps), cold-shock proteins (Csps), ice nucleators and antifreeze proteins, cold-adapted enzymes, and RNA degradosomes are some of the cold tolerance mechanisms known to be active in microorganisms and are discussed herein.

4.1 Cell Membrane-Associated Changes

Since low temperatures primarily affect the lipid bi-layer of the bacterial cell rendering it impermeable to diffusion of solutes, the fluidity of the lipid bi-layer has to be maintained for the cell to function properly. It has been well established that microorganisms adjust their cell membrane constituents in accordance to their growth temperatures to ensure membrane functions such as solute transport (Russell et al. 1995; Mastronicolis et al. 1998). The most common changes in the cell membrane at cold temperature are the unsaturation of fatty acids, by desaturases situated in the membrane itself. In the anaerobic bacterium *Clostridium botulinum*, a decrease in temperature from 37 to 8°C leads to an increased level of unsaturation from 27 to 40% (Russell et al. 1995). This is achieved by an increase in the amount of branched fatty acid and reduction in the concentration of cyclic fatty acids and increases the monounsaturated straight chain fatty acids. The outcome of increasing fluidity may be attributed to the shortening of average fatty acid chain length owing to fewer carbon–carbon interactions between neighboring chains (Evans et al. 1998).

Other mechanisms include reduction in the average fatty acid chain length which is observed in the psychrophilic organism bacterium *Micrococcus cryophilus* (McGibbon and Russel 1983). A similar mechanism is also encountered in the yeast *Zygosaccharomyces bailii* at low temperature (Baleiras-Couto and Huis-In't-Veld 1995). An increase in the amount of branched fatty acids and reduction of the amount of cyclic fatty acids are observed in *Salmonella* spp. (Russell 1984) and *C. botulinum* (Evans et al. 1998). In *Listeria monocytogenes*, the major change that takes place as the temperature falls below optimum (e.g., 7°C) is the enhancement in amounts of $C_{15:0}$ at the expense of $C_{17:0}$. Such a reduction in fatty acid chain length reduces the melting temperature and aids in maintenance of the membrane fluidity at low temperatures. Moreover, there is also a small increase in $C_{18:1}$ which adds to fluidization of membrane at cold temperatures (Puettman et al. 1993; Annous et al. 1997). *B. subtilis* alters its membrane composition by enhancing the level of anteiso-branched fatty acid contents and decreasing the isobranched ones (Klein et al. 1999).

Maintenance of membrane fluidity is a major mode of survival of cold-adapted rhizobia since the symbiotic proteins (p Sym Nod), which are major determinants of nodule competitiveness, are membrane associated (Denarie et al. 1992). The induction of *nod* FE gene in cold-adapted *R. leguminosarum* bv. viciae was found to result in the de novo synthesis of phospholipids with specific polyunsaturated fatty acids (Geiger et al. 1993). Theberge et al. (1996) observed that the proportion of *cis*-vaccenic acid, the major unsaturated fatty acid increased by 30% as growth temperature of two cold-adapted *R. leguminosarum* bv. *viciae* strains were lowered. Drouin et al. (2000) observed that low temperature conditions affected fatty acid composition of all rhizobial strains, regardless of their cold adaptation level. The proportion of unsaturated fatty acids also increased significantly with the decrease in the growth temperature from 25 to 5°C. A specific

fatty acid (*cis*-12 octadecanoic acid) was detected in artic rhizobial strains during growth at 5°C.

4.2 Role of Cryoprotectants in Cold Tolerance

Cold-tolerant microorganisms are endowed with the ability to synthesize several cryoprotectants compounds, such as glycine betaine (a bacterial cryoprotectant), glycerol, trehalose, sorbitol, manintol, glucose, and fructose, to overcome the ill effects of cold temperature induced stress. Such cryoprotectants are thought to act as chemical chaperones at cold temperatures (Margesin and Schinner 1999; Russell 1998). Glycine betaine a cryoprotectant of bacterial origin was detected in the food borne pathogen *L. monocytogenes*, which survives at low temperatures and high osmolarity (Angelidis and Smith 2003). The exact mechanism of action of glycine betaine is not yet clear. However, it is thought to function as a chemical chaperone, which prevents the aggregation of cellular proteins during stress conditions. Chatopadhyay (2002) proposed that the possible function of glycine betaine is to regulate the fluidity of membrane at lower temperatures.

Trehalose is a nonreducing disaccharide (α-D-glucopyranosil-1, 1-α-D-glucopyranoside) found in many prokaryotic and eukaryotic organisms, known to be an important protectant against heat-shock and osmotic stress in microorganisms (Kandror et al. 2002). The main function of trehalose is the stabilization of the cell membrane and proteins by replacing water and preservation of intracellular water structure (Sano et al. 1999). Exogenous trehalose helps to protect a variety of organisms against freezing and the maximum protection happens when trehalose is present on both sides of the cell membrane (Herbraud and Potier 1999). The increased viability of *E. coli* under cold-shock conditions is attributed to the enhanced accumulation of trehalose (Kaasen et al. 1992; Mitta et al. 1997). Trehalose synthesis is regulated by the genes *otsA* and *otsB* that encode the enzymes, trehalose-6-phosphate synthases and trehalose-6-phosphatase, respectively (Kaasen et al. 1992).

4.3 Cold Acclimation Proteins

Cold-tolerant bacteria produce a set of ~20 permanent proteins called the cold acclimation proteins (Caps) during continuous growth at low temperatures. The Caps are fundamental to life in the cold and ensure improved protein synthesis at low temperature (Margesin et al. 2007). Some of the Caps identified in cold-adapted bacteria function as Csps in mesophiles, a typical example being the RNA chaperone CspA. It has been proposed that cold acclimatization proteins are essential for the maintenance of both growth and cell cycle at low temperatures, but their function is still poorly understood. A cold acclimation protein (Hsc 25) produced

in the ice-nucleating bacterium, *Pantoea ananas* KUIN-3, was found to be capable of refolding enzymes, which were denatured by heat, cold, and guanidine hydrochloride, but it had high affinity for cold denatured enzymes than for heat-denatured enzymes (Kawahara et al. 2000).

4.4 Cold-Shock Proteins

A sudden decrease in temperature from the mesophilic range to cold temperatures (10–15°C) creates a stress situation. Microbial cells respond to such a situation by a specific adaptative mechanism, which allows their survival and subsequent growth at lower temperatures. Although such rapid down shifts are unlikely to occur in natural environments, it provides interesting laboratory conditions that have largely contributed to the elucidation of the molecular mechanisms by which cells responds to cold (Herbraud and Potier 1999). A sudden decrease in temperature initiates the cold-shock response (Jones et al. 1987; Graumann et al. 1996; Jones and Inouye 1996), which is evidently not confined to psychrophilic (cold loving) and psychrotrophic (cold-tolerant) microorganisms but constitutes the beginning of cold adaptation in all microbes. It involves the induction and synthesis of Csps for the regulation of protein synthesis and mRNA folding. Bacterial Csps consist of a single nucleic acid-binding domain, called the cold-shock domain (CSD). The CSD is considered to be an ancient molecule present even prior to the advent of single cell life and is the most evolutionarily conserved nucleic acid-binding domain within prokaryotes and eukaryotes (Graumann and Marahiel 1998). Owing to the design of prokaryotic transcriptional machinery, the cold-induced RNA secondary structure may impose premature transcription termination. The functional significance of bacterial Csps is therefore directly related to the formation of stable secondary RNA structures in response to low temperature stress (Polissi et al. 2003). The cold-shock proteins Csp A, Csp C, and Csp E were confirmed to possess in vivo and in vivo transcription antitermination activity (Bae et al. 2000). CspA has been proposed to function as an RNA chaperone at low temperature and has been implicated in transcriptional regulation of two cold-shock genes *hrs* and *gyr A*. The 5′ end of the Csp A mRNA contains a regulatory sequence (cold box), which may be responsible for the cold-shock induction (Jiang et al. 1997).

The number of Csps seems to increase with the severity of the cold shock. The major Csps accounts for more than 10% of total protein synthesized during the acclimation phase of *E. coli* (Goldstein et al. 1990). Radiolabelling of total cellular proteins of *Pseudomonas* spp. 30-3 revealed the elevated expression of an 8 kDa protein at 4°C, which suggests that the protein Cap B plays a pivotal role in survival and tolerance at cold and subzero temperatures (Panicker et al. 2002). Among the bacterial species, the Csps of *E. coli* are the most studied and a fair degree of homology has been observed with the Csps from other microorganisms such as *B. subtilis* and *Streptomyces clavuligerus*. Similarly, a 248 bp DNA

fragment in *Pseudomonas* spp. 30-3 that was amplified using Cap B gene specific primers showed a 98% amino acid sequence homology with Cap B of *Pseudomonas fragi* and 62% homology with Csp A of *E. coli* (Michel et al. 1997; Panicker et al. 2002).

Drouin et al. (2000) isolated cold-adapted strains of *R. leguminosarum* bv. *viciae* from the legumes *Lathyrus japonicus* and *Lathyrus pratensis* in northern Quebec (Canada). When these strains were compared with a poorly adapted strain and a cold sensitive strain for freezing survival, protein induction, and fatty acid composition following a cold shock from 25°C to 10, 5, and 0°C, a common 6.1 kDa Csp was induced in all the strains, but the total number of Csps synthesized at 0°C was higher in the cold-adapted strains than in the cold sensitive strains. Csps have also been detected in several other agriculturally important bacterial species (Table 1).

The regulation of the expression of Csps and their homologues is complex involving autoregulation and is controlled at the level of transcription and translation as well as by the stability of mRNA and proteins. Response to cold shock might be controlled at the transcriptional or translational level, though the two possibilities are mutually exclusive (Horn et al. 2007).

Table 1 Major cold-shock proteins identified in some agriculturally important psychrotrophic bacterial species

Organism	Cold-shock protein	Biochemical features	References
Bacillus megaterium ATCC 14581	Unnamed	Responsible for the hyper induction of desaturase	Fujii and Fulco (1977)
Bacillus cereus WSBC 10201	Csp A	M.W. 7.5 kDa	Mayr et al. (1996)
Bacillus subtilis	Csp B	M.W. 7.365 kDa	Schindelin et al. (1993)
	Csp C	M.W. 8.0 kDa	
	Csp D	M.W. 13.0 kDa	Fujii and Fulco (1977)
	Csp 7.4		Schnuchel et al. (1993)
Rhizobium sp. (Temperate strains)	Unnamed	M.W. 56.1,37.1,34.4, 17.3,11.1 kDa	Cloutier et al. (1992)
Rhizobium sp. (Artic strains)	Unnamed	M.W. 52.0,38.0,23.4,22.7 and 11.1 kDa	Cloutier et al. (1992)
Pseudomonas fragi	C 7.0	M.W. 7.0 kDa	Hebraud et al. (1994)
	C 8.0	M.W. 8.0 kDa	
Listeria monocytogenes	Unnamed	M.W. 48 kDa M.W. 21.1 kDa M.W. 19.7 kDa M.W. 18.8 kDa	Bayles et al. (1996)
Streptococcus thermophilus	Unnamed	M.W. 7.5 kDa M.W. 21.5 kDa	Perin et al. (1999)
Lactococcus lactis	Unnamed	M.W. 7 kDa	Wouters et al. (1999)
Streptoccocus thermophilus	Clp L	M.W. 75 kDa	Varcamonti et al. (2006)

4.5 Role of Ice Nucleators and Antifreeze Proteins in Cold Tolerance

Ice nucleators are proteins which either limit super cooling or induce freezing at temperatures below 0°C by mimicking the structure of an ice crystal surface. They impose an ice crystal like arrangement on the water molecule with their surface and reduce the energy necessary for the initiation of ice formation. Ice-nucleating agents either facilitate cold-protection due to the released heat of fusion or establish protective extracellular freezing in place of lethal intracellular freezing (Zachariassen and Kristiansen 2000). The "ice plus" bacteria posses Ina protein (Ice nucleation-active protein) on the outer bacterial wall which acts as the nucleating center for ice crystals (Lee et al. 1995). This facilitates ice formation at high subzero temperatures, while "ice minus" bacteria do not posses Ina proteins and therefore lower the ice nucleation temperature. Very potent ice nucleators, active at high subfreezing temperature, are produced by bacteria such as *Erwinia herbicola* (Kozloff et al. 1983). Other bacterial genera viz., *Pseudomonas*, *Pantoea* (*Erwinia*), and *Xanthomonas* can nucleate the crystallization of ice from supercooled water (Lindow et al. 1978; Maki et al. 1974; Obata et al. 1990).

Another possible strategy used by microorganisms to survive freezing temperature is the production of antifreeze proteins (AFPs), a structurally diverse group of proteins that have the ability to modify ice crystal structure (Raymond and DeVries 1977) and inhibit recrystallization of ice (Knight et al. 1988). AFPs inhibit further binding of water molecules and affect the shape of ice crystal, even at very low concentrations. The Arctic plant growth promoting rhizobacteria *Pseudomonas putida* GR 12-2 secretes an AFP that enhances its survival at subzero temperature. Expression of *afp A* gene of *P. putida* in *E. coli* yielded an intracellular 72 kDa protein that exhibited lower levels of antifreeze and ice nucleation activities. The AfpA sequence was most similar to cell wall associated proteins and less similar to ice nucleation proteins (INPs). Hydropathy plots revealed that the amino acid sequence of AfpA was more hydrophobic than those of the INPs in the domain that forms the ice template, thereby suggesting that AFPs and INPs interact differently with ice (Muryoi et al. 2004).

4.6 Cold-Adapted Enzymes

The most important selective pressure of low temperatures is exerted toward chemical reaction rates, most of which exponentially drop with decreasing temperature. Despite this, psychrophiles produce cold-adapted enzymes that have high specific activities at low temperatures. The commonly accepted hypothesis for this cold adaptation is the activity–stability–flexibility relationship, which suggests that psychrophilic enzymes increase the flexibility of their structure to compensate for the "freezing effect" of cold habitats (Johns and Somero 2004). This increased

flexibility might concern the entire protein or might be restricted to parts of the structure; especially those implicated in catalysis and are probably also responsible for the generally observed low stability of cold-adapted proteins (D'Amico et al. 2003).

4.7 Role of RNA Degradosomes

The degradosome, a protein-complex of several ribonucleases, is the major determinant factor for stability of cellular RNA. The degradosome of an antarctic bacterium *Pseudomonas syringae* has been found to contain an endoribonuclease RNAse E and a RNA helicase. But instead of polynucleotide phosphorylase, the exoribonuclease found in *E. coli*, the degradosome of the antarctic bacterium contains another exoribonuclease, called RNAse R. In *E. coli*, this enzyme is known to play an important role in ensuring the quality control of rRNA. The significance of the association of this enzyme with RNAse E in the Antarctic bacterium is not definitely known. But it is believed that RNAse R can degrade RNA molecules with extensive secondary structures. This eliminates the necessity of ATP, required by helicase, thereby helping the cell conserve energy at low temperatures (Purusharth et al. 2005).

4.8 Other Mechanisms of Cold Tolerance in Rhizobia

In *Rhizobium* strain DDSS69, it was observed by Sardesai and Babu (2001b) that the specific activities of key enzymes of the pentose phosphate pathway, viz., glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, were enhanced under cold stress to rapidly generate energy to overcome the stress. They also reported diversity in the switching mechanisms of carbon metabolism among cold-acclimated and noncold-acclimated *Rhizobium* isolates. In another study, they detected a rapid induction of two high molecular weight membrane polypeptides of 135 and 119 kDa within 15 min of exposure to 5°C in the *Rhizobium* strain DDSS69. PAGE membrane protein profiles of stressed and nonstressed cells revealed differential regulation of genes (Sardesai and Babu 2001b).

5 Agricultural Importance of Cold-Tolerant Microorganisms

Microorganisms play a major role in sustaining the production and productivity of any agro-ecosystem through a myriad of roles that extend form nitrogen fixation, nutrient solubilization, nutrient mobilization, plant growth promotion, and the suppression of harmful pathogens and insects. A unique feature of temperate

agro-ecosystems around the world is the short growing periods, which are interspersed by suboptimal temperatures. Under such a scenario most microbial processes are bound to slow down or worse even come to a standstill, thereby have an adverse effect on the productivity. This effect is most pronounced in the case of nutrient transformations, where microbes play an enormous role. In such a scenario where time and temperatures are crucial determinants of both crop growth and microbial growth, cold-tolerant microbes which retain their activity in suboptimal temperature conditions are indispensable. But unfortunately not many efforts have been undertaken in understanding the nature and properties of these microbes and the quantum of information on the tolerance mechanisms of other agriculturally important microorganisms is very scarce.

5.1 Plant Growth Promotion by Cold-Tolerant Microbes

The volume of soils surrounding roots is influenced chemically, physically, and biologically by the plant root and is commonly referred to as the rhizosphere. This is a highly favorable habitat for the proliferation of microorganisms which exert a potential impact on plant health and soil fertility. The plant growth promoting rhizobacteria (PGPR), which are an important component of the rhizosphere microbial community, were first defined by Kloepper and Schroth (1978). In recent years, the term has been modified as Plant Growth Promoting Bacteria (PGPB) to accommodate other strains that are nonrhizospheric in origin (Andrews and Harris 2003). In temperate climates, the growth and activity of such rhizospheric communities are highly dependent on the root zone temperature since most physiological processes that influence plant growth virtually come to a standstill at suboptimal temperatures. In such a scenario, it's important that the root colonizing bacteria retain their metabolic versatility at low temperatures, since plant growth promotion is achieved by the action of several metabolic intermediates and end products. One of the major mechanisms of plant growth promotion is the production of the stimulatory phytohormones, by PGPR/PGPB within the root zone; these hormones stimulate the density and length of root hairs resulting in the enhanced uptake of water and mineral nutrients from soil (Volkmar and Bremer 1998). Apart from phytohormone production, plant growth promotion is known to be mediated by a variety of mechanisms including siderophore production (Katiyar and Goel 2004), antagonism toward deleterious root microorganisms (Misaghi et al. 1982), deamination of the precursor molecule of the phytohormone ethylene whose accumulation in root tissue is known to be detrimental to root growth and development (Glick et al. 1998), and induction of systemic resistance to plant pathogenic microorganisms (Lavania et al. 2006).

The auxin, indole-3-acetic acid (IAA), is an important phytohormone produced by PGPR, and treatment with auxin-producing rhizobacteria has been shown to increase the plant growth (Patten and Glick 2002). The IAA producing capability of microorganisms is useful in their identification and provides a valuable marker

when examining the physiological roles or ecological significance of IAA in the establishment and persistence of the organisms (Bric et al. 1991). Auxin production in bacteria is regulated though the proline-linked pentose phosphate pathway (McCue et al. 2000). Selvakumar et al. (2008a, b) reported the occurrence of cold-tolerant plant growth promoting bacterial strains *Pantoea dispersa* strain 1A and *Serratia marcescens* strain SRM from the North-Western Indian Himalayas. These strains retained their IAA producing ability at 4 and 15°C. Seed bacterization with these bacterial strains significantly enhanced plant biomass and nutrient uptake of wheat seedling grown at cold temperatures. The genus *Pseudomonas* is an important component of the rhizospheric microbial community and often plays an important role in plant growth promotion. Mishra et al. (2008, 2009a) described the cold tolerance and IAA production by *Pseudomonas* sp. strain PGERs17 and NARs9 at cold temperature. Seed inoculation with these strains enhanced the seed germination, root and shoot lengths of wheat seedlings grown at low temperatures. Considering the metabolic versatility of Pseudomonads, it is possible to unearth a whole of cold-tolerant plant growth promoting bacterial species in the future.

Another bacterial mechanism that positively influences plant growth is the production of the enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This enzyme plays a significant role in the regulation of the plant hormone, ethylene and thus, influences the growth and development of plants. Bacterial strains containing ACC deaminase can, in part, at least alleviate the stress-induced ethylene-mediated negative impact on plants. Like many other abiotic and biotic factors, accelerated ethylene production under high and chilling temperatures has widely been reported by researchers both in plant tissues and microbial species in the rhizosphere. Plants with ACC deaminase expression may cope with this unfavorable situation by lowering ethylene level like that under other environmental stresses (Saleem et al. 2007). A psychrotolerant ACC deaminase producing bacterium *P. putida* UW4 was found to promote canola plant growth at low temperature under salt stress (Cheng et al. 2007). Considering the role of ethylene in stress physiology, it can be rightly said that much more efforts are needed to decipher the role of ACC deaminase producing bacterial strains in plant growth promotion under cold temperature conditions.

Iron the fourth most abundant element in the earth's crust is required for growth of nearly all forms of life (Howard 1999). However, its availability to the organism is very limited due to the rapid oxidation of ferrous (Fe^{++}) to ferric (Fe^{+++}) state. Ferric ion is highly insoluble under physiological conditions and makes its acquisition by microorganisms a considerable challenge (Neilands 1995). Microorganisms have evolved specialized mechanisms for the assimilation of iron, including the production of low molecular weight iron chelating compounds, known as siderophores, which transport this element into their cells. Siderophores have been implicated for both direct and indirect enhancement of plant growth by rhizospheric microorganisms (Neilands 1981). Siderophores provide an advantage in survival of both plants and bacteria of because they mediate competition that results in exclusions of fungal pathogens and other microbial competitors in the rhizosphere by a reduction in the availability of iron for their survival (Masalha et al. 2000,

Wang et al. 2000). The role of siderophores in biocontrol of plant pathogens was first demonstrated with pseudobactin, the siderophore produced by plant growth promoting *Pseudomonas* strain B_{10} (Kloepper et al. 1980). A cold-tolerant mutant of *Pseudomonas fluorescens* with a 17-fold increase in siderophore production and increased rhizosphere colonization was developed by Katiyar and Goel (2004). This mutant strain promoted growth of *Vigna radiata* plants at 25 and 10°C. Studies on siderophore-mediated growth promotion by psychrotolerant bacteria still remain in its infancy and need to be probed further.

An important facet of the competitiveness of a biocontrol agent is its ability to persist and proliferate. However, it is often difficult to predict the behavior of a particular microbe in the soil since the soil persistence of a bacterium may be influenced by a number of different factors including soil temperature. Many fungal phytopathogens are most destructive when the soil temperature is low, hence it is reasonable to expect that the biocontrol agents are also cold-tolerant. McBeath (1995) reported the isolation of several strains of *Trichoderma* sp. that acted as biocontrol agents at low temperatures (i.e., 4–10°C) against a range of different pathogenic fungi. Negi et al. (2005) have characterized a group of cold-tolerant Pseudomonads from the Garhwal region of the Indian Himalaya. These strains produced siderophores and exhibited plant growth promotion activity at temperatures ranging from 4 to 25°C. Seed inoculation with these isolates resulted in the suppression of major root borne diseases of garden pea. A novel bacterium *Exiguobacterium acetylicum* strain 1P isolated from a high altitude soil in the N.W. Indian Himalaya, which has ability to produce siderophores at 4°C and inhibited the growth and development of *Rhizoctonia solani, Sclerotium rolfsii, Phythium*, and *Fusarium oxysporium* under in vitro and pot culture conditions was described by Selvakumar et al. (2009c). Recently, Malviya et al. (2009) have isolated antagonistic, chitinolytic, psychrotolerant strains of *Streptomyces* from glacial sites of the Indian Himalayas. These stains were found to inhibit the growth of several plant pathogenic fungi. In the present scenario where the demand for pesticide free food products is on the rise, much more research efforts are required for identifying cold-tolerant strains of biocontrol agents for use in the temperate growing regions.

Freezing injury in plants is particularly complex because of the nonuniform behavior of different plant parts, e.g., stem, leaf, bud, flowers, etc. Ice nucleation in plants is frequently not endogenous, but is induced by catalytic sites present in microbial parasites, which can be found on leaves, fruits, or stems (Lindow 1983). Ice nucleating strains of *P. syringae* increase the frost susceptibility of tomato and soybean when sprayed on leaves prior to low temperature stress in addition to being a pathogen of these plants (Anderson et al. 1982). Recognition of the gene associated with ice nucleation in *P. syringae* first led to the synthesis of an "ice-minus" mutant, which was found to be inactive in promoting ice nucleation in plants leaves (Xu et al. 1998). Reducing the numbers of ice nucleating bacteria by different approaches is an effective and environmentally safe method of controlling freezing injury in plants and is considered a classic example of displacement of a bacterial pathogen by a biocontrol agent.

5.2 Diazotrophy Under Cold Temperature Conditions

Nitrogen fixation by symbiotic and asymbiotic bacterial genera is one of the major means by which life is sustained in this planet. But this process is hugely affected by cold temperature stress. The effects of low temperature on the activities of rhizobia include depression of nodule competitiveness and nodule functioning. The production of Nod metabolites by *Rhizobium leguminosarum* bv. *trifolii* is reduced by lowering the temperature, which in turn may affect the nodulation and yield of host legumes (McKay and Djordjevic 1993). Many studies have shown that suboptimal temperatures affect the competitiveness of rhizobia for nodulation, delay root infection, and inhibit nodule function (Lynch and Smith 1994). It has been estimated that under temperate conditions, the establishment of an effective symbiosis a week earlier in the crop-growing season could double the amount of nitrogen fixed and thus increase legume crop productivity (Sprent 1979). Therefore, it is imperative to select cold-adapted strains of rhizobia to overcome the cold induced stress. In a major step in this direction, Prevost et al. (1999) selected cold-adapted rhizobia from Canadian soils with the aim of improving the productivity of legumes that are subjected to low temperatures during the growing season. For this purpose, they used rhizobia associated with legume species indigenous to arctic and subarctic regions. The candidate rhizobia were *Mesorhizobium* sp. isolated from *Astragalus*/*Oxytropis* spp. and *Rhizobium leguminosarum* from *Lathryrus* spp. These rhizobia are considered psychrotrophs due to their ability to grow at 0°C. The advantages of cold-adapted arctic *Mesorhizobium* in improving legume symbiosis were demonstrated with the temperate forage legume sainfoin (*Onobrychis viciifolia*). In laboratory and field studies, arctic rhizobia were found to be more efficient than temperate (commercial) rhizobia in improving growth of sainfoin and were more competitive in forming nodules. Biochemical studies on cold adaptation revealed higher synthesis of Csps in the cold-adapted rhizobia, than their mesophilic counterparts. Since the arctic *Mesorhizobium* could not nodulate agronomically important legumes, the nodulation genes and the bacterial signals (Nod factors) were characterized as a first step to modifying the host specificity of nodulation.

Another approach was to screen for cold-adapted rhizobia naturally associated with agronomic legumes cultivated in temperate areas. It has been shown that the environment from which rhizobia are isolated, relates to their ability to enter into symbiosis with legumes under specific environmental conditions. Rhizobia originating from the cooler climes of North America were able to positively influence the nodulation and nitrogen fixation of soybean, compared with their counterparts originating from the warmer southern climes (Zhang et al. 2003). A superior strain of *Sinorhizobium meliloti* adapted for nodulation of alfalfa at low temperatures was selected and was found efficient in improving growth of alfalfa in laboratory and field studies. This strain also performed well in improving growth of alfalfa after over wintering under cold and anaerobic (ice encasement) stresses, indicating a possible cross-adaptation of selected rhizobia for various abiotic stresses inherent to

temperate climates (Prevost et al. 2003). Ideal candidate rhizobia for temperate legumes would, therefore, require a high degree of nodule competitiveness and nitrogen fixing abilities combined with cold-tolerant traits. Such rhizobia would retain their membrane fluidity at low temperatures, thereby enabling the synthesis and activity of membrane-associated Nod factors that play a major role in the nodulation and host specificity.

Azospirillum is an associative symbiotic plant growth promoting bacterium that is predominantly associated with the grasses and cereal crops of the tropics. Tripathi and Klingmuller (1992) proposed that growth, survival, and activity of the bacterium are highly dependent on temperature. Kaushik et al. (2001) postulated that a low or nonsignificant effect of *Azospirillum* inoculation in winter crops has discouraged the large-scale use of this bacterium. Kaushik et al. (2000) selected Tn5::lacZ mutants isogenic to wild type *Azospirillum brasilense* that were capable of growing at cold temperatures. In field studies, they observed that two strains of *A. brasilense* were able to influence wheat growth at suboptimal temperatures (Kaushik et al. 2002). Though the temperature regime at which the isolates were evaluated for their plant response was not strictly temperate, this is one of the few studies on field performance of *Azospirillum* under suboptimal temperatures. Considering its agronomic significance *Azospirillum* is a candidate bacterium for the potential for exploration and development of cold-tolerant isolates.

5.3 Phosphate Solubilization by Cold-Tolerant Bacteria

Phosphate solubilization by rhizospheric microflora is one of the most important means of achieving plant growth promotion. Bacterial mineral phosphate solubilization has been mainly attributed to the activity of glucose dehydrogenase; a membrane-bound enzyme that is involved in the direct oxidation of glucose to gluconic acid (Goldstein 1995). Subsequently, gluconic acid is enzymatically converted to 2-ketogluconic acid and 2,5-diketogluconic acid. The 2-ketogluconic acid is more effective than gluconic acid in solubilizing phosphate (Kim et al. 2002). Earlier studies on this phenomenon were restricted to mesophilic temperatures (Chung et al. 2005; Chen et al. 2006). The first report of P solubilization at low temperatures was made by Das et al. (2003) who studied cold-tolerant *Pseudomonas* mutants for their phosphate solubilization activity at low temperature (10°C). They found that all the cold-tolerant mutants were more efficient than their respective wild type counterparts for phosphate solubilization activity at 10°C as compared with 25°C. P solubilization by *Pseudomonas* mutant's at the psychrotolerant range has also been reported (Katiyar and Goel 2003; Trivedi and Sa 2008). But considering the environmental stability of mutant strains, for commercial inoculant production it would be prudent to scout pristine environments for naturally occurring psychrotolerant strains. Most progress has been made in this direction, mainly from the Indian Himalayan Region.

Pandey et al. (2006) isolated a cold-tolerant phosphate solubilizing and antagonistic strain of *P. putida*, from a subalpine location of Indian central Himalaya. This strain solubilized phosphate in the temperature range of 4–28°C. Phosphate solubilization by a cold-tolerant strain of *P. fragi* was reported by Selvakumar et al. (2009a). This is a novel discovery since *P. fragi* is generally associated with spoilage of dairy foods under refrigerated conditions. This strain solubilized phosphate at temperatures ranging from 4 to 30°C, besides significantly increasing the percent germination, rate of germination, plant biomass, and nutrient uptake of wheat seedlings under cold temperature conditions. A rhizosphere competent phosphate solubilizing strain of *Acinetobacter rhizosphaerae* was isolated from the cold deserts of the Indian Himalayan region by Gulati et al. (2009).Though phosphate solubilization at cold temperatures by this bacterium was not described, this is an early report on the occurrence of this bacterium in cold environments. Vyas et al. (2009) screened 19 efficient phosphate-solubilizing fluorescent *Pseudomonas* isolates from the cold deserts of the trans-Himalayas, for tolerance against temperature, alkalinity, salinity, calcium salts, and desiccation-induced stresses. Phylogenetic analysis based on 16S rRNA gene sequencing placed these bacteria under three groups with 14 strains in Group I including *Pseudomonas trivialis* and *P. poae*, two strains in Group II together with *Pseudomonas kilonensis* and *P. corrugata*, and three strains in Group III along with *Pseudomonas jessenii* and *P. moraviensis*. In a recent study, Selvakumar et al. (2009b) reported that the genetic clustering of cold-tolerant phosphate solubilizing Pseudomonads was affected by their geographical origin. Repetitive element PCR profiles revealed that isolates originating from the warmer southern slopes formed a distinct cluster, while their counterparts from the cooler north facing slopes formed the second cluster. The studies that have been mentioned above are mostly of exploratory nature, while the real need of the hour is the development of a commercially viable cold-tolerant PSB inoculant that can be profitably used in temperate agriculture.

5.4 Induction of Resistance to Cold Stress by PGPB

Cold temperature stress affects the metabolic activity of plants in multiple ways and causes significant yield reduction. To overcome this, several exploratory studies using microbial strains have been carried out. In vitro inoculation of *Vitis vinifera* cv. Chardonnay explants with a plant growth-promoting rhizobacterium, *Burkholderia phytofirmans* strain PsJN, increased grapevine growth and physiological activity at a low temperature. There was a relationship between endophytic bacterial colonization of the grapevine plantlets and their growth at both ambient (26°C) and low (4°C) temperatures and their sensitivities to chilling. The major benefits of bacterization were observed on root growth (11.8 and 10.7-fold increases at 26°C and 4°C, respectively) and plantlet biomass (6 and 2.2-fold increases at 26°C and 4°C, respectively). The inoculation with PsJN also significantly improved plantlet cold tolerance compared with that of the nonbacterized control. Moreover, relative

to the noninoculated controls, bacterized plantlets had significantly increased levels of starch, proline, and phenolics. These increases correlated with the enhancement of cold tolerance of the grapevine plantlets (Barka et al. 2006).

Mishra et al. (2009b) examined the effect of seed inoculation with 12 cold-tolerant plant growth promoting *Pseudomonas* strains on wheat growth and physiological changes under green house conditions at 10 ± 2°C. It was observed that bacterization with Pseudomonads significantly improved root length (27.9–70.5%), shoot length (4.7–26.1%), dry root biomass (1.69–3.19-fold increases), dry shoot biomass (1.27–1.66-fold increase) compared with nonbacterized control. Bacterized wheat plants showed enhanced levels of total chlorophyll, anthocyanin, free proline, total phenolics, and starch contents, while a decrease was observed in the Na^+/K^+ ratio and electrolyte leakage vaues. These parameters are critical to the plant's ability to tolerate cold stress conditions. In another study, they observed that inoculation with cold-tolerant bacterium *Pseudomonas* spp. strain PPERs23 significantly improved root length (41%), shoot length (11.9%), dry root biomass (44.4%), dry shoot biomass (53.8%), total chlorophyll (3.1%), total phenolics (37.3%), and amino acid (39.4%) content of wheat seedlings. In this study also, increased levels of physiologically available iron, protein, anthocyanin, proline and relative water contents coupled with a decrease in Na^+/K^+ ratio and electrolyte leakage values were observed in bacterized wheat plants. These parameters indicate the ability of bacterium to alleviate cold induced stress in wheat seedlings (Mishra et al. 2009c).

6 Industrial Potential of Psychrotolerant Microorganisms

The unique properties of cold-tolerant microorganism make them potential candidates for exploitation in industry. Microbial cryoprotectants like trehalose have immense biotechnological potential and can be used in a wide range of applications (Lillford and Holt 2002). Similarly, the Antifreeze Proteins (Afps) from bacteria can be used in a wide variety of ways (Tange et al. 2003). Cold active proteases are used for the industrial peeling of leather by proteases at tap water temperatures instead of heating to 37°C. During cold storage β-galactosidases are used to remove lactose in milk, while cold active pectinases are used for the clarification of fruit juices. Another interesting application is the use of a heat labile alkaline phosphatase, which does not interfere with end labeling of polynucleotide kinase after heat treatment. Cold-tolerant microbes and enzymes can be used for the bioremediation of polluted soils and waste waters during winter in temperate regions, when the degradative capacities of the endogenous microflora is impaired by low temperatures (Feller and Gerday 2003).The development of transgenic plants with increased frost tolerance is another exciting application. The introduction of genes from microorganisms or even whole biosynthetic pathways in plants has already been shown to improve freeze tolerance.

Arabidopsis thaliana plants transformed with the *codA* gene encoding choline oxidase and accumulating glycine betaine in the chloroplast showed a significant improvement in freeze tolerance (Sakamoto et al. 2000).

7 Conclusion

Cold-tolerant microorganisms are widely distributed in the agro-ecosystem and play a variety of roles extending from nitrogen fixation, plant growth promotion, and alleviation of cold stress in plants. Though most research work conducted so far has largely focused on rhizobia, it is a welcome sign that many agriculturally important resourceful microbes are being described from various parts of the earth. But serious attempts are needed to study the activity of enzymes such as nitrogenases in cold-adapted microorganisms. Another interesting area where research needs to be focused is the identification of cold active decomposing microorganisms, since temperature is a major determinant of decomposition, and most decomposition processes come to a standstill at suboptimal temperatures. If research efforts succeed in identifying consortia of potential decomposers that retain their enzymatic potential at lower temperatures, it would be of immense use in agriculture the world over.

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The Role of Plant Growth Promoting Rhizobacteria in Sustainable and Low-Input Graminaceous Crop Production

Stephen P. Cummings and Caroline Orr

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Abstract Plant growth stimulating rhizobacteria that improve the yield of graminaceous crops have been studied since the 1930s. Increases in crop yield have often been inconsistent, reflecting a lack of understanding by which PGPR exert their effects. Many PGPR are able to fix N_2, which was initially assumed to boost crops by supplementing soil N. Subsequently, it became clear that for most free-living PGPR other mechanisms affecting root development and nutrient uptake can explain the increased crop yields. Endophytic bacteria have demonstrated some potential to contribute to the N budget of certain graminaceous crops but require more robust assessment of their potential. Here, we review the current state of our understanding of PGPR in graminaceous crop cultivation, identifying their potential

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contribution to more sustainable agricultural practices but also highlighting issues that need to be addressed before this technology can be appropriately assessed as a replacement for inorganic N addition.

1 Introduction

The latter part of the twentieth century saw a remarkable rise in the productivity of agricultural systems; today, the global production of food is 145% greater than that of 1960 (Pretty 2008). In parallel with increases in agricultural output, the world population has doubled from three to six billion over the same period; nevertheless, on a per capita basis there is 25% more food for each individual compared to 50 years ago. China has demonstrated the most significant developments, increasing overall food production fivefold and per capita production threefold since 1960. In contrast, Africa has observed a 10% decline in per capita food production over the same period (Pretty 2008), Currently, the global population could be fed by the present level of agricultural output; however, the regional inequalities reflect continuing political, economic, and social challenges preventing agriculture to feed the current population, 1.2 billion of whom live in crippling poverty (Hazell and Wood 2008).

The gravest issue for sustainable food security is the predicted increase in global population, which is projected to rise from the current 6.8 billion and surpass 9 billion people by 2050. The burden of feeding these additional people will be felt most keenly by developing countries, whose populations are projected to rise from 5.6 billion in 2009 to 7.9 billion in 2050 (https://www.unfpa.org/public/).

Most developing countries have environmental constraints that will impede the development of agricultural systems able to meet this challenge. These include lack of water, desertification and insufficient cultivable land. Moreover, the increase in urbanisation of the global population poses additional challenges. That will require a 500% increase in economic activity driven by a 300% increase in both energy consumption and manufacturing activity (Miller 2008). For many people in rapidly developing economies, an increased disposable income coincides with the adoption of a diet with a greater consumption of meat and processed cereal products. To meet this demand, livestock will need to be raised intensively on a diet of cereals and oils (Pretty 2008). This, in turn, will place an increased pressure on the available agricultural land and how it is farmed. As a result, it has been argued that current models of low-input agriculture relying on biological nitrogen fixation (BNF) and requiring large areas of land will be unlikely to provide the annual requirement of an extra 15 million tonnes of protein by 2050 to stave off widespread hunger (Jenkinson 2001; Smil 2001).

The technological advances that have enabled agriculture to intensify its production practices in order to keep pace with the increasing demand have relied on the application of huge amounts of inorganic N fertiliser that effectively meets the demand of crops throughout the growing season and removes the requirement for a

fertility enhancing cycle in crop rotations. The exploitation of inorganic N fertiliser has contributed to a 4% increase in aggregate global cereal grain production in the 40 years since 1960, during which period fertiliser consumption increased from 10.8 to 85.6 Mt N $year^{-1}$ (Crews and Peoples 2004). The significance of inorganic N fertiliser and the Haber–Bosch process that generates has been contextualised by Smil (2001) who asserts that by 2050 over half of the human population will owe its existence to synthetic N fertilisers.

While the application of inorganic N has had significant benefits for agricultural food productivity and global food security in the short term, there are increasing concerns around the sustainability of this technology to provide a long-term solution to ensure that food production keeps pace with the burgeoning population. The management of agricultural soil is fundamental to ensuring a sustainable agricultural system; however, it is becoming clear that intensive agricultural systems leads to the degradation of agricultural soils as a result of, among other factors, the loss of organic matter, compaction and increased salinity, and leaching of inorganic nitrate, along with associated costs such as fuel requirements and the loss of water resources (Kibblewhite et al. 2008; Peoples et al. 1995; Smil 2001).

Consequently, there is increasing interest in developing agricultural management systems that embrace the principles of sustainability. While such concepts are not novel, there is an increasing urgency in developing and implementing them because of increasing alarm that current conventional agricultural management systems cannot continue linearly increasing their reliance on fertilizer consumption, pesticide application, the expansion of agricultural land and machine usage indefinitely, without detriment to the environment (Kitzes et al. 2008).

Pretty (2008) articulated three factors that defined sustainable agricultural practices and technologies:

1. They have no adverse effects on the environment.
2. They are accessible and effective for farmers.
3. They lead to improvements in food productivity and have positive effects on environmental goods and services.

The aim of this chapter is to map the contribution of plant growth promoting rhizobacteria (PGPR) that are indigenous or inoculated into soils to the sustainable cultivation of graminaceous crops. We examine how this technology have and may continue to contribute to more sustainable approaches to the production of these key crops in the context of increasing population growth and other environmental pressures.

2 The Role of PGPR in Low-Input and Sustainable Agriculture

PGPR have been extensively studied and used as inoculants on graminaceous crops, to increase yield and simultaneously reduce the requirement for inorganic fertiliser application. There are numerous studies, utilising a range of bacterial taxa, which explore the effects of PGPR inoculation on such crops. In Table 1, a summary of the

Table 1 A summary of the effects of PGPR on graminaceous crops under laboratory and field conditions

PGPR	Crop	Effect of inoculation	Proposed mode of action	Reference
Azospirillum; *Flavobacterium*; *Pseudomonas*	Rice (*Oryzia sativa* L.)	Maximum rice yields were observed when PGPR inoculum was combined with inorganic fertiliser (30 kg N ha^{-1})	More efficient utilisation of fertilizer N	Ali et al. (1995)
Isolates from rice rhizosphere		Isolate inoculation resulted in a significant increase in germination rates, plant height, root length and dry matter production in rice seedlings.	Produce phytohormones and solubilise phosphate	Ashrafuzzaman et al. (2009)
Azospirillum lipoferum		Increased root length, root surface area and root volume	Phytohormone synthesis and siderophore production	Boyer et al. (2008)
Azospirillum		Grain yield and N content was improved	Increased N fixation	Pedraza et al. (2009)
Pseudomonas spp.		Increased IAA levels	Phytohormone synthesis	Karnwal (2009)
Azospirillum; Enterobacter; *Aeromonas veronii*		Increase in root area, plant biomass and N fixation.	Increased N fixation and phytohormone synthesis	Mehnaz et al. (2001)
Pseudomonas spp.; *Azospirillum*		Increased shoot biomass and grain yield	Increased N fixation and phytohormone production	Mirza et al. (2006)
Herbaspirillum sp. strain B501 gfp1		Increased dry and fresh weight	Increased N fixation and phytohormone synthesis	Zakria et al. (2007)
Burkholderia vietnamiensis		Increased shoot and root weight and leaf surface	Mechanism is not addressed but hypothesize that increased N fixation and phytohormone production are involved	Van et al. (2000)
Isolates from wheat rhizosphere	Wheat (*Triticum aestivum* L.)	Improved yield and higher N content in grain and straw when used in combination with recommended chemical fertilizers.	Improved N use efficiency	Akhtar et al. (2009)
Azospirillum brasilense		Increased the quantity of photosynthetic pigments resulting in greener plants.	Enhanced photosynthetic pigment production.	Bashan et al. (2006)
Isolates from wheat rhizosphere (reference strains *Azospirillum* sp.)		Increased shoot and root biomass	Siderophore or phytohormone production	Fischer et al. (2007)

Pseudomonas cepacia R55, R85, *P. aeruginosa* R80, *P. fluorescens* R92; *P. putida* R104		Increased root dry weight but results were very inconsistent	Interaction with AMF alters nutrient and water uptake but leads to inconsistent results	Germida and Walley (1996)
Pseudomonas fluorescens		Significant increase in yield	Regulate production of ethylene and elongate roots by hydrolyzing 1-aminocyclopropane-1-carboxylic acid	Naveed et al. (2008a)
Pseudomonas fluorescens		Plant protected from soil borne fungal pathogens	Suppress soil borne fungal pathogens	Okubara and Bonsall (2008)
Pseudomonas fluorescens SBW25; *Paenibacillus brasilensis* PB177		In some combinations increased plant dry weight	Protection from pathogenic fungus	Jaderlund et al. (2008)
30 Isolates taken from wheat rhizosphere		Increased growth and yield	Increased phytohormone biosynthesis	Khalid et al. (2004)
Pseudomonas spp.; *Burkholderia caryophylli*		Significant root elongation, root weight and grain and straw yields.	Increased ACC-deaminase activity, chitinase activity, phytohormone production and P solubilization	Shaharoona et al. (2008)
Pseudomonas fluorescens		Significantly improved growth when increased NPK are also added to the soil	ACC deaminase activity	Shaharoona et al. (2008)
Pseudomonas fluorescens	Rye (*Secale cereale*)	Significant increase in foliar dry mass	Siderophore production and suppression of fungal pathogens	Kurek and Jaroszuk-Scisel (2003)
Bacillus licheniformis RCO2; *Rhodobacter capsulatus* RCO4; *Paenibacillu polymyxa* RCO5; *Pseudomonas putida* RCO6	Barley *(Hordeum vulgare* L.)	Increased root and shoot weight and increased uptake of Fe, N, Mn and Zn	Increased N fixation and production of phytohormones	Cakmakci et al. (2007)
Commercially available Plant Growth Activator (PGA)	Maize (*Zea mays* L.)	Greater plant height	More efficient uptake of N and P	Adesemoye et al. (2008)

(*continued*)

Table 1 (continued)

PGPR	Crop	Effect of inoculation	Proposed mode of action	Reference
Azospirillum brasilense Az39; *Bradyrhizobium japonicum* E109	Maize (*Zea mays* L.)	Seed germination and nodule formation were promoted	Production of phytohormones	Cassan et al. (2009)
Bacillus sp.; *Ochrobactrum* sp.		Significantly increased shoot and root dry weight	Suppressed fungal pathogens	Principe et al. (2007)
Azospirillum lipoferum CRT1		Root growth was enhanced	No explanation given	El Zemrany et al. (2006)
Rhizobium spp.; *Sinorhizobium* spp.		Increased shoot and root dry biomass	Production of phytohormones and siderophores.	Hossain and Martensson (2008)
Bacillus megaterium; . *B subtilis*; *Pseudomonas corrugata*		Increase in grain yield	Increase in fixed nitrogen, production of phytohormones, phosphate solubilization, production of antibiotics and siderophores	Kumar et al. (2007)
Pseudomonas spp.		Increased grain yield and nutrient uptake	Hydrolyses ACC	Naveed et al. (2008a)

effects of PGPR inoculation on a range of graminaceous crops including rice, wheat and maize are presented, derived from studies that have been conducted at both a laboratory and field scale. What is striking is the number of PGPR that have N_2-fixing ability but also the additional array of mechanisms of action, proposed to account for the enhanced plant growth observed. Many of the studies report a response of the crop under study and link it, often without robust data sets, to a particular bacterial activity such as N_2 fixation or production of phytohormones. It is clear that the issues around the lack of reproducibility of response to PGPR inoculation require a far more systematic approach before the technology can be effectively deployed in the field. Such studies need, among other things, to be designed to robustly test the mechanism(s) by which the plant responds to PGPR inoculation and its reproducibility from one growing season to the next, a consistent measure of the plants response (Vessey 2003) and an appraisal of the persistence of the inoculated PGPR in the soil (Strigul and Kravchenko 2006).

3 Mechanisms of Action of PGPR

In much of the earliest work on the exploitation of PGPR in graminaceous crop production, the mechanism of action of the bacteria was presumed to be a result of the increased input of fixed nitrogen into the soil. Many of the taxa identified as being effective PGPR are capable of fixing atmospheric nitrogen (Table 1). Subsequent work has revealed that there are a variety of mechanisms through which plant growth can be facilitated, including hormone production, enhanced nutrient acquisition, pathogen suppression and N_2-fixation, often working in parallel to produce the observed response. These effects have been extensively studied and reviewed. Here, we summarise the key findings suggesting that PGPR frequently exert their effect through multiple mechanisms working simultaneously.

3.1 Biological Nitrogen fixation

Biological Nitrogen fixation (BNF) can occur in bulk or rhizospheric soil. Fixed nitrogen can then be acquired through root uptake and contribute to the nitrogen budget of the crop. The earliest large-scale experiments, exploiting PGPR potential to enhance crop productivity used N_2-fixing bacteria, with the implicit assumption that it was this activity that was producing the enhanced crop yields. For example, large-scale field trials in the 1950s used N_2-fixing bacteria, principally *Azotobacter chroococcum* as an inoculum on several million hectares of graminaceous crops including wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) (Cooper 1959); however, owing to inconsistent results the trials were abandoned in the 1960s (Andrews et al. 2003). Other bacterial taxa, including *Azospirillum* spp. and *Agrobacterium radiobacter*, were also extensively studied and trialled as potential

substitutes for N fertiliser (Zavalin et al. 2001). One study in Russia to test the potential of a strain of *A. radiobacter*, isolated from the rhizosphere of rice (*Oryza sativa* L.), on winter wheat and spring barely appeared to give significant increases (5–30%) in yield in 2 out of 3 years. At the same time, it was estimated that the contribution of N_2 fixation to total N assimilation was between 23 and 32% (Bairamov et al. 2001). However, the lack of consistency in the results from one year to the next reflected that of the earlier studies (Andrews et al. 2003). More significantly, in this example, *A. radiobacter*, now reclassified as *Rhizobium radiobacter* (Young et al. 2001), was a taxa that had never demonstrated the ability to fix N_2. Subsequent studies on this strain demonstrated unequivocally that, as with all members of this taxon, *R. radiobacter* was not capable of fixing atmospheric N_2, nor did it form a physical association with the roots of barley. The plant growth promoting substances it produced were most probably responsible for the increase in yields of graminaceous crops (Humphry et al. 2007).

N_2-fixing activity has been confirmed in PGPR in many other cases. *Azospirillum* species have, for example, been implicated in the enhancement of rice (Pedraza et al. 2009), maize (Montanez et al. 2009) and wheat (Sala et al. 2007) through BNF mechanisms. As many of the PGPR exhibit N_2-fixing abilities, it will always remain a temptation to invoke this activity to explain some of the perceived enhancement of yields observed when such bacteria are used as inoculants on graminaceous crops (Andrews et al. 2003). However, it is apparent by careful analyses of the literature that their mechanisms of action in enhancing crop yields are often due to a range of other activities which ironically, can reduce soil N rather than supplement it. What is clear is that none of the PGPR effects, studied to date, can match N fertiliser application as a consistent replacement for soil N deficiency (Andrews et al. 2003).

In contrast to free-living PGPR diazotrophs in soil, there is evidence that endophytic bacteria, particularly *Azoarcus* spp. in Kallar grass (*Leptochloa fusca* (L.) kunth.) and *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp. in sugar cane (*Saccharum* spp.) may play a major role in N_2 fixation (Hurek et al. 2002; Baldani et al. 1997). Endophytic bacteria colonise plants without causing damage to the host and fix nitrogen in situ. In graminaceous crops, most interest has focussed on the potential of endophytic BNF to replace or reduce the requirement for inorganic N fertiliser largely as a result of intensive studies on the effects of acetic acid bacteria, particularly *G. diazotrophicus*. This bacterium has been shown to make a substantive contribution to the nitrogen requirement of sugar cane in several regions where this crop is cultivated, most notably in Brazil (Boddey et al. 1991). The contribution of this bacterium to the nitrogen balance of sugar cane has been convincingly demonstrated with ^{15}N isotope dilution and ^{15}N natural abundance studies (James 2000). It has also been isolated from other tropical grasses, including kallar grass (Malik et al. 1997), rice (Muthukumarasamy et al. 2005) and Cameroon grass (Reis et al. 1994). Moreover, the closely related taxa *G. azotocaptans* was isolated from maize, and *Swaminathania salitolerans* and *Acetobacter peroxydans* from wild and wetland rice, respectively (Loganathan and Nair 2004; Muthukumarasamy et al. 2005). The ecology and contribution of *G. diazotrophicus* and other taxa to BNF in graminaceous crops has been

extensively reviewed (Pedraza 2008; Saravanan et al. 2008); however, it is worth summarising some of the key findings identified from such literature surveys. The mechanisms, by which colonisation of the host by *G. diazotrophicus* is achieved, remains to be thoroughly characterised; the bacterium evades the host defences and the plant may control the initial colonization event through specific signalling events (Nogueira et al. 2001; Vargas et al. 2003).

The interaction between *G. diazotrophicus* and sugar cane does not always result in demonstrable BNF activity; data from Australia, South Africa and Japan indicated no significant BNF from sugar cane colonised with *G. diazotrophicus* (Saravanan et al. 2008). It is also clear that as well as BNF other plant growth stimulating activities, including hormone synthesis, nutrient mobilisation and pathogen suppression, (Saravanan et al. 2007, 2008 and references therein) play a role in the enhancement of plant growth. The potential of endophytes on the cultivation of more economically significant graminaceous crops has yet to be thoroughly investigated. However, endophytic *Azoarcus* species have been shown to colonise the interior roots of rice and express nitrogen-fixing systems (Hurek and Reinhold-Hurek 2003).

3.2 Production of Phytohormones

The early large scale studies of PGPR using *Azotobacter* (in the 1930s–1950s) and *Azospirillum* spp. (between 1976 and late 1980s) demonstrated that, in field trials, it was possible to observe significant increases in yields with a number of graminaceous crops (Andrews et al. 2003 and references therein). However, results were inconsistent and as a result the technology was never adopted. The original hypothesis, that the increased crop yields were due to BNF by the PGPR increasing the soil N budget, could not be substantiated. However, subsequent work has demonstrated that there are a range of mechanisms at play, the most significant of which is the production of phytohormones that increase root weight, length and surface area (Vessey 2003).

There are a number of studies in which the inoculation of PGPR, along with the addition of inorganic N fertiliser, results in an increase in crop yields comparable or greater than that observed when conventional quantities of inorganic N are applied. A study on wheat demonstrated maximum increases in yields of grain and straw were observed in treatments where PGPR were used in combination with recommended dosages of inorganic fertiliser (Akhtar et al. 2009). A further study indicated that PGPR, which demonstrated ACC-deaminase activity, such as *Pseudomonas fluorescens* and *Pseudomonas putida*, could both improve wheat and maize yield and reduce the dependence on inorganic N by 25%, while giving an increase in wheat grain yield to 96% (Naveed et al. 2008a, b). Other workers have demonstrated positive responses on wheat yields with reductions in the requirement for inorganic fertiliser with strains of *P. fluorescens* and *Azospirillum brasilense*

(Shaharoona et al. 2008; Sala et al. 2007). Such work needs to be rigorously followed up for several seasons.

The mechanism of crop yield enhancement and the reduction on the requirement for inorganic N may reflect short-term enhancement of N uptake from the pool already present in the soil complementing that provided by the fertiliser. Over time, as the residual N in the soil is depleted such applications of PGPR, with reduced levels of inorganic N addition, may result in deficits for crop growth. A consequence could be a reduction or inconsistent response of yield to this protocol, typical of those that bedevilled earlier attempts to develop PGPR as a tool for enhancing sustainability in agriculture.

3.3 Enhanced Nutrient Availability

A number of studies have proposed that the addition of PGPR to crops can enhance yields by increasing uptake of nutrients, including nitrogen, phosphorus, potassium and iron. The uptake of nutrients by plants represents an interaction between the plant root, the physical and chemical environment of the soil and the rhizospheric microbial community. PGPR may increase the surface area or roots through the production of phytohormones, enabling greater uptake of key nutrients (Cakmakci et al. 2007). *G. diazotrophicus* has been shown to solubilise Zn, an essential micronutrient, a deficiency of which is common in sugar cane plants in which this bacterium is an endophyte (Saravanan et al. 2008) or may mobilise key nutrients by the production of siderophores (Fischer et al. 2007). Frequently, the mechanisms underlying the observed crop growth enhancement are not understood and, as a result, are attributed to a specific activity of the organism involved. In the case of free-living diazotrophs, the additional provision of N to the plant is assumed to be significant in observed increases in yields; however, such organisms do not seem able to directly release fixed N to the plant and this occurs only through the turnover of the microbial biomass (Richardson et al. 2009). In tandem with the N_2 fixation, many PGPR also produce phytohormones that have a significant effect on the crop root biomass and surface area, as seen in studies on rice (Mirza et al. 2006) and maize (Kumar et al. 2007). As a consequence, the increases in grain yield may reflect the indirect enhancement of plant nutrition through the increased root surface area, as opposed to a direct effect of increased fixed N being available to the plant form the diazotrophic bacteria. The effect of phytohormones on crop root growth probably explains the increased N use efficiency in rice (Van et al. 2000) and wheat (Akhtar et al. 2009) inoculated with PGPR.

Similarly, studies on rice, wheat and maize have all demonstrated that bacteria with P-solubilising activity can have a positive effect on plant growth (Bashan et al. 2006; Adesemoye et al. 2008). However, the mechanisms remain ambiguous and whether these organisms mobilise sufficient P to make a substantive contribution to plant nutrition has not been resolved and phytohormones may once again play a role in the positive increase in crop yields. Certainly, field studies have failed to

consistently demonstrate such a response and few studies attempt to address the significance of P solubilisation by demonstrating a negation of the response when higher concentrations are applied (Richardson et al. 2009).

3.4 Enhanced Stress Tolerance

Large areas of agricultural land have been degraded by poor irrigation practice, resulting in damage such as salinization which affects 20% of total irrigated areas. Moreover, climate change appears to be a contributing factor to increased variability in rainfall (Hazell and Wood 2008). As a result, the impact of environmental stresses, such as drought and salinity, on crop yields is significant (Kibblewhite et al. 2008).

There is some evidence that the inoculation of crops with PGPR enhances the tolerance of crops to such environmental stress. *Pseudomonas* spp. inoculated on legumes were shown to ameliorate the effects of drought stress on the growth and yield of the crop (Arshad et al. 2008). However, effective inoculation of crops cultivated in soils subject to environmental stress requires that the bacteria deployed can tolerate these conditions and remain effective in promoting plant growth. Paul and Nair (2008) demonstrated that *P. fluorescens* MSP-393, used as biocontrol agent of soil pathogens, remained capable of effectively colonising plant roots even in high salinity soils. However, development of PGPR inocula for soils subjected to one or several environmental stresses need to validate that they remain effective under such conditions.

3.5 Indirect Effects

The application of PGPR to graminaceous crops may result in improved yields because of other indirect effects. The most widely studied is the ability of many such bacteria to suppress plant pathogens present in the rhizosphere. These effects have been extensively reviewed (Francis et al. 2010; Richardson et al. 2009; Vessey 2003). Here, we report some examples indicating that such effects are as applicable to graminaceous cultivation as they are to legume and vegetable crops. *Azotobacter* and *Azospirillum* strains have been shown to inhibit *Rhizoctonia solani* in the wheat rhizophere (Fatima et al. 2009) and *Pseudomonas* spp. have demonstrated similar activity in rice and maize against a range of fungal pathogens (Lawongsa et al. 2008). This response can be due to the production of antimicrobial compounds or competitive exclusion of the pathogen, as well as by inducing systemic resistance in plants, but typically due to multiple mechanisms (Francis et al. 2009).

PGPR have also been shown to promote the interaction of beneficial fungi with the plant host, for example, *A. brasilense* stimulated the root colonisation of maize by arbuscular mycorrhizae that enhance the uptake of various soil nutrients.

4 Application of PGPR to Soil

The use of bacterial inoculants to enhance crop production has been widely practised in the cultivation of legumes for many years. As a result, there are well-established technologies to add bacterial inoculants either as a liquid to coat the seeds or directly to the soil, typically using a carrier, such as peat or other materials like perlite, composted cork or bagasse (Albareda et al. 2008). In agricultural applications, peat carriers have been the most widely used on a commercial scale; they have a number of advantages, including, a long shelf life and better survival of the bacteria compared to liquid inoculants added directly to the seed. However, they have frequently resulted in inconsistent effects on crop yield, because of either the quality of the inoculant being low (Brockwell and Bottomley 1995) or the bacteria being unable to survive in the soils to which they are added as a result of either adverse environmental conditions, competition from native bacterial flora (Catroux et al. 2001) or a combination of these two factors.

The use of PGPR on graminaceous crops is a different issue to their use on legumes, the mechanisms of action may occur in the rhizosphere (phytohormone production, pathogen suppression, enhanced nutrient uptake) or be associated with the colonization of the plant roots (phytohormones, BNF). In the first case, the aim of the inoculation process is to engineer the rhizosphere to accommodate the bacteria. The competitiveness of the introduced bacteria will reflect how well it adapts to soil conditions and competes with the indigenous flora. Studies utilizing genetically engineered *P. putida* strains in the wheat rhizosphere, inoculated by broth culture application to the seed coat, have shown a rapid decrease in the numbers of introduced bacteria by five orders of magnitude between sowing and harvesting (Viebahn et al. 2003). The experiment was conducted over two growing seasons, in the first some perturbation of the indigenous microbial flora was observed but not in the second. Moreover, the effect of the genetically modified PGPR on increased plant growth was no greater than that observed after a conventional crop rotation event.

A recent study on the impact of inoculation of rice seeds with *A. brasilense* on the diversity of bacteria in the phyllosphere showed no significant impact (Pedraza et al. 2009). In another study, *Azospirillum lipoferum* was shown to significantly shift the rhizosphere population of field grown maize up to 35 days after sowing (Baudoin et al. 2009).

The influence of the plant genotype on the microbial community of the rhizosphere has been understood for almost 40 years, following studies using several wheat lines (Neal et al. 1970). This reflects the differential rhizodeposition of different plant species and varieties. Ryan et al. (2008) have recently reviewed data from a number of studies indicating the differential population of *P. fluorescens* found in the rhizospheres of both different wheat varieties (Mazzola et al. 2004) and plant hosts (Bergsma-Vlami et al. 2005).

The application and fate of inoculants on field-grown crops needs to be carefully validated to ensure that they can produce some demonstrable benefit to yields.

Recently, attempts have been made to mathematically model PGPR inoculation into the rhizosphere (Strigul and Kravchenko 2006). Such approaches are welcome as they enable the impacts of the different abiotic and biotic factors on PGPR survival to be considered. Strigul and Kravchenko (2006) demonstrated, through mathematical simulations, that the most significant factor affecting PGPR survival was the competition for limiting resources with indigenous flora, followed by the compatibility between the rhizodeposition of compounds by the plant host and the ability of the inoculated bacteria to utilise them. Such work is useful in framing ongoing studies in the use of PGPR, enabling a prediction of the success of a PGPR inoculation in a particular soil with a specific variety of crop to be made.

5 Future Work

Most of the PGPR inhibit the deleterious phytopathogens by involving proteins, peptides, etc. Their gene manipulation may help in engineering proteins, etc., which ultimately diffuse out in the rhizosphere. PGPR or bacterial inoculums adapted to a specific soil and crop varieties are in the form of 'bespoke inocula' proved beneficial in increasing yield.

5.1 Engineering the Rhizosphere

Engineering the rhizosphere of crops to improve productivity and plant health has been studied through a number of mechanisms, including manipulating the plants to modify their rhizosphere to promote nutrient availability, suppress pathogens or encourage PGPR bacterial growth (Ryan et al. 2008). Similarly, the inoculation of soil with a PGPR leading to enhancement of crop yields implies that the bacteria have become established in the rhizosphere of the plant and are exerting a stimulatory effect via one or several mechanisms described above. As a result, there is an implicit assumption that the rhizosphere has been manipulated or engineered by the inoculation process. Such a response can be demonstrated in the field; for example, *A. lipoferum* inoculated onto the seed of field grown maize produced a statistically significant shift in the composition of the indigenous rhizobial community (Baudoin et al. 2009). However, several studies including a field-based study on wheat have indicated that such inoculation effects are transient as a result of a rapid decline in inoculant numbers after the bacteria are added (Viebahn et al. 2003). Advances in our understanding of the ecological effects of inoculation will also be significant in enabling more effective modelling of the inoculation. Recent studies indicate invading bacteria might release anti-competitor toxins or parasitic phage to overcome the barrier presented by the resident flora in the rhizosphere (Brown et al. 2006). More explicit manipulation has been demonstrated by engineering PGPR strains to enhance their ability to suppress pathogens or inhibit the production of

stress hormones by the plant (Ryan et al. (2008)). It is unlikely that genetically engineered strains offer a realistic mechanism to exploit PGPR effectively in the short and medium term, as they would have to satisfy stringent regulatory criteria, demonstrate a reproducible positive impact on crop yield and in some areas significant public antipathy to such technology.

5.2 Bespoke Inocula

The effective utilisation of PGPR in the future will demand that there is a much more rational approach to the choice and delivery of the particular bacterium into the field. This will depend on a range of variables that require consideration (Trivedi et al. 2005). The development of 'bespoke inocula' that are adapted to specific soil and crop varieties is essential if the full benefit of PGPR increase in crop yields is to be realised (Cummings and Andrews 2003). However, a consequence of such parochial inoculants is that the cost of development and production may outweigh the benefits in terms of increased yields and reduce the size of the potential market for such products such that they are not economically viable.

6 Conclusions

At the outset of this discussion, we aimed to map the potential contribution of PGPR to the sustainable cultivation of graminaceous crops. We described the three factors that defined sustainable agricultural practices and technologies (Pretty 2008):

1. They have no adverse effects on the environment.
   PGPR represent a less significant threat to the environment than the use of inorganic N or pesticide application. However, in the longer term, the consequence of inoculation of soils with PGPR on microbial soil diversity is unknown. Most studies indicate that such bacteria rapidly reduce in competition with the indigenous flora. Genetically engineered strains are possible but remain an expensive and potentially more controversial approach to the technology. However, until it has been demonstrated to be a robust and reproducible method of crop yield enhancement this approach does not appear to be viable.
2. They are accessible and effective for farmers.
   The technology has had a long and chequered history, while the production of inoculants is relatively cheap, until they can be proven to produce a return for the additional cost it is unlikely to be widely taken up by farmers. Inoculant technology has developed significantly in recent years, in terms of scale and quality, particularly for legumes. The mechanisms by which PGPR seem to exert their most significant effect on crop growth is by enhanced nutrient uptake.

However, they do not offer significant reproducible gains in graminaceous crop yield year on year. More systematic approaches to research questions should be adopted to determine how PGPR can be most effectively deployed to improve agricultural productivity

3. They lead to improvements in food productivity and have positive effects on environmental goods and services.
   Questions remain whether PGPR inoculation of graminaceous crops could offer a long-term increase in productivity – the evidence does suggest that the key response of graminaceous crops to PGPR inoculation is an improvement in nutrient uptake from the soil and a number of studies have shown (Table 1) that as a result more efficient utilisation of inorganic fertiliser can be observed resulting in the requirement for the application of lower amounts to the soil without compromising yield – and whether this is sufficient to offset the additional cost of the inoculation itself requires systematic study, but this would give a positive effect on sustainability.

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Rice Endophytic Diazotrophic Bacteria

Janpen Prakamhang, Nantakorn Boonkerd, and Neung Teaumroong

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Abstract The extension of nitrogen-fixing symbioses to cultivated rice has been a long-standing goal in the field of biological nitrogen fixation. Endophytic bacteria have been found in virtually every plant studied, where they colonize the internal tissues of their host plant and do not cause any harmful effect to their host plant. Therefore, there is a need to use endophytic diazotrophic bacteria that can make biologically fixed nitrogen available for the growth of rice plants. However, prior to introducing any selected endophytic diazotrophic strain into rice plant, the port of entry of the endophytic bacteria, the interaction via this bacteria and their host plant should be clarified. Furthermore, the complexity of bacterial community such as the behavior of native species inside the rice tissue and their interaction with inoculated endophytic strain should be clearly demonstrated. Moreover, the mechanism of plant response to those of bacteria should also be revealed. Consequently, the diversity of endophytic diazotrophic bacteria, the colonization sites and infection pathways, the effect of diazotrophic bacteria on rice growth, as well as the complexity of endophytic diazotrophic bacteria community structure will be reviewed and discussed in this chapter.

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1 Introduction

Rice (*Oryza sativa*) is the most important staple crop in the developing world. In the next three decades, the world will need to produce about 60% more rice than today's global production to feed the extra billion people (Ladha and Reddy 2003). Nitrogen is the most important input required for rice production. In order to make rice cultivation sustainable and less dependent on chemical nitrogen fertilizer, it has been shown that the proportion of plant growth promoting bacteria, which is bacterial endophytes, is higher than the case of bacteria found on the rhizoplane or in the rhizosphere (Hallmann et al. 1997). Therefore, an endophytic diazotrophic bacterium is the high potential group of biofertilizers that can be used for rice cultivation.

The term "endophyte" is defined as an organism, inhabiting plant organs that at some time in its life can colonize internal plant tissue without causing apparent harm to the host (Petrini 1991). Endophytes have been discovered in high numbers within different tissues of various plants. Various endophytic nitrogen fixing bacteria, named "endophytic diazotrophs" have been detected most frequently in the nonsymbiotic roots and vascular tissues of several nonleguminous plants (Hallmann et al. 1997). Endophytic diazotrophs have been proposed to be responsible for the supply of biologically fixed nitrogen to their host plant such as *Pantoea* sp. and *Ochrobactrum* sp. to deep-water rice (Verma et al. 2004), *Herbaspirillum* sp. B501 to wild rice (You et al. 2005), *Pantoea agglomerans* YS19 (Feng et al. 2006) and *Azoarcus* sp. strain BH72 (Reinhold-Hurek et al. 2006) to rice. These endophytes do not cause damage to the host organism but they promote plant growth by the production and secretion of plant growth regulators (Verma et al. 2001), the antagonistic activity against phytopathogens (Downing and Thomson 2000) and the supply of biologically nitrogen fixation (Ladha et al. 1997). Therefore, cultivated rice fields are considered to be ideal niches for biological nitrogen fixation (BNF), especially for endophytic diazotroph bacteria.

It is well known that a remarkable diversity of N_2-fixing bacteria is naturally associated with field-grown rice (Balandreau 1986). However, in the case of wetland rice, even when specific varieties have been shown to fix N_2 (Ladha et al. 1997), it will be extremely difficult to isolate the organisms responsible, because approximately 90% of the bacteria isolated from surface-sterilized rice plants (several species and varieties, plus some related genera) using N-deficient media are nondiazotrophs (Barraquio et al. 1997). In addition, the culturable diazotrophic population is extremely varied, and so far virtually uncharacterized (Stoltzfus et al. 1997). Also, the behavior of native species inside rice tissues, and the natural association and endophytic interaction of diazotrophs with rice are considered very promising. The microbial community in rice is inherently complex, and assessments performed with such a complex population do not always reveal its specific components. Moreover, the community structure of the bacterial population, both culturable and unculturable strains, inside the rice should be considered especially in relation to the actual rice field soil.

It is widely recognized that endophytic diazotroph inoculum is capable of fixing N more efficiently than diazotrophs that remain in the rhizosphere or on the rhizoplane. This may be due to the fact that the plants directly provide the endophytic diazotroph bacteria with their nutrient requirement. Therefore, they do not need to compete with other soil microbes for scarce resources. In return for providing this niche, the bacteria provide fixed N and/or plant growth-promoting compounds to the host plant.

In the present chapter, we focus on the diversity of endophytic diazotrophic bacteria, and the colonization sites and infection pathways will be discussed.

2 The Diversity of Endophytic Diazotrophic Bacteria

Diverse endophytic diazotrophic bacteria have been isolated from rice plants. The endophytic niche offers protection from the environment for those bacteria that can colonize and establish in planta. These bacteria generally colonize the intercellular spaces, and they have been isolated from all plant compartments including seeds (Kaga et al. 2009).

Bacteria belonging to the genera *Azospirillum*, *Herbaspirillum* and *Azoarcus* are found as endophytes of rice mostly from the tropical regions. *Azospirillum* has been found in the elongation and root hair zones of roots, and some strains of both *A. lipoferum* and *A. brasilense* are either facultatively or obligately endophytic (Baldani et al. 1997). Strains of *A. brasilense* can colonize plant tissues differently; some strains live only on root surfaces, whereas others colonize cortical intercellular spaces or even the vascular tissue (James and Olivares 1998). Besides, the ability of fixing nitrogen, both *A. brasilense* and *A. lipoferum* can produce plant growth hormone auxin (Costacurta and Vanderleyden 1995). Nitrogen-fixing bacteria belonging to the genus *Azoarcus* has been found mainly in roots of Kallar grass (*Leptochloa fusca*) and rice in the intercellular spaces, xylem vessels, and dead root cells. *Azoarcus* has been demonstrated to spread systemically within the plant via the xylem vessels (Hurek et al. 1994). In addition to the plant roots, this bacterium has been discovered in close interaction with a rhizosphere fungus (Hurek et al. 1997). The genus *Herbaspirillum* contains an unusual group of endophytes in the respect that these bacteria may become pathogenic to their host under certain conditions. *H. seropedicae* strain Z67 colonized mainly subepidermal regions of rice roots (Roncato-Maccari et al. 2003). There are several bacterial species, in addition to the most well studied root endophytes, which have been isolated from gramineous plants, whereas certain species are less studied, but connected by their ability to fix nitrogen (James and Olivares 1998). Genera *Burkholderia* and *Klebsiella* are preferably regarded as endophytes (Baldani et al. 2000; Palus et al. 1996). The isolation of presumptive endophytic diazotroph bacteria from rice has also been reported. For example, *K. oxytoca* and *Enterobacter cloacae* have been isolated from the rhizosphere of wetland rice (Fujii et al. 1987). *Serratia marcescens* IRBG500 have been observed within rice root, stem, and leaves and could also

increase the root length and root dry weight of the inoculated plants (Gyaneshwar et al. 2001). Teaumroong et al. (2001) found that five endophytic bacteria isolates from Thai rice showed a high N_2-fixation potential and three strains were able to produce the plant growth promoting substance IAA. *P. agglomerans* (Remus et al. 2000), *Alcaligenes faecalis* (You and Zhou 1989), and a few other bacteria belonging to the genera *Pseudomonas*, *Enterobacter* and *Bacillus* (Lindberg et al. 1985; Persello-Cartieaux et al. 2003; Watanabe and Lin 1984) have also been considered as endophytic bacteria.

A recent study published by Minamisawa et al. (2004) reported the existence of anaerobic N_2-fixing consortia (ANFICOs) in many gramineous plants consisting of N_2-fixing clostridia and diverse nondiazotrophic accompanying bacteria which were phylogenetically dispersed in the β- and γ-*Proteobacteria* and the high C+G content and low G+C content Gram-positive lineages (Fig. 1a). The phylogenetic analyses of 40 anaerobic N_2-fixing isolates from various origins categorized them exclusively into clusters I and XIVa among the 17 clusters of *Clostridium* spp. on the basis of their 16S rRNA gene sequences (Fig. 1b). The clostridial isolates were further subdivided into groups I and II in cluster XIVa and groups III, IV, and V in cluster I. These clusters and groups were not clearly correlated with the plant species, plant tissue, or location of isolation. Their work indicated that clostridia should be candidates for diazotrophic endophytes in grasses, and also demonstrated a new principle in environmental microbiology, i.e., consortium of bacteria, rather than monocultures, may be responsible for a particular activity within a very complex environment. Recently, Prakamhang et al. (2009) reported the population of viable endophytic bacterial communities within each plant part and growth stage of rice under different soil conditions in cultivated rice (*O. sativa* L. cultivar KDML-105), single isolates from each diazotrophic consortium were shown to be capable of both the inhibition and promotion of N-fixation and found closely related to *E. dissolvens, Brevundimonas aurantiaca, P. agglomerans*, *Pseudomonas* spp., *Rheinheimera* sp. and Enterobacteriaceae. This is the first report of diazotrophic nature of *Rheinheimera* strain, although it has been reported that this bacteria is associated with spores of the arbuscular mycorrhizal fungi (Roesti et al. 2005) and have association to the root of the tomato plant (Kim et al. 2006).

3 Colonization Sites and Infection Pathways

According to Dobereiner's report (1997), endophytic diazotrophs, by inhabiting the interior of the plants, can avoid the competition with rhizospheric bacteria and derive nutrients directly from the host plants. In return, as the plant interior may provide an environment conducive to N-fixation by being low in O_2 and relatively high in carbon, the bacteria can fix N more efficiently to the host (James and Olivares 1998). The stele of plants has been considered to be colonized by pathogens only (Campbell and Greaves 1990) or by saprophytes (Gagné et al. 1987). Vessels of nondiseased plants were thought to be sterile. This is not true for

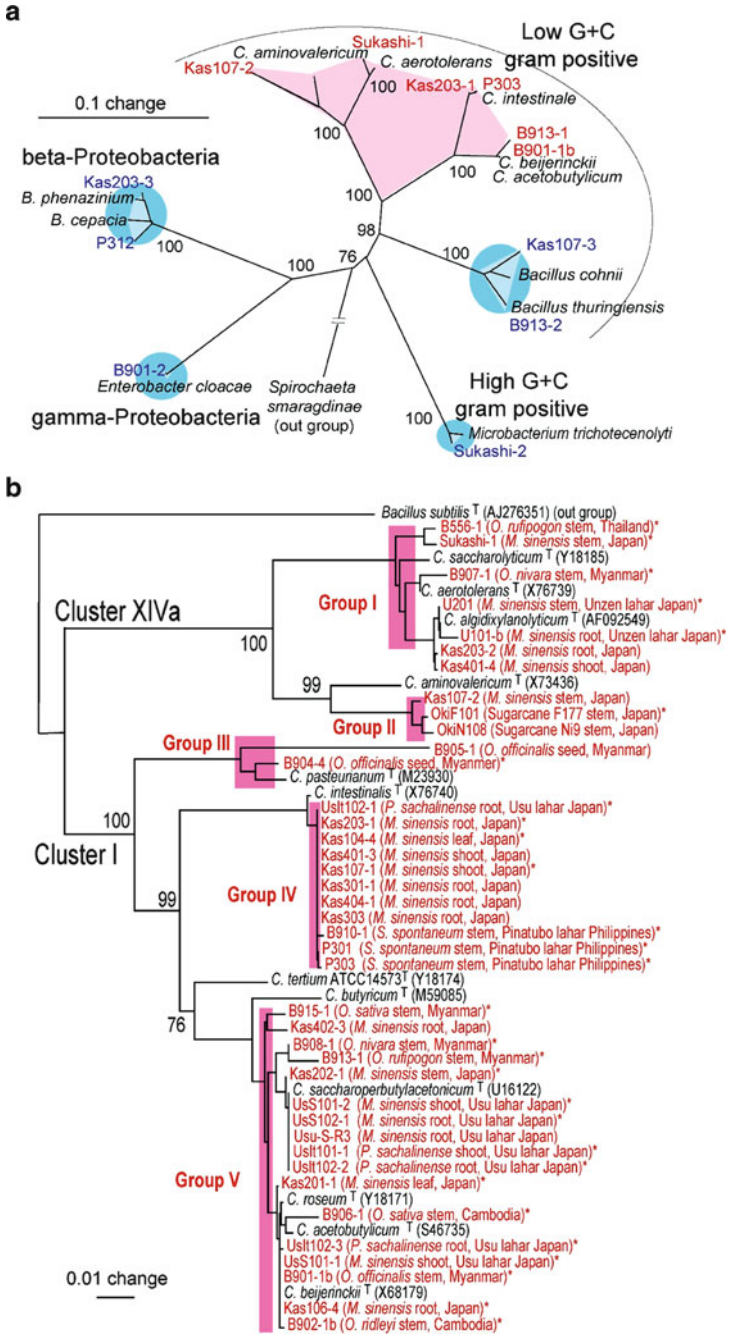

Fig. 1 Phylogenetic tree of anaerobic nitrogen-fixing bacteria and accompanying bacteria from various origins and representative close relatives by 16S rRNA gene sequences (Minamisawa et al. 2004). (**a**) Representative members of ANFICOs; *grey area* indicated nitrogen-fixing bacteria,

endophytic diazotrophs, as first found in *Azoarcus* sp. BH72 in Kallar grass and rice. The bacteria were present in vessels of roots in gnotobiotic cultures demonstrated by immunogold-labeling using genus-specific antibodies (Hurek et al. 1991, 1994). Microscopical studies using immunological approaches and reporter genes have clearly shown similar colonization patterns for several nitrogen-fixing grass endophytes, such as *Azoarcus* sp. BH72 (Hurek et al. 1994), *H. seropedicae* (James and Olivares 1998), *Gluconacetobacter diazotrophicus* (Cavalcante and Dobereiner 1988) and certain strains of *Azospirillum* spp. (Schloter and Hartmann 1998).

In plants showing no symptoms of disease, *Azoarcus* sp. BH72 colonizes the original host Kallar grass and also rice seedlings in a similar way. Outer cell layers, epidermis and the root cortex are colonized inter- and intracellularly within 2–3 weeks, the aerenchyma which occurs in waterlogged plants being the main site for large microcolonies (Egener et al. 1999; Hurek et al. 1994). The main intercellular colonization pattern raises questions on the delivery of nutrients, especially carbon sources for the bacteria (Hurek et al. 1994). Rarely, the bacteria penetrate deeply into plant roots into the stele where they may present in the parenchyma and in xylem vessels. The detection of *Azoarcus* sp. in stelar parenchymatic cells of the culm and in vessels of Kallar grass and rice (Hurek et al. 1991, 1994) suggested that systemic spreading into shoots may be mediated through the transport in vessels. However, shoot colonization of *Gramineae* appears to be more obvious in *G. diazotrophicus* (James and Olivares 1998) and *H. seropedicae* (Gyaneshwar et al. 2002). Kaga et al. (2009) hypothesized that endophytic bacteria are considered to originate from the external environment. To examine this hypothesis, endophytic bacteria were isolated from the rice (*O. sativa*, cultivar Kinuhikari) seeds, the shoots, remains of the seeds, and roots of rice seedlings that were aseptically cultivated in vitro from surface-disinfected seeds. Of the various bacterial strains isolated, the closest relatives, identified by 16S rRNA gene sequencing, were; *Bacillus firmus*, *B. fusiformis*, *B. pumilus*, *Caulobacter crescentus*, *Kocuria palustris*, *Micrococcus luteus*, *Methylobacterium fujisawaense*, *Me. radiotolerans*, and *P. ananatis*. The latter three species have been detected frequently inside both rice seedlings and mature rice plants. These results indicate that rice seeds are an important source of endophytic bacteria. The bacteria that colonize the seed interior appear to infect the subsequent generation via seeds and become the dominant endophytic species in the mature plant. The presence of diazotrophic bacteria was also detected in roots, stems and leaves (Prakamhang et al. 2009). The location of

Fig. 1 (continued) *accompanying bacteria.* ***(b)*** *Tree of 40 isolates of anaerobic nitrogen-fixing bacteria from various pioneer plants and wild rice species, including M. sinensis, S. spontaneum* (wild sugarcane), *Polygonum sachalinense, Saccharum* hybrid sp. (sugarcane), *Oryza sativa* (cultivated rice), and *O. rufipogon, O. nivara, O. officinalis,* and *O. redleyi* (wild rice species). Grey box indicated the nitrogen-fixing bacteria. The trees are based on >1.2 kb of DNA sequences and were constructed by the neighbor-joining method. Bootstrap values (percentages from 1,000 replications) are indicated. Bold letter indicated the reference strain, regular letter represent isolated strain. The utilization of carbon sources was tested for 30 isolates, which are indicated with *asterisks* (Adapted from Minamisawa et al. 2004)

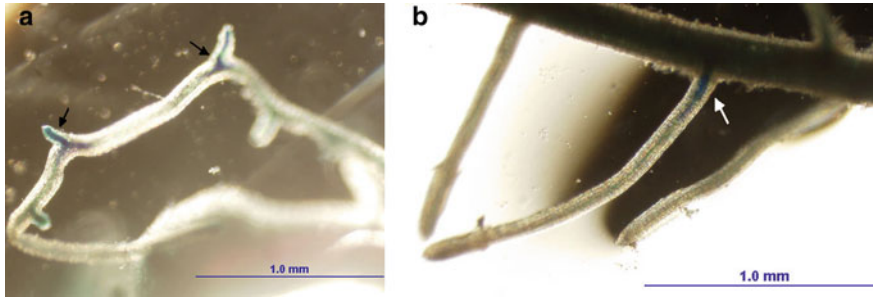

Fig. 2 Light micrographs of GUS-stained roots, stems, and leaves of rice at 5 days after inoculation with strain VFR5-3 marked with GUS. *Arrows* indicate the most intense GUS activity on the lateral roots (**a**), root junction (**b**)

the *Rheinheimera* sp. J3-AN42 in root of rice on the fifth day after inoculation (DAI) was observed with the most intense color development on the lateral roots (Fig. 2a) and root junctions (Fig. 2b).

Endophytic microorganisms differ remarkably from highly developed root nodule symbioses, in which rhizobia enter the plants through root hairs via infection threads. The infection of grasses by endophytes is similar to the crack entry. One site of primary colonization is the points of emergence of lateral roots, where bacterial microcolonies can readily be detected, and bacterial cells have been found between the cell layers of the lateral root and the cortex of the main root. Another route of entry is the root tip at the zone of elongation and differentiation. The bacteria can invade intercellular and intracellular and may penetrate into the central tissues (Hurek et al. 1991, 1994), with the exception of *Azospirillum* spp. which is mainly regarded as a rhizoplane colonizer (Steenhoudt and Vanderleyden 2000). The entry of bacteria into the root is most likely an active process, which might be mediated by enzymes degrading plant cell wall polymers. Two types of cellulolytic enzymes, cellobiohydrolase and β-glucosidase have been detected in *Azoarcus* sp. BH72 (Reinhold-Hurek and Hurek 1998), pectinase and cellulase production were also detected (Prakamhang et al. 2009). Further insights into the cellular machinery for plant invasion, and a comparison with pathogens and symbionts, will be fostered by the genome analysis.

4 Complexity of Endophytic Diazotrophic Bacteria Community Structure

While the search for a natural association and endophytic interaction of diazotrophs with rice is considered very promising, the microbial community in rice is also inherently complex, and assessments performed with such a complex population do not always reveal its specific components. The PCR–DGGE analysis conducted

directly on rice tissue samples obtained in Thailand using 16S rDNA primers was used to elucidate the structure of the endophytic bacterial communities (Fig. 3). Almost all of the samples contained two major bands of DGGE–PCR products except that from reproductive stage of rice. Each sequence retrieved from the bands a–d showed similarity to different strains. While band a has a high similarity to *E. dissolvens,* band b showed similarity to *B. aurantiaca,* band c to *P. agglomerans*, and band d to *Pseudomonas* sp. (Prakamhang et al. 2009).

Recently, the endophytic–endophytic consortium interaction within rice plant has been reported. Since the N-fixing activities of other single culture occurred more than those of the original combinations (Prakamhang et al. 2009), this suggests the presence of the accompanying bacteria that produced specific metabolites of consortium that induced/reduced the N-fixation as well as association of nondiazotrophic endophytes in culture. The surface-sterilized rice plant materials were mechanically macerated and then cultured in N-free semisolid medium for determinations of the N-fixing bacteria as a consortia or original mixed culture. Each single isolate was tested for inhibition/promotion to the other isolate in the same consortium. This suggested that one single isolate affected other single isolates in the same consortium by producing agents that can kill the bacteria (bactericidal effect) as shown by the clear zone in the bacterial layer (Fig. 4). However, some consortia do not show any clear zone around the spotted culture filtrate of each single isolate. Perhaps one single isolate of this consortium produced agents that can inhibit only N-fixing activity (bacteriostatic effect). Similarly, a major feature of ANFICOs is that N- fixation by the anaerobic clostridia is supported by the consumption of oxygen by the accompanying bacteria in the culture and the presence of unknown metabolites (Minamisawa et al. 2004).

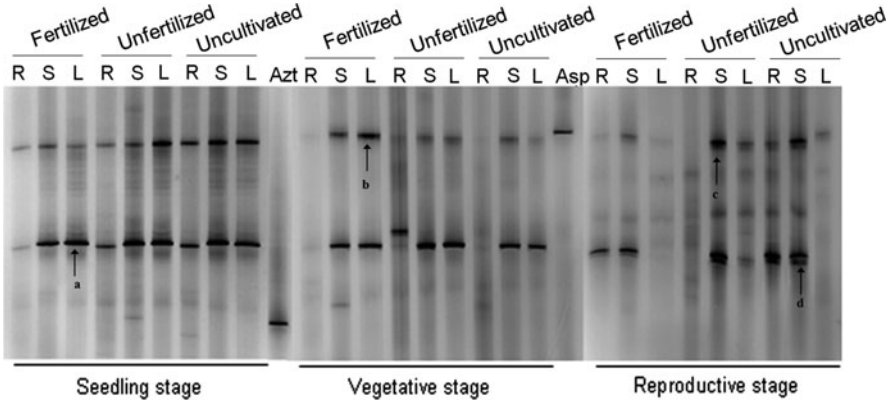

Fig. 3 PCR–DGGE analysis of 16S rRNA gene banding patterns from rice endophytic bacteria in samples from plants grown in different types of soil (fertilized paddy soil, unfertilized paddy soil and uncultivated forest soil). *Arrows* show excised and sequenced bands (a–d). Azt., *Azotobacter* sp.; Asp., *Azospirillum* sp.; R, root; S, stem; L, leaf. (Adapted from Prakamhang et al. 2009)

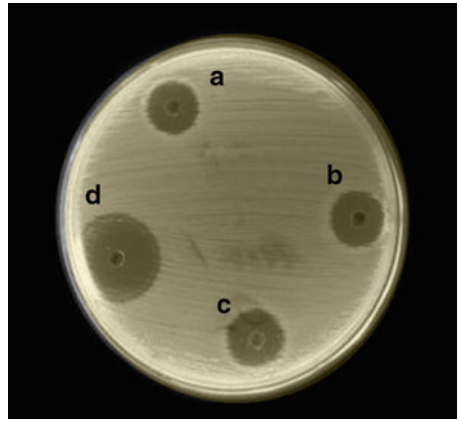

Fig. 4 Antimicrobial activities of culture filtrate of strain VFR3-1 corresponding to bacterial growth as show by the inhibition zones in the bacterial layer. The N-free plate was cultured with VFR3-1-1 inoculated with 1 µl (**a**), 2 µl (**b**), 3 µl (**c**), and 4 µl (**d**) of single culture VFR3-1-2

5 Rice Growth and Endophytic Bacteria

Microbial promotion of plant growth may be the outcome of several additional factors besides the nitrogen fixation. For example, an indirect plant growth promotion is the production of phytohormones, which has been considered to be the main function in the symbioses. Almost all root endophytes also fix nitrogen (Baldani et al. 1997). However, the benefit of their nitrogen fixing ability for the plant has not been demonstrated indisputably (James 2000). *P. agglomerans* can infect and colonize in the rice roots and produce IAA and have been shown to be a potent biological control agent against fungal disease (Verma et al. 2001). Production of auxins and gibberellins is also typical for many root associated endophytic bacteria such as *Azospirillum* sp., *G. diazotrophicus*, and *H. seropedicae* (Bastián et al. 1998). The flavonoids, quercetin and diadzein, significantly increased the endophytic colonization ability of *Serratia* sp. than growth hormones. The induced colonization of *Serratia* sp. due to quercetin proportionally increased the *in planta* nitrogenase activity which is reflected in the increased plant height, protein and chlorophyll contents of rice seedlings (Sandhiya et al. 2005). However, apart from the roots, the importance of the microbial production of phytohormones has been evaluated to be low, and the significance of these products for the plant has remained ambiguous (Zinniel et al. 2002). Therefore, endophytic function which is considered distinctively beneficial for the plant appears to be the protection of the host against pathogens. As, not all endophytes are responsible for producing antagonistic substances, their role is yet to be discovered. Nevertheless, it appears that the function of an endophyte may be composed of several diverse factors that may together have a positive influence on the plant.

Nitrogen fixation is catalyzed by the enzyme nitrogenase complex. More than, 20 genes have been identified as controlling the structure and function of the nitrogenase system, and much functional detail has been defined. A substantial molecular diversity of N fixing bacteria has been detected in field grown rice based on retrieval of *nifH* gene fragments from root DNA (Ueda et al. 1995). The diazotrophic

endophyte of rice, *Serratia* sp., was marked with enhanced green fluorescence protein (egfp)-Km marker gene by biparental mating, and was used for colonization studies in rice. The conjugants established themselves endophytically in rice root, stem and leaves, with the stem being most colonized (Sandhiya et al. 2005). The expression of *nif* genes of *H. seropedicae* LR15 strain occurred in roots, stems and leaves as detected by the GUS reporter system, and the colonization of plant tissue by *H. seropedicae* did not depend on the nitrogen-fixing ability (Roncato-Maccari et al. 2003). To detect N-fixing bacteria in a plant without using culture methods, *nifH* gene segments were amplified with degenerate primers from DNA extracted from stems and leaves of rice plant. Furthermore, the study of Ueda et al. (1995) demonstrated the extent of phylogenetic diversity of diazotrophic bacteria associated with rice roots by characterizing 23 *nifH* gene sequences derived directly from rice roots without culturing the organisms. This study also showed a variety of significant components of the diazotrophic community dominated mainly by proteobacteria. Similar results were obtained by Prakamhang et al. (2009), in which nested PCR–DGGE analysis with *nifH* primer demonstrated less diazotrophic bacterial diversity in the roots of rice cultivated in paddy soil amended with nitrogen fertilizer than in unfertilized and previously uncultivated soil, and plant tissue type was found to dictate the endophytic diazotrophic community structure rather than the type of soil or fertilizer amendment (Fig. 5). Furthermore, most isolates were detected both by culturable approach and by DGGE, suggesting that the molecular approach directly reported culturable endophyte bacteria. However, some isolates such as those from leaves with no-fertilizer soil of all stages of growing were not detected by ARA procedure, suggesting that these endophytic bacteria are nonculturable (or not yet cultured) endophytic bacteria from rice plants. The dendrogram constructed from the PCR–DGGE of *nifH* gene band pattern of endophytic diazotrophic bacteria within each parts (root, stem, and leaf) of rice

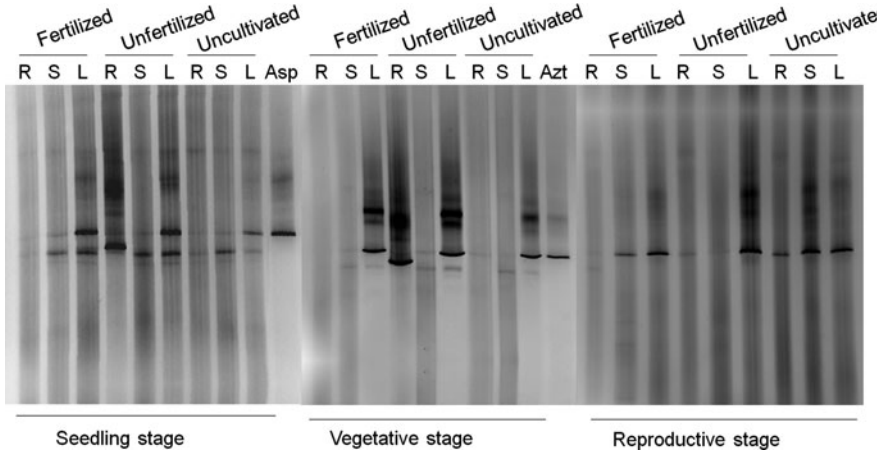

Fig. 5 Nested PCR–DGGE of *nifH* banding patterns from rice endophytic bacteria. R, root; S, stem; L, leaf; types of soil as in Fig. 3

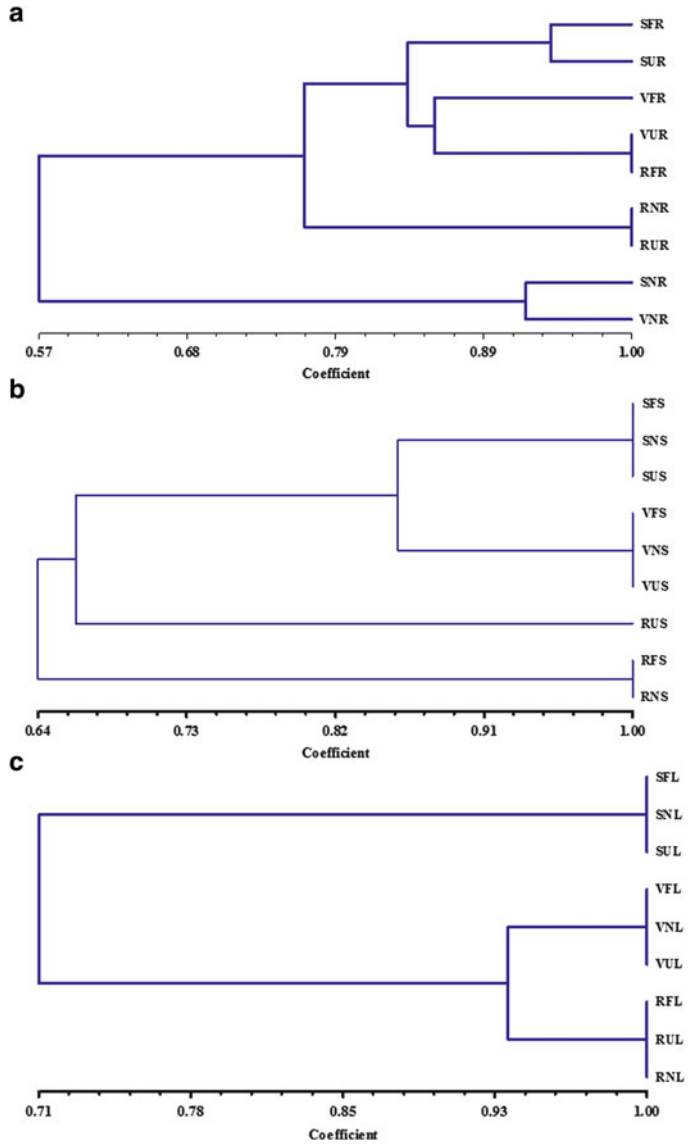

Fig. 6 Dendrogram obtained from UPGMA cluster analysis of PCR–DGGE of *nifH* gene band pattern of rice endophytic diazotrophic bacteria within stage of growth and part of rice plant; seedling stage (**a**), vegetative stage (**b**), and reproductive stage (**c**). *Abbreviations*, First letter is growth stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is type of soil (F; fertilized, N; unfertilized, U; uncultivated soil), and third letter is rice part (R; root, S; stem, L; leaf)

plant represented high complexity of community in the rice root (Fig. 6a). On the other hand, in stem and leaf, stages of growth seem to dictate endophytic diazotrophic bacteria community. For example, in stem and leaf, bacteria of each stages

of growth were grouped together (Fig. 6b, c). This result suggested that plant tissue types may dictate the endophytic diazotrophic community structure rather than the type of soil or fertilizer amendment.

However, only the presence of nitrogenase gene does not indicate that bacteria are actively fixing nitrogen (James 2000). Culturing techniques have been used to determine the type of individual species present, but these techniques yield biased results and a misrepresentation of the types of bacterial species that are active in the environment. The reverse transcriptase PCR (RT-PCR) makes it possible to assay for cells that are actively expressing specific gene at the time of sampling and it has been used recently to detect *nifH* expression of *Azoarcus sp* BH72 in Kallar grass and rice (Hurek et al. 2002), and *Herbaspirillum* sp. B501 associated in the shoot (leaf and stem) of wild rice (You et al. 2005). Recent study of Prakamhang et al. (2009) showed that the *nifH* gene expression could be differently detected in each part and growth stage of rice plants as well as could be influenced by soil nitrogen status (Fig. 7). The expression level of the *nifH* gene in all roots from plants grown in N-fertilized soil was the lowest among the treatments studied. The results confirm the complexity of the endophytic diazotrophic bacterial community, and indicate that the type of plant tissue seems to influence the community structure.

In addition to fertilization with nitrogen, variations in the growth stage and part of rice plant and the environmental conditions caused large differences in the population structure of endophytic diazotrophs, as demonstrated in a culturable approach. Nitrogen fertilization has been reported as a leading repression factor of nitrogenase genes and inactivation of nitrogenase activity in most diazotrophic bacteria (Egener et al. 1999; Fuentes-Ramirez et al. 1999; Martin and Reinhold-Hurek 2002). Nevertheless, the diazotrophs abundant in rice plants may be either rapidly decaying or overgrown by others after fertilizer application. Colonization of maize plants by diazotrophic bacteria was inhibited by high N-fertilization (18.5 mg kg^{-1}) during the early stages of growth but not during subsequent stages (Roesch et al. 2006), whereas the inhibitory effect of high N fertilization (148 mg kg^{-1}) on diazotrophic bacterial numbers could be reduced by the application of

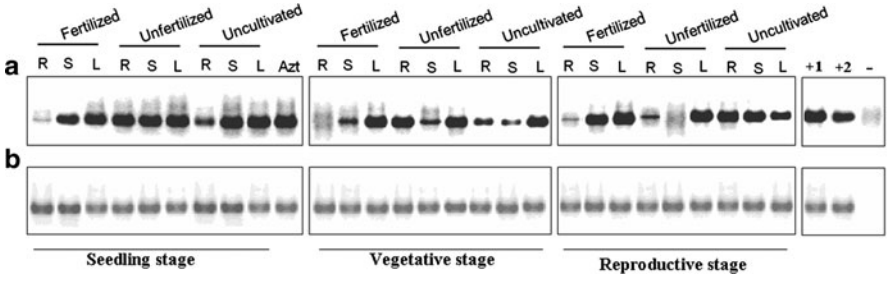

Fig. 7 Gel electrophoresis analysis of RT-PCR for *nifH* gene expression. Reverse transcription and nested PCR amplification was performed using *nifH* primers (**a**). The 16S rRNA gene was used as a standard to calibrate the amount of RNA (**b**). R, root; S, stem; L, leaf; types of soil as in Fig.3 Azt., *Azotobacte*r sp.; +1, rice inoculated with *Azotobacter* sp. as positive control; +2, Positive Control; −, negative control (no-template)

compost (Muthukumarasamy et al. 2007). In the case of cultivated rice in Thailand, the *nifH* pattern indicated lower diversity in plants grown in soil fertilized according to local custom (35.9 mg kg^{-1}) and in uncultivated soil than that in plants from unfertilized soil (Prakamhang et al. 2009). Since the N-fertilization did not affect the diazotrophic bacterial population in all stages of growth, the observed effect does not seem to be a direct negative effect of the fertilizer on the bacteria. *Herbaspirillum* spp. was also found to occur both in N-fertilized and unfertilized samples (Muthukumarasamy et al. 1999). Nitrogen alters the physiological state of the plant, and this subsequently affects its association with the diazotrophic bacterial population (Muthukumarasamy et al. 1999; Reis et al. 2000). This suggests that the original diazotrophic community consists mainly of autochthonic bacteria on which N-depletion conferred a selective advantage. These results also demonstrate that rice represents a highly dynamic microenvironment for bacteria.

6 Conclusion

In the present chapter, we have shown that there is a high diversity in endophytic diazotrophic bacteria community. The presence of diazotrophic bacteria was detected in roots, stems, and leaves in the colonization sites. The complexity of the endophytic diazotrophic bacterial community revealed that the type of plant tissue seems to influence the community structure. The understanding about how and when endophytic communities form in rice plants and about their interaction is essential for an investigation of the ability of endophytic diazotrophic bacteria, especially the culturable strains, to compete with other endophyte strains, and to contribute nitrogen to the host plants prior to applying endophytic diazotroph bacteria as rice biofertilizer for rice farming. Thus, it is strongly proposed that endophytic diazotrophic bacteria provide an agricultural benefit which is of definite ecological and economic significance.

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Bacillus and *Paenibacillus* spp.: Potential PGPR for Sustainable Agriculture

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Abstract The Gram-positive aerobic endospore-forming bacteria (AEFB) belonging to the genus *Bacillus* and *Paenibacillus* are essentially ubiquitous and occur abundantly in most rhizospheric soils. In the rhizosphere, species of these two genera are involved in atmospheric nitrogen fixation, solubilization of soil phosphorus and uptake of micronutrients, and production of phytohormones and antimicrobial metabolites. Multiple species of *Bacillus* and *Paenibacillus* affect the crop growth and its health by three different ecological mechanisms viz, promotion of host plant nutrition and growth, antagonism against fungal, bacterial, nematode pathogens and insect pests, and stimulation of host defence mechanisms. Specific strains of both *Bacillus* and *Paenibacillus* spp. are known to elicit induced systemic

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resistance (ISR) similar to that of *Pseudomonas* spp. which leads to the stimulation of host defence mechanisms against multiple pathogens on diverse crop plants. Several species of *Bacillus* and *Paenibacillus* are the major source of broad spectrum peptide antibiotics that are active against various microbial and nematode pathogens. Endophytic colonization and biofilm formation by these two genera are also reported. These plant growth promoting abilities of *Bacillus* and *Paenibacillus* can make them suitable plant growth promoting rhizobacteria for their application in sustainable agriculture.

1 Introduction

The microbial world is the largest unexplored reservoir of biodiversity and act as a major resource for agricultural, industrial, and medicinal applications (Handelsman et al. 1998; Daniel 2005; Lorenz and Eck 2005). Bacteria are the most dominant group of this diversity which exists in diverse ecological niches, including extreme environments present in both lithosphere and hydrosphere, where their metabolic abilities play a critical role in geochemical nutrient cycling (Daniel 2005). Rhizosphere, as coined by Hiltner (1904), is one such well-characterized ecological niche consisting of layer of soil with highest bacterial population and their activities are much influenced by the surrounding plant roots. The bacterial populations in the rhizosphere is 100–1,000 times higher than in bulk soil, and up to 15% of root surface is covered by microcolonies of a variety of bacterial species (Gray and Smith 2005). The rhizosphere effect is due to the fact that a substantial amount of carbon fixed by the plant as photosynthates (5–21%) is secreted in to rhizosphere mainly as root exudates that can be utilized as nutrients by bacterial populations. In return, the metabolic activities of these bacteria in the rhizosphere stimulate mineral nutrient delivery and uptake by plant roots (Glick 1995). These beneficial bacterial populations of rhizosphere are commonly called as plant growth promoting rhizobacteria or PGPR (Kloepper and Schroth 1978; Glick 1995). They promise plant growth promotion by secreting a variety of metabolites and employing various mechanisms (Glick et al. 1999).

A number of different bacterial groups being considered as plant growth promoting rhizobacteria include *Acinetobacter*, *Agrobacterium*, *Arthobacter*, *Azotobacter*, *Azospirillum*, *Burkholderia*, *Bradyrhizobium*, *Rhizobium*, *Frankia*, *Serratia*, *Thiobacillus*, Pseudomonads, and Bacilli (Glick 1995; Vessey 2003). Among them, *Bacillus* and *Paenibacillus* of aerobic endospore-forming bacteria (AEFB) are essentially ubiquitous in agricultural systems. The native populations of these two genera occur abundantly in most rhizosphere soils and plant tissues are differently colonized by distinct subpopulations (Mahaffee and Kloepper 1997; Seldin et al. 1998). Multiple species of *Bacillus* and *Paenibacillus* can promote plant growth and health in a variety of ways. Some species can promote plant growth directly by synthesizing plant hormones or increasing mineral nutrient uptake by fixing atmospheric nitrogen, solubilization of soil phosphorus, and other methods. Some populations suppresses plant pathogens and insect pests by producing

antibiotic metabolites, while others stimulate plant host defenses prior to pathogen infection (Glick et al. 1999; Van loon 2007; Govindasamy et al. 2008), which indirectly contributes to increased crop productivity. Published reports on endophytic colonization and biofilm formation by *Bacillus* and *Paenibacillus* spp. have suggested that the endophytic colonization and biofilm formation improves the bacterium ability to act as a biocontrol agent against plant pathogens (Hallman et al. 1997; Davey and O'Toole 2000; Timmusk et al. 2005). In recent years, *Bacillus* and *Paenibacillus* spp. attracted considerable attention because of their advantages over other PGPR strains in inoculant formulations, stable maintenance in rhizosphere soil, and greater potentials in sustainable agriculture.

2 Taxonomy and Phylogeny of Genus *Bacillus* and *Paenibacillus*

Taxonomically, the genus *Bacillus* and *Paenibacillus* is coming under gram-positive, aerobic, or facultative endospore-forming bacteria. The genus *Bacillus* has undergone considerable taxonomic changes. Early attempts at classification of *Bacillus* species were based on two characteristics: aerobic growth and endospore formation. Starting off with two prominent and truly endospore-forming species, *Bacillus anthracis* and *B. subtilis*, the number of species allocated to this genus increased to an incredible 146 in the fifth edition of *Bergey's Manual of Determinative Bacteriology* (Bergey et al. 1939). Meticulous comparative studies on 1,114 strains of AEFB helped to reduce this number to 22 well-defined species in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons 1974). In 1980, with the publication of the *Approved Lists of Bacterial Names* 38 species of AEFB were listed, of which 31 were allocated to the genus *Bacillus* and 7 to other aerobic endospore-forming genera (Skerman et al. 1980). In Bergey's Manual of Systematic Bacteriology (1st edn., 1986), the G+C content of known species of *Bacillus* ranges from 32 to 69%. This observation, as well as DNA hybridization tests, revealed the genetic heterogeneity of the genus. Not only was there variation from species to species, but there were sometimes profound differences in G+C content within strains of a species. For example, the G+C content of the *Bacillus megaterium* group ranged from 36 to 45% (Claus and Berkeley 1986).

In Bergey's Manual of Systematic Bacteriology (2nd edn., 2004), phylogenetic classification schemes landed the two most prominent types of endospore-forming bacteria, clostridia, and bacilli, in two different Classes of Firmicutes, Clostridia, and Bacilli. Clostridia includes the Order *Clostridiales* and Family *Clostridiaceae* with 11 genera including, *Clostridium*. Bacilli included in the Order *Bacillales* and the Family *Bacillaceae*. In this family, there are 37 new genera on the level with *Bacillus*. This explains the heterogeneity in G+C content observed in the 1986 genus *Bacillus*. Their taxonomic hierarchy (Table 1) is Kingdom: Bacteria;

Table 1 Systematic position of the gram-positive aerobic endospore-forming bacteria (AEFB) based on 16S rRNA/DNA sequences as per Bergey's Manual of Systematic Bacteriology (2nd edn., 2004)

Systematic position/taxonomic hierarchy	No. of genus	No. of species/subsp.
Domain: Bacteria		
PhylumBXII: Firmicutes phy nov		
Class III: Bacilli		
OrderI: Bacillales		
Family I: Bacillaceae	17	
Key genus: *Bacillus*		88/2
Family II: Alicyclobacillaceae	3	
Key genus: *Alicyclobacillus*		8/2
Family III: Caryophanaceae	1	
Key genus: *Caryophanaon*		1
Family IV: Listeraceae	2	
Key genus: *Listera*		1
Family V: Paenibacillaceae	7	
Key genus: *Paenibacillus*		45/2
Family VI: Planococcaceae	5	
Key genus: *Planococcus*		1
Family VII: Sporolactobacillaceae	2	
Key genus: *Sporolactobacillus*		3
Family VIII: Staphylococcaceae	5	
Key genus: *Staphylococcus*		2
Family IX: Thermoactinomycetaceae	1	
Key genus: *Thermoactinomyces*		6
Family X: Turicibacteraceae	1	
Key genus: *Turicibacter*		1

Phylum: Firmicutes; Class: Bacilli; Order: Bacillales; Family: Acyclobacillaceae (genus: *Acyclobacillus*); Family: Bacillaceae (genus: Bacillus, *Geobacillus*); Family: Paenibacillaceae (genus: *Paenibacillus*, *Brevibacillus*); Family: Planococcaceae (genus: *Sporosarcina*). The phylogenetic approach to *Bacillus* taxonomy has been accomplished largely by the analysis of 16S rRNA molecules by oligonucleotide sequencing. This technique, of course, also reveals phylogenetic relationships. Surprisingly, *Bacillus* species showed a kinship with certain non-spore-forming species, including *Enterococcus*, *Lactobacillus*, and *Streptococcus* at the Order level, and *Listeria* and *Staphylococcus* at the Family level. Otherwise, some former members of the genus *Bacillus* were gathered into new families, including *Acyclobacillaceae*, *Paenibacillaceae,* and *Planococcaceae*, now on the level with *Bacillaceae*. All in all, today (2004) over 200 species of AEFB allocated to about 25 genera have been validly published. Notable former members of the genus *Bacillus* that have been moved to new families and/or genera are given in Table 2.

Taxonomy of the genus *Bacillus* consists of two groups of organisms vernacularly called the *B. subtilis* group and the *B. cereus* group. Species of the *B. subtilis* group are closely related and thus not easily distinguishable which included the two subspecies of *B. subtilis* (*B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*), *B. pumilus*, *B. licheniformis*, *B. amyloliquefaciens*, *B. mojavensis*, *B. sorensis* and

Table 2 Important species reassignments in the Genus *Bacillus* as per the recent approved lists of bacterial names (1986–2004)

Sl. no.	Bergey's manual of systematic bacteriology (1st edn., 1986)	Bergey's manual of systematic bacteriology (2nd edn., 2004)
1	*Bacillus acidocalderius*	*Acyclobacillus acidocalderius*
2	*Bacillus agri*	*Brevibacillus agri*
3	*Bacillus alginolyticus*	*Paenibacillus alginolyticus*
4	*Bacillus amylolyticus*	*Paenibacillus amylolyticus*
5	*Bacillus alvei*	*Paenibacillus alvei*
6	*Bacillus azotofixans*	*Paenibacillus azotofixans*
7	*Bacillus brevis*	*Brevibacillus brevis*
8	*Bacillus globisporus*	*Sporosarcina globisporus*
9	*Bacillus larvae*	*Paenibacillus larvae*
10	*Bacillus laterosporus*	*Brevibacillus laterosporus*
11	*Bacillus lentimorbus*	*Paenibacillus lentimorbus*
12	*Bacillus macerans*	*Paenibacillus macerans*
13	*Bacillus pasteurii*	*Sporosarcina pasteurii*
14	*Bacillus polymyxa*	*Paenibacillus polymyxa*
15	*Bacillus popilliae*	*Paenibacillus popilliae*
16	*Bacillus psychrophilus*	*Sporosarcina psychrophilia*
17	*Bacillus stearothermophilus*	*Geobacillus stearothermophilus*
18	*Bacillus thermodenitrificans*	*Geobacillus thermodenitrificans*

B. vallismortis. Even more loosely attached to this group are the species *B. firmus*, *B. lentus*, and *B. sporothermodurans*, which are clearly distinguishable from the other species of this group (Claus and Berkeley 1986). The species of *B. cereus* group includes closely related species such as *B. cereus*, *B. thuringiensis* (both motile), *B. mycoides,* and *B. pseudomycoides*. The species *B. weihenstephanensis* seems to consist of strains of *B. mycoides* and *B. cereus* (Jackson et al. 1999).

The genus *Paenibacillus* was created by Ash et al. (1993) to accommodate the former "group 3" of the genus *Bacillus*. It comprises over 30 species of facultative anaerobes and endospore-forming, neutrophilic, periflagellated heterotrophic, low G+C gram-positive bacilli. The name reflects this fact, in Latin *paene* means *almost*, and therefore the *Paenibacillus* is almost a *Bacillus*. Comparative 16S rRNA sequence analyses revealed that rRNA "group 3" bacilli represents a phylogenetically distinct group and exhibit high intragroup sequence relatedness and is only remotely related to *B. subtilis,* the type species of the genus *Bacillus*. The taxon contains various species such as *B. alvei*, *B. amylolyticus*, *B. azotofixans*, *B. gordonae*, *B. larvae*, *B. macerans*, *B. macquariensis*, *B. pabuli*, *B. polymyxa*, *B. pulvifaciens,* and *B. validus* (Ash et al. 1993). Phenotypically, species of this group react weakly with gram's stain and even young cultures appear gram-negative. They differentiate into ellipsoidal spores which distinctly swell the mother cell. The combination of morphology and physiology is sufficient to distinguish rRNA "group 3" bacilli from all other mesophilic species of *Bacillus* with the exception of *B. circulans*, *B. lautus*, *B. lentimorbus,* and *B. popilliae*. The latter four species are, however, phylogenetically only remotely related to *B. polymyxa* and its relatives and the described rRNA "group 3" specific gene probe provides an

unequivocal method for distinguishing these taxa (Ash et al. 1991). The genus *Bacillus* of which, *B. subtilis* is the type is and an established model organism for research on gram positive bacteria. Recently, the genome of *B. subtilis* was sequenced completely and it represents the first published genome for a soil-living bacterium (Kunst et al. 1997; Wipat and Harwood 1999). Among the 51,713 Firmicutes sequences listed in Ribosomal Database Project (RDP) II, *Paenibacillaceae* comprises 1,057 16S rRNA sequences with 74 as *P. polymyxa* (as on January 2008). Complete sequencing of the genome of the plant growth promoting strain *P. polymyxa* E681, isolated from winter barley roots, is in progress.

3 Ecology and Distribution of *Bacillus* and *Paenibacillus* spp.

The species of *Bacillus* and *Paenibacillus* are metabolically diverse; the primery habitat of genus *Bacillus* is the soil and associated plants, rivers, and estuarine waters, although some species are pathogenic for mammals (e.g., *B. anthracis*) and insects (e.g., *B. sphaericus*, *B. thuringiensis*). The species of *Paenibacillus* inhabits different niches such as soils, roots, and rhizosphere of various crop plants including wheat, maize, sorghum, sugarcane and barley, and forest trees such as lodgepole pine, douglas fir, and marine sediments etc (Holl and Chanway 1992; von Der Weid et al. 2000). Multiple *Bacillus* and *Paenibacillus* spp. can be readily cultured from both bulk and rhizosphere soils. Culturable counts of these bacteria generally range from log 3 to log 6 cells per gram fresh weight, with soil counts typically exceeding those obtained from the rhizosphere (Mahaffee and Kloepper 1997; Seldin et al. 1998). Standard isolations on complex media typically yield multiple isolates of phylogenetically and phenotypically similar species related to *B. subtilis* and *B. cereus*. Most distinctive among these morphologically is *B. mycoides*, which often confound attempts to accurately enumerate cultured populations by virtue of their rapid mycelial-like growth patterns on agar media. *B. megaterium* has been reported to be one of the most abundant in some soils (Liu and Sinclair 1992), but it seems unlikely that a single species will dominate numerically in most soils. While multiple species of *Paenibacillus* can be detected in the soils and rhizosphere (Seldin et al. 1998), less work has been done to indicate which might be the most commonly isolated species.

Some species were initially defined based on the extreme physical or chemical conditions under which they were first isolated (e.g., *B. psychrophilus*), but few examples of obligate extremophiles exist (e.g., *B. stearothermophilus*, which are typically isolated from thermophilic composts) (Priest 1993). Instead, niche specificity and important ecological activities in *Bacillus* and *Paenibacillus* spp. appear to span phylogenetic boundaries. Most species can survive as saprophytes in soils, which are considered the primary reservoirs of these bacteria; however, most viable cells probably occur as inactive spores at any given time (Nicholson 2002). Culture-independent analyses of soil DNA have confirmed the presence of

the easily cultured species and revealed additional, uncultured diversity in both the *Bacillus* and *Paenibacillus* rRNA lineages (Felske et al. 1999; Smalla et al. 2001; Garbeva et al. 2003). However, contradictory evidence exists on the relative abundance of cultured and uncultured representatives of these genera in different soils. Some reports indicated that the large majority of *Bacillus*-like sequences cloned from soils were highly similar to known species. But, others report that the dominant *Bacillus* sequences present in a different soil are not the same as those present in easily cultured isolates (Smalla et al. 2001; Garbeva et al. 2003). Interestingly, the substantial effort leading to the isolation of this previously uncultured lineage (referred to as DA001) also led to the isolation of even more microdiversity that had not been previously directly detected in DNA clone banks of sequences obtained from the same soil (Felske et al. 1999).

At the species level, most *Bacillus* and *Paenibacillus* are globally distributed and such widespread occurrence of more defined subspecies of *B. subtilis* and *B. cereus* with the capacity to suppress plant pathogens has also been reported (Priest 1993; Stabb et al. 1994; Pinchuk et al. 2002). Other studies have reported only a limited degree of geographic endemicity in *B. thuringiensis* (Chak et al. 1994; Bravo et al. 1998) and *Paenibacillus azotofixans* (Seldin et al. 1998) over spatial scales. Recently, ribosomal sequences amplified from environmental samples have been used to characterize the relative distribution of *Bacillus* and *Paenibacillus* spp. between soils and plant tissues. Overall, the structure of soil bacterial communities is known to vary with soil type more than with management regime (Garbeva et al. 2003); however, the magnitude of such variation may be relatively small for *Bacillus* and *Paenibacillus* spp.

4 PGPR Potentials of *Bacillus* and *Paenibacillus* spp.

Bacillus and *Paenibacillus* spp. are known to have wide PGPR potentials; in the rhizosphere, they are involved in nitrogen fixation, soil phosphorus solubilisation, the production of antibiotics, chitinase, hydrolytic enzymes, and exopolysaccharides and in the enhancement of soil porosity. Numerous *Bacillus* and *Paenibacillus* strains express these activities which promote plant growth and suppress soilborne plant pathogens. A number of these strains already have been developed commercially as plant growth promoters and biocontrol agents (Table 3) and their use in agriculture has recently been reviewed (Lacey et al. 2001; Paulitz and Belanger 2001). Similarly, many strains of *Paenibacillus* were isolated and characterized functionally (Table 4) for their potential use in agriculture as plant growth promoters (Timmusk and Wagner 1999; Timmusk et al. 2003; Senthilkumar et al. 2007a). Improvements in plant growth and productivity by the applications of *Bacillus* and *Paenibacillus* spp. are mediated by three different ecological mechanisms: promotion of host plant nutrition and growth, antagonism against plant pathogens and insect pests, and stimulation of plant host defense mechanisms.

Table 3 Commercially available *Bacillus* spp. based plant growth promoters and biocontrol products

Bacillus species/strains	Activity/function	Product name
Bacillus polymyxa and other species	Atmospheric nitrogen fixation	Wide variety of products
Bacillus megaterium and *B. coagluans*	Mineral phosphate solubilization	Phosphobacter
B. subtilis QST 713	Fungi and bacteria on vegetables and fruit	Serenade
B. licheniformis	Fungi on turf	Ecoguard
B subtilis GB03	Fungi on cotton and soybeans	Kodiak
B. pumilis GB34	Fungi on soybeans	Yield Shield
B. amyloliquefasciens and *B. subtilis* GB122	Fungi on bedding plants	BioYield
B. subtilis MBI600	Fungi on cotton and soybeans	Subtilex
B. subtilis MBI600 and *Rhizobium*	Fungi on soybeans	HiStick

4.1 Promotion of Host Plant Nutrition and Growth

Bacillus and *Paenibacillus* spp. promote plant growth directly by providing nitrogen to the host plant. They also solubilize insoluble phosphates in soil by various mechanisms and secrete phytohormones. Such activities lead to induced plant growth and development.

4.1.1 Biological N_2 Fixation

Biological nitrogen fixation by soil prokaryotic microorganisms is considered one of the major mechanisms by which plant benefit from the association of microbial partners. One of the benefits that diazotrophic bacteria provide to plants is fixed nitrogen in exchange of fixed carbon released as root exudates. Isolates of nitrogen-fixing bacilli from plant rhizospheres were determined by an acetylene reduction assay (ARA) for nitrogenase activity and by amplifying and sequencing part of *nifH* gene. Xie et al. (1998) reported that the following species were nitrogen-fixing bacteria based on nitrogenase activity: *Bacillus megaterium*, *Bacillus cereus*, *Bacillus pumilus*, *Bacillus circulans*, *Bacillus licheniformis*, *B. subtilis*, *Bacillus brevis,* and *Bacillus firmus*. The three former Bacillus species, *Paenibacillus azotofixans*, *Paenibacillus macerans,* and *P. polymyxa*, were nitrogen fixers, based on nitrogenase activity (Seldin et al. 1984). Recently, *Paenibacillus odorifer*, *Paenibacillus graminis*, *Paenibacillus peoriae,* and *Paenibacillus brasilensis* have been described as nitrogen fixers (Berge et al. 2002; von der Weid et al. 2002). However, *nifH* gene was only detected in the following *Paenibacillus* species*: P. azotofixans*, *P. macerans*, *P. polymyxa*, *P. graminis,* and *P. odorifer* (Berge et al. 2002). Ding et al. (2005) isolated and identified nitrogen-fixing bacilli from plant

Table 4 Functional characteristics of isolated *Paenibicillus polymyxa*

Sl. no.	Isolates/strains	Source	Activity/functions	References
1	*P. polymyxa* strain B1 and B2	Wheat rhizosphere	Nitrogen fixation and formation of biofilm	Lindberg et al. (1985), Timmusk et al. (2005)
2	*P. polymyxa* strain B2	Wheat rhizosphere	Cytokinin production	Timmusk et al. (1999)
3	*P. polymyxa* strains B2, B3 and B4	Wheat rhizosphere	Increased resistance to plant pathogens (biotic stress) and drought resistance(abiotic stress)	Timmusk and Wagner (1999)
4	*P. polymyxa* strains B5 and B6	Wheat rhizosphere	Biocontrol of the oomycete plant pathogens, *Phytophora palmivora* and *Phytim aphanidermatum*	Timmusk et al. (2003)
		Soil around peanut roots	Production of exopolysaccharides, biocontrol against *Aspergillus niger* in roots and seeds of peanut plants	Haggag and Timmusk (2008)
5	*P. polymyxa* PMD66, PMD112, PMD128, PMD216 and 230	Wheat rhizoplane, rhizosphere, soil	Production of auxin and other indolic and phenolic compounds	Labuhn et al. (1997)
	P. polymyxa		Production of chitinase	Mavingui and Heulin (1994)
6	*P. polymyxa* SCE2	Soil(Brazil)	Proteases production, production of antimicrobial compounds active against human pathogenic microorganisms	Seldin et al. (1999)
	P. polymyxa T129	Soil	Biocontrol against *Fusarium oxysporum*	Dijksterhuis et al. (1999)
7	*P. polymyxa* JB115, *P. polymyxa* 1460 and *P. polymyxa* BY-28	Soil	Production of β-glucan, lectin, and flocculants production	Jung et al. (2007), Karpunina et al. (2003), Gong et al. (2003)
8	*P. polymyxa* strains CM5-5 and CM5-6	Barley rhizosphere	Production of hydrolytic enzymes, multitarget and medium-independent type of fungal antagonism	Nielsen and Sorensen (1997)
	P. polymyxa E681	Winter barley roots	Fusaricidin biosynthesis, biocontrol of fungal pathogens on sesame plants	Choi et al. (2007), Ryu et al. (2006)
9	*P. polymyxa* HKA-15	Root and nodule tissues of soybean	Biocontrol against charcoal rot pathogen *R. bataticola* and production of cyclic and depsipeptides	Senthilkumar et al. (2007a, b)
10	*P. polymyxa* OSY-DF	Fermented foods	Coproduction of polymyxin E1 and lantibiotic	He et al. (2007)
	P. polymyxa P13	Fermented sausages	Polyxin production and biosorption of heavy metal	Piuri et al. (1998)

rhizospheres in Beijing region and reported that *nifH* gene exists in both genera *Bacillus* and *Paenibacillus*. Nitrogen-fixing ability by *P. polymyxa* was demonstrated by Guemori-Athmani et al. (2000). These authors measured nitrogenase activity of some representative isolates of *P. polymyxa* recovered from Algerian soil by ARA. Results showed that only 14 of the 23 strains tested were able to reduce acetylene. Some of them were very active: strain SGH1 reduced C_2H_2 at a similar rate to *P. azotofixans* ATCC 35681T, which is a very efficient nitrogen-fixing bacterium (Seldin and Penido 1986). In India, numerous reports are available on the application of free-living diazotrophs, including *Bacillus* spp., for increased yield of various crops.

4.1.2 Solubilization of Phosphorus and Uptake of Minor Nutrients

Phosphorus (P) is a major growth-limiting nutrient, and unlike the case for nitrogen, there is no large atmospheric source that can be made biologically available (Ezawa et al. 2002). Microorganisms enhance the P availability to plants by mineralizing organic P in soil and by solubilizing precipitated phosphates (Fig. 1) (Kucey et al. 1989; Pradhan and Sukla 2005; Chen et al. 2006). Inorganic forms of P are solubilized by a group of heterotrophic bacteria excreting organic acids that dissolve phosphatic minerals and/or chelate cationic partners of the P ions, i.e., PO_4^{3-} directly, releasing P into solution. Bacterial biomass assimilates soluble P, and prevents it from adsorption or fixation. These bacteria in the presence of labile carbon serve as a sink for P by rapidly immobilizing it even in low P soils (Bünemann et al. 2004; Khan and Joergensen 2009). Subsequently, phosphate-solubilizing bacteria (PSB) become a source of P to plants upon its release from their cells. PSB are being used as biofertilizer since 1950s and release of P by PSB from insoluble and fixed/adsorbed forms is an important aspect regarding P availability in soils (Igual et al. 2001).

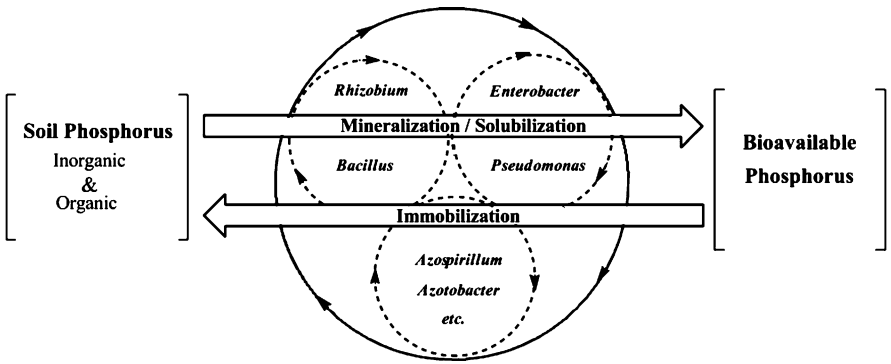

Fig. 1 Schematic diagram of soil phosphorus mineralization, solubilization and immobilization by rhizobacteria

Phosphate solubilization takes place through various microbial processes/mechanisms including organic acid production and proton extrusion. Inorganic P is solubilized by the action of organic and inorganic acids secreted by PSB in which hydroxyl and carboxyl groups of acids chelate cations (Al, Fe, and Ca) and decrease the pH in basic soils. The PSB dissolve the soil P through the production of low molecular weight organic acids, mainly gluconic and keto gluconic acids (Goldstein 1995; Deubel et al. 2000), in addition to lowering the pH of rhizosphere. Among the soil bacterial communities, ectorhizospheric strains from *Pseudomonas* and Bacilli, and endosymbiotic rhizobia have been described as effective phosphate solubilizers. *Bacillus megaterium*, *B. circulans*, *B. coagulans*, *B. subtilis*, *B. polymyxa*, *B. sircalmous,* and *Pseudomonas striata* could be referred as the most important strains (Subbarao 1988; Kucey et al. 1989). Gluconic acid and 2-ketogluconic acid seems to be the most frequent agent of mineral phosphate solubilization. Other organic acids, such as glycolic, oxalic, malonic, and succinic acid, have also been identified among phosphate solubilizers. Strains of *Bacillus* were found to produce mixtures of lactic, isovaleric, isobutyric, and acetic acids in addition to the major organic acids. Phophorus-solubilizing activity of *B. megaterium* enhanced the number of nodules, dry weight of nodules, yield components, grain yield, nutrient availability, and uptake in soybean crop and enhanced the seedling length of *Cicer arietinum* (Son et al. 2006; Sharma et al. 2007), while coinoculation of *Bacillus* spp. along with other PGPR strains reduced P application by 50% without affecting corn yield (Yazdani et al. 2009). Inoculation with phosphate-solubilizing *B. megaterium* increased sugarcane yield by 12.6% (Sundara et al. 2002). Phosphate-solubilizing *B. subtilis* strains have been reported to synergistically increase plant nitrogen and phosphate-accumulation when coinoculated with *Glomus intraradices*. Toro et al. (1997) evaluated the interactive effect of Phosphate-solubilizing bacteria (*Bacillus subtilis*) and arbuscular mycrorrhizal (AM) fungi (*Glomus intraradices*) on onion with a soil of low P content. Coinoculation of these both significantly increased the vegetative biomass and N, P accumulation in onion tissues. Combined inoculation of arbuscular mycorrhiza and Phosphate-solubilizing *Bacillus* and *Paenibacillus* spp. give better uptake of both native P from the soil and P coming from the phosphatic rock (Goenadi et al. 2000; Cabello et al. 2005).

Almost half of the microorganisms in soil and plant roots possess P mineralization potential under the action of phosphatases. The largest portion of extracellular soil phosphatases is derived from the microbial population (Dodor and Tabatabai 2003). Alkaline and acid phosphatases use organic phosphate as a substrate to convert it into inorganic form. Principal mechanism for mineralization of soil organic P is the production of acid phosphatases by rhizobacteria (Hilda and Fraga 2000). Three out of the four strains investigated were identified as *B. amyloliquefaciens* and were able to degrade extracellular phytate (*myo*-inositol hexakisphosphate). The highest extracellular phytase activity was detected in strain FZB45, and diluted culture filtrates of this strain stimulated growth of maize seedlings under phosphate limitation in the presence of phytate (Idriss et al. 2002). Mixed cultures of phosphate-solubilizing rhizobacteria including *Bacillus* and *Paenibacillus* spp. are most effective in mineralizing organic phosphate.

Most studies are indicating that PGPR isolates may increase the mobility and availability of micronutrients including iron (Fe) by the formation of high-affinity siderophores. Chemically, siderophores are low molecular weight compounds of either catecholate or hydroxamate types that complex with Fe^{2+} and render it available to crop plants (Leong 1986). The widespread production of siderophores by diverse rhizobacterial genera included as *Bacillus*, *Rhizobium*, *Pseudomonas,* and *Agrobacterium* at low iron levels are reported by Neilands (1986). Numerous plants are capable of using rhizobacterial Fe siderophore complexes as a means of obtaining Fe from soil (Wang et al. 1993). This view is supported by the findings of Hughes et al. (1992) who reported enhanced Fe uptake in oats because of siderophore production. *Bacillus* and *Paenibacillus* spp. produces both types of siderophores; the bacterium *B. megaterium* ATCC 19213 is known to produce two hydroxamate siderophores, schizokinen and N-deoxyschizokinen, under iron-limited conditions. In addition to their high affinity for ferric ions, these siderophores also chelate aluminum (Hu and Boyer 1996). Wilson Melissa et al. (2006) reported that three *B. anthracis* strains (USAMRIID, 7702, and 34F2) and *B. cereus* ATCC 14579 excrete two catecholate siderophores, petrobactin (which contains 3,4-dihydroxybenzoyl moieties) and bacillibactin (which contains 2,3-dihydroxybenzoyl moieties). However, the insecticidal organism *B. thuringiensis* ATCC 33679 makes only bacillibactin. More details on the production of siderophores by *Bacillus* and *Paenibacillus* spp. and their role in enhancing Fe uptake have been reported by different researchers in variety of crop plants.

4.1.3 Production of Phytohormones and Growth Stimulants

Plant hosts may also be affected by hormones known to be produced by various microbial species, including *Bacillus and Paenibacillus*. There are five classes of well-known phytohormones, namely, auxins, gibberellins, cytokinins, ethylene, and abcisic acid and soil microorganisms, particularly the rhizosphere bacteria, are potential sources of these hormones (Patten and Glick 1996; Arshad and Frankenberger 1998). These phytohormones are known to mediate processes such as plant cell enlargement, division, and extension in symbiotic as well as nonsymbiotic roots. Among these hormones, most attention has focused on auxins in which the most common and well characterized is indole-3-acetic acid (IAA), which is known to stimulate both rapid (e.g., increase in cell elongation) and long-term (e.g., cell division and differentiation) responses in crop plants. Gutierrez-Manero et al. (2001) isolated *B. pumilus* and *B. licheniformis* from the rhizosphere of *Alnus glutinosa* shown to produce physiologically active gibberellins which had strong growth promoting activity on alder. The hormone ethylene production and its effect on plant growth by *Bacillus licheniformis*, *Bacillus subtilis,* and *Bacillus mycoides* also had been reported (Fukuda et al. 1989).

The production of plant growth promoting compounds by *P. polymyxa,* similar in activity to indole-3-acetic acid (IAA), has been suggested to stimulate growth in crested wheatgrass (Holl et al. 1988). It also releases iso-pentenyladenine and

one unknown cytokinin-like compound during its stationary phase of growth which promotes seed germination, *de novo* bud formation, release of buds from apical dominance, stimulation of leaf expansion and reproductive development and retardation of senescence in wheat (Mok 1994). Some strains of *B. subtilis*, and *B. amyloliquefaciens*, promote plant growth by releasing volatiles such as 2,3-butanediol and acetoin. The highest level of growth promotion was observed with mutants of *B. amyloliquefaciens* IN937a and *B. subtilis* GB03, blocked in the biosynthesis of these compounds, were inactive in plant growth promotion (Ryu et al. 2003). More recently, Zhang et al. (2008) found that *B. subtilis* GB03 increases the photosynthetic efficiency and chlorophyll content of *A. thaliana* through the modulation of endogenous signalling of glucose and abscisic acid sensing. Enzyme cellulase (CMCase) activities were also shown in *Bacillus pumilus*, *Bacillus sphaericus,* and *Bacillus circulans*, which showed that most plant-associated microorganisms might have cellulase activity for adoption or establishment of a plant microbe interaction (Emitizi et al. 2007). The effect of inoculation with *P. polymyxa* on growth parameters of wheat and spinach plants and the activities of enzymes present in the leaves of these plants such as glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione reductase, and glutathione S-transferase were also observed (Cakmakci et al. 2007).

4.2 Antagonism Against Plant Fungal and Bacterial Pathogens

Bacillus and *Paenibacillus* spp. suppress phytopathogens by producing various antifungal metabolites and impart induced systemic resistance (ISR) against insects and nematodes.

4.2.1 Control of Fungal Pathogens

Direct antagonism against plant fungal pathogens by *Bacillus* spp. has been well exploited in agriculture as biocontrol agents. The most thoroughly studied of these include *B. subtilis* (Leifert et al. 1995; Asaka and Shoda 1996; Pinchuk et al. 2002). Additionally, a number of studies have reported direct antagonism by other species including *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. mycoides*, and *B. pumilus* as well as isolates of unidentified species from the genus (Handelsman et al. 1990; Leifert et al. 1995; Liu and Sinclair 1992). Although less frequently reported in the literature, some isolates of *P. macerans* and *P. polymyxa* may also be antagonistic to plant pathogens (Timmusk and Wagner 1999). Most of these studies focused on control of fungal and oomycete pathogens. The culture filtrate of *B. amyloliquefaciens* RC-2 showed activity against *Colletotrichum demantium*, *Rosellina necatrix*, *Pyricualria oryzae*, *Agrobacterium tumefaciens,* and *Xanthomonas campestris* pv. campestris (Yoshida et al. 2001). A soil bacterium *Bacillus* sp. strain BC121 isolated from the

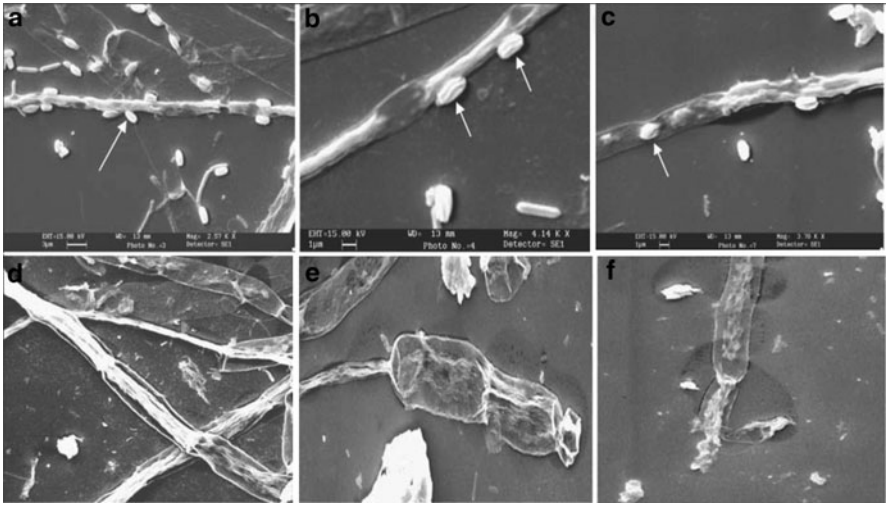

Fig. 2 Scanning electron microphotographs (SEM) showing the antagonistic interaction of *Paenibacillus polymyxa* HKA-15 against charcoal rot pathogen *Rhizoctonia bataticola* (Senthilkumar et al. 2007b)

rhizosphere of sorghum showed high antagonistic activity against *Curvularia lunata*. The strain produces chitinase protein which showed clear hyphal lysis in in vitro observations (Basha and Ulaganathan 2002). *Bacillus subtilis* strain PRBS-1 and AP-3 inhibited five soybean seed pathogenic fungi viz., *Rhizoctonia solani*, *Colletotrichum truncatum*, *Sclerotinia sclerotium*, *Macrophomina phaseolina,* and *Phomopsis* spp. under in vitro conditions (Araujo et al. 2005). *B. amyloliquefaciens* strains conferred protection of oil seed rape (*Brassica napus*) toward all fungal pathogens such as *Alternaria brassicae*, *Botrytis cinerea*, *Leptosphaeria maculans*, and *Verticillum longisporum* (Danielsson et al. 2006).

The in vitro antagonistic activity of *P. polymyxa* against the fungus *Gaeumannomyces graminis* var. *tritici* that causes take-all of wheat and the plant pathogenic fungus *Fusarium oxysporum* that causes *Fusarium* wilt disease has been reported by Heulin et al. (1994). Ryu et al. (2006) demonstrated that *P. polymyxa* strain E681 effectively controlled preemergence and postemergence damping-off diseases on sesame plants. *Paenibacillus polymyxa* HKA-15 was active against *R. bataticola* causing charcoal rot in soybean (Senthilkumar et al. 2007a). Many workers have carried out light microscopic studies on the effect of biocontrol isolates on fungal hyphal morphology. The necrotrophic effect and sequential lysis of *R. bataticola* fungal hyphae by *Paenibacillus polymyxa* HKA-15 cells under light and scanning electron microscope was demonstrated (Fig. 2) (Senthilkumar et al. 2007a). Microscopic analysis on the effect of antagonist on *Magnoporthe grisea* revealed the inhibition of spore germination under light microscope (Tendulkar et al. 2007). Recently, Zhou et al. (2008) isolated *Paenibacillus* strain HT16 from locusts, which showed strong inhibition to *Penicillium expansum* and produced antifungal protein with the molecular weight of 4,517 Da.

4.2.2 Control of Bacterial Pathogens

The role of lipopeptides produced by *Bacillus* sp. against *Xanthomonas campestris*, has been widely studied (Monteiro et al. 2005; Salerno and Sagardoy 2003). However, the effect of metabolites on bacterial cell morphology has also been reported (Hashizume et al. 1996; Nakao et al. 1981). Antagonistic activities of epiphytic bacteria from soybean leaves against *Pseudomonas syringae* pv. glycineae in vitro and in planta was tested. In in planta assay, *Pseudomonas syringae* pv. glycineae and each isolate were simultaneously inoculated in to wounds of pin-pricked leaves of greenhouse-grown plants. Out of 82 isolates, 19 isolates were able to suppress the pathogen. The mixtures of isolate and pathogen were inoculated at ratios >1 (May et al. 1996). Under green-house conditions, inoculation of the isolate *Bacillus subtilis* 210, 72 h before the inoculation of the pathogenic bacteria, significantly reduced the number of lesions caused by *X. campestris* (Salerno and Sagardoy 2003). The species of *Bacillus* and *Paenibacillus* also showed effective antagonism against other bacterial plant pathogens of economically important crops.

4.3 Antagonism Against Insect Pests and Nematode

Antagonism of insect pests and pathogen populations by *Bacillus* sp. and closely related AEFB takes many forms. Some species are pathogens of insects or nematodes (Siddiqui and Mahmood 1999; Lacey et al. 2001). Perhaps, the most studied of the insect pathogens are those classified as *B. thuringiensis*. This species is distinguished from the common saprophytic species *B. cereus* by the occurrence of plasmids that encode pathogenicity factors that make the strains pathogenic to various invertebrates. The production of the crystalline inclusion bodies (Cry proteins) within their spores allow for opportunistic growth when consumed by soil invertebrates. While the crystalline proteins are widely known to be disruptive to the digestive tracts of numerous Lepidopertera and Diptera larvae, evidence also exists for their toxicity to nematodes (Wei et al. 2003). The wide variation in *cry* gene structure and the known occurrence of tolerance to the protein toxins produced by various isolates indicates that a range of virulence exists in nature. *B. sphaericus* are pathogenic to various Diptera species, but the species appears to be more effective at controlling insects that bite animals and humans rather than those that damage crops. *B. sphearicus* also produce protein toxins, but these are deposited outside the spore coat by the mother cell. *P. popilliae* and *P. lentimorbus* cause milky disease in the larvae of some beetles (Order: Coleoptera) including those that can damage crops. Antagonistic activity of *P. polymyxa* was also demonstrated against the root-knot nematode, *Meloidogyne javanica*. The inoculation of *P. polymyxa* alone or together with *Rhizobium* increased lentil plant growth both in *M. javanica*-inoculated and uninoculated plants (Siddiqui et al. 2007).

4.4 Stimulation of Plant Host Defense Mechanisms Through Induced Systemic Resistance

Recently, "induced resistance" to diseases, or plant "immunization," has received increasing attention (Uknes et al. 1992). This refers to a process in which plants exhibit an increased level of resistance to infection by a pathogen after appropriate stimulation. Induced resistance can be triggered by, e.g., infection with a necrotizing pathogen, or by treatment with certain chemicals, e.g., salicylic acid (SA). This response is referred to as systemic acquired resistance (SAR). Induced resistance can also be a result of root colonization by PGPR (Alström 1991; Wei et al. 1991). The latter response is called induced systemic resistance (ISR), and has been shown to protect against disease in several plant species (Thomashow and Weller 1995; van Wees et al. 1997). Elicitation of ISR by plant-associated bacteria was initially demonstrated using *Pseudomonas* spp. and other gram-negative bacteria. Fewer published accounts of ISR by *Bacillus* spp. are also available. The specific strains of the species *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus* elicit significant reductions in the incidence or severity of various diseases on a diversity of hosts (Kloepper et al. 2004). Elicitation of ISR by these strains has been demonstrated in greenhouse or field trials on tomato, bell pepper, muskmelon, watermelon, sugar beet, tobacco, *Arabidopsis* sp., cucumber, loblolly pine, and two tropical crops (long cayenne pepper and green kuang futsoi) (Van Loon 2007). Protection resulting from ISR elicited by *Bacillus* spp. has been reported against leaf-spotting fungal and bacterial pathogens, systemic viruses, a crown-rotting fungal pathogen, root-knot nematodes, and a stem-blight fungal pathogen as well as damping-off, blue mold, and late blight diseases (Van Loon et al. 1998). Reductions in populations of three insect vectors have also been noted in the field: striped and spotted cucumber beetles that transmit cucurbit wilt disease and the silverleaf whitefly that transmits *Tomato mottle virus*. In most cases, *Bacillus* spp. that elicits ISR also promotes plant growth (Zehnder et al. 1997, 2000).

Many individual bacterial components induce ISR, such as LPS, flagella, salicylic acid, and siderophores (Van Loon 2007). More recently, cyclic lipopeptides, the antifungal factor Phl, the signal molecule acyl homoseine lactone (AHL), and volatile blends produced by *B. subtilis* GB03 and, to a lesser extent, the individual volatiles acetoin and 2,3-butanediol have been added to the list (Ryu et al. 2004). Studies on mechanisms indicate that elicitation of ISR by *Bacillus* spp. is associated with ultrastructural changes in plants during pathogen attack and with cytochemical alterations. Investigations into the signal transduction pathways of elicited plants suggest that *Bacillus* spp. activate some of the same pathways as *Pseudomonas* spp. and some additional pathways (Fig. 3). For example, ISR elicited by several strains of *Bacillus* spp. is independent of salicylic acid but dependent on jasmonic acid, ethylene, and the regulatory gene *NPR1* – results that are in agreement with the model for ISR elicited by *Pseudomonas* spp. (Van Loon 2007). However, in other cases, ISR elicited by *Bacillus* spp. is dependent on salicylic acid and independent of jasmonic acid and *NPR1*. In addition, while ISR by *Pseudomonas* spp.

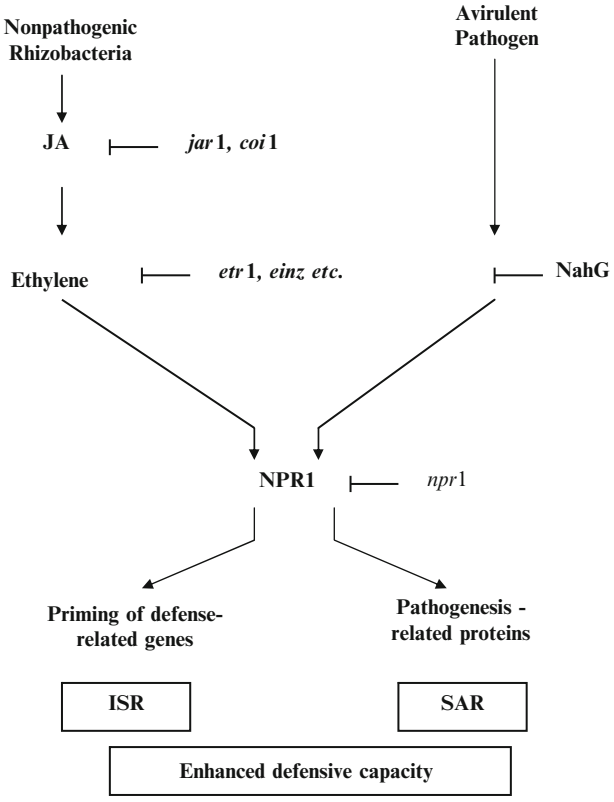

Fig. 3 Current model of signal-transduction pathways leading to pathogen-induced systemic acquired resistance (SAR) and rhizobacteria-induced systemic resistance (ISR). Some species of *Bacillus* spp. may trigger a SA-dependent signaling pathway that leads to a state of induced resistance resembling SAR (Van Loon 2007)

does not lead to accumulation of the defense gene *PR1* in plants, in some cases, ISR by *Bacillus* spp. does. For example, elicitation of ISR in sugar beet by *B. mycoides* strain Bac J and *B. pumilus* strains 203-6 and 203-7 was associated with enhanced peroxidase activity and increased production of one chitinase isozyme and two isozymes of β-1,3-glucanase (Bargabus et al. 2002, 2004). In the tobacco blue mold system, Zhang et al. (2002) reported that plants treated with *B. pumilus* strain SE34 had greatly increased levels of salicylic acid, compared with that of nontreated plants or plants treated with two gram-negative bacteria, 1 day after challenge-inoculation with the pathogen. In a recent study, Timmusk and Wagner (1999) reported that natural isolates of *P. polymyxa* B_2 induces changes in *Arabidopsis thaliana* gene expression and confers significant resistance to plant pathogen *Erwinia caratovora* upon challenge inoculation. This isolate also induces drought tolerance and these effects were observed in both gnotobiotic and soil systems. Similarly, *P. polymyxa* isolates B_2, B_3, and B_4 induces ISR toward Oomycete plant pathogens *Phytophthora palmivora* and

Pythium aphanidermatum causing damping-off in *Arabidopsis thaliana* (Timmusk et al. 2003).

5 Production of Peptide Antibiotics by *Bacillus* and *Paenibacillus* spp.

Several species of *Bacillus* and *Paenibacillus* are known to produce toxins that are inhibitory to the growth and/or activities of fungal, bacterial, and nematode pathogens of plants. Catabolic enzymes (e.g., proteases, chitinases, and glucanases), peptide antibiotics, and small molecules can be secreted by various species (Priest 1993) and may all contribute to pathogen suppression. *Bacillus* spp. and its related genera have been identified as potential biocontrol agent as they produce wide range of peptide antibiotics (Fig. 4) active against various microorganisms

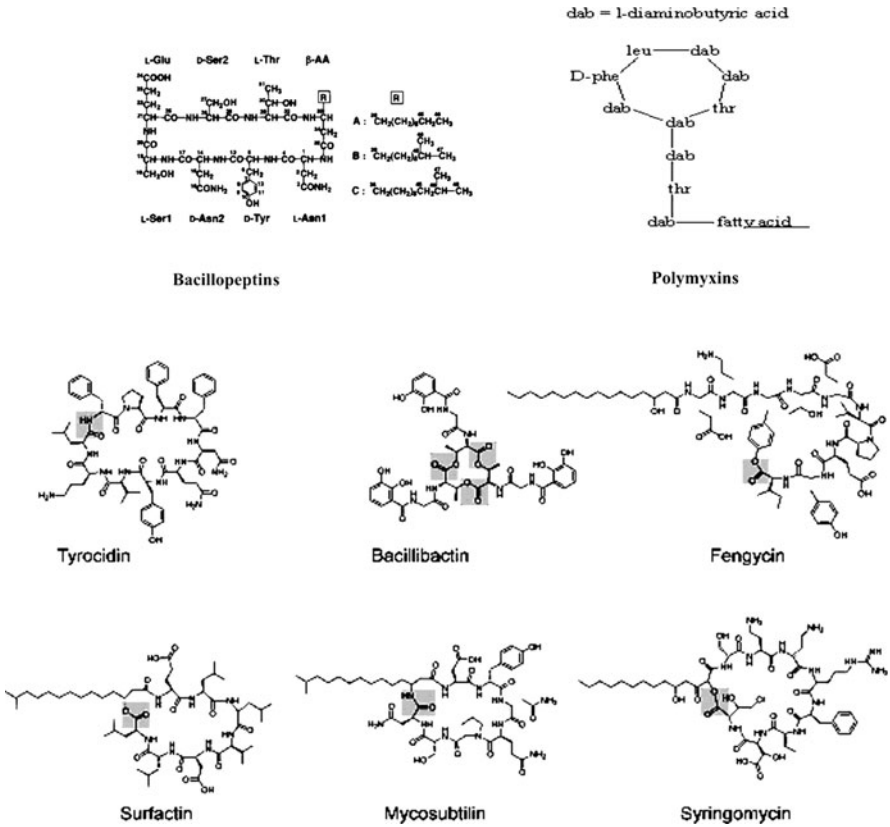

Fig. 4 Chemical structures of important peptide antibiotics produced by *Bacillus* and *Paenibacillus* spp. (Selim et al. 2005)

(Kim et al. 2003). On the basis of the chemical nature, these peptides may be classified into two broad groups viz., cyclic and linear peptides. The identification and characterization of these peptides from different strains showed that they are Bacillopeptins (Kajimura et al. 1995), fusaridicin group of peptides (Beatty and Jensen 2002), matacin (Polymyxin M) (Martin et al. 2003), Gavaserin and Saltavalin (Pichard et al. 1995), and Polymyxin B (Selim et al. 2005).

Iturin A2, a cyclic peptide was identified by NMR and FAB-MS analysis from the culture filtrate of *B. amyloliquefaciens* RC-2 (Yoshida et al. 2001). Crude antibiotic extracts of *B. cereus* were purified and the active fraction $X_{16s}I$ was stable at a wide range of temperature, pH, and polar organic solvents (Safdi et al. 2002). Bacillomycin F, a new family of iturin group antibiotics was isolated from *Bacillus subtilis* (Mhammedai et al. 1982). Acid hydrolysis of the antibiotic gave a peptide moiety which contains 7 mol of amino acids and a lipid moiety which is a mixture of two main long-chain β-aminoacids. Tendulkar et al. (2007) reported biologically active fractions isolated from the culture filtrate of *B. licheniformis* BC 98. These were further fractionated by RP-HPLC and characterized by 500 MHz ^{1}H NMR analysis and identified as surfactin with the molecular mass of 1,035 Da. Two active methanol fractions viz., KB-8A and KB-8B were extracted from the culture filtrate of *Bacillus polymyxa*. The purified fraction KB 8A had minimum inhibitory concentration (MICs) of 12.8 μg/ml for *Fusarium oxysporum* and *Alternaria mali* (Hyun et al. 1999).

Two new antibacterial substances viz., Gavaserin and Saltavalin were isolated from *Bacillus polymyxa* with the molecular mass of 911 and 903 Da, respectively (Pichard et al. 1995). Bacillopeptins, a new iturin group antifungal antibiotic was isolated from *B. subtilis* FR-2 from the rhizosphere of garlic suffering from basal rot caused by *F. oxysporum*. Their structures were elucidated to be cyclic lipopeptides similar to bacillomycin L (Kajimura et al. 1995). Bacillomycin F produced by *Bacillus subtilis,* isolated from honey, showed antagonism against *Byssochlamys fulva* H25 and its structure had varying lengths of the fatty acid chain moiety from C14 to C16. Tamehiro et al. (2002) reported a novel phospholipid antibiotic (bacilysocin) produced by *Bacillus subtilis* 168 and the structure of Bacilysocin elucidated was 1-(12-methyltetradecanoyl)-3-phosphoglyceroglycerol using NMR and Mass Spectrometry analysis. The antimicrobial peptide cerein 8A was isolated from *B. cereus* and its purified substance corresponded to 26 κDa peptide band (Bizani et al. 2005). The importance of antibiotic production to plant disease suppression by *Bacillus* spp. has been demonstrated. *B. subtilis* strains that produce the lipopeptide antibiotics iturin A and surfactin could suppress damping-off in tomato while mutants could not (Asaka and Shoda 1996). And, in *B. cereus*, production and resistance to zwittermicin A have been correlated to suppression of damping-off in alfalfa (Raffel et al. 1996). Pueyo et al. (2009) showed a large group of lipopeptides produced by soil bacterium *B. megaterium* and their antagonistic activity similar to surfactins, lichenysins, itrurin A, and fengycins.

Physicochemical characterization of antimicrobial metabolite produced by *Paenibacillus peoriae* strain NRRL BD-62 showed that the compound retained the activity after autoclaving at 121°C for 10 min. The compound was stable after

treatment with organic solvents and hydrolytic enzymes, and its activity was preserved at a wide range of pH (Weid et al. 2003). *Paenibacillus* sp. strain B2, isolated from the mycorrhizosphere of sorghum colonized by *Glomus mosseae*, produced three active antagonistic compounds. The first peptide compound had the same retention time as polymyxin B1 with the molecular mass of 1,184.7 and contains a 2,3-didehydrobutyrine residue with a molecular mass of 101 Da replacing a threonine at the A_2 position of polymyxin side chain and this could explain the broader range of antagonistic activity of this peptide compared to that of polymyxin B (Selim et al. 2005). Most studies on the biocontrol activity of *P. polymyxa* have been concentrated on the production of different antibiotic substances. Fusaricidin, a peptide antibiotic consisting of six amino acids, has been identified as a potential antifungal agent from *P. polymyxa* E681 (Choi et al. 2007). Various analogs of fusaricidins were isolated and characterized from *P. polymyxa*; these included LI-F03, LIF04, LI-F05, LI-F06, LI-F07, and LI-F08 as well as fusaricidins A–D (Kajimura and Kaneda 1996, 1997).

Fusaricidins have an excellent antifungal activity against plant pathogenic fungi such as *Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus oryzae*, *Penicillium thomii,* and fusaricidin B has particularly antagonistic activity against *Candida albicans* and *Saccharomyces cerevisiae*. Fusaricidins also have an excellent germicidal activity to gram-positive bacteria such as *Staphylococcus aureus* (Kajimura and Kaneda 1996, 1997). *Paenibacillus polymyxa* PKB1 produces fusaridicin peptides with molecular masses of 883, 897, 948, and 960 Da. The characterization of 897 Da component was determined to be cyclic depsipeptide and has antifungal activity against *Leptosphaeria maculans*, which causes black root rot of canola (Beatty and Jensen 2002). The antifungal metabolite produced by *Paenibacillus polymyxa* strain HKA-15 showed strong antagonism against *Rhizoctonia bataticola* causing charcoal rot disease in soybean. Two bioactive fractions collected from the culture filtrate of *Paenibacillus polymyxa* strain HKA-15 by preparative HPLC were characterized as cyclic peptide and depsipeptide (Senthilkumar et al. 2007b). *Paenibacillus lentimorbus* strain WJ5, a soil isolate, produced antifungal metabolite which was extracted with *n*-butanol. The FT-IR spectrum of the antifungal metabolite confirmed the presence of the peptide and glycosidic bonds. (Lee et al. 2008).

6 Endophytic Colonization and Biofilm Formation by *Bacillus* and *Paenibacillus* spp.

Some bacteria and fungi present in the rhizosphere are capable of entering the plant as endophytes that do not cause harm and could establish a mutualistic association. Plants constitute vast and diverse niches for endophytic organisms. Endophytic bacteria have been isolated from a large diversity of plants and most likely, there is not a single plant species devoid of endophytes (Hallman et al. 1997). In general,

endophytic bacteria occur at lower population densities than rhizospheric bacteria or bacterial pathogens. Endophytic populations, like rhizospheric populations, are conditioned by biotic and abiotic factors but endophytic bacteria could be better protected from biotic and abiotic stresses than rhizospheric bacteria (Hallman et al. 1997). The population density of endophytes is highly variable, depending mainly on the bacterial species and host genotypes but also on the host developmental stage, inoculum density, and environmental conditions. Endophytic bacteria in a single plant host are not restricted to a single species but comprise several genera and species (Araujo et al. 2002). The presence of different endophytic species in soybean depended on the plant genotype, the plant age, the tissue sampled, and also on the season of isolation. It seems that the bacteria best adapted for living inside plants are naturally selected. Mavingui et al. (1992) found that there are different populations of *Bacillus polymyxa* in rhizosphere soil and rhizoplane, and that wheat roots select specific populations. The analysis by genomic fingerprinting of the diversity of *B. pumilus* isolated from surface-disinfected leaves showed that populations inside citrus do not seem to be clones derived from a single genotype (Araujo et al. 2002).

Similar to rhizosphere bacteria plant growth stimulation mechanisms by endophytic bacteria is also a consequence of nitrogen fixation or by enhancing availability of minerals or the production of phytohormones, biocontrol of phytopathogens in the root zone through production of antifungal or antibacterial agents, siderophore production, nutrient competition, and induction of systematic acquired host resistance (Sessitsch et al. 2004; Rosenblueth and Martínez-Romero 2006). Endophytic N_2-fixing bacteria seem to constitute only a small proportion of total endophytic bacteria and increasing N_2-fixing populations in plants has been considered as a possibility to increase nitrogen fixation. Nitrogen-fixing bacteria were identified in sweet potato in N-poor soils with an analysis that consisted of amplifying nitrogenase (*nifH*) genes by polymerase chain reaction and the resulting sequences, presumably derived from endophytic *Paenibacillus odorifer* (Reiter et al. 2003). The endophytic *B. megaterium* isolated from maize, sweet corn, carrot, and citrus plants is known to solubilize insoluble phosphates (McInroy and Kloepper 1995; Surette et al. 2003). Bacterial endophytes were also isolated from root, nodule, and stem tissues of wild (*Glycine soja*) and cultivated (*Glycine max*) soybean varieties. Many were phytohormone indole acetic acid (IAA) producers and 33% of them secreted extra cellular enzymes cellulase and pectinase (Hung et al. 2007). Bacterial endophytes are capable of suppressing the proliferation of fungal and nematode pathogens, and this may benefit other crops in rotation with the host plants. Nine of the soybean bacterial endophytes belong to *Bacillus* spp., reported to have antifungal activity against major soilborne plant pathogens like *Rhizoctonia bataticola*, *Macrophomina phaseolina*, *Fusarium udam*, and *Sclerotium rolfsii*. These endophytes suppressed the pathogens under in vitro plate assay shown to possess biocontrol traits such as production of hydrogen cyanide (HCN), siderophores, hydrolytic enzymes, and antibiotics. The endophytic *Bacillus* sp. HKA-121 was reported as effective suppressor of charcoal rot disease as well as plant growth promotion in soybean (Senthilkumar et al. 2009).

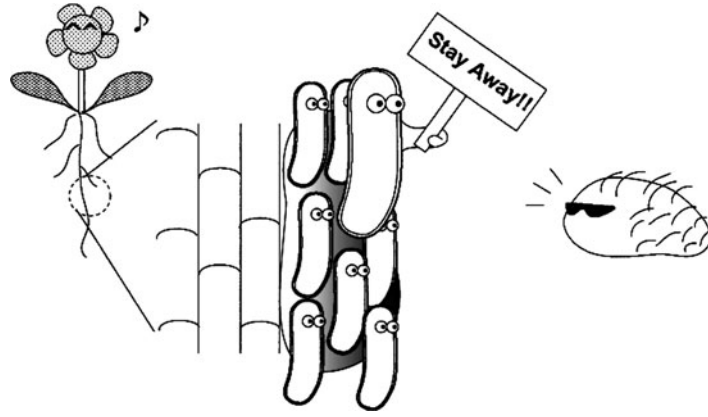

Fig. 5 Schematic diagram showing protection of plant roots against pathogen infection by bacterial biofilm formation on roots

The elucidation of these mechanisms promoting plant growth by bacterial endophytes will help favor species and conditions that lead to greater plant benefits.

It is now commonly known that bacteria persist in their natural environments by forming biofilms. Biofilms are highly structured, surface-attached communities of cells encased in a self-produced extracellular matrix. Bacteria seems to initiate biofilm formation in response to specific environmental cues, such as nutrient and oxygen availability, biofilms undergo dynamic changes during their transition from free-living to sessile biofilm cells, including the specific production of secondary metabolites and a significant increase in the resistivity to biological, chemical, and physical assaults (Davey and O'Toole 2000). Recent studies have suggested that the biofilm formation is important for the bacteriums' ability to act as a biocontrol agent against plant pathogens (Fig. 5). Bacterial biofilms established on plant roots could protect the colonization sites and act as a sink for the nutrients in the rhizosphere, hence reducing the availability of root exudate nutritional elements for pathogen stimulation or subsequent colonization on the root (Weller and Thomashow 1994). In addition, these biofilm-forming bacterial species can produce a variety of antimicrobial metabolites which include broad spectrum lipopeptides of *Bacillus* and *Paenibacillus*, such as surfactins that are potent biosurfactants and important for maintaining the aerial structure of biofilms (Bais et al. 2004). The presence of surfactin-producing *B. subtilis* 6051 biofilms is expected to prevent the planktonic cells of other microbes colonizing biological surfaces including plant roots. Bais et al. (2004) have reported that the biocontrol of *Pseudomonas syringae* by biofilm-forming *B. subtilis* 6051 is related to surfactin production on the surface of the root. Upon root colonization, *B. subtilis* 6051 forms a stable, extensive biofilm and secretes surfactin, which acts together to protect plants against infection by other pathogenic bacteria.

The biofilm-forming strains of *B. thuringiensis* suppress the quorum-sensing-dependent virulence of the plant pathogen *Erwinia carotovora* through a new form

of microbial antagonism called signal interference (Dong and Zhang 2004). *E. carotovora* produces and responds to AHL quorum sensing signals to regulate antibiotic production and the expression of virulence genes, whereas *B. thuringiensis* strains posses AHL-lactonase, which is a potent AHL-degrading enzyme. *B. thuringiensis* does not seem to interfere with the normal growth of *E. carotovora*; however, it abolishes the accumulation of the AHL-signal when they are cocultured (Zhang and Dong 2004). In plants, *B. thuringiensis* significantly decreases the incidence of *E. carotovora* infection and symptom development of potato soft rot caused by the pathogen (Dong and Zhang 2004). In the recent studies, Timmusk et al. (2005) reported that the natural isolates of plant growth promoting rhizobacterium *P. polymyxa* B_1 and B_2 forms biofilms in *Arabidopsis thaliana*. They studied intracellular colonization of these isolates by tagging with plasmid-borne green fluorescent protein (GFP). Fluorescence microscopy and scanning electron microscopy indicated that the bacteria colonized predominantly the root tip, intercellular spaces outside the vascular cylinder where they formed biofilms (Fig. 6). Similarly, the intracellular colonization and biofilm formation in root tips and nodules of soybean by a biocontrol bacterium *P. polymyxa* HKA-15 tagged with GFP was also

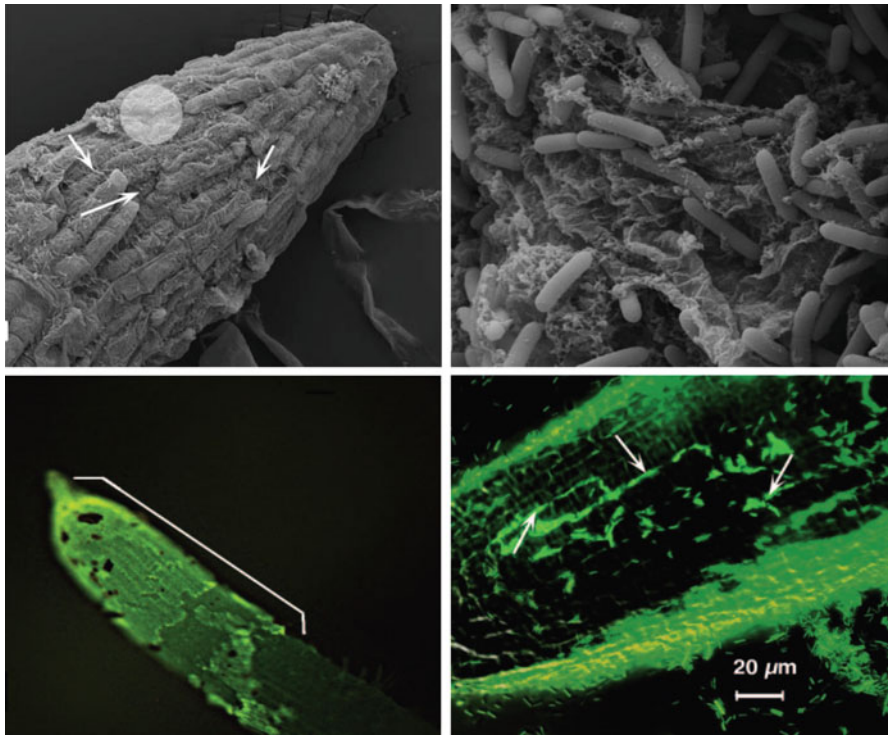

Fig. 6 Scanning electron microphotographs (*top row*) and fluorescence micrographs (*bottom row*) showing the biofilm formation by Gfp tagged isolates of *Paenibacillus polymyxa* B1 on roots (Timmusk et al. 2005)

observed under fluorescence microscopy (data not published). The biofilm-forming *P. polymyxa* on plant roots protected the plant from pathogen infection. Two *P. polymyxa* isolates B_1 and B_2 characterized to form biofilms prevented root colonization and infection by *Aspergillus niger* causing crown rot disease in peanuts (Haggag and Timmusk 2008).

7 Conclusion

The various plant growth promoting properties, together with endospore-forming ability of *Bacillus* and *Paenibacillus*, enable the strain formulations to resist a wide range of environmental stresses. Strains of *Bacillus subtilis* and *Paenibacillus polymyxa* are well-established model organisms for research on molecular plant–microbe interactions. The complete genome of *Bacillus subtilis* has been published and genome sequencing of root colonizing bacterium *Paenibacillus polymyxa* is underway. This genomic information will be available to investigate molecular responses of *Bacillus* and *Paenibacillus* in soil and the crop rhizosphere in particular. Further, biotechnology can be applied to create transgenic strains with multiple mechanisms of action and strains with specific formulation qualities (stability of inoculants and better root colonization). Continued research with endophytic colonization and biofilm formation by these bacterial genera also holds potential for developing biofertilizer and biocontrol agents that may be self-perpetuating within the colonizing host plants. Focusing research in these areas will make *Bacillus* and *Paenibacillus* spp. as promising/potential PGPR. Their applications will significantly reduce the use chemical fertilizers and pesticides which will be essential for achieving sustainable crop yield in agriculture.

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The Role of ACC Deaminase Producing PGPR in Sustainable Agriculture

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Abstract The plant rhizosphere is a multidimensional and dynamic ecological environment of complicated microbe–plant interactions for harnessing essential macro and micronutrients from a limited nutrient pool. Certain plant growth

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promoting rhizobacteria (PGPR) contain a vital enzyme, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (EC 4.1.99.4), which regulates ethylene production by metabolizing ACC (an intermediate precursor of ethylene biosynthesis in higher plants) into α-ketobutyrate and ammonia. The microbial enzyme 1-aminocyclopropane-1-carboxylate deaminase cleaves ACC irreversibly, this being the immediate precursor of ethylene in plants. ACC deaminase-expressing PGPR protect plants against the growth inhibition that might otherwise result following flooding, extremes of temperature, the presence of organic and inorganic toxicants, phytopathogens, drought or high salt concentrations. Organisms containing ACC deaminase genes have been reported to be useful in promotion of early root development from either seeds or cuttings, increasing the life of horticultural flowers, protecting plants against a wide range of environmental stresses, facilitating the production of volatile organic compounds responsible for aroma formation and phytoremediation of contaminated soils.

1 Introduction

Certain plant growth promoting rhizobacteria (PGPR) contain a vital enzyme, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (EC 4.1.99.4), which regulates ethylene production by metabolizing ACC (an intermediate precursor of ethylene biosynthesis in higher plants) into α-ketobutyrate and ammonia. This pyridoxal phosphate (PLP) enzyme was first isolated in 1978 from *Pseudomonas* sp. strain ACP and from the yeast *Hansenula satrunus* (Honma and Shimomura 1978) since then, it has been detected in fungi and in a number of other bacteria. When ACC deaminase-containing plant growth-promoting bacteria (PGPB) are bound to a plant, they act as a sink for ACC ensuring that plant ethylene levels do not become elevated to the point.

Conceptually, PGPR can have an impact on plant growth and development in two different ways: indirectly or directly. The indirect promotion of plant growth occurs when bacteria decrease or prevent some of the deleterious effects of a phytopathogenic organism by one or more mechanisms. On the other hand, the direct promotion of plant growth by PGPR generally entails providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of nutrients from the environment (Glick 1995; Glick et al. 1999). Rhizosphere bacteria multiply to high densities on plant root surfaces where root exudates and root cell lysates provide ample nutrients. Sometimes, they exceed 100 times to those densities found in the bulk soil (Campbell and Greaves 1990). Certain strains of these plant-associated bacteria stimulate plant growth in multiple ways: (1) they may fix atmospheric nitrogen, (2) reduce toxic compounds, (3) synthesize phytohormones and Siderophores, or (4) suppress pathogenic organisms (Bloemberg and Lugtenberg 2001). Research on the "biocontrol" activity of rhizobacteria has seen considerable progress in recent years. Disease suppression of soilborne pathogens includes competition for nutrients and production of antimicrobial compounds or lytic

enzymes for fungal cell walls or nematode structures (Persello-Cartieaux 2003). By contrast, systemic resistance can also be induced by rhizosphere-colonizing *Pseudomonas* and *Bacillus* species where the inducing bacteria and the challenging pathogen remained spatially separated excluding direct interactions (Van Loon et al. 1998; Ryu et al. 2004).

Etiolated pea seedlings are very sensitive to ethylene. The most widely renowned example of the effect of ethylene on plant growth is the classical "triple" response in etiolated dicot seedlings in the presence of ethylene. This effect consists of three distinct morphological changes in the shape of seedlings, inhibition of stem elongation, increase in stem diameter and horizontal growth (Akhtar et al. 2005; Khalid et al. 2006). This "triple" response reaction of etiolated seedlings has been a reliable bioassay for ethylene action (Guzman and Ecker 1990). Shaharoona et al. (2007) observed the effect of inoculation with ACC utilizing and ethylene-producing rhizobacteria and compared through highly ethylene specific classical "triple" response bioassay. In this study, the effect of inoculation with rhizobacteria having different ACC-deaminase activities on extenuating the classical "triple" response in etiolated pea seedlings was investigated.

ACC deaminase-containing PGPB up-regulate genes involved with plant growth and protein production while down-regulating plant genes involved with ethylene stress and defence signaling pathways (Hontzeas et al. 2004a). The ACC deaminase-containing PGPB, in part, alleviate the need for the plant to actively defend itself against various environmental stresses (Hontzeas et al. 2004b; Van Loon and Glick 2004). The crystal structure has been determined for the yeast (Minami et al. 1998), and recently for the bacteria (Karthikeyan et al. 2004) ACC deaminase enzymes; the biochemical and thermodynamic properties of the ACC deaminase from *Pseudomonas putida* UW4 have been measured (Hontzeas et al. 2004b).

ACC deaminase from *Pseudomonas* sp. ACP, *P. putida, P. fluorescens* (Glick 1995), *Enterobacter cloacae* CAL2 and UW4 (Shah et al. 1998), *Kluyvera ascorbata* SUD165 (Burd et al. 1998), *Hansenula saturnus* (Honma and Shimomura 1978), and *Penicillium citrinum* (Jia et al. 2006) have been reported.

This enzyme facilitates plant growth as a consequence of the fact that it sequesters and cleaves plant produced ACC, thereby lowering the level of ethylene in the plant. In turn, decreased ethylene levels allow the plant to be more resistant to a wide variety of environmental stresses, all of which induce the plant to increase its endogenous level of ethylene; stress ethylene exacerbates the effects of various environmental stresses. The ACC deaminase-containing soil bacteria decrease a significant portion of the physiological damage to plants following environmental stresses including phytopathogen infection, exposure to extremes of temperature, high salt, flooding, drought, exposure to metals and organic contaminants, and insect predation. For many plants a burst of ethylene is required to break seed dormancy but, following germination, a sustained high level of ethylene can be inhibitory to root elongation. PGPB that contain the enzyme ACC deaminase, when bound to a plant root or to the seed coat of a developing seedling, may act as a mechanism for insuring that the ethylene level within the plant's tissues does not become elevated to the point where root (or shoot) growth is impaired. By facilitating the formation of

longer roots and shoots, these bacteria may enhance the survival of some seedlings, especially during the first few days after the seeds are planted.

2 Ethylene Biosynthesis in Higher Plants

Ethylene, which is produced in almost all plants, mediates a range of plant responses and developmental step. Ethylene is involved in seed germination, tissue differentiation, formation of root and shoots primordial, root elongation, lateral bud formation, flowering initiation, anthocyanin synthesis, flower opening and senescence, fruit ripening and degreening, production of aroma, leaf and fruit abscission and response of plant to biotic and abiotic stresses. (Saraf and Tank 2005). Ethylene is a potent plant growth regulator that affects diverse developmental processes, including fruit ripening, senescence, and stress responses (McKeon and Yang 1987; Reid 1987). Chemical inhibitors of ethylene synthesis or action completely block ripening in fruits and senescence in flowers of many plant species.

At a molecular level, ethylene is known to induce expression of a number of genes involved in ripening (Lincoln and Fischer 1988) and pathogen response (Ecker and Davis 1987). In some instances, ethylene is stimulatory while in others it is inhibitory.

When plants are exposed to conditions that threaten their ability to survive, the same mechanism that produces ethylene for normal development instead produces "stress ethylene" which may be defined as an acceleration of ethylene biosynthesis associated with biological and environmental stresses, and pathogen attack (Abeles et al. 1992; Hyodo 1991; VanLoon 1984). Ethylene is synthesized from S-adenosyl L-methionine (AdoMet) by way of the intermediate ACC (McKeon and Yang 1987).

While working on the ethylene biosynthesis pathway, Adams and Yang (1979) found that when ACC was applied to various plant organs, an increase in ethylene production was obtained. From their observations, ACC, as a key intermediate that linked the methionine cycle and ethylene biosynthesis, was deemed to be the direct precursor of ethylene production with its level directly controlling ethylene synthesis in plants (Fig. 1).

Ethylene biosynthesis consists of three steps (1) L-methionine is converted to AdoMet, a reaction catalyzed by methionine S-adenosyl transferase. AdoMet is also utilized in other cellular reactions such as ethylation and polyamine synthesis, (2) The conversion of AdoMet to ACC which is catalyzed by ACC synthase. The ACC synthase step is considered to be the rate-limiting step in the pathway (3) ACC is further metabolized to ethylene, carbon dioxide and cyanide by ACC oxidase.

Since all plants respond differently to stress, it has been difficult to detail the functioning of stress ethylene. Increased ethylene levels in plants exposed to various types of stress including chilling, heat, wounding, pathogen infection, salt, metals and nutritional stress, with increased damage as the result has been documented. Stress ethylene, though its role is unclear, is deleterious to plants in many instances (Saravanakumar and Samiyappan 2007).

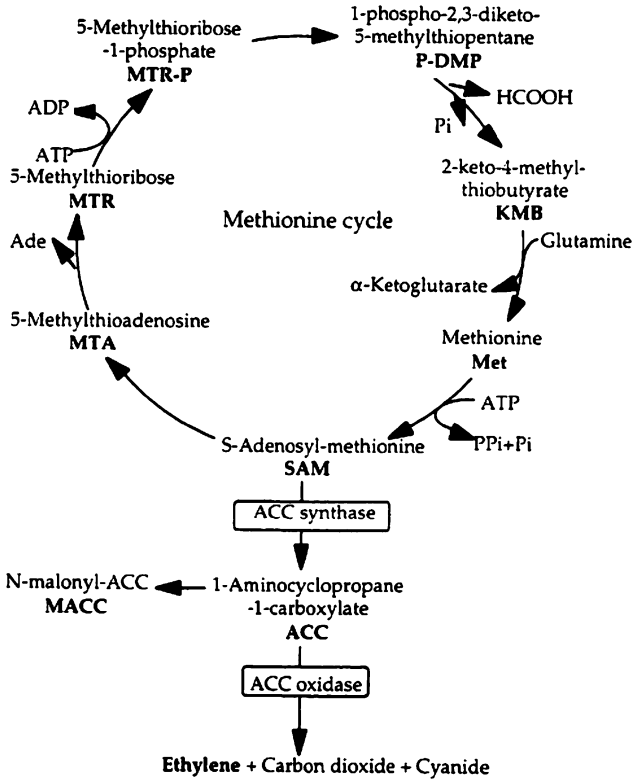

Fig. 1 Pathway of ethylene biosynthesis from the methionine cycle in higher plants. Modified figure adapted from the source reference Li (1999)

3 Characteristics of ACC Deaminase Enzyme

Enzymatic activity of ACC deaminase is assayed by monitoring the production of either ammonia or α-ketobutyrate, the products of ACC hydrolysis. ACC deaminase has been found only in microorganisms, and there are no microorganisms that synthesize ethylene via ACC (Fukuda et al. 1993). ACC Deaminase is a multimeric enzyme (homodimeric or homotrimeric) with a subunit molecular mass of approximately 35-42 kDa. It is a sulfhydral enzyme in which one molecule of the essential cofactor PLP is tightly bound to each subunit. Interestingly, this enzyme is cytoplasmically localized so that the substrate ACC must be exuded by plant tissues and subsequently taken up by an ACC deaminase-containing microorganism before it is cleaved (Glick et al. 1998).

The enzyme–substrate relationship demonstrates Km values of ACC deaminase for ACC estimated at pH 8.5, in all instances examined, to be approximately 1.5–17.4 mM indicating that the enzyme does not have a particularly high affinity for ACC (Honma and Shimomura 1978). Moreover ACC levels in plants are

typically in μM range, therefore in most plant tissues the ACC concentration will be dramatically below the Km of ACC deaminase for this substrate so that based on the Michaelis–Menton rate equation for enzyme catalyzed reaction a small increase in the ACC concentration will result in a parallel increase in the rate of ACC cleavage.

4 Crystal Structure of 1-Aminocyclopropane-1-Carboxylate Deaminase

PLP-dependent enzymes catalyze many important reactions that act upon amino acids, including transamination, decarboxylation, β,γ-replacement/elimination, and racemization. In all of these reactions (except in the case of the glycogen phosphorylase family), the two basic chemical properties of the PLP are conserved; it forms an external aldimine between its aldehyde group and the α-amino group of the substrates and withdraws electrons from the substrate by serving as an electron sink. As a PLP-dependent enzyme, the ACCD's ring opening reaction starts with a transformation reaction from an internal aldimine between the PLP and the enzyme to an external aldimine. These enzymes have been classified based on their three dimensional structure, into four folding types: (1) tryptophan synthase, (2) aspartate aminotransferase, (3) D-amino acid aminotransferase and (4) alanine racemase. In most of the PLP-dependent enzymes, the next step is the nucleophilic abstraction of the α-substituent, either an α-proton or a carboxylate group, to form an α-carbanionic intermediate. This reaction mechanism cannot be applied to ACCD because the substrate (ACC) does not contain α-hydrogen and the carboxyl group is retained in the product. Therefore, the ring-opening reaction of ACC must be initiated without obvious accessibility to an α-carbanionic intermediate, which is, for PLP-dependent enzymes, the common entry for catalysis. One proposed reaction mechanism is the nucleophilic addition to Cγ followed by the cleavage of the Cα–Cγ bond and β-proton abstraction. As PLP, acts as an electron sink, external aldimine is fairly electrophilic, and the nucleophilic addition to Cγ to rupture the cyclopropane ring of ACC is mechanistically feasible (Yao et al. 2000) (Fig. 2).

5 Mechanism of ACC Deaminase Action

A model is proposed to explain how ACC deaminase-containing PGPB can lower plant ethylene levels and in turn stimulate plant growth (Glick et al. 1998), especially under stress conditions. PGPB bind to the surface of either the seed or root of a developing plant in response to tryptophan and other small molecules in the seed or root exudates the PGPB synthesize and secrete the auxin, Indoleacetic acid (IAA), some of which is taken up by the plant. This IAA together with endogenous plant IAA can stimulate plant cell proliferation and elongation, or it can induce the activity

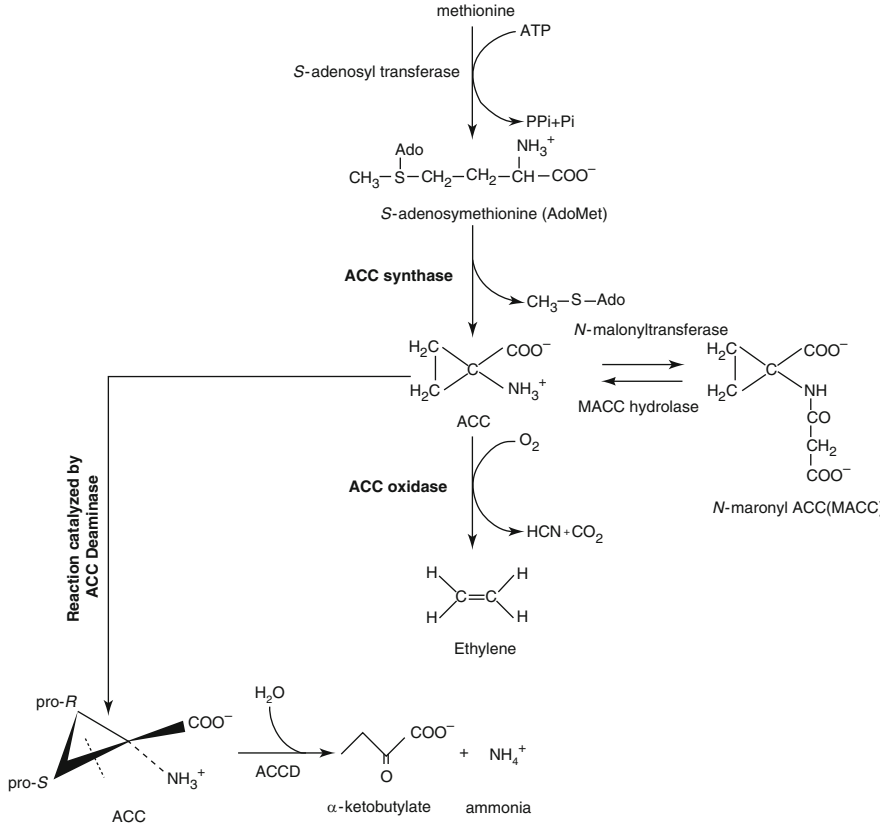

Fig. 2 The enzymatic reaction catalyzed by ACCD. Modified figure adapted from the source reference Ose et al. (2003)

of ACC synthase to produce ACC (Penrose and Glick 2001). Some of the plant's ACC will be exuded along with other small molecules such as sugars, organic acids and amino acids. The exudates may be taken up by the bacteria and utilized as a food source of the rhizosphere bacteria. ACC may be exuded together with the other components of the root or seed exudates. ACC may be cleaved by ACC deaminase to form ammonia and α-ketobutyrate, compounds that are readily further metabolized by the bacteria (Holguin and Glick 2001). The presence of the bacteria induces the plant to synthesize more ACC than it would otherwise need and also, stimulates the exudation of ACC from the plant (some of which may occur as a consequence of plant cell wall loosening caused by bacterial IAA). Thus, PGPB are supplied with a unique source of nitrogen in the form of ACC that enables them to proliferate/survive under conditions in which other soil bacteria may not readily flourish (Hontzeas et al. 2006). As a result of acting as a sink for ACC and lowering its level within the plant, the amount of ethylene that is produced by the plant is also

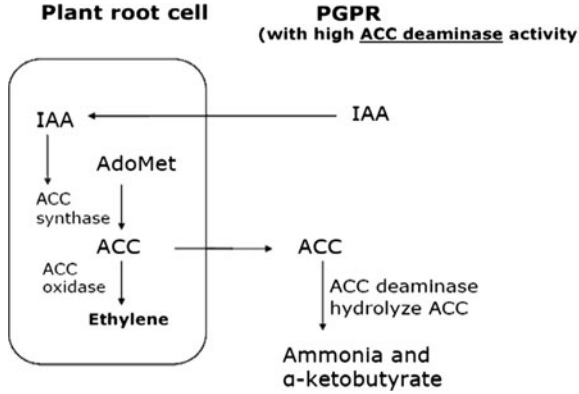

Fig. 3 The ACC deaminase in PGPR degrades the ethylene precursor ACC. The ACC deaminase in PGPR lowers ethylene level in plants by degrading ACC to ammonia and α-Ketobutyrate. Lowering ethylene in plants can alleviate stress and thereby improve plant growth. Some PGPR can also produce plant regulator IAA and further stimulate plant growth. Modified figure adapted from the source reference Glick and Pasternak (2003)

reduced. Thus, the inhibition of plant growth by ethylene (especially during periods of stress) is decreased and these plants generally have longer roots and shoots and greater biomass (Fig. 3).

6 Role of Bacterial ACC Deaminase Under Stress Agricultural Conditions

PGPR containing ACC Deaminase activity eliminates heavy metal toxicity, imparts resistant to drought, other abiotic stresses such as salinity, extremes of temperature and pH in soil apart from antagonism against phytopathogens. Ethylene regulation in plants due to PGPR is now well established (Table 1).

6.1 Pathogenicity Stress

Pathogenic microorganisms are a major and serious threat to food production and ecosystem stability worldwide. PGPR mediated biocontrol in terms of competition for an ecological niche or a substrate and producing allelo-chemicals and inducing systemic resistance (ISR) in host plants to a broad spectrum of pathogens (Compant et al. 2005).

ACC deaminase bacteria, apart from directly antagonizing pathogens, support the plant resistance against pathogen attack. Beneficial rhizobacteria do not obviously damage their host/cause localized necrosis, therefore, the eliciting factors

Table 1 List of ACC deaminase producing bacteria

Strain	ACC deaminase activity (nM αKB $mg^{-1}h^{-1}$)	Reference(s) or Sources
Achromobacter xylosoxidans A551	400 ± 4	Belimov et al. (2001, 2005)
A. xylosoxidans Bm1	90 ± 4	Belimov et al. (2001, 2005)
Achromobacter sp. strain CM1	130 ± 3	Belimov et al. (2001, 2005)
Acidovorax facilis 4p-6	3,080 ± 120	Belimov et al. (2001, 2005)
Azospirillium brasilense Cd1843	–	Holguin and Glick (2003)
Enterobacter aerogenes CAL3	16 ± 12	Shah et al. (1998)
Pseudomonas putida UW4	3,030 ± 60	Hontzeas et al. (2006)
P. syringae GR12-2	3,470 ± 30	Belimov et al. (2001, 2005)
P. brassicacearum Am3	5,660 ± 12	Belimov et al. (2001, 2005)
P. putida BM3	3,780 ± 32	Belimov et al. (2001, 2005)
P. marginalis DP3	4,054 ± 27	Belimov et al. (2001, 2005)
Rhizobium leguminosarum128C53K	5 ± 1	Belimov et al. (2001, 2005)
R. hedysari ATCC 43676	20 ± 0.1	Ma et al. (2003)
R. leguminosarum 99A1	8 ± 3	Ma et al. (2003)
Rhodococcus sp. strain Fp2	7,320 ± 400	Belimov et al. (2001, 2005)
Rhodococcus sp. strain 4N-4	12,970 ± 440	Belimov et al. (2001, 2005)
Serratia quinivirans SUD165	12 ± 15	Belimov et al. (2001, 2005)
Variovorax paradoxus 3P-3	3,700 ± 90	Belimov et al. (2001, 2005)
V. paradoxus 5C-2	4,322 ± 100	Belimov et al. (2001, 2005)
V. paradoxus 2C-1	3,588 ± 26	Belimov et al. (2001, 2005)
P. putida ATCC17399	–	Shah et al. (1998)
Schizosaccharomyces pombe	–	Wood et al. (2002)
Hansenula saturnus	–	Honma and Shimomura (1978), Minami et al. (1998)
Penicillium citrinum	–	Jia et al. (2006)
Yersinia pestis	–	Parkhill et al. (2001)
Caulobacter crescentus	–	Nierman et al. (2001)
Bacillus anthracis	–	Read et al. (2002)
Mesorhizobium loti	–	Sullivan et al. (2002)
Burkholderia fungorum	–	NCBI microbial genome annotation project

produced by ISR-triggering rhizobacteria must be different from elicitors of pathogens. Expression of ISR is similar to systemic acquired resistance (SAR) upon challenge inoculation with pathogen wherein disease severity is reduced; the number of diseased plants also diminishes. This reduction is associated with decreased growth of the pathogen and reduced colonization of induced tissues which reflects upon the ability of plant to resist the pathogen (Dobbelaere et al. 2003). Salicylic acid is an important signaling molecule in both locally and systemically induced resistance responses; however, research on rhizobacteria mediated ISR signaling has demonstrated that jasmonic acid and ethylene play the key roles. Thus, expression of ISR is phenotypically quite similar to SAR, and relies not only on a different type of biological induction but occurs also through different defense-related

activities (Domenech et al. 2006). It is emphasized that ISR-inducing PGPR is a useful tool to reduce diseases caused by pathogens that are sensitive to jasmonic acid and ethylene-dependent defenses. Rasche et al. 2006 reported that ACC deaminase bacteria were capable of antagonizing at least one of the two potato pathogens *Ralstonia solanacearum* and *Rhizoctonia solani*.

6.2 Remediation of High/Heavy Metal Concentration

High metal concentrations in soil have also been shown to cause increased ethylene production and inhibition of root development, to reduce CO_2 fixation and limit sugar translocation. ACC deaminase and siderophore producing PGPB can help plants to overcome many of the effects of high levels of metal (Burd et al. 1998, 2000). Phytoremediation of metals poses a significant challenge because most metal contaminants are tightly bound by soil particles and are not readily bioavailable to plants. Moreover, although many plants can tolerate the presence of excess metals in the soil, most will experience a decrease in plant growth and viability due to either the synthesis of stress ethylene and/or iron depletion. PGPR can alleviate some of the effects of metal toxicity in plants via several different mechanisms. For example bacterial siderophore bind iron with extremely high affinity and plants are able to take up and utilize the iron from these complexes. Thus PGPR are able to protect plants against the inhibitory effects of high concentration of metals by providing the plants with sufficient iron. Belimov et al. (2005) reported 11 cadmium-tolerant strain of PGPR isolated from the rhizosphere of *Brassica juncea* grown in cadmium-containing soils. *Variovorax paradoxus, Rhodococcus* sp. and *flavobacterium sp*. all stimulated root elongation in untreated and Cd-treated soils.

6.3 Drought Stress

Drought is one of the major environmental stresses that limit the growth of plants and the production of crops. The inhibitory effects of ethylene induced by drought stress might have been eliminated through the ACC deaminase activity of the PGPR. Inoculation of plants with PGPR containing ACC deaminase partially or completely eliminated the "drought stress imposed effects" on root and shoot growth, fresh and dry weights, and number of leaves per plant of peas. This might be due to suppression of the stress-induced accelerated synthesis of ethylene by the ACC deaminase activity of these PGPR in the inoculated roots. Sharp increases in ACC levels and, consequently, ethylene synthesis in plants under drought stress conditions has been frequently reported. (Apelbaum and Yang 1981). The rhizobacteria having ACC deaminase activity are effective in promoting plant growth and water use efficiency under drought conditions, by lowering the ethylene or ACC accumulation whose higher levels have inhibitory effects on root and shoot

growth. It is highly likely that rhizobacteria containing ACC deaminase might have decreased the drought-stress induced ethylene in inoculated plants, which resulted in better growth of plants even at low moisture levels. Therefore, inoculation with rhizobacteria containing ACC deaminase could be helpful in eliminating the inhibitory effects of drought stress on the growth of plants. Dodd et al. (2005) investigated the physiological responses of pea (*pisum sativum* L.) to inoculation with ACC deaminase bacteria *V. paradoxus* 5C-2 under moisture stress and watering condition. The bacterial effects were more pronounced and more consistent under controlled soil drying (moisture stress conditions).

6.4 Organic Contaminants Stress

Many organic contaminants are recalcitrant and highly persistent in the environment, making them particularly difficult to remediate. Many of these compounds are hydrophobic and are bound tightly to soil particles. A few studies have revealed an accelerated production of ethylene in soil and plants treated with organic contaminants (Coupland and Jackson 1991). Reed and Glick (2005) have studied the growth of canola (*Brassica napus*) seeds treated with PGPR in copper-contaminated and creosote-contaminated soil. In creosote-contaminated soils, the native bacterium was the least effective, and the transformed encapsulated ACC deaminase bacterium was the most effective in growth promotion.

6.5 Waterlogging Stress

Waterlogging enhances the biosynthesis of ethylene in roots and stem of plants. In flooding, ACC, which is synthesized in roots, is transported to plant shoots where it is converted to ethylene by ACC oxidase (Bradford and Yang 1980). The molecular basis for the increase in ethylene production observed in shoots of flooded tomato plants is due to an increase in the activity of both ACC synthase in the submerged roots and ACC oxidase in the shoots (Chao et al. 1997). The accelerated production of ethylene in the shoots of flooded tomato plants is responsible for the phenotype to demonstrate abnormal growth under flooding conditions (Jackson 1997).

6.6 Temperature Stress

The heat stress in terms of so-called global warming is a serious threat to world agriculture (Mendelsohn and Rosenberg 1994). A fluctuation in temperature leads to hormonal imbalances in plants and thus their growth is significantly affected

(Cheikh and Jones 1994). It has been reported that PGPR containing ACC deaminase activity performs better when subjected to diurnal temperature regime. *Bacillus globiosporus* was inoculated to analyze the effect of diurnal temperature regime (i.e., 25°C days and 5°C night) on root and shoot length, fresh and dry weight were significantly increased in comparison to *B. subtilis* and magnesium sulphate controls (Ghosh et al. 2003).

6.7 Flower Senescence

Ethylene is a key signal in the initiation of willing in most plants. Typically flowers produce minute amount of ethylene until an endogenous rise of the phytohormone, which is responsible for flower senescence to occur (Mol et al. 1995). However, the senescence symptoms that are covered by ethylene differ from plant to plant. The use of ACC deaminase containing PGPR to lower ACC levels in cut flowers might be an environmentally friendly alternative to the available use of silver thiosulphate. An important characteristic of PGPR containing ACC deaminase activity has been shown to be the enhancement of shelf life of flowers incubated in suspension form (Nayani et al. 1998). On a commercial scale, shelf life of flowers could be increased to manifold by treating them with suspensions of PGPR containing ACC deaminase activity, which portends great prospects for the application of this biotechnological approach to commercial floriculture.

6.8 Salinity Stress

Salinity is one of the most severe environmental stresses on plants (White and Broadley 2001; Tester and Davenport 2003; Munns and Tester 2008). Salt primarily limits plant growth in three ways: (1) osmotic effects that lower the ability of plants to take up water from the soil, (2) ion-specific damage of excess Na^+ and Cl^-, and (3) nutrient deficiencies because elevated levels of Na^+ compete with the uptake of other nutrients by interfering with ion transporters (Tester and Davenport 2003). Symptoms of damage to plants include: growth inhibition, leaf discoloration, anatomical and morphological changes such as changes in cell wall structure (Tester and Davenport 2003). Highly saline soil (ECe $>$ 16 dS/m) can severely interfere with seed germination and growth of plants. As water and nutrients move from areas of low salt concentration to areas of high salt concentration, soil salinity prevents plant roots from taking up water and other nutrients, resulting in osmotic and nutrient imbalances that impair proper plant growth. A sudden increase in soil salinity will cause plant cells to shrink due to water loss and immediate changes in expansion rates resulted from the osmotic effects of salt around the roots (Cramer and Bowman 1991; Munns 2002; Neumann 1993). After several hours, plant cells can restore their original shape; however, a decrease in cell elongation rates is

observed in both leaves and roots (Hsiao and Xu 2000; Munns 2002). Continued exposure for a few days results in a decrease in plant growth (i.e., slower cell division and impaired cell elongation). In this case, leaves are often more sensitive to salinity than roots (Hsiao and Xu 2000; Munns 2002). Changes in plant cell dimension are observed more for an area than depth, therefore, leaves appear to be smaller and thicker (Munns and Tester 2008). The effects of salinity become more apparent after a few weeks of exposure (Munns and Tester 2008). Yellowing or death of older leaves may be visible in salt-sensitive plants, where salt levels are high, due to increase uptake or inability to store salt in vacuoles (Karley et al. 2000; Munns and Tester 2008; Tester and Davenport 2003). Only the salt-tolerant plants are able to grow for several months under moderate salinity; but showed early flowering or decreased production of florets (Munns 2002).

Salinity stress boosts endogenous ethylene production in plants, which in most cases serves as a stress hormone (Blumwald 2000). It is very likely that reducing salinity-induced ethylene by any mechanism could decrease the negative impact of salinity on to plant growth. Recent studies have revealed that plants inoculated with PGPR containing ACC deaminase were able to thrive better through the salinity stress while demonstrating a normal growth pattern. Tank and Saraf (2010) have reported that increase in the salinity is directly proportional to the ACC deaminase activity which increases survival rate in saline soils. As the uptake and hydrolysis of ACC by the PGPR decreases the ACC level in plants, the biosynthesis of the "stress ethylene" is impeded, facilitating plant growth under stress conditions (Glick et al. 1998). It has been shown that PGPR promotes plant growth under saline conditions. The presence of PGPR with ACC deaminase may lower the levels of ethylene in developing or stressed plants, enhance the survival of some seedlings and facilitate the formation of longer roots.

6.9 Ethylene–IAA Cross-talk

It is well known that IAA can activate the transcription of ACC synthase (Kende 1993; Kim et al. 1992) but it is less well known that ethylene may inhibit IAA transport and signal transduction (Pratiyon et al. 2006). This feedback loop of ethylene inhibition of IAA synthesis and/or functioning limits the amount of ACC synthase, ACC and ultimately, ethylene following every stressful event in the life of the plant. When an ACC deaminase containing PGPB lowers the ethylene concentration in plant roots, these relieve the ethylene repression of auxin response factor synthesis, and indirectly increase plant growth. Thus ACC deaminase containing PGPR facilitate plant growth by decreasing ethylene inhibition and permitting IAA stimulation without the negative effects of increasing ACC synthase and plant ethylene levels.

6.10 Air Pollutants Stress

It is very likely that PGPR can be utilized as a gene source for genetic modification of plants expressing the enzyme ACC deaminase against plant damage by air pollutants. Air pollution, in addition to damaging plants, inhibits many enzyme systems and metabolic processes of plants (McCune 1975). Increased ethylene evolution by plants exposed to various environmental stresses i.e., air contaminants has been well documented (Wang et al. 2002) and this hormone is now considered a major regulator of plant defense reactions, including cell death, in response to pathogen attack and air contaminant stresses, i.e., O_3 exposure. Many researchers reported that the inhibition of ethylene biosynthesis resulted in a significant reduction of O_3-induced leaf lesion formation (Moeder et al. 2002). In this direction, the role of ACC deaminase in alleviation of air contaminants stresses has not been studied.

6.11 Rhizobial Infection

Considerable evidence suggests that the ethylene that is produced following infection of legumes with *rhizobia* is inhibitory to the process of nodulation. The latest evidence has demonstrated that PGPR containing ACC deaminase activity promotes nodulation in legumes through inhibition of ethylene biosynthesis and consequently, they enhance symbiosis and nitrogen fixation in plants (Okazaki et al. 2004). Uchiumi et al. (2004) reported that an up regulated gene in bacteroids, mlr5932, and encoding ACC deaminase activity was involved in enhanced nodulation in *Lotus japonicus*. Pandey et al. (2005) isolated an endophytic ACC deaminase bacterium capable of modulating nodulation in *Mimosa pudica*. Coinoculation with *Bradyrhizobium* plus ACC deaminase rhizobacteria increased nodulation in mung bean compared to inoculation with *Bradyrhizobium* spp. alone (Shaharoona et al. 2006).

7 Microbe–Microbe Interactions Benefiting Sustainable Agro-Ecosystem Development

Direct interactions occurring between members of different microbial types often result in the promotion of key processes benefiting plant growth and health. It is obvious that all interactions taking place in the rhizosphere are, at least indirectly, plant-mediated (Azcon-Aguilar and Barea 1992). However, this section will deal with direct microbe–microbe interactions themselves, with the plant as a

"supporting actor" in the rhizosphere. Three types of interactions have a major role to play in bacteria–plant health development because of their relevance to the development of sustainable agro-ecosystems. These are (1) the cooperation between ACC deaminase producing PGPR and *Rhizobium* for improving N-fixation, (2) microbial antagonism for the biocontrol of plant pathogens, and (3) interactions between rhizosphere microbes and AM fungi to establish a functional mycorrhizosphere (Barea et al. 2005).

8 ACC Deaminase Gene-Containing Transgenic Plants

Transgenic plants express a bacterial ACC deaminase under the control of either the *35S* (constitutive) or *rolD* (root-specific) promoter as a treatment with ACC deaminase containing bacteria, although ethylene levels have been reported to be decreased by more than 95% in some ripen transgenic tomato fruit. Transgenic plants that express ACC deaminase are also significantly protected against the potentially deleterious effects of a variety of stresses including drought, flooding (Grichko and Glick 2001), high salt (Sergeeva et al. 2006), phytopathogens (Robison et al. 2001), arsenic (Nie et al. 2002), and several different metals (Grichko et al 2001). In all instances, transgenic plants, in which ACC deaminase was under the control of the *rolD* promoter, performed significantly better than the nontransformed plants (regardless of whether the plant was tomato, canola or tobacco) and the transgenic lines in which the ACC deaminase gene was under the control of the *rolD* promoter, yielded significantly more root and shoot biomass than either the nontransformed plants or transgenic plants in which the ACC deaminase gene was under the control of the *35S* or *prb-1b* (stress-specific) promoter. Transgenic plants in which ACC deaminase is under the control of the *rolD* promoter appear to mimic the behavior of nontransgenic plants treated with ACC deaminase-containing PGPB. However, the performance of plants treated with ACC deaminase-containing PGPB is almost always superior to the performance of transgenic plants expressing ACC deaminase under the control of the *rolD* promoter. This likely reflects the fact that the bacteria do more than merely lower plant ethylene levels. They also provide the plants with other "benefits" such as plant hormones and siderophores.

9 Conclusions and Future Trends

There is considerable experimental evidence that certain microorganisms are able to colonize the root–soil environments where they carry out a variety of interactive activities known to benefit plant growth and health, and also soil quality. Given the current reluctance of many consumers worldwide to embrace the use as foods of genetically modified plants, it may be advantageous to use PGPB as a means to

promote growth by lowering plant ethylene levels or reduce disease through induction of resistance, rather than genetically modifying the plant itself to the same end.

Rhizobacteria having ACC deaminase activity are effective in promoting plant growth and water use efficiency under drought conditions, by lowering the ethylene or ACC accumulation whose higher levels have inhibitory effects on root and shoot growth. From the previous demonstrations, it is established that the microorganisms that possess ACC deaminase activity have the selective advantage over other bacteria during biotic and abiotic stress conditions. Besides the activity of ACC deaminase in alleviating ethylene-mediated abiotic and biotic stresses, the ecology of bacterium and physiology of the plant may also interact with plant system to increase resistance to stress. However, the defined mechanisms involved in the use of plant growth-promoting rhizobacteria which decrease the damage to plants that occurs under stress conditions is a potentially important adjuvant to agricultural practice in locales where stress is a major constraint.

From the agricultural and ecological viewpoints, the aims will be to increase food quality, and to improve sustainable plant productivity, while maintaining environmental quality. However, to achieve this, basic and strategic studies must be undertaken to improve our understanding of microbial interactions in the rhizosphere. Only then can the corresponding agro-biotechnology be applied successfully. Hence, future investigation in the field of microbial cooperation in the rhizosphere will include: (1) advances in visualization technology; (2) analysis of the molecular basis of root colonization; (3) signaling in the rhizosphere; (4) functional genomics; (5) mechanisms involved in beneficial cooperative microbial activities; (6) engineering of microorganisms for beneficial purposes; and (7) biotechnological developments for integrated management.

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The Role of the C:N:P Stoichiometry in the Carbon Balance Dynamics of the Legume–AMF–Rhizobium Tripartite Symbiotic Association

Vincent M. Gray

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Abstract Synergistic or additive interactions among the partners of the legume tripartite symbiotic association (*Rhizobium*–Arbuscular mycorrhizal fungi–legume) have been shown in most instances to increase legume productivity. Arbuscular mycorrhizal fungi (AMF) promote increased legume biomass production and photosynthetic rates by increasing the ratio of P to N accumulation. An increase in the P content in legume tissue due to the AMF symbiotic association has been consistently associated with an increase in N accumulation and N productivity in legumes with or without a *Rhizobium* association. Photosynthetic N use efficiency, irrespective of the inorganic source of N is usually enhanced by increased P supply because of the AMF association. Both light-saturated photosynthetic rates and

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quantum yields increase in legumes in response to increasing N supply due to the *Rhizobium* symbiotic association. However, the maximum levels achieved for both light-saturated photosynthesis and quantum yield as a function of N supply concentration depend on both P and CO_2 supply rates. The N:P supply ratio controls the legume's growth and photosynthetic response to elevated atmospheric CO_2 concentrations. These findings indicate that the N:P:C supply ratio as influenced by the tripartite symbiotic associations plays a fundamental role in controlling the legume's photosynthetic rate and biomass productivity.

1 Introduction

Resource acquisition and allocation in legumes are dependent on a complex set of exchanges between the three members of the legume–*Rhizobium*–mycorrhizal tripartite symbiotic association (Fig. 1). The biological basis for the superiority

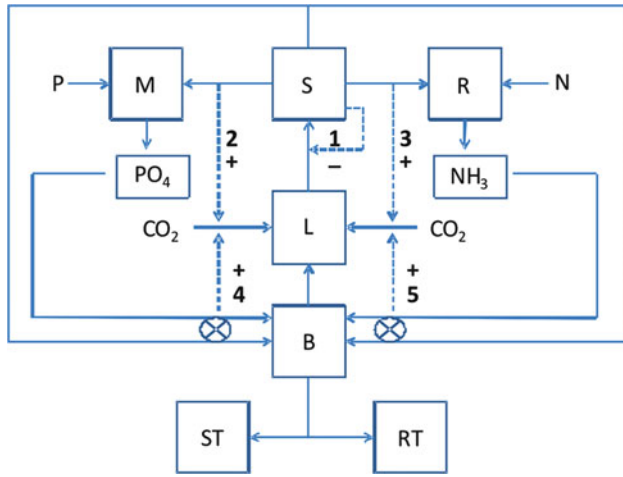

Fig. 1 Model of the three-way resource exchange system between the three symbiotic partners of the legume tripartite association. The *arrows* represent the fluxes or exchanges of C, N and P between partners of the tripartite symbiotic association. M, AM fungi; R, rhizobial nodule system; S, substrate carbon (C) storage pool; L, leaves which function as source of the carbon substrate; B, plants metabolic pool in which the processes of catabolism and anabolism take place; ST, represents the plant stems and RT represents the plant root system. The *broken arrows* with *plus* sign represent stimulation of photosynthesis by (a) C fluxes from the carbon storage pool to the AM fungi (2) and to the rhizobial nodules (3) both promote photosynthetic CO_2 assimilation; (b) fluxes of P (4) and N (5) into the metabolic pool promote the flux of substrate carbon (C) from the carbon storage pool into the metabolic pool which in turn promotes photosynthetic CO_2 assimilation. Build up of C in the carbon storage pool represses (*broken arrow* 1 with *minus* sign) photosynthetic CO_2 assimilation. The *cross* in the *circle symbol* indicates that the flux of C into the metabolic pool is controlled by the supply of N and P. Biosynthesis of the biomass polymers from C takes place in the metabolic pool

of legume crops derives from the three-way resource exchanges among members of the tripartite symbiotic association (Bethlenfalvay and Newton 1991; Barea et al. 1992). AMF and *Rhizobium* play an important role as microbial endosymbionts in the supply of P and N, respectively, to legumes growing in nutrient deficient soils (Azcón et al. 1979). In exchange for P or N, the two microbial symbionts receive C from the legume host. Thus, the formation of the tripartite symbiotic association (legume–AMF–*Rhizobium*) is codependent on a complex three-way source–sink relation involving C exchanges for P and C exchanges for N (Brown and Bethlenfalvay 1988). In most reported instances, these exchanges have had a positive influence on legume growth (Azcón et al. 1979; Paul and Kucey 1981; Harris et al. 1985; Brown and Bethlenfalvay 1988; Jia et al. 2004).

In general, the legume has been shown to have the capacity to fully compensate for any internal carbon deficit resulting from photosynthate transfers to the microbial endosymbionts (Jia et al. 2004; Kaschuk et al. 2009). Furthermore, in general, the evidence indicates that the C:P and C:N exchanges between the host and the two microbial symbionts under P and N limiting conditions do not diminish legume productivity relative to the productivity of plants that are not nutrient limited (Azcón et al. 1979; Paul and Kucey 1981; Harris et al. 1985; Brown and Bethlenfalvay 1988; Gray 1996). Plant growth is usually co-limited by both N and P supply (Jia and Gray 2004a, b). This observation is consistent with recent studies that have investigated the relationship between N:P stoichiometries and yield maximization in various crops (Ågren 2004; Sadras 2006). In previous studies, we have shown that the ratio of P to N was a major factor in determining the level of productivity in *Vicia faba* L (Jia et al. 2004 Jia and Gray 2004a, b). Recent studies have shown that legumes that were partners in the tripartite symbiotic association (*V. faba*–AMF–*Rhizobium*) had significantly higher elemental P to N ratio compared to plants with no symbiotic association (Jia et al. 2004). These results also confirmed the original observations of Brown and Bethlenfalvay (1988) that plants colonized by both AMF and *Rhizobium* had significantly higher photosynthetic nitrogen-use efficiencies and photosynthetic phosphorus-use efficiencies. Both P and N use efficiency has been shown to be strongly dependent on the P to N supply ratio (Jia and Gray 2004a, b, 2007). In N and P supply studies involving *V. faba* L for plants without any microbial symbiotic associations, it was found that the optimal values for the different photosynthetic parameters such as photon saturated net photosynthetic rates (P_{max}), quantum efficiency (α), intercellular CO_2 concentrations (C_i) and carboxylation efficiency (CE) were dependent on both N supply rate and leaf nitrogen content (Jia and Gray 2004a). It was also found that the level of N accumulation and the optimal values for the above photosynthetic parameters were positively influenced by the level of P supply (Jia and Gray 2004b; Jia and Gray 2007). These results indicate that the P or N exchanges from the microbial symbiont for host C also have a stimulatory effect on leaf photosynthetic capacity (Jia et al. 2004).

2 The Legume Tripartite Symbiotic Association

Growth of legumes under limiting nitrogen and phosphorus regimes is facilitated by the resource acquisitions efficiencies and capacities of the two microsymbionts, *Rhizobium* and AMF. AMF symbiotic associations with plant roots generally improve plant growth by enhancing the uptake of inorganic phosphorus (Jayachandran et al. 1992). Each of the symbiotic partners in the legume tripartite system has a specific source and sink function with regard to C, N and P exchanges. In response to C, N and P demand or supply, each of the tripartite symbiotic partners plays a specific role in this three-way source–sink exchange system (Fig. 1). It has been proposed that this three-way resource exchange may be subject to intersymbiont competition (Bayne et al. 1984; Bethlenfalvay 1992). The two microsymbionts may be regarded as the primary sources of P and N for legumes growing in soils deficient in plant-available forms of these two nutrients (Azcón et al. 1979, 1988; Barea and Azcón-Aguilar 1983; Piccini et al. 1988; Cihacek 1993). From the legume side of the symbiotic association, the exchange of resources involves the allocation of carbon to nodules in exchange for reduced N and to the AM fungi in exchange for P (Fig. 1). Phosphorus, the major plant growth limiting factor apart from N, is required for photosynthesis in the leaves of the legume and also for nitrogen fixation in the root nodules (Israel 1987; Haaker 1988; Bethlenfalvay and Newton 1991). The contribution of AM fungi to the tripartite symbiotic association is particularly significant for nodulated legumes growing under a soil regime where available inorganic nitrogen in the form of ammonium or nitrate is limiting, the reason being the high P requirement for nodulation (Daft 1978; Betlenfalvay and Yoder 1981) and N_2 fixation (Bergersen 1971).

With respect to the above ground and below ground source–sink interactions in legume systems, the roots, nitrogen-fixing nodules and mycorrhizal fungi all compete for a share of the below ground carbon allocation. An appreciation of this potential three-way below ground competition for carbon in legumes brings a new perspective to the conceptualization of source–sink dynamics in legumes. It has been reported that 42% or more of daily net photosynthate can be allocated to the belowground legume–*Rhizobium*–mycorrhizal association (Paul and Clark 1989). Paul and Kucey (1981) reported that 60% of the photosynthetic carbon flux was partitioned into the below ground root-nodule–mycorrhizal association. This below ground fraction of daily carbon allocation is nearly evenly distributed (12, 13 and 17%) to nodules, the root, and the mycorrhizal fungi (Paul and Clark 1989). In one report regarding the photosynthate allocation schedule in growing alfalfa plants, the partitioning of the carbon in the following proportions to the major organ systems was observed: 26.2% to the main stem; 12.7% to shoot apex unexpanded leaves on the main stem; 0.8% to the fully expanded leaves on the main stem; 27.1% to the auxiliary bud shoots on the main stem; 6.5% to the crown shoots; 3.8% to the crown; 19.1% the roots and 3.5% to the nodules (Cralle et al. 1987). If the

crown is included as part of the taproot, then 73.3% of photosynthate is allocated to the shoot, and the remaining 26.7% of fixed carbon is allocated to the roots and nodules (Cralle et al. 1987). The carbon allocation to mycorrhizal fungi, which is an obligate symbiont, can constitute between 4 and 20% of host photosynthate as indicated in single host–fungus combinations (Azcón and Ocampo 1984; Douds et al. 1988; Pearson and Jakobsen 1993). Observations on the supply of photosynthate in legume systems confirm that photosynthate production is in excess of carbon demand by the nodules (Gordon et al. 1985; Kouchi et al. 1985; Hostak et al. 1987; Vance and Heichel 1991; Kaschuk et al. 2009).

While there is excess photosynthate supply capacity in legumes such as alfalfa as evidenced in the accumulation of starch in the taproots in these legumes (Vance and Heichel 1991), the above examples do demonstrate that legume root microsymbionts represent substantial carbon sinks. Taken together, a series of observations (Hostak et al. 1987; Walsh et al. 1987) indicate that the growth of legumes such as alfalfa or soybean was not limited by source photosynthetic capacity but rather by microsymbiont sink strength for carbon. This becomes especially significant if the flux of carbon to the microsymbionts is regulated by the plant in exchange for P or N. This idea of microsymbiont carbon sink strength defined in terms of carbon demand being coupled to the microsymbionts capacity to supply N or P to the legume needs to be more fully developed. Which of the possible two resource exchanges (C:P or C:N) is most limiting or constraining with respect to legume growth? Is it possible that the carbon demand by the microsymbiont sinks could limit or constrain legume growth? Is it possible that AMF microsymbiont's capacity to accumulate, transport and mobilize P in exchange for C could limit legume growth? Or alternatively is it possible that the rhizobial microsymbiont' capacity to fix and mobilize N in exchange for C and P could limit legume growth? Given that the supply of P or N or both are factors that in general limit plant growth, then all factors influence that effect and the C, N, and P exchange dynamics among symbionts of the tripartite association will have an impact on legume productivity. These questions will be explored in this chapter. There is evidence that the relative amounts of carbon allocated for storage or growth depend on the supply of N and P to the legume (Greenwood et al. 1991).

In Fig. 1, photosynthetic CO_2 assimilation is controlled by the flux of substrate carbon out of the carbon storage pool (Gray 2000). This flux is promoted by the C:P and C:N exchanges between the legume host and the two microsymbionts, AMF and *Rhizobium* respectively. Various consequences of the relationships and dynamics depicted in Fig. 1 will be investigated in this chapter. The focus of this investigation is the status of the hypothesis that the photosynthetic capacity of the legume host and its growth rate are constrained by the supply of N and P. In addition, it will be argued that the sink demand for C or the flux of C to the two microsymbionts does not limit legume growth as its photosynthetic capacity can be increased to compensate for C losses to the two microsymbionts.

3 AMF Carbon Economy

In an analysis of the carbon economy of the tripartite soybean–*Glomus*–*Rhizobium* symbiotic association, Harris et al. (1985) found that carbon was allocated in the following proportions: 30.49% to leaves, 20.52% to stems and petioles, 6.3% to shoot respiration, 7.8% to roots, 2.0% to nodules, 2.7% to AMF, 5.2% to root and soil respiration, 13.7% to AMF respiration and 9.4% to nodule respiration. In that study, it was reported that approximately 42.6% of photosynthate was allocated to below ground sinks. This below ground carbon allocation was distributed between the various sinks as follows: 38.6% to the AMF, 26.6% to nodules and 30.6% to roots. When compared to the case of nonsymbiotic plants with nonlimiting supplies of P and N, it appears that the complex C, N and P three-way source–sink relations between the members of the tripartite symbiotic association do not limit plant productivity (Brown and Bethlenfalvay 1988). This supports the hypothesis that the carbon demand by the microbial symbionts does not limit plant growth.

In the case of AMF, the sink demand created by fungal colonization could account for an extra 4–26% drain of photosynthate from the AM-infected host plant (Kucey and Paul 1982a, b; Snellgrove et al. 1982; Koch and Johnson 1984; Harris et al. 1985; Douds et al. 1988; Wang et al. 1989; Jakobsen and Rosendahl 1990; Black et al. 2000). The maximum hypothetical photosynthetic allocation to the AMF association may well be as high as 40–50% of the total photosynthate production (Stribley et al. 1980). Hence, it has been suggested that the carbon demand by the AMF symbiont has the potential to limit plant growth, and thereby bring about a decline in the plant growth efficiency (Buwalda and Goh 1982, Jia et al. 2004; Jia and Gray 2008; Kaschuk et al. 2009). It has been proposed that increases in the photosynthetic rate of the AM-infected host plant fully compensates for carbon losses from the plant because of increases in AM carbon demand (Brown and Bethlenfalvay 1988; Fitter 1991; Wright et al. 1998a, b; Kaschuk et al. 2009). If N and P status of AM-infected plant and nonmycorrhizal plants were similar, it would be found that mycorrhizal plants had higher photosynthetic rates but similar biomass to the nonmycorrhizal, indicating that the additional photosynthate had been allocated to the fungal symbiont (Wright et al. 1998a, b) as supported by the fact that increases in the photosynthetic rate of AM-infected plants growing under low P conditions may be mainly due to mycorrhizal-dependent increases in the plant P status (Allen et al. 1981; Fredeen and Terry 1988; Azcón et al. 1992; Black et al. 2000). Further increase in photosynthetic rates in AM-infected plants may be due to the combined effects of enhanced P status and the AM-dependent carbon sink (Black et al. 2000). An increase in the photosynthetic rate in the leaves of AM-infected cucumber was found to be due to an increase in the leaf P status and not because of compensatory increases in photosynthesis in response to increase in the mycorrhizal sink demand for assimilates (Black et al. 2000). In barley where there was no difference in P status between AM-infected plants and nonmycorrhizal plants, the AM-infected plants had enhanced photosynthetic rates, indicating a compensatory response to mycorrhizal colonization

(Fay et al. 1996). Also, at equivalent P:N ratios, AM-infected *Andropogon gerardii* had higher overall photosynthetic rates compared to the nonmycorrhizal plants (Miller et al. 2002). Enhancement of photosynthetic rates in mycorrhizal plants was found not to be indirectly related to the plant P or N status but directly related to compensatory responses to fungal colonization (Miller et al. 2002) as also evidenced by Wright et al. (1998a, b).

A number of attempts have been made to quantify the stoichiometric exchange of mycorrhizal acquired phosphorus for photosynthate (Douds et al. 1988; Eissenstat et al. 1993; Pearson and Jakobsen 1993). Schwab et al. (1991) have proposed a model involving the exchange of one triose-phosphate for one inorganic phosphate molecule. This gives a carbon/phosphorus exchange ratio of 3. A positive correlation has been observed to exist between the capacity of AMF to stimulate the growth of the host plant and the radiant flux density incident on the plant (Same et al. 1983; Son and Smith 1988). Thus, it could be argued that any enhancement of additional photosynthetic capacity above the levels of P unlimited nonmycorrhizal plants would be strongly dependent on the P supply from AMF.

4 Rhizobial Carbon Economy

In the case of the rhizobial endosymbiont, dinitrogen (N_2) fixation and nodule growth also like AMF create a sink demand for photosynthate (Bethlenfalvay and Brown 1985; Brown and Bethlenfalvay 1986). A number of estimates evaluating the carbon energetic cost of N_2-fixation have been attempted (Salsac et al. 1984; Twary and Heichel 1991; Vance and Heichel 1991). Total energy costs including energy utilized for the growth of nodulated roots, maintenance respiration of nodules, N_2-fixation and ammonia assimilation range from 0.4 to 19.4 g C g^{-1} N (Salsac et al. 1984). If the energetic costs associated with the growth and maintenance of nodules are omitted, then the estimates of the energy costs for N_2-fixation range from 2.5 to 8.0 g C g^{-1} N (Atkins 1984; Salsac et al. 1984, Minchin and Witty 2005). Gross and net carbon requirements for N_2-fixation in alfalfa range from 3.5 to 11.9 g C g^{-1} N and 2.5 to 8.8 g C g^{-1} N, respectively (Twary and Heichel 1991). The average value from over 35 estimates for the carbon cost of N_2-fixation was approximately 6.0 g C g^{-1} N (Vance and Heichel 1991).

5 N and P Control of Legume Photosynthesis

It is likely that the microbial symbionts exert a positive influence on the legumes photosynthetic capacity via control of the N and P supply to the host (Fig. 1). For example, inorganic nitrogen supply rates exert a direct influence on photon saturated net photosynthetic rates, quantum efficiency, intercellular CO_2 concentrations and carboxylation efficiency (Jia and Gray 2004a, b). In addition, the

optimal values attained for the above photosynthetic parameters in response to N supply are in turn strongly modulated by P supply (Jia and Gray 2004a, b, 2007). As leaf nitrogen concentration increased, the α converged onto a maximum asymptotic value of 0.0664 ± 0.0049 μmol (CO_2) μmol (quantum)$^{-1}$. Also, as leaf nitrogen concentration increased, the C_i value fell to an asymptotic minimum of 115.80 ± 1.59 μmol mol^{-1}, and CE converged onto a maximum asymptotic value of 1.645 ± 0.054 μmol (CO_2) m^{-2} s^{-1} Pa^{-1} and declined to zero at a leaf nitrogen content equal to 0.596 ± 0.096 g(N) m^{-2}. Quantum efficiency fell to zero for a leaf nitrogen content of 0.660 ± 0.052 g(N) m^{-2}. In *V. faba* L as leaf nitrogen content increases, the value of photon saturated photosynthesis converged onto a maximum asymptotic value of 33.400 ± 2.563 μmol (CO_2) m^{-2} s^{-1}. Photon saturated photosynthesis fell to zero for a leaf nitrogen content equal to 0.710 ± 0.035 g(N) m^{-2}. Increased P supply increased the photosynthetic N use efficiency in terms of P_{max} and α. Increased P supply was also associated with an increase in CE and a decrease in C_i.

It has been observed that the responses of plant communities to global warming and elevated CO_2 were influenced by leaf N:P ratios (Hedin 2004), which are in turn dependent on soil N and P supply. This proposal has received support from experiments which exhibited increases in biomass production in plants acclimatized to elevated CO_2 (440 and 600 CO_2 μL L^{-1}) relative to that in control plants (280 CO_2 μL L^{-1}) depending on the level of NPK supply (Grünweig and Korner 2003). These results are consistent with the observations that photosynthesis is co-limited by both N and P supply (Jia and Gray 2004a, b, 2007; Jia et al. 2004). In *Pinus pinaster* the magnitude of growth, photosynthetic rates and N partitioning into ribulose-1, 5-bisphosphate carboxylase oxygenase (RubisCO) in response to increasing N supply were also positively modulated by P supply (Warren and Adams 2001). This modulation of the photosynthetic response to N supply by P may take place either directly or indirectly. Direct modulation of photosynthetic activity by P may be facilitated through the influence of P on RubisCO activation (Marcus and Gurevitz 2000). Alternatively, indirect control of photosynthetic rates by P supply could be exerted through the chloroplast phosphate shuttle. Phosphate recycling between the chloroplast and cytoplasm has been observed to modulate the photosynthetic rate by influencing the rate of export of photosynthate from the chloroplast (Cockburn et al. 1967a, b; Usuda and Edwards 1982; Mächler et al. 1984; Rao and Terry 1989; Rao et al. 1989a, b; Usuda and Shimogawara 1991; Rao and Terry 1995). P supply may also indirectly modulate photosynthetic rate by influencing sink demand for photosynthate (Pieters et al. 2001). In general, the proposal that photosynthesis is usually co-limited by both N and P supply is consistent with the observations of recent studies that yield maximization in various crops was influenced by N:P supply stoichiometries (Ågren 2004; Sadras 2006).

Photosynthesis and plant growth are co-limited by N and P supply (Sterner and Elser 2002; Ågren 2004). Maintenance of high photosynthetic rates and plant growth depends on the capacity to mobilize accumulated substrate carbon (C) from the storage pools into anabolic and catabolic metabolic pathways. Mobilization of C from the substrate storage pools into the catabolic and anabolic metabolic pathways

is tightly coupled to N and P supply (Fig. 1). When N and P supply becomes limiting, photosynthetic generated substrate carbon accumulates. Accumulation of storage carbon results in the repression of photosynthesis. Under these conditions, plant growth is not limited by carbon supply. However, the growth of the two heterotrophic microbial symbionts is always limited by C supply. In an idealized legume tripartite symbiotic system, the N and P supply rates from the microbial symbionts to the legume will be dependent on C:N and C:P exchange stoichiometries between the legume and the two microsymbionts. What would be the optimal N:P supply ratio for the achievement of maximum photosynthetic and plant growth rates? Also would the supply of CO_2 become limiting when N and P supply is nonlimiting with respect photosynthesis and growth? While an extensive literature now exists on the optimal N:P stoichiometries for crop growth (Sadras 2006), not much is known about the optimum C:N:P ratios for photosynthesis or plant growth. For the legume, the C in the C:N:P ratio would represent CO_2, the inorganic C substrate for plant growth. In the case of the microsymbionts, C represents organic carbon from the plant's substrate storage pool. The study of Grünweig and Korner (2003) does suggest that with long-term exposure to elevated CO_2, plant growth becomes co-limited by both N and P supply. Plants exposed to elevated CO_2 when adapted to ambient CO_2 levels show responses consistent with photosynthesis being co-limited by CO_2, N and P supply (Jia and Gray 2007). In addition, if the photosynthetic catalytic machinery in terms of leaf N concentration determines the source capacity of the plant canopy and P concentration influences the energetic efficiency of CO_2 assimilation into plant biomass, then the stoichiometric ratio of N:P (as a percentage of dry biomass) will determine plant productivity levels in response to CO_2 supply. Therefore, in general, it could also be argued that with increasing CO_2 supply, the sink demand of actively growing tissues for additional reduced carbon and source capacity for assimilating additional CO_2 would also be controlled by the N:P supply ratio. In addition, it can be further argued that with increasing CO_2 supply, sink demand of the legume's microbial symbiotic associations may be controlled by the N:P supply ratio from the microsymbionts.

In general, the evidence indicates that the C:P and C:N exchanges between the host and the two microbial symbionts under P and N limiting conditions do not diminish legume productivity relative to that of plants that are not nutrient limited (Azcón et al. 1979; Paul and Kucey 1981; Harris et al. 1985; Brown and Bethlenfalvay 1988; Gray 1996). In fact, plant growth is usually co-limited by both N and P supply (Jia and Gray 2004b). This observation is consistent with recent studies that have investigated the relationship between N:P stoichiometries and yield maximization in various crops (Ågren 2004; Sadras 2006). Earlier, it was observed that the ratio of P to N was a major factor in determining the level of productivity in *V. faba L* (Jia et al. 2004). Plants that were partners in a tripartite symbiotic association (*V. faba*–AMF–*Rhizobium*) had a significantly higher elemental P to N ratio compared to plants with no symbiotic association. These results also confirmed the original observations of Brown and Bethlenfalvay (1988) that plants colonized by both AMF and *Rhizobium* had significantly higher photosynthetic nitrogen and phosphorus-use efficiencies. Both photosynthetic P and N use

efficiencies have been shown to be strongly dependent on the P to N supply ratio (Jia and Gray 2004b).

6 Microbial Symbiont Effect on Legumes

In the case of legumes, there have been several studies on maintenance respiration and growth yield (McCree and Silsbury 1978; Irving and Silsbury 1987); however, these studies did not include the effects of microbial symbiotic partners. Also, while many other studies have focused on the apparent carbon costs induced by the microsymbiotic partners in the tripartite symbiotic association, they did not directly investigate the impact of these costs on the growth yield (Azcón et al. 1979; Paul and Kucey 1981; Harris et al. 1985; Douds et al. 1988). What possible effects do microbial symbiotic associations have on the host growth yield (Y_g) and maintenance respiration under P and N limiting conditions? Increases in legume productivity correspond to increases in the growth yield. For various legumes the average values reported for Y_g are 0.75 for *Trifolium. subterraneum* L (McCree and Silsbury 1978), 0.7 for field bean, 0.72 for Lucerne and 0.68 for chick pea (Irving and Silsbury 1987). In the study undertaken with *V. faba* L, plants with both rhizobial and AMF associations under both low N and P supply conditions achieved values for $Y_g > 0.7$ (Jia and Gray 2008). Hence, the legume partner in the tripartite symbiotic complex can overcome the constraints on growth efficiency that arise as a consequence of low N and P concentrations in the soil, even with the concomitant C losses to the microsymbiotic partners. In broad bean, depending on the presence of microbial symbiotic associations, the Y_g values ranged from 0.44 to 0.78 (Jia and Gray 2008). Growth yield were found to be significantly higher in plants that had one or more microbial symbiotic association (Jia and Gray 2008). It was observed that all the legumes with one microbial symbiotic had similar Y_g values irrespective of the N supply level. Plants with two microbial associations had significantly higher Y_g values than plants with all N and P treatments but without any microbial association.

7 Microbial Effects on Legume Maintenance Respiration

Maintenance respiration (m) estimates obtained for field bean, lucerne, chick pea, pea and kidney bean have been as follows: 21.35, 24.11, 28.65, 26.53 and 16.51 mg CO_2 $(gDM)^{-1}$ day^{-1}, respectively (Irving and Silsbury 1987). For *Trifolium subterraneum*, maintenance respiration rates ranged from 14 mg CO_2 $(gDM)^{-1}$ day^{-1} grown at 10°C to 64 mg CO_2 $(gDM)^{-1}$ day^{-1} at 35°C (McCree and Silsbury 1978).

No significant differences were found in the maintenance respiration rates between legumes without any microbial association and those with one or two

microbial associations (AMF or rhizobial) (Jia and Gray 2008). Depending on nitrogen supply, the *m* values fell between 12 and 36 $CO_2(gDM)^{-1}$ day^{-1}. Maintenance respiration rates were highest in plants with rhizobial and AMF associations grown under low N supply conditions. As with Y_g, the *m* rates also remained constant over the two harvest intervals for individual treatments. The above growth yield and maintenance respiration results are consistent with the hypothesis that in the legumes potential photosynthetic capacity exceeds the carbon demand of the *Rhizobium*–AMF symbiotic complex.

8 Mycorrhizal C:P Exchange Dynamics

In order to evaluate the impacts of mycorrhizal C demand and P supply on legume growth, two major considerations are (1) the C requirements of the mycorrhizal fungi and (2), the P acquisition efficiency of the extra radicle mycorrhiza hyphal system. Both the carbon requirement of the mycorrhizal symbiont and its efficiency in phosphate acquisition have an impact on the nitrogen and carbon economy of the tripartite legume symbiont system. Mycotrophic growth of plants is most frequently attributed to enhanced P uptake; however, there appears to be an optimal level of mycorrhizal colonization above which the plant receives no additional enhancement in P uptake or growth, yet the plant continues to support mycorrhizal metabolism (Koch and Johnson 1984; Douds et al. 1988). The conditions necessary for the maintenance of mycotrophic growth under steady-state levels of available soil phosphorus can be summed up in the following proposition: The maximum balanced-exponential growth rate under the conditions corresponding to a given steady-state soil phosphorus regime is only achievable for certain values of the following ratios – (a) the plant biomass:mycorrhizal biomass ratio; (b) the mycorrhizal C utilization rate:mycorrhizal P supply rate and (c) the plant specific photosynthetic rate (mg CO_2 g^{-1} dry leave mass s^{-1}):leaf phosphate concentration (% P of dry mass). The values of these specific ratios would represent the optimal values for a given soil phosphorus regime. To get some idea of the order of magnitude with respect to the above ratios, the following values have been reported (they are not necessarily the optimal values): A ratio of 140C/1P (g atom/g atom) corresponding to mycorrhizal C utilization and mycorrhizal P supply for soybean during mycotrophic growth has been reported (Harris et al. 1985). The standard critical leaf concentration for P-deficiency is 0.35% P on a dry mass basis (Scaife et al. 1983); 0.250–0.7 mg P g^{-1} leaf is the leaf P concentration range showing P-deficiency symptoms; 1.0–8.0 mg P g^{-1} leaf is P-sufficiency range for tissue phosphate concentrations (Bould et al. 1983). Leaf phosphate status affects the level of light saturated photosynthesis and plant growth rate (Rao and Terry 1995; Jia and Gray 2004b; Jia et al. 2004).

The regulatory control of carbon allocation to the various below ground sinks which includes the storage, and resource acquisition structural–functional components of the root require some elaboration. The extent of the fungal carbon requirements has not been well investigated; however, it appears that the carbohydrate flux is regulated by the host plant species, and is also dependent on the mycorrhizal fungal species (Sieverding 1991). It is estimated that AM fungi remove for their development and functional activity 1–17% of the carbohydrates allocated by the plant to root biomass production (Sieverding 1991). The maximum hypothetical photosynthate requirement and corresponding potential loss of plant dry matter production to the AM fungal association may well be as high as 40–50% (Stribley et al. 1980). This means that carbon demand by the fungus seems to have the potential to limit plant growth (Buwalda and Goh 1982).

Intraradical hyphae of VA mycorrhiza exhibit four categories of hyphae: (1) intracellular hyphae which exist as coils, often found in the outerlayers of the root cortex; (2) intercellular hyphae; (3) intracellular hyphae with highly ramified and invaginating membrane structures, known as arbuscules and (4), intercellular or intracellular hypertrophic hyphae, called vesicles (Scannnerini and Bonfante-Fasolo 1983). The arbuscules which are the sites of nutrient exchanges are essential for the functioning of VA mycorrhizal associations. The other three intraradical structures are involved in growth and storage functions. Arbuscules are found in the inner root cortex and are formed from a penetrating hypha that invaginates the host plasmalemma and repeatedly bifurcates to form a bushlike structure with progressively thinner branches (Wilcox 1991).

Potential carbon fluxes to mycorrhizal fungi via the arbuscules have been estimated to approach values up to 100 mg C g^{-1} root day^{-1} (Schwab et al. 1983). If the rate of C transfer per unit interfacial area in arbuscules is similar to that in mildew haustoria and C uptake by mycorrhizae occurs only in the arbuscules, then transfer of 5 mg C g^{-1} root day^{-1} to the fungus would require an interfacial surface area of 7×10^3 mm^2 g^{-1} root day^{-1} (Harris and Paul 1987). The magnitude of carbon or phosphate fluxes across the host–fungus interface will be influenced by the area of the arbuscule membrane surface within root cells. It has been calculated that the membrane surface area presented by arbuscules to colonized root cells ranges from 40 to 300 mm^2 of plasma membrane cm^{-1} length of root of onion and maize respectively (Toth and Miller 1984; Toth et al. 1990). Using the data of Cox et al. (1975), Harris and Paul (1987) estimate that 1.6×10^5 arbuscules g^{-1} root dry mass with an arbuscule membrane surface area in the region of 4×10^4 μm^2 $cell^{-1}$ would be necessary to facilitate the uptake of 5 mg C g^{-1} root day^{-1}. If there are approximately 1.4×10^8 cells g^{-1} root (Brown and Broadbent 1950), about 0.1% of cells must contain active arbuscules to facilitate the above daily transfer of C to the mycorrhizae.

Mycorrhizal carbon demand will depend on the carbon requirements necessary to support (1) fungal growth; (2) growth respiration and (3) maintenance respiration. Carbon demand by the fungus will depend on the specific growth rate of the mycorrhizal system. With respect to *Glomus fasciculatum*, Harris et al. (1985) have reported mycorrhizal fungal growth rates of 3.7 mg C day^{-1} when associated with

the soybean–*Rhizobium*–*Glomus* tripartite symbiotic system. Bethlenfalvay et al. (1982) gave a specific growth rate of 0.064 h^{-1} with a doubling time of 11 days for mycorrhizal fungi associated with soybean. Instantaneous specific growth rates ranging from 0.03 to 0.04 day^{-1} have been reported for *G. fasciculatum* in *Glycine max*–*Rhizobium*–*G. fasciculatum* associations (Harris et al. 1985). Growth analysis studies of mycorrhizae have demonstrated that fungal biomass production has an exponential phase (Bethlenfalvay et al. 1982). During exponential growth of the mycorrhizal symbiont, substrate C is partitioned into growth of new fungal biomass; growth respiration and maintenance respiration. A maximum growth yield (Y_{max}) of 0.6 has been found for many aerobic heterotrophs growing on a variety of substrates (Payne 1970). The maintenance respiration coefficient, m, has been defined in terms of Y_{max} and the specific maintenance rate, ($time^{-1}$), by $m = b/Y_{max}$. Accurate estimations of mycorrhizal specific maintenance rate (b) have been difficult to obtain; however, the following respiration rates have been reported: 11.0 mg CO_2 g^{-1} h^{-1} for *Glomus mossease* in leeks (Snellgrove et al. 1982) and 11.7 mg CO_2 g^{-1} h^{-1} for *G. fasciculatum* in soybeans (Harris et al. 1985).

Carbon fluxes into the mycorrhizal association and the growth of AMF, or phosphate fluxes from the mycorrhizal symbiont into the legume root, can be meaningfully analysed as a function of three parameters: (1) the area of the intracellular arbuscule membranal exchange interface; (2) percentage of root cells with active arbuscules and (3) the stoichiometric exchange of mycorrhizal acquired phosphate for photosynthate. A number of attempts have been made to quantify the exchange of mycorrhizal acquired phosphorus for photosynthate (Douds et al. 1988; Eissenstat et al. 1993; Pearson and Jakobsen 1993). Schwab et al. (1991) have proposed a model involving the exchange of one triose-phosphate (TP) for one inorganic orthophosphate (Pi) molecule. The root plasma membrane contains a triose-phosphate:inorganic phosphate translocator similar to the triose-phosphate: inorganic phosphate shuttle system corresponding to the phosphate-translocator of chloroplast membranes. In the case of host–mycorrhizal carbon–phosphate exchange, the TPs would be dephosphorylated in the interfacial matrix with the Pi returned to the host via a proton motive force driven Pi pump. The unchanged trioses in turn will be taken up by the fungus down a gradient of chemical potential. The source of exchangeable Pi in the interfacial matrix would probably be derived from the hydrolysis of polyphosphate granules located in the arbuscular hyphae. Phosphate will be transferred down a concentration gradient from the arbuscular hyphae into the interfacial matrix and then be taken up the root cells in exchange for TPs.

Within a given plant species the level of AM fungal infection is positively correlated with (1) the root content of soluble carbohydrates (Same et al. 1983) and (2) the level of sugar exudation (Azcón and Ocampo 1981, 1984). As the host plant supplies photosynthates for AM fungal growth in exchange for phosphate, there should exist a positive correlation between the efficiency of AM fungi as a supplier of phosphate and the photosynthetic rate of the host. Phosphate supply efficiency depends on radiant flux density incident on the plant (Diedrichs 1983a; Tester et al. 1986; Son and Smith 1988) and the day length to which the plant

is exposed (Diedrichs 1983b). Shading or defoliation depresses mycorrhizal growth (Same et al. 1983). Phosphorus deficiency is often associated with increased exudation of sugars and enhanced VA mycorrhizal infection of roots (Graham et al. 1981).

9 Rhizobial C:N Exchange Dynamics

It remains unresolved whether assimilation of nitrogen via NO_3^- or N_2 reduction is more favourable to growth and dry matter production in legumes (Atkins 1984). Carbon costs of N_2 fixation vary with host species, rhizobial strain, stage of plant development and method of measuring (Rainbird et al. 1984; Salsac et al. 1984; Skot et al. 1986). Reported values range from 1.3 to 22.8 mol C mol^{-1} N (Twary and Heichel 1991). Under steady-state growth conditions, it can be reasonably assumed that the rate of N acquisition by the rhizobium microsymbionts equals the rate of N supply to the legume host, which in turn is a function of host demand for N. With a dynamic functional equilibrium between plant and N_2 fixing symbiont, biomass production in alfalfa was found to be correlated with nodule mass per plant (Cralle et al. 1987). However, an increase in nodulation was associated with an overall increase in biomass, but the actual proportion of photosynthate partitioning to individual components of the plant (stem, leaves, crown, roots, nodules) remains unaltered. Reduction of N_2 by nitrogenase is an energy-intensive process that results in the consumption of 60–80% of nodule ATP (Heytler and Hardy 1984). Dinitrogen (N_2) fixation by legume–rhizobial symbioses is driven by shoot photosynthesis and therefore can theoretically decrease the amount of photosynthate partitioned to economic yield. If there is no compensatory increase in the photosynthetic rate in response to C demand associated with N_2 fixation, then the photosynthate consumed by the processes of N_2 fixation will be unavailable for plant dry matter production. Twary and Heichel (1991) found that dry matter accumulation in alfalfa was unrelated to the C cost of N_2 fixation.

The C consumed in nodule N_2 fixation is partitioned among the component processes of nodule growth and maintenance, reduction of N_2 by nitrogenase and assimilation plus transport from the nodules (Mahon 1983). Net CO_2 evolution by nodule respiration is usually measured to calculate the C costs of N_2 fixation (Schubert and Wolk 1982). A certain proportion of this CO_2 is refixed by phosphoenolpyruvate carboxylase in the nodule (Twary and Heichel 1991). It therefore follows that gross and net C costs of N_2 fixation need to be distinguished when evaluating the C energetic costs of N_2 fixation. Because of refixation of respiratory CO_2 in the nodules, the C costs of N_2 fixation can be decreased by 10–14% (Ryle et al. 1984).

A number of estimates evaluating the energetic cost of nitrogen fixation have been attempted (Phillips 1980; Rawsthorne et al. 1980; Schubert and Ryle 1980; Minchin et al. 1981; Schubert and Wolk 1982; Skot et al. 1986) in terms of the amount of carbon utilized for nitrogen fixation. In general, these estimates sum up

the total costs involved in nitrogen acquisition from N_2 as the nitrogen source. Therefore, the total energetic cost includes the energy utilized in nodulated root growth, maintenance respiration, N_2 fixation and ammonia assimilation. The values range from 0.4 to 19.4 g C g^{-1} N (Salsac et al. 1984). If the energetic costs associated with the growth and maintenance of nodulated roots are omitted, then estimates of the energetic requirements for N_2 fixation range from 2.5 to 8.0 g C g^{-1} N (Atkins 1984; Salsac et al. 1984). Twary and Heichel (1991) observed that the specific N_2 fixation rates in alfalfa depended on the strain of *Rhizobium meliloti*. They reported specific N_2 fixation rates ranging from 3.39 to 16.49 nmol N min^{-1} g^{-1}dry wt nodule. The rhizobium microsymbionts rate of N acquisition would be dependent on the total nodule mass. Selection of alfalfa for greater nodule mass resulted in a proportional increase in the mass of all plant organs (Cralle and Heichel 1986; Cralle et al. 1987). Nodule N_2 fixation occurs unabated throughout both day and night (Vance and Heichel 1991). The carbon energy input that drives N_2 fixation in the nodules appears to be derived entirely from the shoot (Vance and Heichel 1991). During the day, translocation of photosynthate into the nodules drives N_2 fixation and at night mobilization and translocation of shoot carbon storage reserves support N_2 fixation. It would thus appear that the tap root storage reserves are not available for driving N_2 fixation in the nodules (Vance and Heichel 1991). Leaf nitrogen status determines growth potential and if the N input for plant growth is dependent on nodule N_2 fixation then the rate of N_2 fixation becomes the process that limits growth. Under these conditions, the legume growth depends on the plant's nitrogen utilization efficiency and the rate at which fixed nitrogen is supplied to the host. Achievement of maximum growth rates of the nodule system and the plant may be constrained by the exchange rates associated with C export from the plant to the rhizobial nodules and N export from the rhizobial nodules to the plant system.

Under conditions of balance exponential growth, a functional equilibrium relationship with respect to the C:N exchange ratio should exist between the legume and the rhizobium microsymbiont. Under these conditions a system of regulatory control would maintain a dynamic functional equilibrium between the activity of the N_2 fixing nodule system and the photosynthesizing shoot such that the legume's tissue C:N ratio would be kept poised at a constant value during steady-state exponential growth. Given this state of affairs, the legume's specific growth rate would be a function of the nodule:legume mass ratio. Thus, if the nodule's specific N_2 fixation rate and the plant's nitrogen concentration are known, then theoretically the legume's specific growth rate can be determined as a function the legume's nodule:plant mass ratio. Measured mean relative growth rates range from 0.120 g g^{-1} day^{-1} for soybean (Sa and Israel 1995) to 0.237 g g^{-1} day^{-1} for alfalfa shoots (Philippot et al. 1991). On the basis of these relationships, it should be possible to compare predictions for legume specific growth rates, on the basis of nodule N_2 fixation rates, plant nitrogen concentrations and nodule:plant dry mass ratios, with measured mean relative growth rates (RGRs).

Values for nodule:plant mass ratios range from 0.01 to 0.03 for alfalfa (Cralle et al. 1987) and 0.017 to 0.08 for soybean (Ribet and Drevon 1995; Sa and Israel

1995). Assuming the carbon content of alfalfa to be 45%, the C:N ratios vary from 14.5 to 19 (Twary and Heichel 1991). With a plant carbon content of 40%, the C:N values will vary from 8 to 13.4. The *critical nutrient range* (CNR) for tissue nitrogen concentration in alfalfa is about 3% of dry mass and the plant sufficiency levels for tissue nitrogen in alfalfa range from 3.76 to 5.5% of dry mass (Miller and Donahue 1990). Assuming a plant carbon content of approximately 40%, the average C:N ratio for a typical legume such as alfalfa growing on arable soils is 13 (Miller and Donahue 1990, pp 188–189), which is equivalent to 31 mg N g^{-1} dry mass. Nodulated P-sufficient soybeans grown on N-free nutrient media attained the following plant nitrogen concentrations: 40–60 mg N g^{-1} dry mass for leaves; <20 mg N g^{-1} dry mass for stems; ±15 mg N g^{-1} for roots and 60–80 mg N g^{-1} dry mass for nodules (Sa and Israel 1995); averaged over the total plant biomass the mean tissue nitrogen concentration is in the region of 30 mg N g^{-1} dry mass. Reported values for nodule acetylene reduction activity in soybean range from 100 to 300 μmol C_2H_4 g^{-1} nodule dry mass h^{-1} (Parsons et al. 1992; Ribet and Drevon 1995; Sa and Israel 1995). Predictions of N_2 fixation rates based on acetylene reduction assays need to be treated with caution. A major problem in the use of the acetylene reduction based nitrogenase assay to estimate absolute rates of N_2 is the reliability of the C_2H_2 to N_2 calibration. The C_2H_2/N_2 ratio can range from 2.66 to 4.33 (Minchin et al. 1983). For an average C/N ratio value of 13 which corresponds to a tissue nitrogen concentration of approximately 30 mg N g^{-1} dry mass for a plant with a C content of 40%, a specific growth rate of 0.115 g g^{-1} day^{-1} can be calculated for soybean plants having a specific nitrogenase activity of 300 μmol C_2H_4 g^{-1} dry mass h^{-1} and an average nodule:plant mass ratio of 0.06 (given a theoretical average C_2H_2/N_2 ratio of 3.5). The calculated specific growth rate gives a fairly accurate estimation of the actual measured mean RGR, 0.120 g g^{-1} day^{-1} (data for this comparison were derived from Sa and Israel 1991; 1995).

10 Rhizobium Nodule Phosphorus Requirements

Phosphorus deficiency is a major limiting factor for N_2 fixation. Specific nitrogenase activity decreases with the onset of P-deficiency (Sa and Israel 1991). Several physiological and metabolic properties were associated with lower specific nitrogenase activity in nodules of P-deficient plants: Bacteroid mass per unit nodule mass, bacteroid N concentrations, plant cell ATP concentrations and energy charge were significantly lower in nodules of P-deficient plants (Sa and Israel 1991). Because nitrogenase is localized in the bacteroids, lower bacteroid mass per unit nodule mass and N concentration could account for decreased specific nitrogenase activity under P-deficiency (Sa and Israel 1991). Legumes dependent on symbiotic N_2 fixation have a higher internal P requirement for optimum nitrogen assimilation compared to plants dependent on combined inorganic nitrogen in the form of nitrate and ammonium (Israel 1987). Soybean grown with limiting P supply showed a

reduction in nodule numbers; nodule mass; individual nodule mass and plant mass (Israel 1993). The growing nodule is a major sink for P in legumes and in soybean the total nodule P concentration is threefold higher than in the rest of the plant (Sa and Israel 1991). Phosphorus deficiency resulted in decreases of *Rhizobium* bacteroid dry mass per unit nodule dry mass by an average of 20% relative to that of P-sufficient controls in soybean; P and N concentrations in bacteroids from P-deficient plants averaged 9 and 95 mg g^{-1} dry mass bacteroid respectively (Sa and Israel 1991). These P and N concentrations were 25 and 17% lower, respectively, than the P and N concentrations in bacteroids from P-sufficient plants. Nodule nitrogenase activity is decreased by plant P deficiency independently of the effectiveness of the rhizobium strain with nonlimiting P supply (Singleton et al. 1985). The concentration of ATP remained constant in whole nodules of P-sufficient and P-deficient plants with the ATP concentration being three to fourfold greater in P-sufficient plant nodules (Sa and Israel 1991). The magnitude of the specific nitrogenase activity is well correlated with legume tissue phosphorus concentration (Sa and Israel 1995). This empirical correlation provides phenomenological grounds for deriving a functional relationship between specific nitrogenase activity and legume P content.

11 Interrelations Between the C:P and C:N Exchange Dynamics

Exchanges of photosynthate, N and P between the symbiotic systems and the legume have important consequences for the overall plant carbon economy. The source capacity of leaves measured as daily gross photosynthetic output will be dependent on the nitrogen and phosphorus concentrations in the leaf tissues. Leaf nitrogen and phosphorus concentrations in legumes are influenced by the degree of nodulation and intensity of VA mycorrhiza fungal infection and the extent to which the external mycorrhizal mycelium explores the soil volume surrounding the root. The ratio of mycorrhizal P-supply and nodule N-supply to the respective carbon demand can give a quantitative index of host carbon expenditure necessary for the acquisition of nitrogen and phosphorus via these two symbiotic associations (Paul and Clark 1989; Twary and Heichel 1991; Bethlenfalvay 1992).

Photosynthetic rates in the host plant of the *Glycine–Glomus–Rhizobium* symbiont system increased linearly with increasing leaf P or N concentration (Brown and Bethlenfalvay 1988). Brown and Bethlenfalvay (1988) showed that the rate of photosynthetic CO_2 assimilation per unit leaf N or P was significantly greater in symbiotic than in nonsymbiotic plants. The experimental results of Brown and Bethlenfalvay (1988) provide sufficient evidence to counter the argument that the enhancement of photosynthetic nutrient-use efficiencies (N and P) in plants with microsymbionts can be explained as being exclusively due to increased stomatal conductance resulting from VA mycorrhizal colonization of the roots as suggested by Koide (1985). They found that soybean plants which had an association with one

(*Glomus* or *Rhizobium*) or both microsymbionts always had greater photosynthetic rates per unit leaf N or P than nonsymbiotic plants with similar leaf N or P concentrations. Also efficient nutrient utilization by the N- and P-deficient symbiotic plants relative to the N- and P-sufficient nonsymbiotic plants is shown by higher CO_2 assimilation rates in the former. The thresholds of N- and P-deficiency for youngest mature soybean leaves have been defined as < 40 mg N and <1.5 mg P per gram of leaf dry mass (De Mooy et al. 1973). Brown and Bethlenfalvay (1988) reported that most of the leaves of their symbiotic plants were below these values. The mechanism responsible for the greater photosynthetic N- and P-utilization efficiencies that have been observed in N-and P-deficient symbiotic plants remains obscure.

The magnitude or degree of *benefits* for the host measured in terms of enhanced growth potential or elevated photosynthetic rates resulting from the exchange of C for N supplied by the N_2-fixing nodules, and P supplied by the mycorrhizal system depends on two sets of efficiencies: (1) host nutrient utilization efficiencies which include the plant's nitrogen utilization efficiency and phosphate utilization efficiency and (2) microsymbiont nutrient acquisition efficiencies which include the nodules' N_2-fixation efficiency, to be called in this context the nitrogen acquisition efficiency and the mycorrhiza's phosphate acquisition efficiency. The nitrogen use efficiency of the legume is given by the derivative, dW_{plt}/dW_N, which defines the increment of biomass production per unit of N supply, and the legume's phosphorus utilization efficiency can be similarly expressed as dW_{plt}/dW_P, where W_{plt} is plant host's structural dry mass, W_N is mass of N in plant tissue and W_P is mass of P in plant tissue.

A formal-analytical approach may help elucidate the fundamental conceptual issues underlying plant nutrient-utilization efficiencies. One approach for achieving this involves the *decoupling* of photosynthesis and growth; photosynthesis involves nonstructural carbon or photosynthate (starch and sucrose) production from growth as structural biomass production. Nutrient use efficiencies, for N and P, can be construed as follows: N-utilization efficiency (NUE) is the differential of substrate carbon production (W_C) per unit nitrogen (dW_N)

$$\text{NUE} = dW_C/dW_N \tag{1}$$

and P-utilization efficiency (PUE) which is the differential of substrate carbon utilization for biomass production per unit phosphate (dW_P)

$$\text{PUE} = dW_C/dW_P \tag{2}$$

Using the above equations the following two differential equations for photosynthate production and growth can be derived:

$$dW_C/dt = (dW_C/dW_N)(dW_N/dt) - dW_{plt}/dt \tag{3}$$

or

$$dW_{plt}/dt = (dW_C/dW_P)(dW_P/dt) \quad (4)$$

In the above equations, the rate of W_C production is given as the product of the photosynthetic NUE and the rate of N acquisition uptake. In second equation, the rate of structural biomass (W_{plt}) is given as product of PUE and the rate of P acquisition.

12 Nitrogen Utilization in Legume Biomass Production

Under any given ambient CO_2 partial pressure, the upper limit of light-saturated photosynthetic rates is fixed by a relatively small set of physical and biochemical factors, for example, stomatal conductance, concentration of activated rubisco catalytical sites, steady-state concentrations of sugar–phosphate intermediates of the Calvin cycle and chlorophastic orthophosphate concentration. Ågren (1985a, b) provides a useful summary of the potential rates of biomass production theoretically achievable when the rate of CO_2 assimilation is calculated as a function of irradiance, water supply, ambient CO_2 or nitrogen. He concluded that nitrogen tissue concentration sets the upper limit to plant productivity. In order to develop a meaningful analysis of how nitrogen contributes to plant productivity, it is useful to begin with a quantitative analysis of the physical characteristics of the leaf photosynthetic system.

The results of a number of quantitative analyses have been used to construct the physical features characterizing the photosynthetic system of an "average" C_3 leaf. For an idealized leaf the following physical estimates or vital statistics have been derived (Lawlor 1987): 5.5×10^{11} chloroplasts m^{-2} leaf; total chloroplast volume per m^2 of leaf is 1.8×10^{-5} m^3; total volume of thylakoid lumen m^{-2} leaf is 1.2×10^{-6} m^3; 16.8 cm^3 stromal volume per m^2 of leaf; concentration of rubisco catalytic sites is 4 mM; inorganic phosphate concentration is 100 mM and ribulose 1,5-bisphosphate concentration is 0.1–2 mM; these values are from the data for spinach, tobacco and wheat given by different workers (Esua 1958; Heath 1969; Nobel 1974; Mühlethaler 1977). Rubisco accounts for up to 50% of the soluble leaf protein in C_3 plants (Schmitt and Edwards 1981).The molecular weight of rubisco is 557,000 Da with each molecule of rubisco containing eight potential catalytic sites. In one mole of rubisco, there is 83,550 g of N (assuming that the average fraction of N in the 20 different amino acids is approximately 0.15) and if each mole of rubisco contains 8× Avogadro's number (6.022×10^{23}) catalytic sites, then the ratio of catalytic sites per gram N of rubisco is 5.77×10^{19} rubisco catalytic sites per gram N of rubisco or 9.6×10^{-2}mmoles rubisco catalytic sites per gram N of rubisco.

The general response of light saturated rates of photosynthesis to increasing leaf nitrogen (g N m^{-2}) is generally curvilinear with two critical response regions: (1) a

lower limit for photosynthetic rates corresponding to a certain threshold of leaf nitrogen concentration below which photosynthetic rates approach zero and (2) an upper limit for photosynthetic rates which corresponds to threshold leaf nitrogen concentrations above which no increase in photosynthetic rate occurs (Sinclair and Horie 1989). In soybeans the rate of photosynthesis is zero at 1.0 g N m^{-2} and increases linearly for leaf nitrogen concentration between 1.0 and 2.4 g N m^{-2}; above 2.4 g N m^{-2} the response is curvilinear reaching a maximum of 1.6 mg CO_2 m^{-2} s^{-1} (Sinclair and Horie 1989). Using the above data, an average hypothetical soybean or C_3 plant with a leaf nitrogen content of 2.4 g N m^{-2} (rubisco accounting for 50% of the leaf N) should have approximately 6.92×10^{19} or 0.115 mmol of rubisco catalytic sites per m^2 leaf. In order to compare the calculated A_{max} with the measured light saturated values, let the Vc_{max} of the rubisco carboxylation reaction be equal to the product kcat$[R_{sites}]^a$, where kcat is the catalytic turnover time for rubisco (2 s^{-1}) and $[R_{sites}]^a$ is the concentration of activated catalytic sites (catalytic sites g^{-1} rubisco N) while *a* stands for activated sites. The rate of RuBP carboxylation under light saturated conditions when rubisco catalytic sites are saturated with RuBP depends on the concentrations of CO_2 and O_2 in the stroma and A_{max} can be taken as being equal to Farguhar and von Caemmerer's (1982) expression:

$$A_{max} = (\text{kcat}[R_{sites}]^a)(\text{Ca} - \ni)/(\text{Ca} + K_c(1 + O/K_o)) - R_d \quad (5)$$

where, Ca is the ambient CO_2 concentration; $\ni$ is the CO_2-light compensation value (4–5 Pa CO_2 in 21 kPa O_2) and is equal to $(0.5Vo_{max}K_cO/Vc_{max}K_c)$, and Vo_{max} is the maximum velociy of the RuBP oxygenase reaction; K_c and K_o are Michaelis–Menten constants for CO_2 (12–20 μM for C_3 plants) and O_2 (250 μM), respectively; R_d is "dark" respiration in the light.

At standard atmospheric pressure (101,325 Pa) and with a leaf temperature of 25°C, the ambient CO_2 and O_2 concentrations are 13.9 mmol m^{-3} and 8.6 mol m^{-3} respectively. An average light saturated CO_2 compensation $\ni$ value for C_3 plants is approximately 1.6 mmol CO_2 m^{-3}. On the basis of assumptions concerning the proportion of rubisco N with respect to total leaf N values, A_{max} can be calculated for C_3 leaves using the above equation. An average hypothetical C_3 leaf consists of 16.8 cm^3 chloroplast stromal volume per m^2 of leaf tissue. A leaf with an N concentration of 2.4 g N m^{-2} will have a rubisco catalytic site concentration in the region of 6.85 mM if 50% of leaf N is partitioned into rubisco. All the data necessary to generate A_{max} with (5) are summarized in Table 1. Substitution of Table 1 data into (5) gives a net A_{max} value of 55.8 μmol CO_2 m^{-2} s^{-1} or 2.46 mg CO_2 m^{-2} s^{-1} for a hypothetical soybean leaf with a leaf N concentration of 2.4 g N m^{-2}.

The above calculation assumes that ribulose 1,5 bisphosphate is at substrate saturating levels in light saturated leaves and that rubisco is 100% activated. The calculated A_{max} value for the theoretical soybean leaf gives an over estimation of 1.54 times the measured value. If only 65% of rubisco is in the activated state under light saturating conditions, then the calculated and the measured rates are the same.

Table 1 Gas and kinetic data for calculation of A_{max} using (5)

Variable or parameter	Units
Kcat	$2\ s^{-1}$
$[R_{sites}]$[a]	$0.115\ mmol\ m^{-2}$
Ca	13.9×10^{-3} mM
∋	1.6×10^{-3} mM
O	8.6 mM
K_c	1.0×10^{-3} mM
K_o	0.250 mM
Specific leaf mass	$22\ g\ m^{-2}$
Maintenance respiratory coefficient[a]	3.18×10^{-6} g $CO_2\ g^{-1}DM\ s^{-1}$

[a]Amthor (1986)

Therefore, it is reasonable to assume that the factor that contributes greatest to the calculated overestimation of A_{max} is the estimated percentage activation state of rubisco in leaves. The percentage of activated sites is dependent on the size of the carbon substrate storage pool, N supply and P supply (Fig. 1).

13 Phosphorus Utilization in Legume Biomass Production

Plant nutrient sufficiency levels for phosphorus in alfalfa on a percent dry mass basis ranges from 0.26 to 0.7% (Miller and Donahue 1990 pp 370–371). Higher P tissue concentrations result in toxic effects. An interesting consideration is the relationship or correlation between the legume's RGR and the capacity of the external hyphal system of the AM fungi to acquire and transfer phosphorus from the surrounding soil volume into the plant root system. It has been proposed that only 20% of the total root mass is involved in nutrient acquisition (Robinson 1986), and it is most probable that the AM fungal–root association is restricted to this fraction of the total root mass. The fraction of root dry mass attributable to mycorrhizal fungi ranges from 12 to 14% for AMF colonized legume roots (Sieverding 1991). The specific activity of the AMF can be expressed as mg P cm^{-1} hypha day^{-1}. Functional relationships should exist between a legume's specific growth rates and the corresponding uptake rate per unit root mass of any mineral nutrient (Garnier et al. 1989). When plants are grown at steady-state nutritional supply rate, it has been shown for P (Eissenstat et al. 1993) and for N (Cromer and Jarvis 1990; Ingestad and Ågren 1991) that the slope of the RGR vs plant nutrient concentration is linear until a maximum *RGR* is reached. With steady-state conditions of phosphate supply during exponential growth, tissue phosphorus concentration remains constant, i.e., $dP_c/dt = 0$, where $P_c = W_p/W_{plt}$ is the tissue phosphorus concentration, W_p equals plant phosphorus mass and W_{plt} is the plant dry mass.

For sustained steady-state exponential growth, a dynamic functional equilibrium must exist between the size and the specific activities of the microbial symbiotic

associations and the plant. Maintenance of this dynamic functional equilibrium involves the regulatory control by the plant of the proportion of carbon lost in exchange for the quantity of phosphate necessary for sustaining exponential growth. Therefore, with regard to phosphate-limited growth, the maintenance of a constant tissue phosphorus concentration above a P-deficiency threshold is a necessary condition for sustained steady-state exponential growth. The constraint that plant tissue P-concentration imposes on plant growth is mediated via the effects that P has on key growth driving metabolic and physiological processes such as the Calvin cycle and phloem loading. Low-P treatment (plants grown on 0.05 mM phosphate) has a greater impact on plant biomass production (60% reduction compared to P-sufficient plants) than on the rate of photosynthesis; low-P treatment effected photosynthesis much less at low irradiances than at high radiances relative to P-sufficient plants (plants grown on 1.0 mM phosphate), light saturated rates in leaves of P-deficient plants were decreased by 35% (Rao and Terry 1989). P-deficiency may decrease light saturated rates of photosynthesis by decreasing RuBP regeneration capacity and/or decreasing the concentration of activated rubisco (Brooks 1986; Brooks et al. 1988). Under low-P conditions, RuBP concentrations declined to half the rubisco binding site concentration (Rao et al. 1989a, b). The activities of the phosphatases, FBPase and SBPase increased with P-deficiency in leaves, possibly to promote more rapid *recycling* of phosphate, and the activities of the enzymes associated with RuBP regeneration, i.e., PGA kinase, NADP-G3P dehydrogenase and Ru5P kinase, declined with P-deficiency in leaves (Rao and Terry 1989). The concentrations of Calvin cycle intermediates declined in P-limited leaves while starch increased to levels higher than in control leaves (Rao et al. 1990). Starch, sucrose and glucose concentrations increased in P-deficient leaves, resulting in a higher accumulation of leaf photosynthate compared to P-sufficient plants (Rao et al. 1990; Rao and Terry 1995). Low-P treatment decreases leaf ATP concentrations considerably (Rao et al. 1989a, b) and it is well known that the loading of sugars into the phloem has a large requirement for ATP (Giaguinta 1983), and therefore, there may be insufficient ATP in P-deficient leaves to maintain the rates of photosynthate export necessary to support high growth rates (Rao et al. 1990).

Maintenance of optimal rates of photosynthetic carbon assimilation, photosynthate translocation and nitrogen fixation during exponential growth of legumes requires the maintenance of an optimal constant concentration of phosphate in the leaf tissue and root nodules. Put differently, in the legume symbiotic system, phosphorus uptake rates must satisfy the metabolic phosphate demands of the Calvin cycle, phloem phosphate-loading system and the N_2-fixing nodule system, which are the necessary P-demands for sustaining optimal RGRs. Consistent with the premises of the model depicted in Fig. 1, tight coupling between the RGR and nitrogen supply rates has been shown to exist (Hirose 1986, 1988; Hirose et al. 1988; MacDuff et al. 1993); also the same tight coupling applies between growth rates and phosphate supply rates.

A definition for phosphate demand and utilization efficiency as a function of optimal inherent growth capacity can be developed as follows: Under steady-state

exponential growth, given mass flow and mass conservation, the following equalities hold: rate of phloem loading = rate of phloem unloading = rate of biomass production in sink organ. In this case, the sink organ can be conceptualized as a biomass growth sink such as growing leaves, elongating nodes and growing root systems. Given this scenario of events, it is proposed that phloem loading is the rate limiting step for biomass production. Phloem loading is an energetically uphill process requiring the consumption of ATP and if ATP is treated as a P equivalent, then phloem loading can expressed as a function of tissue phosphate concentration. Phloem loading can be treated as a two substrate enzyme reaction involving sucrose and ATP. Sucrose is usually present at the phloem loading site at substrate saturating levels and ATP can be treated as the rate limiting substrate for the phloem loading process. Metabolic demand for phosphate is linked to its role in ATP synthesis for phloem loading. The maximum rate of phloem loading is proportional to product of the concentration of phloem loading sites, (sites g^{-1} dry mass) and the turnover time of the loading site, (sites per second). In the model depicted in Fig. 1, the close coupling between P supply and C mobilization into the metabolic pool for biomass synthesis could be linked to its role as the "high energy phosphate ester" in ATP driven phloem loading. In addition, the close coupling between P supply and C utilization in the metabolic pool for biomass synthesis could also be linked to its role, either direct or indirect, as the "high energy phosphate ester" in ATP driven polymerization reactions (protein synthesis and polysaccharide synthesis).

14 Conclusion

In this chapter, an analysis of how legume growth is influenced by C:N:P stoichiometries has been put forward. The supply of N and P by the two microsymbionts plays a major role in the control of photosynthetic CO_2 assimilation and in the control of conversion of photosynthate into plant biomass (Fig. 1). Resource (C, N and P) allocation dynamics have been investigated in terms of (1) C:P exchange dynamics with respect to the AMF symbiotic partner, (2) C:N exchange dynamics with respect to the *Rhizobium* symbiotic partner and (3) how the C:N:P supply ratio controls photosynthetic rates and the rate of conversion of photosynthate into plant biomass. The existence and maintenance of balanced exponential growth or steady-state exponential growth of all plant components and microbial symbiotic associations in the tripartite complex have been an underlying functional assumption in this investigation. Furthermore it has been assumed that resource allocation strategies within the tripartite complex ensure the maintenance of functional equilibrium between plant components and microbial symbiotic associations. The intensities of metabolic activities of different plant components and microbial associations represent the resource acquisition or resource utilization capacities of the plant components and the microbial associations. If during balanced exponential growth of the tripartite complex a

functional equilibrium has been maintained between the steady-state rates of resource acquisitions (C, N, P), then it logically follows that the concentrations of various substrates (C, N, P) within the plant tissues will remain constant and the RGRs of the various components and associations can be expressed as a function of one or more of these substrate concentrations. This will greatly help in simplifying any effort to model the resource allocation and growth dynamics of the tripartite complex. The internal concentration values of the different resources (C, N and P) within the tripartite complex will at any given time be determined by the rate of resource acquisition from the external environment. Rate of acquisition will depend on the available supplies of the resources. Given the realities of global climate change arising as a consequence of increasing levels of atmospheric CO_2, it has become relevant to formulate plant–microbe resource allocation optimization problems in terms of C:N:P stoichiometries. For example with respect to the legume tripartite association, what would be the optimal microbial symbiotic to plant mass ratio that ensures maximum specific legume growth rates for a given supply of soil nitrogen and phosphate at elevated levels of ambient CO_2 concentration? How does N and P availability affect plant productivity at elevated levels of ambient CO_2 concentration?

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Regulation of Nitrogen Assimilation in Foliar Fed Legume Plants at Insufficient Molybdenum Supply

Marieta Hristozkova, Maria Geneva, and Ira Stancheva

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Abstract Formation and function of N_2-fixing systems between bacteria from *Rhizobiaceae* family and legume plants from *Fabaceae* family are especially sensitive to molybdenum (Mo) deficiency. The hypothesis of the present work was that nitrogen fixation and assimilation in Mo deficient pea and alfalfa plants are enhanced when the nutrients were supplied through the foliage. It was established that foliar fertilization resulted in the increase of nitrogen fixation and biomass accumulation in the absence of Mo. The positive effect of foliar fertilization at insufficient Mo supply on the nitrogen uptake is better expressed in garden pea than in alfalfa. Otherwise, alfalfa was more sensitive to Mo starvation than the pea plants. Insufficient Mo supply leads to significant reduction in plant Mo content and nitrogen fixing activity, while stress induced free amino acids increased repeatedly. The negative effect of Mo exclusion from the nutrient media on nitrogen assimilation and biomass accumulation diminished through the foliar absorbed nutrients.

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1 Introduction

Molybdenum (Mo) is among the micronutrients that are very essential for the plant growth and is required in small amounts. The symptoms associated with Mo deficiency are closely related to nitrogen metabolism (Gupta and Lipsett 1981), and nitrogen assimilatory processes in plants tissues strongly depend on the plant Mo levels (Kaiser et al. 2005). Mo is an important constituent of several enzymes catalyzing different chains of nitrogen metabolism: nitrate reductase (EC 1.6.6.1), nitrogenase (EC 1.18.6.1), xanthine dehydrogenase (EC 1.1.1.204), etc. (Mendel and Haensch 2002). Loss of Mo-dependent activity (directly or indirectly through low internal Mo levels) impacts nitrogen metabolism and eventually plant development.

Amino acids in plant tissues are determined by complex interplay of factors, which may be influenced by nutrition, developmental stage, and environmental conditions (Foyer and Noctor 2002). In legumes, ammonia can be formed by direct fixation of atmospheric dinitrogen atoms within root nodules (Lam et al. 1996). It is established that the major pathway for ammonia incorporation into nontoxic organic compounds occurs through glutamine synthetase-glutamate synthase (GS-GOGAT) cycle (Ireland and Lea 1999). The GS-GOGAT cycle converts ammonia by the combined activity of the two enzymes to produce two molecules of glutamate. Amino groups are then transferred out of the GS-GOGAT cycle, predominantly via glutamate to other amino acids, such as aspartate and alanine and a range of transamination products are formed. Leaf amino acid content increased with enhanced supply of nitrogen during growth. In legumes, fluctuations in the amino acid proportion might reflect changes in the source of nitrogen for growth (Peoples et al. 1987) or in the effectiveness of symbiosis (Rosendahl and Jakobsen 1987).

According to Streeter (1981), mineral nitrogen availability in the legumes rhizosphere is a limiting factor for nodule formation and nitrogen fixation. Foliar application of nutrients, including N, allows avoiding the harmful direct action of inorganic nitrogen on symbiotic processes (Marschner 1995). Foliar uptake of N is not only supplementary but can influence the N assimilation of the whole plant. The significance of foliar fertilizer application may lie in the localization and regulation of the enzyme systems involved in nitrogen assimilation. Although the effect of foliar application of several nitrogenous fertilizers has already been investigated, information on the effect of foliar application on the content of amino acids has been limited.

There are not enough data about the localization and regulation of nitrogen assimilatory enzymes and amino acid accumulation in case of changing the site of primary N assimilation through the foliar feeding especially when plants are inoculated with the respective nitrogen fixing bacteria strain under conditions of insufficient Mo supply.

Experiments on the effects of additional foliar feeding on the nitrogen fixation and free amino acid accumulation in pea and alfalfa plants (temperate legumes with

amide compounds transport), inoculated with *Rhizobium leguminosarum bv. Vicae* and *Sinorhizobium meliloti*, respectively, were carried out under the influence of Mo deficiency.

2 Foliar Application of Nutrient Elements: Connection with Function of Symbiotic Systems and Nitrogen Assimilation

Symbiotic systems *Medicago sativa* L. – *S. meliloti* and *Pisum sativum* L. – *R. leguminosarum* bv. *viciae*, form indeterminated nodules with apical permanently functioning meristem. In these plants from temperate latitudes, the fixed and reduced nitrogen is transported toward the xylem as amide compounds – asparagine and glutamine (Atkins 1991). It was known that nitrates present in the nutrient media depressed the root nodule formation and symbiotic nitrogen fixation (Schulze et al. 1998). One of the ways to diminish such negative influence is to change the place of uptake and assimilation of exogenous supplied mineral nitrogen through the foliar application (Boote et al. 1978; Poole et al. 1983). Foliar fertilization (or foliar feeding) entails the application via spraying of nutrients (minor and major nutrients, plant hormones, stimulants and other beneficial substances). Observed effects of foliar fertilization have included yield increases, resistance to diseases and insect pests, improved drought tolerance, and enhanced crop quality (Kuepper 2003).

Foliar fertilization caused the plant to pump more sugars and other exudates from its roots into the rhizosphere. Beneficial microbial populations in the root zone are stimulated by the increased availability of these exudates. On the other hand, absorption of water and nutrients by the roots toward xylem increased (Alexander 1986). The uptake of nutrients through the foliage is affected by a number of interacting factors (Kuepper 2003; Wójcik 2004) of which a few have been known recently (Table 1).

Table 1 Influences determining the efficacy of foliar nutrient sprays (Clarkson 1985)

Plant	Environment	Spray solution
• Epicuticular wax	• Temperature (max. 30°C)	• Concentration
• Cuticular wax	• Light	• Application rate
• Age of the leaf	• Photo period	• Application technique
• Stomata and guard cells	• Air movement	• pH (5.5–8.5)
• Leaf hairs	• Humidity	• Polarity
• Adaxial and abaxial leaf sides	• Time of day	• Hygroscopicity
• Leaf turgor	• Nutrient ratio	• Sticking ability
• Surface moisture		• Carriers, penetrates
• Cation exchange capacity		• Chelates
• Root osmotic potential		
• Nutritional status		
• Species and cultivar		

Two related plant processes are associated with the effectiveness of foliar application of fertilizers (Doring and Gericke 1986). The first relates to the rates of absorption for each of the nutrients. The second is concerned with the mobility or the extent to which each foliar absorbed nutrient is translocated out of the leaves to the other parts of the plant including the stem, roots, flowers, and seeds. The structure of epicuticular wax helps limit penetration of water molecules and ions across the membrane (Marschner 1995).

The flow of cations through the cuticular membrane is much easier than that of anions. It is estimated that cation ability to penetrate the cuticular membrane is 1,000 times higher than for the anions (Mengel 2002). Generally, the movement of low-molecular-weight solutes (e.g., ions, organic acids, amino acids) from the leaf surface to the epidermal cell wall is a nonmetabolic process driven by diffusion and electrochemical potential formed by a negative charge increase across the cuticular membrane (Kannan 1980; Tyree et al. 1992). Selective transport of nutrients across the plasma membrane requires energy, specific carriers, permeases and channels and may also be a passive process driven by diffusion.

Much interest in foliar feeding has centered on the use of nitrogen. Plants respond to foliar sprays of nitrates, ammonium compounds and urea (Wójcik 2004). Generally, it seems that the ability of leaves to absorb different N forms depends considerably on plant species. The leafy absorbed nitrogen has to be metabolized rapidly before it is involved into high molecular compounds. Andrews (1986) reported that the parts of the plants above the ground are the main place of nitrogen assimilation. It was established (Peuke et al. 1998) that under nitrate nutrition the highest incorporation of N was found in the roots of *Ricinus communis* L., but when ammonium was sprayed, it was in the growing leaves. It was not surprising that N uptake was significantly higher in ammonium-sprayed leaves than in nitrate-sprayed leaves (Garten and Hanson 1990). It seems that the positively charged ammonium was more easily transported across the cuticle than was the negatively charged nitrate.

Many authors showed a beneficial effect of foliar fertilization on the nitrogen fixing plants on the different developmental stages (Wojcieska and Kocon 1997; Palta et al. 2005). Hanway (1979) reported about a significant increase of soybean seed yields that received nitrogen through the leaves as urea. Application of urea resulted in avoiding early fall of leaves and senescence. Similar research with soybean showed that foliar fertilization with macronutrients at early vegetative stages enhanced nutrient uptake through the roots, photosynthetic rates and seed yields (Haq and Mallarino 2000). The authors suggested that low input of N, P, and K could stimulate growth without inhibiting nodulation.

Da Silva et al. (1993) have concluded that bean plants (*Phaseoulus vulgaris* L.) are capable for nitrogen fixation in the case of the exogenous nitrogen supplied through the leaves which resulted in the increase of seed yields with high content of nitrogen.

It is likely that foliar fertilization alleviated problems with early nutrient uptake, which sometimes occur even in high-testing soils. Positive effect of foliar fertilization on the legume plants growth increased in the case of application of combined

foliar fertilizers containing macro- and micronutrients. Complex foliar fertilizers are absorbed easily with high efficiency (Garcia and Hanway 1976; Schon and Blevins 1990; Fenn et al. 1995; Haq and Mallarino 2000).

Despite many studies carried out on mineral nutrient absorption by leaf tissues many aspects of foliar fertilization are still unknown. At present, it is believed that such fertilization of plants is a valuable complement to the application of nutrients to the soil. It is proposed that this treatment should be recommended in the integrated plant production because it is environment friendly and increases productivity and yield quality (Wójcik 2004).

3 Molybdenum Importance for Plant Nitrogen Metabolism

Among the micronutrients that are essential for plant growth, Mo is required in the smallest amounts. Plant species significantly differ in their requirements for Mo. Grasses from nonlegume plants contained less Mo (0.2–1.0 ppm) than legume plants (0.5–20 ppm) per gram dry weight (Gupta and Lipsett 1981). Its mobility is proved by the translocation of foliar supplied molybdenum. The form in which Mo is translocated is unknown, but its chemical properties indicated that is most likely transported as MoO_4^{2-}, rather than a complex ion (Marschner 1995).

Arnon and Stout (1939) first reported about Mo deficiency symptoms in tomato plants. The visual Mo deficiency symptoms were shown in cauliflower (Davies 1945; Mitchell 1945) alfalfa (Anderson 1942), clover (Anderson 1946), and grape (Williams et al. 2004). The symptoms associated with the deficiency of Mo are closely related to metabolism of nitrogen. In plants only a few enzymes have been found to contain molybdenum as cofactor. These include enzymes that catalyzed different chains of nitrogen metabolism. The molybdenum requirements of higher plants, therefore, depend on the mode of nitrogen supply. Under Mo deficiency conditions, plant molybdoenzymes can be broken down to those involved in nitrogen reduction and assimilation (Kaiser et al. 2005). Harper and Paulsen (1969) observed significant accumulation of nitrates in wheat seedlings starved for Mo and a negative correlation between nitrate content and nitrate reductase activity. On the other hand, it could be expected that under molybdenum-deficiency conditions, application of nitrogen in a form different from nitrate-N, for example NH_4^+-N do not influence so strongly, nitrogen assimilation and plant development (Notton 1983).

The other notable influence of Mo on plant nitrogen metabolism is in nitrogen-fixing legumes. The symbiotic bacterial enzyme nitrogenase is comprised of two subunits one of which is the MoFe protein directly involved in the reduction of N_2 to NH_3. What is known, with respect to molybdenum and legume nitrogen fixation, is that Mo availability is closely correlated with nodule development and Mo requirement of nitrate reductase is lower than for nitrogenase (Anderson 1956; Kaiser et al. 2005). Depending on the plant species, the critical deficiency levels of molybdenum vary between 0.1 and 1.0 ppm leaf dry weight (Gupta and Lipsett

1981). Frame et al. (1998) showed that critical Mo levels for alfalfa are 0.5–0.9 ppm in plant dry weight.

Anderson (1956) clarified the association of deficiency symptoms with seed reserves and pointed it out as a probable reason for the relative absence in early experience of deficiency of large-seeded legumes. Jongruaysup et al. (1997) suggested that in case of high Mo seed reserves deficiency symptoms were unlikely even on low Mo soils. Hagstrom and Berger (1965) observed that large-seeded crops, such as peas (*P. sativum* L.,) responded to soil application of Mo when the seeds contained less than 0.2 ppm Mo, but not when they contained enough Mo (0.5–0.7 ppm) to supply the Mo needs of the crop. Most frequently occurred Mo deficiency symptoms are chlorosis, golden-yellow coloration of older leaves along the apex and the apical leaf margins as well as necrotic areas extending back along the apex and the apical leaf margins (Agarwala et al. 1979; Gupta 1997). Plants had short internodes and reduced foliage (Gupta and Lipsett 1981). It would appear that nodules accumulate significantly more Mo than what is required in order to support bacterial nitrogenase activity and symbiotic nitrogen fixation (Kaiser et al. 2005).

Mo distribution among the plant organs strongly depended on the Mo soil reserves. In case of poor Mo supply, Mo transport is directed to the roots and nodules while in the case of Mo excess Mo is accumulated mainly in the leaves (Becking 1961; Franco and Munns 1981).

Mo content in plant samples was determined with inductively coupled plasma spectrometry. The seeds, roots and shoots of nitrogen fixing pea and alfalfa plants were analyzed (Table 2) (Hristozkova et al. 2009). As the alfalfa plants are more sensitive than pea to Mo contents in the nutrient media, Mo reserves in alfalfa seeds were ten times higher than those in pea seeds. The content of Mo in Mo deficient organs was obviously lower than the content in normally supplied plants and this trend appeared more clearly in alfalfa. Thus, Mo contents in plants grown in the absence of Mo significantly decreased – with 99% in the shoots and 98% in the roots in comparison with relevant Mo adequate plants. In this connection, typical Mo deficiency visual symptoms expressed as chlorosis of the young mature leaves as in alfalfa plants only (Fig. 1). Higher amounts of Mo were accumulated into the shoots in case of pea and in roots of Alfalfa. This trend was observed both in deficient plants and those supplied with Mo. Higher levels of Mo in the pea shoots coincided with higher nitrogen content in shoots (data not shown).

Total Mo content (Table 2) (Hristozkova et al. 2009) in the alfalfa Mo deficient plants (roots and shoots) is relevant to the initial Mo level in the seeds and is much

Table 2 Molybdenum content in plant organs (ppm) (Hristozkova et al. 2009)

Plant organs	Pea	Alfalfa
Seeds	0.17	1.73
Shoots (+Mo)	4.94	23.8
Roots (+Mo)	0.72	64.5
Shoots (−Mo)	0.69	0.31
Roots (−Mo)	0.17	1.20

Fig. 1 Alfalfa plants grown at reduced Mo supply

higher in roots. Jongruaysup et al. (1997) showed that in Mo deficient nitrogen fixing plants roots are the main storage organ for Mo accumulation, necessary for the forming seeds. According to Brodrick and Giller (1991) when plants suffer from Mo shortage, Mo become more mobile and its transport is orientated from the shoots toward roots and nodules in order to support nitrogen fixing activity.

4 Plant Biomass Accumulation, Number of Nodules and Nitrogen Fixing Activity

Significant changes in plant metabolism related to nitrogen assimilation and biomass accumulation of pea and alfalfa, grown under conditions of Mo deficiency were observed. Under conditions of optimal Mo supply, the favorable effect of foliar fertilization on root and shoot dry biomass accumulation was expressed and more distinct in pea plants. Foliar fertilization resulted to increase of shoot dry biomass with 67% and root dry biomass with 10% in comparison with the control. In alfalfa plants, only shoot biomass increased with 33% (Table 3).

Under Mo deficiency conditions, additional foliar nutrition also enhanced shoot and root dry biomass of both plant species as compared with the root nutrition. According to the obtained results excluding Mo from the nutrient media resulted to a less reduction of plant biomass in case of additional nutrient supply though the foliage. Therefore, plant dry biomass accumulation of Mo deficient foliar fed plants were close to plant biomass with root nutrition and sufficient Mo supply.

Table 3 Effect of foliar feeding on the dry biomass accumulation of nitrogen fixing 35-days old pea and alfalfa plants grown at different Mo supply

Variants	Dry biomass (g plant^{-1})		Dry biomass (g plant^{-1})	
	Shoots	Roots	Shoots	Roots
	Pea		Alfalfa	
F1+Mo-control	0.600 ± 0.019^{fb*}	0.480 ± 0.021^{c}	0.03 ± 0.002^{b}	0.022 ± 0.0015^{c}
F2+Mo	1.000 ± 0.044^{d}	0.530 ± 0.016^{e}	0.04 ± 0.002^{c}	0.019 ± 0.0014^{b}
F1−Mo	0.501 ± 0.015^{f}	0.420 ± 0.017^{b}	0.02 ± 0.0011^{f}	0.013 ± 0.0011^{f}
F2−Mo	0.559 ± 0.022^{a}	0.494 ± 0.015^{c}	0.029 ± 0.0012^{a}	0.015 ± 0.0013^{a}

F1 – root nutrition; F2 – combined root and foliar nutrition

*Different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis

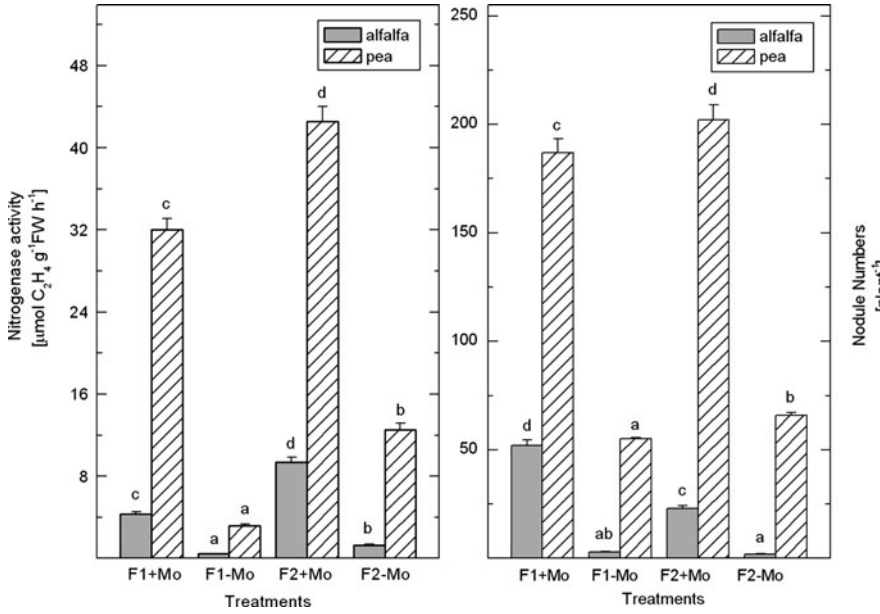

Fig. 2 Nitrogenase activity and nodule number in pea (Hristozkova et al. 2007a) and alfalfa (Hristozkova et al. 2009) plants grown at different Mo supply: (F1+Mo)-control Mo supplied plants with root nutrition; (F2+Mo)-Mo supplied plants with root and foliar nutrition; (F1−Mo)-Mo deficient plants with root nutrition; (F2−Mo)-Mo deficient plants with root and foliar nutrition. Different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis

The number of nodules in the pea plants (Hristozkova et al. 2007a) grown without Mo declined and this reduction is expressed to a less extent in foliar fed (F2−Mo) plants (Fig. 2). The exclusion of Mo from the nutrient media resulted in the reduction of nodule numbers with 63% for the plants with root nutrition and 15% for those with root and foliar nutrition. A depression of nitrogenase activity

(NG) with 98% was observed in Mo deficient pea plants with root nutrition in comparison with the relevant Mo supplied plants (Fig 2) (Hristozkova et al. 2007a). Nitrogenase activity in Mo deficient pea plants with root and foliar nutrition decreased to a less extent (with about 68%) in comparison with relevant Mo adequate plants.

However, the number of nodules in alfalfa plants with additional foliar feeding declined in comparison with the plants with root nutrition both in the presence and absence of Mo (Fig. 2). Nitrogenase activity in foliar fed plants – treatments F2+Mo and F2−Mo was higher than the activity in root fed plants – treatments F1+Mo and F1−Mo (Hristozkova et al. 2009). Therefore, the number of nodules was not relevant to their NG activity (Fig. 2). The lack of correspondence between the nodule number and nitrogen fixing activity was also suggested by Puppo et al. (2005).

5 Free Amino Acid Composition

Connection between Mo deficiency and nitrogen metabolism strongly affected protein synthesis. Low molecular nitrogen compounds accumulated (amino acids, amides) as a result of high rubo nuclease and low aminotransferase activities (Marschner 1995). In temperate legumes with amide compounds transport, both fixed and supplied inorganic nitrogen are assimilated into amino acids glutamine (Gln), glutamate (Glu), asparagine (Asn) and aspartate (Asp), which serve as important nitrogen carriers in plants (Ta et al. 1984). When the pea plants were supplied with normal Mo concentration the highest content of Asp/Asn was found in the roots, especially in the plants with foliar fertilization (F2+Mo) – 35% of total amino acid content (Fig. 3). Relatively high content of proline (Pro) in root fed plants (F1+Mo) and in foliar fed plants (F2+Mo) was observed both in the shoots and roots followed by the content of alanine (Ala). The content of Asp/Asn and Glu/Gln in the shoots of Mo supplied plants (F1+Mo, F2+Mo) was lower than in the roots (Fig. 3). Ta et al. (1984) suggested that in legumes with amide compounds transport, Asn is a major nitrogen transport compound. Rosendahl and Jakobsen (1987) have studied the concentration of major amino acids and amides in the root xylem sap of *P. sativum* in relation to the efficiency of various strains of *Rhizobium*. The authors concluded that Asn contents were clearly higher than the Gln in the most efficient symbiosis. According to Fougére et al. (1991) in alfalfa roots, Glu and γ-aminobutyrate (GABA) are predominantly higher and represented 25% and 18% of the total amino acid fraction, respectively. We observed high levels of GABA in the shoots (Fig. 3) of Mo supplied pea plants (F1+Mo, F2+Mo). GABA amino acid is found in plants as a significant component of the free amino acid pool. In higher plants GABA is synthesized primarily from L-glutamate (Bown and Shelp 1997). This is in correspondence with lower Glu/Gln levels in the shoots (Fig. 3). Some authors suggested that GABA might play a role in signaling (Kathiresan et al. 1997).

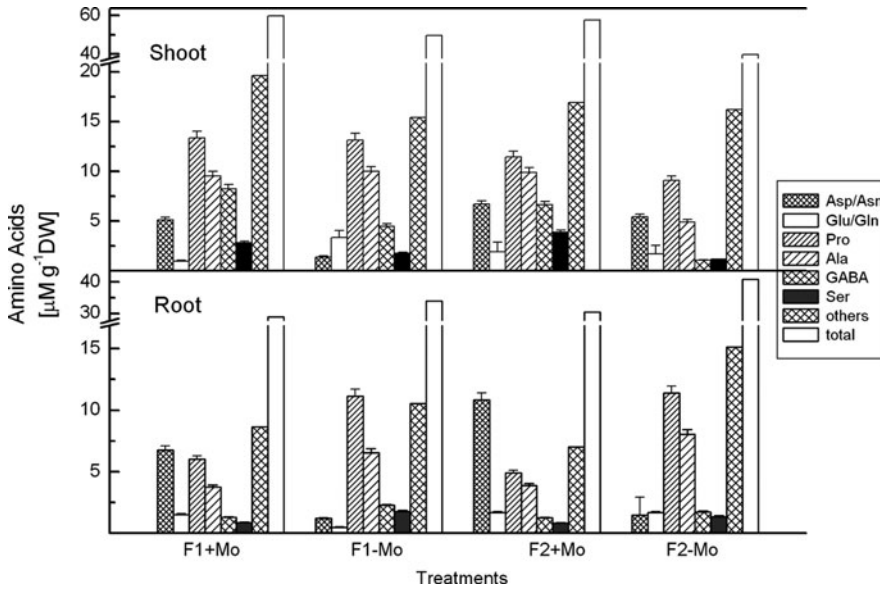

Fig. 3 Shoot and root amino acids content in pea plants (Hristozkova et al. 2007b). Treatments: F1+Mo; F1−Mo; F2+Mo; F2−Mo are described in detail at Fig. 2. Bars represent the standard error of the mean. Reported values were averaged from three independent extractions ($n = 3$)

In the variants without Mo (F1−Mo, F2−Mo) the highest Pro content in the free amino acid pool was measured both in pea shoots and roots, and varied from 22 to 32% of the total amino acid fraction (Fig. 3). The addition of foliar nutrients resulted in lower proline content in the pea shoots. The proline content in the roots of Mo deficient plants was higher than the Mo adequate pea plants, while in the shoots significant changes were not observed. Marked increase in free proline occurs in many plants during moderate or severe water and salt stress; this accumulation, mainly as a result of increased proline biosynthesis, is usually the most outstanding change among the free amino acids (Fougére et al. 1991). Hence, Mo exclusion from the nutrient media could be considered as stress factor for the pea plants. The content of Asp/Asn in Mo deficient plants is low with the exception of the value in the shoots of foliar supplied F2−Mo pea plants (Fig. 3). The Asp/Asn concentration in F2−Mo treatment is close to the value in the shoots of Mo supplied plants with root nutrition (F1+Mo). Therefore, the transport of major nitrogen compound Asp/Asn toward the leaves is not suppressed in Mo deficient plants in case of foliar nutrient application. High content of alanine (Ala) of Mo deficient pea shoots and roots were accounted for and reached values between 12 and 20% of the total amino acid pool (Fig. 3). Ta and Joy (1986) pointed out that Ala has a major involvement in photorespiration and those other amino acids and amides such as Asn are also involved although to a lesser extent. The synthesis of alanine may occur at the expense of the acidic amino acids, glutamate and aspartate (Stewart and Larher 1980), and occurs concomitantly with the accumulation of GABA (Wallace

et al. 1984; Ratcliffe 1995). We observed similar correlation regarding the shoots of Mo supplied pea plants (F1+Mo, F2+Mo). In plants grown in Mo restrictive media such correlation was not found.

The content of total free amino acids (Hristozkova et al. 2009) in alfalfa roots was significantly higher in the plants grown in Mo absence compared with the relevant treatments when Mo was supplied (Fig. 4). The lowest free amino acids content was established in F2+Mo treatment. High content of alanine (Ala) was found in the all treatments. In the roots of F1−Mo plants, the main nitrogen carriers' aspartate/asparagine (Asp/Asn) and glutamate/glutamine (Glu/Gln) content decreased in comparison with F1+Mo treatments.

The levels of Asp/Asn and Glu/Gln in the roots of F2−Mo alfalfa plants were higher than in F2+Mo. The highest content in the roots of Ala, γ-aminobutyrate (GABA), proline (Pro), threonine (Thr) and serine (Ser) was observed in F1−Mo treatment. In the shoots of Mo deficient plants, the total content of free amino acids exceeded three times than that of Mo supplied – F1+Mo and F2+Mo plants (Fig. 4). Additional foliar nutrition did not significantly change the total amount of free amino acids independently on Mo supply. In the shoots of F1−Mo and F2−Mo treatments, the level of stress induced amino acids Ala, GABA, Pro, Thr, and Ser mainly increased in comparison with the controls (F1+Mo). Enhanced Asp/Asn and Glu/Gln levels were observed in Mo deficient plants with root and foliar nutrition in comparison with Mo supplied treatments.

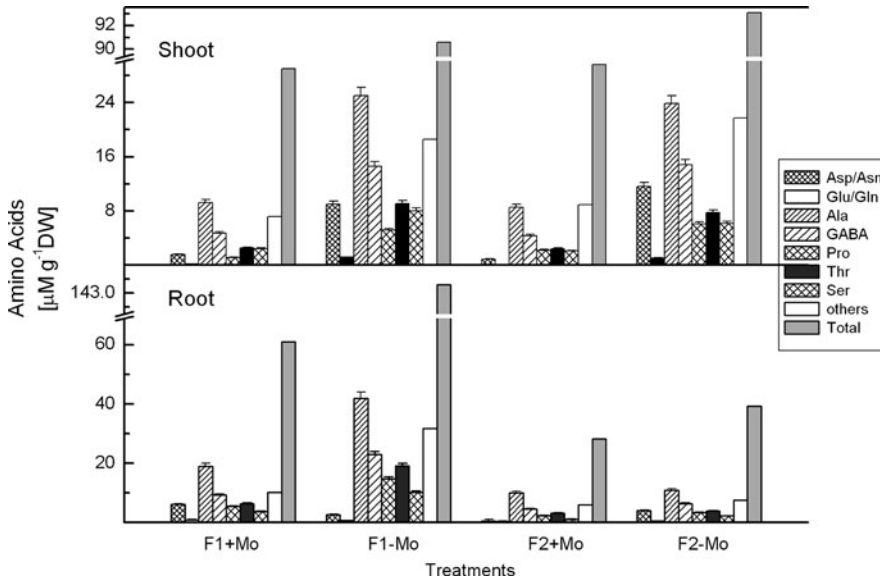

Fig. 4 Shoot and root amino acids content in alfalfa plants (Hristozkova et al. 2009). Treatments: F1+Mo; F1−Mo; F2+Mo; F2−Mo are described in detail at Fig. 2. Bars represent the standard error of the mean. Reported values were averaged from three independent extractions ($n = 3$)

Molybdenum starvation resulted in an increase of total content of free amino acids especially in the alfalfa roots in comparison with the Mo supplied plants. Influence of foliar feeding was appeared in free amino acids accumulation mainly in the roots especially in Mo deficient plants. The increase of free amino acids is a result of enhanced flow of assimilates toward the roots in foliar supplied plants.

Increased levels of stress induced amino acids such as Ala, GABA, Trh, Pro, and Ser in the roots and shoots of Mo deficient plants indicated that alfalfa plants are very sensitive to insufficient Mo supply (Fig. 4). The main nitrogen transport compounds Asp/Asn increased at insufficient Mo supply especially in the shoots. In the all treatments, Asp/Asn content in the shoots and roots was higher than the Glu/Gln content, and its values varied in dependence on Mo presence in the nutrient media as well as additional foliar nutrition. On the other hand, Lea et al. (2007) observed accumulation of Asn in plant tissues during the periods of suppressed protein synthesis. Foliar fertilization resulted in lowering of total free amino acid content predominantly in the roots both in the Mo supplied and Mo deficient plants compared to the plants with root nutrition. It was also observed that the foliar fertilization reduced the inhibitory effect of Mo shortage on the aspartate/asparagine content in the pea shoots.

6 Conclusion

Biological nitrogen fixation provides a large proportion of plant nitrogen requirements and contributes to agricultural sustainability. Symbiotic nitrogen fixation by the legume–*Rhizobium* symbiosis is a finely regulated process. Mechanisms for the regulation of symbiotic N_2 fixation under conditions of nutrient deficiency may be very different in the different symbiotic types. Molybdenum is now known to be essential in the fixation of nitrogen by the symbiotic bacteria associated with leguminous plants. It was established that Mo shortage in the nutrient media resulted in nodule number and biomass reduction, lowered nitrogenase activity and suppressed plant biomass accumulation of pea and alfalfa plants. The composition of free amino acids and amides changed under Mo deficiency conditions – the main nitrogen transport compounds aspartate/asparagines and glutamate/glutamine decreased, while stress induced amino acids as alanine, GABA, threonine, proline, and serine accumulated. It was shown that alfalfa was more sensitive to Mo starvation than the pea plants.

The results connected with nitrogen assimilation and biomass accumulation of pea and alfalfa showed that efficiency of nitrogen fixation and assimilation could be improved through the application of Agroleaf® in 0.3% concentration. The positive effect of foliar nutrition on nitrogen assimilation was better expressed in pea plants under Mo deficiency conditions. In addition it was observed that the negative effect of Mo deficiency on the nitrogen fixation and assimilation in pea and alfalfa plants (both temperate legumes with amides nitrogen transport) was lowered through the foliar absorbed nutrients.

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